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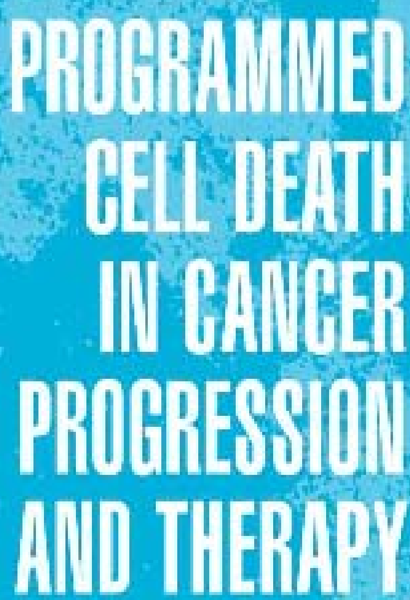

PROGRAMMED CELL DEATH IN CANCER PROGRESSION AND THERAPY

Edited by
Roya Khosravi-Far
and Eileen White

Programmed Cell Death in Cancer Progression and Therapy

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Dedication

We dedicate this book to three deserving groups. First, our families for their support and encouragement, Simin, Ghasem, Reza and Ali Khosravi-Far, and Greg, Jason and Melissa Diamond. Second, to our students, fellows and assistants, who with their hard work pave the road to discovery. Last but not least, to anyone who has been touched by cancer, as they are our motivation for this work and for our research.

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Foreword

Apoptosis is a tightly regulated cell-suicide program that plays an essential role in development and maintenance of tissue homeostasis by eliminating unnecessary or harmful cells. Impairment of this native defense mechanism of the cell promotes uncontrolled growth and frequently confers chemoresistance to tumor cells. Substantial progress has been made in the elucidation of several of the underlying mechanisms of apoptotic signaling and their dysregulation in cancer. These advances have facilitated the identification of new drug targets for promising apoptosis-inducing therapeutic strategies. Several of the novel therapeutic agents directed against these targets demonstrate enhanced apoptotic killing and sensitize resistant cancer cells to antineoplastic agents. As a number of these agents have entered the clinic and more are in the pipeline, this is an exciting time for reaping the benefits of years of basic science discoveries through their translation into cancer therapies.

In this book, the regulation of apoptotic signaling in normal cells and the means by which this protective response is suppressed in cancer cells will be discussed. In addition, the novel apoptosis-inducing therapeutic strategies will be summarized. We hope that this book will be a useful source for scientists and clinicians.

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Chapter 1
Cell Death: History and Future

Zahra Zakeri* and Richard A. Lockshin

Abstract Cell death was observed and understood since the 19th century, but there was no experimental examination until the mid-20th century. Beginning in the 1960s, several laboratories demonstrated that cell death was biologically controlled (programmed) and that the morphology was common and not readily explained (apoptosis). By 1990, the genetic basis of programmed cell death had been established, and the first components of the cell death machinery (caspase 3, bcl-2, and Fas) had been identified, sequenced, and recognized as highly conserved in evolution. The rapid development of the field has given us substantial understanding of how cell death is achieved. However, this knowledge has made it possible for us to understand that there are multiple pathways to death and that the commitment to die is not the same as execution. A cell that has passed the commitment stage but is blocked from undergoing apoptosis will die by another route. We still must learn much more about how a cell commits to death and what makes it choose a path to die.

Keywords apoptosis, autophagy, autophagic cell death, history, lysosome

1 Cell Death has Long been Recognized as an Important Biological Problem

Cell death was seen and reported as early as 1842 by Carl Vogt (see Clarke and Clarke, 1996) although at that time it was not called cell death. Cell death was recognized almost as soon as the normal form of a living cell was understood, i.e., by the middle of the 19th century. If a living being can die, it is reasonable to watch a cell

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die. Thus, the first histologists recognized dying cells. They even recognized morphologies of death that one would today describe as apoptosis. Much of the work in the 19th century relied on the histological identification of dying cells with limited recognition of its importance and regulation. This history is well described by Clarke and other authors in a number of reviews (Clarke and Clarke, 1995, 1996; Häcker and Vaux, 1997; Lockshin and Zakeri, 2001). What is more interesting is to see when scientists began to appreciate that death was not an accident. In other words, at a certain time the phrase “cells that die are replaced by mitosis” was transformed into “cells die, and they are then replaced by mitosis” (the emphasis changed: it is no longer that an accident is repaired by an organized act, but that the organized step, i.e., cell suicide, is followed by a repair process). With the change in emphasis also came the observation that cell death could not be an abnormal event.

It became obvious that the death of cells seen during metamorphosis of amphibians or insects could not be considered abnormal (Terre, 1889; Janet, 1907; Pérez, 1910). However, the idea that the death was under some sort of control came much later. The great insect physiologist V. B. Wigglesworth well understood that the growth and disappearance of muscles in the blood-sucking insect *Rhodnius prolixus* depended on molting and thus on molting hormones (Wigglesworth, 1972). But the importance and generalization of that idea dates only from the middle of the 20th century.

2 The Mechanism of Cell Death Becomes a Question of Interest

Much of the realization and generalization of the appearance of cell death was reported by the developmental biologists. Starting in the 1950s, A. Glücksmann assembled a long list of instances of cell death, which he classified according to their function (Glücksmann, 1951, 1965). His classifications were heavily teleological, based on the presumed value to the organism (elimination of vestigial organs, scaffolding or basis for construction of a secondary organ, metamorphic loss of structure, etc.). The value of these reports was that they established the commonness and reproducibility of cell death as a biological activity. Certainly, the implication was that the deaths derived in some manner from the organization of the animal, but he did not specifically argue a physiology of cell death. It was John Saunders who really began the experimental phase. His experiments were very simple but revealing. From his transplantation experiments using the wing of a chick embryo, he concluded that the cells in the designated area were condemned to die but were neither dead nor moribund: hence he noted: “The death clock is ticking” (Saunders, 1966).

At the same time, Richard Lockshin, working in the laboratory of Carroll M. Williams, on the disappearance of muscles in the large American silkmoths, noted the activation of lysosomes (at the time recently discovered) just before the death of the muscles and the dependency of both the death and the activation of lysosomes on the action of hormones (Lockshin and Williams, 1964, 1965a–d). It was

evident that the death of the muscles followed a biological plan and they described the plan as a program and the process, programmed cell death. Later, following the lead of Jamshed Tata (1966), who studied pieces of tadpole tail in culture, they observed a need for protein synthesis for the execution of death (Lockshin, 1969), and Jacques Beaulaton and Richard Lockshin described the morphology of dying cells and of those protected from death (Beaulaton and Lockshin, 1977, 1978; Lockshin and Beaulaton, 1974a, b, 1979). The phrase programmed cell death was very much in style, and was readily accepted by the handful of biologists who worked on the subject.

A few years after the establishment of the term programmed cell death, John Kerr, an Australian pathologist working with A.R. Currie, and a postdoctoral fellow named Andrew Wyllie noted that dying cells in tadpole tail, epidermis, thymus, tumors, and other tissues resembled each other: they were rounded, dense, with blebs, and with rounded or fragmented nuclei, and the chromatin was very condensed and pushed against the nuclear membrane. The curiosity was striking for not only did they resemble each other, but also their morphology was difficult to explain. (Kerr, 1971; Kerr et al., 1972; Kerr and Harmon, 1991).

A necrotic cell (to use today's terminology) is easily explicable: without oxygen or energy, the cell ferments. Lactate accumulates in the cell and draws in water by osmotic pressure. Soon the cell explodes. It is much more difficult to explain how a cell shrinks. The shrinkage presumes either a loss of osmoles or an expulsion of water by hydrostatic pressure (Lockshin and Beaulaton, 1981). Later, it was calculated that the force exerted by the cytoskeleton was not enough to expel the water, and John Cidlowski explained the loss of osmolality (Bortner and Cidlowski, 2002). Kerr, Wyllie, and Currie chose the name "apoptosis" for that generalized form of death, thus indicating three things: (1) the form of death was general and common; (2) it suggested a very interesting physiology of death; and (3) death perhaps followed a ritual as well disciplined as birth, i.e., mitosis (Kerr et al., 1972). Still the field remained quiet. What catapulted the field of studying cell death was the recognition of its role and the morphology of apoptosis in cancer, which is the focus of this book. After 36 years, we readily accept that, for homeostasis to function, it is certainly necessary for both birth and death to be regulated.

It is not worth the trouble to insist too much on the distinction between "programmed cell death" and "apoptosis." At first, "programmed cell death" described a process, whereas "apoptosis" described a morphological conformation. The former term was used primarily in development, whereas the latter often referred to pathological situations. The implication of a requirement for synthesis of protein or mRNA for programmed cell death was vehemently argued for the case of apoptosis. Today, however, both terms are used in an essentially interchangeable fashion, and to insist like a scholastic on the purity of the terms no longer makes sense. It is also true that to explain a phenomenon by saying "the loss of cells is by apoptosis" does not say anything more than "the loss of cells is by cell death." As described below, we now understand that apoptosis is perhaps the most efficient means of cell destruction, but there are others; not all deaths are apoptotic, and if apoptosis is blocked, the cell may default to an alternative

pathway. Today it is not only acceptable but also fashionable to describe some deaths as nonapoptotic or to use other names to describe cell death.

It is therefore more important to explain how apoptosis occurs and the trigger that launches it. For that story we are indebted to Robert Horvitz and his little worms.

3 The Genetics of Cell Death Reveals the Physiology

The first big step was taken when Brenner, Sulston, and others traced the embryonic descent of each cell in the nematode worm *Caenorhabditis elegans*. Horvitz and colleagues, among others, showed that all the cell deaths in the embryo (13% of the cells, 131 total) were under the control of a handful of genes, which they named ced (cell death defective, after the mutant phenotype). The activity of the *ced* genes was regulated by *ces* (cell death selection) genes. These results were very interesting, but the discovery, shortly thereafter, that one gene encoded a type of restriction protease, a CASPase (cysteinyl protease cleaving at the carboxyl side of an aspartic acid) was earthshaking. First, one now had the first mechanism of death; and second, the gene was conserved from worm to mammal (Horvitz, 2003). Its function was therefore obviously important. This recognition was quickly followed by the identification of substrates and homologous genes in mammals and the realization that mutations of these and other cell death genes were at the origin of different cancers. Thus began the excitement that led by mid-2006 to over 180,000 publications. But the explosion of interest is also due to the realization that cell death is an important component of diseases such as neurodegenerative diseases (Lang-Rollin et al., 2003; Tolkovsky et al., 2004), acquired immune deficiency syndrome (AIDS) (Ameisen and Capron, 1991), cancers (Yonish-Rouach et al., 1993), and immunologic diseases (Golstein et al., 1995a, b; Nagata and Golstein, 1995; Golstein, 1997).

4 The Activation of Cell Death is a Decision by a Cell, but the Decision is made Based on the Type of Cell; Activities by its Neighboring Cells; Nutrients, Kinins, Growth Factors, and Other Components of its Environment; and the Past History of the Cell

We know today the events taking place during the process of apoptosis, and the sequence of the activation of enzymes and the molecular partners that encourage or block apoptosis. These stories are worth telling and constitute a major section of this book. However, they are far from the entire story, because in many situations either the components do not change in amount or only a fraction of supposedly

identical cells die in response to challenge. The decision to commit suicide is always very delicate and depends on many factors beyond the machinery itself. B-cell lymphoma can be caused by the transposition and activation of the antiapoptotic gene *bcl-2*, but in the lymphoma the pre-caspases are still present and can be activated by more intense challenge. This is also true for the p53-type cancers: the cancerous cells persist certainly not because of the failure of apoptosis, but because apoptosis is invoked only at a very high threshold of challenge. We have much to learn about the establishment of the threshold. By the same token, in cell culture, when cells are subjected to a modest challenge, the destiny of sister cells is always very variable as a function of time or sensitivity to dose of toxin or other inducer of cell death. As the 19th-century physiologist Claude Bernard remarked: "Life is the result of contact between the organism and the milieu; we cannot comprehend it by the organism itself, no more than by the environment alone." The activation of the death pathway is a type of positive feedback in which the threshold is vigorously defended, but once passed, death progresses without recourse. This threshold may be marked by the activation of caspases or of caspase-3. Thanks to many investigators, we know the partners of the apoptosome and the competition that determines their assembly and activation, but what do we still not know? What is the role of adenosine triphosphate (ATP), guanidine triphosphate (GTP), ceramide, NO, prostaglandin, and other resources of metabolism? How do the more sensitive and less sensitive cells differ? What makes in the same environment one cell more sensitive and the other more resistant? Why is it that much of our effort to block cell death only delays the event and that 100% block of cell death is less attainable?

5 It is Easy to Categorize, but Harder to Live with Intermediates or Ambiguity

We human beings love names and categories. A child who asks five times per minute "What is that?" is generally satisfied if one gives him or her the name of the object. We readily classify everything: "Is it a boy or a girl? Animal or vegetable?" "Are his politics to the left or the right?" We tolerate ambiguity very poorly. Thus, we had apoptosis vs necrosis. Then we found that there are intermediates, and more complex deaths. There have been several efforts to reduce confusion by describing and defining intermediate or alternative forms of cell death (see, e.g., Jaattela, 2004; Sperandio et al., 2004; Kroemer et al., 2005). While these efforts have some value in clarifying concepts, occasionally there is a sense that square pegs are being forced into round holes, and that the focus really needs to be on the process and the physiology. When all is said and done, what we are examining is more often than not a corpse, and this corpse may be the result of concurrent or sequential events, some of which may have been aborted or failed to conclude. One very common error is to suppose that there are only one, two, or three forms of death. But one does not need an instruction manual to die. If caspases are inhibited when cells are exposed to a very strong toxin, the cells will die, but the death demonstrates neither

the existence of a second pathway to death nor the absence of a caspase pathway. If a toxin blocks, for example, all sources of energy, or the possibility of protein synthesis, the cell is going to die. For example, there are many reports that an embryo, before the point at which it begins to synthesize its own RNA, resists apoptosis (Hensey and Gautier, 1997, 1999; Negron and Lockshin, 2004). We find that such an embryo, exposed to cycloheximide, does not display a single morphological sign of apoptosis. Nevertheless, it activates caspase 3 at the same time as an older embryo would, though the cells of an older embryo would become apoptotic. The difference is that the cells of the younger embryo burst, necrotic, immediately after the activation of caspase 3, whereas the older cells survive another 90 min and thus have the time to transform themselves into apoptotic cells. It is therefore a weakness (of uncertain origin) of the younger cells so that they cannot reach the stage of apoptosis (Negron and Lockshin, 2004).

What is possible is that the most efficient pathway is the one preferred by the menaced cell and is determined by the nature of the cell, the agent that induces cell death, the environment and history of the cell, and much more. We see cells that consume the bulk of their cytoplasm before dying, in a form of death that is called "autophagic cell death" but we do not know if the autophagy in this situation differs in character from the autophagy seen in a starving cell (a protective autophagy, not necessarily fatal). We also do not know if death by autophagy is not more correctly described as an autophagy that continues without resolution, perhaps terminated by an apoptotic death. In the case of insect metamorphosis, it is possible that an activated autophagic process terminates by apoptosis. For example, the metamorphic death of labial glands or salivary glands (homologous organs in moths and flies, respectively) is well known as an autophagic type II cell death (Zakeri et al., 1993). At the beginning of metamorphosis, there is an activation of lysosomes and an expansion of the autophagic compartment. The bulk of the cytoplasm is eliminated without intervention of phagocytes, and without indication of any sign of apoptosis – no DNA fragmentation, no coalescence or margination of chromatin, no exteriorization of phosphatidylserine, no activation of caspases (even though the cells contain caspase genes). The death is therefore purely manifested by increased activity of autophagy, for instance, the death of a cell of mammary epithelium (Zakeri et al., 1995). But at the end (4th of 5 days for disappearance of the labial gland of *Manduca sexta*; 12th of 13.5 h for disappearance of the labial gland of Drosophila) one sees: cleavage of DNA, exteriorization of phosphatidylserine, coalescence and margination of chromatin, cleavage of caspase substrates. It would seem as if, for bulky, cytoplasm-rich cells, outside of the mitotic cycle, the elimination of cytoplasm is the priority, and this occurs by autophagy. For the moment, we do not know if this autophagy differs from an autophagy provoked by the lack of nourishing substrates in the milieu. In this case, autophagic death would not be death by autophagy, but autophagy activated by an unknown failure of the cell, with apoptosis being activated only when the autophagy had carried the cell beyond the point at which it could survive. For instance, in sympathetic neurons deprived of nerve growth factor, the autophagy and the threatened death of the cell is reversible until the mitochondria have been consumed (Xue et al., 1999, 2001; Zakeri and Lockshin,

2002, 2004; Lockshin and Zakeri, 2004a–c; C.O. B. Facey and R.A. Lockshin, in preparation). This argument would be consistent with observations of others emphasizing the protective role of autophagy: that inactivation of autophagy genes disrupts the formation of dauer larvae in *Caenorhabditis* (Melendez et al., 2003); the neonatal death of mice that lack Atg5, during the time that they must switch from placental nourishment to milk (Kuma et al., 2004); and the importance of autophagy in prolonging the life of cells deprived of growth factors (Boya et al., 2005; Lum et al., 2005). If such cells are not rescued, they die with a morphology typically described as autophagic cell death, with DNA fragmentation occurring very late if at all (Okada and Mak, 2004; Kroemer et al., 2005). One draws from this discussion the following arguments. First, apoptosis or programmed cell death is a very important and well-regulated process; it is not the event once considered to be passive. Second, in an acute situation a temporary protection against cell death, or in which one wishes to kill certain cells, interference with apoptosis promises a good outcome. But in more chronic situations, such as neurodegeneration, diseases such as AIDS or autoimmune disease, blocking apoptosis only allows alternative pathways to be exposed, and at the end of the day the cell will die. What is threatening the cell, and the limit to which it can be pushed before it invokes the death sequence, are questions that still must be resolved. Similarly, when one wishes to activate cell death as in cancer, the cell resources become a major consideration and one must examine very carefully the issue of specific targeting.

6 Where are We Now? Do We Know Where We are Going?

The aim of this chapter is not to review the over 180,000 publications on cell death, but to emphasize that several of our most favored arguments are based on somewhat tenuous ground and that we should not avoid the ambiguities. The value of this book is that the several authors confront these ambiguities and the options that we perceive today. The longer one works as a scientist, the more suspicious one becomes of predictions: there are always surprises, and we all remember instances such as very renowned professors being oblivious to about-to-break ideas such as clonal selection. Thus, it seems inappropriate if not self-destructive to attempt to imagine the next 5 or 10 years. Nevertheless, there are several themes that can be recognized as important for current research:

- The regulation of cell death is an important factor in disease. An important and even determining factor in many cancers is the reluctance of the affected cells to die on schedule, usually by mutation of genes in the cell death pathways; and in other diseases, the pathology is exacerbated or caused by the suicide of cells that appear to be capable of surviving.
- One cannot address these deaths simply by focusing on the direct apoptosis pathway. In many situations such as the p53- and bcl-2-driven cancers, the effectors (initiator and effector caspases) are in place but are not activated at

the appropriate time. In other situations, the affected cell is in dire straits and blocking caspases simply allows the cell to die by other means, or to persist in a nonfunctional zombie-like state, alive but incapable of fulfilling its physiological role.

- It is important therefore to consider apoptosis, or cell death in general, as more of a symptom or result than as a process, and to learn more about what in the environment or the history of the cell initiates the process. After all, in a community, murder and suicide rates are statistics, but they are only statistics revealing an underlying social pathology. Controlling access to guns may have value, but if this move simply diverts the pathology to knives and poison, the issue is not resolved. Similarly, blocking apoptosis, particularly in the acute situation, may provide real benefit, but more often the problem will persist. If, for instance, bystander cells cross the threshold to suicide in AIDS, will more aggressive positive support such as lymphokines help keep them below the threshold? Can one disrupt the Fas–FasL interaction that triggers receptor-mediated death? What effect will such disruption have on the physiology of the organism?
- Similarly, in situations such as neurodegenerative disease, cells are clearly agonizing over extended periods of time before they ultimately fail. Almost certainly, if one blocks the immediate executioner, the cells will still be agonizing and will probably die using other pathways, or remain alive in a weakened and poorly functional state. The question is far more to disrupt the process: to recognize the causes of stress on the cell, and to relieve the stress or support the cell so that it can better resist the stress.
- Activation of apoptosis as an oncolytic intervention will require considerable subtlety. Certainly, specific activation of apoptosis in cancer, especially disseminated cancer, is theoretically very interesting, because it promises to be considerably less toxic than systemic antimetabolites or antimitotics. However, most cancer cells are not dangerous because they have lost the death effector machinery; for one reason or another, they have increased their threshold to activating it. Thus, any efforts to address these cancers will have to reach the malignant cells in a highly specific and targeted manner.
- Large, postmitotic, cytoplasm-rich cells including postweaning mammary epithelium and postcastration prostatic epithelium undergo substantial autophagy prior to dying, in what has been called autophagic cell death. We need to understand whether the autophagy is a death process or an agony, and what the threshold and point of no return are. If the autophagy represents an agony, we need to know why, for instance, insect larval tissue appears to be agonizing during metamorphosis, when the blood is filled with available nutrients. If we can recognize and relieve agony in threatened cells that it is desirable to maintain, we will have accomplished much without directly manipulating the cell death pathway.
- Perhaps the nicest element of the following chapters is that they emphasize process rather than cell death itself. This seems to be the pathway leading to the most important growth in the field, since we still have much to learn.

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Chapter 2
Caspase Mechanisms

Guy S. Salvesen* and Stefan J. Riedl

Abstract The main effectors of apoptosis encompass proteases from the caspase family, which reside as latent precursors in most nucleated animal cells. The apoptotic caspases constitute a minimal two-step signaling pathway. The apical (initiator) caspases are activated within oligomeric signaling complexes in response to apoptotic stimuli. Their mechanism of activation probably results from proximity-induced clustering to the dimeric active forms. Once activated, the apical caspases directly activate the executioner (effector) caspases by limited proteolytic cleavage. The distinct activation mechanisms explain how an apoptotic stimulus is converted to proteolytic activity, and how this activity is amplified to allow for limited proteolysis of the dozens of protein substrates whose cleavage is required for efficient apoptosis.

Keywords apoptosome, caspase, DISC, IAP, inhibition, protease, zymogen

1 Apoptosis and Limited Proteolysis

Apoptosis is a mechanism to regulate cell number, and is vital throughout the life of all metazoan animals. Although several different types of biochemical events have been recognized as important in apoptosis, perhaps the most fundamental is the participation of the caspases[1–3] – a family of proteases found in multicellular animals. The name caspase comes from cysteine-dependent aspartate-specific protease,[4] thus their enzymatic properties are governed by a stringent specificity for protein substrates containing Asp, and by the use of a Cys side chain for catalyzing peptide bond cleavage. The use of a Cys side chain as a nucleophile during peptide bond hydrolysis is common to several protease families. However, the primary

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specificity for Asp turns out to be very rare among proteases throughout biotic kingdoms. Of all known mammalian proteases only the caspase activator granzyme B, a serine protease, has the same primary specificity.[5,6] Caspases cleave a number of cellular proteins,[7,8] and the process is one of limited proteolysis where a small number of cuts, usually only one, are made. Sometimes cleavage results in activation of the protein, sometimes in inactivation, but presumably not in degradation since their substrate specificity distinguishes the caspases as among the most restricted of endopeptidases. This is an important distinction from the other cytosolic proteases such as the proteasome, which permits signaling by wholesale destruction of regulatory proteins such as IκB in NFκB signaling and PDS1 in anaphase promotion,[9] or calpains, whose specificity has pretty much defied analysis in vivo so far.[10]

2 Caspase Signaling

A consensus view of caspases places them in two main groups. First are the cytokine activators (inflammatory caspases) related to caspase-1, probably including mouse caspase-11 and its orthologs caspase-4 and caspase-5 in humans. Their role is to respond to infection by rapidly converting active cytokines (IL-1β, IL-18) from intracellular stores. Confirmation of the important roles of the caspases in the inflammatory cytokine response comes from gene ablation experiments in mice. Animals ablated in caspase-1 or caspase-11 are deficient in cytokine processing,[11,12] but without any overt apoptotic phenotype. The second group constitutes the apoptotic caspases that transduce and execute death signals. The phenotypes of these knockouts are very gross, usually antiapoptotic, and vary from early embryonic lethality to perinatal lethality to relatively mild with defects in the process of normal oocyte ablation.[13,14] Researchers in the area have placed the apoptotic caspases in two converging pathways, such that some are activated by others (Fig. 2.1). The core pathways probably represent the minimal apoptotic program, and certainly its simplicity is complicated by cell-specific additions that help to fine-tune individual cell fates. Nevertheless, the basic order and at least some of the essential functions and, importantly, the catalytic and activation mechanisms are known.

2.1 Caspase Activation

In common with most proteolytic enzymes, caspases reside as latent forms (zymogens) that are usually activated by limited proteolysis. It is relatively easy to imagine that the caspases operating at the bottom of the pathway are activated by ones above. But the question of how the first caspase in a pathway became activated, how the first death signal was generated, was initially perplexing.

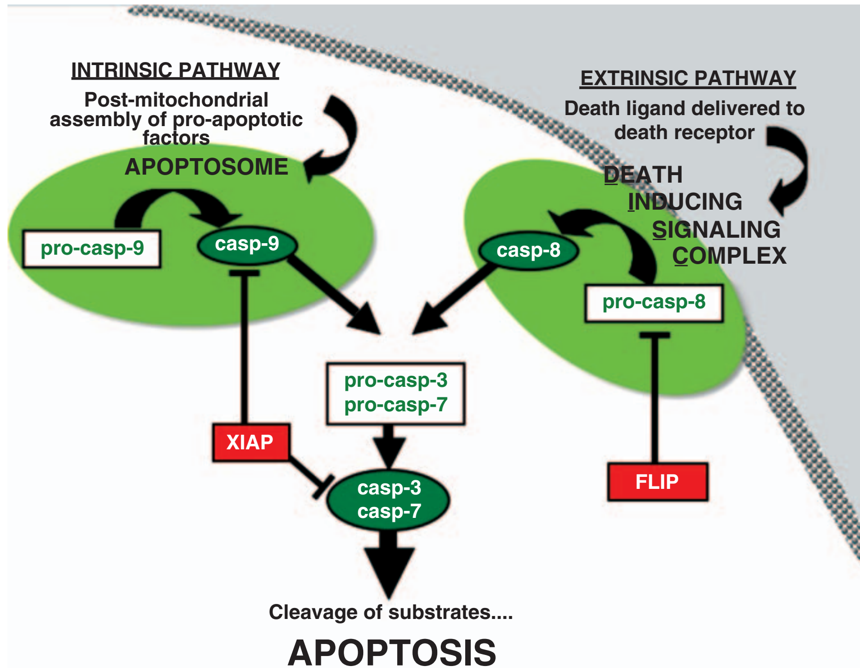

Fig. 2.1 The framework of apoptosis. Death may be signaled through ligand enforced clustering of receptors at the cell surface via the extrinsic pathway, which leads to the activation of apical caspase-8.[54] This caspase then directly activates the executioner caspase-3 and caspase-7 (and possibly 6), which are primarily responsible for the limited proteolysis that defines apoptotic dismantling of the cell. Irreparable damage to the genome caused by mutagens, pharmaceuticals that inhibit DNA repair, or ionizing radiation – transmitted by a mechanism thought to involve the release of cytochrome C from mitochondria via the intrinsic pathway – engages the same executioner caspases.[55] The latter events progress through the apical caspase-9 and its cofactor Apaf-1.[26] Activation of the extrinsic pathway is regulated by FLIP, which modulates the recruitment of caspase-8 to its adapters.[56] The execution phase is regulated through direct caspase inhibition by XIAP, which can also regulate the active form of caspase-9. In turn, the IAPs are under the influence of antagonist proteins that compete with caspases for IAPs.[44] Though other modulators may regulate the apoptotic pathway in a cell-specific manner, this framework is considered common to most mammalian cells

How exactly does a recruited zymogen become active? To understand this one must understand the unusual properties of caspase zymogens that set them apart from most other proteases. For, unlike most other proteases, simple ectopic expression of caspase zymogens in *Escherichia coli* usually results in their autolytic cleavage by limited proteolysis within a “linker segment” that separated the large (~20 kDa) and small (~10 kDa) subunits of the catalytic domain.[15, 16] This processing is a consequence of intrinsic proteolytic activity residing in the caspase zymogens. It is not due to *E. coli* proteases since catalytically disabled caspase mutants fail to undergo processing. In vitro, apical caspase zymogens can be induced to become

active either by self-association (dimerization), and executioner caspases by proteolytic processing, reviewed by Fuentes-Prior and Salvesen.[17] These distinct requirements activation are at the heart of the processes that generate caspase activity in vivo.

2.1.1 The Activation Complexes

The seminal discovery that apoptotic signaling via ligation of death receptors required, in its most basic form, simply a transmembrane receptor, an adapter molecule and a caspase[18, 19] uncovered a solution to the perplexing problem of how the first proteolytic signal was generated during apoptosis, since it implicated a caspase directly in the triggering event, as outlined below.

Extracellular ligands such as FasL and TRAIL that bind in a conventional manner to the extracellular domains of transmembrane receptors trigger the extrinsic pathway, reviewed by LeBlanc and Ashkenazi.[20] The death signal is transmitted to the cytosol by receptor clustering followed by recruitment of the apical caspase-8 (Fig. 2.1). The caspase-8 paralog, caspase-10, is also an initiator in death-receptor-mediated cell death, at least in humans (mice apparently lack a caspase-10 gene), although there is controversy in the literature regarding the ability of caspase-10 to functionally substitute for caspase-8 in death receptor signaling.[21] Structural information on the conformation adopted by the receptors in this complex is very sketchy, but recent data on the adaptor protein FADD,[22] and homologues of caspase-8[23, 24] suggest that activation of caspase-8 occurs at the cytosolic face of the cell membrane by an induced proximity mechanism (see below). The exact process of ligand binding and receptor oligomerization may require receptor internalization in addition to clustering,[25] but it is clear that the death-inducing signaling complex (DISC) represents a common example of a typical ligand-dependent transmembrane signaling receptor.

The receptor of the intrinsic pathway – the apoptosome – in contrast is not a typical transmembrane signaling receptor.[26] In the apoptosome, the cytosolic protein Apaf-1[27] senses the release of ligand, cytochrome C, which, upon binding to Apaf-1, triggers its oligomerization. As a "soluble" receptor, Apaf-1 lacks the two-dimensional arrangements of transmembrane domains spanning the cell membrane, and uses another mechanism to generate a two-dimensional surface, or platform, for signaling. This process has been reviewed,[28] and involves a mechanism-based oligomerization that uses the specialized AAA+ domain of Apaf-1 to generate a ring with sevenfold symmetry for the recruitment of caspase-9, the apical caspase of the intrinsic pathway.

2.1.2 Apical Caspases: Induced Proximity

Having formed a seven-membered recruitment platform, the apoptosome must now activate pro-caspase-9. In common with other caspases, caspase-9 is a dimer in its active form.[29] However, pro-caspase-9 exists in cells at a concentration of ~20 nM,[30]

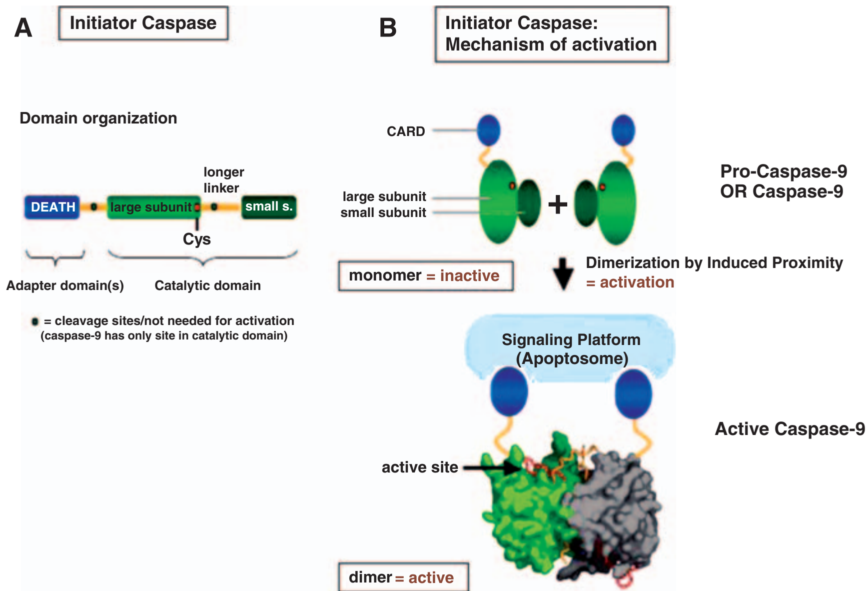

Fig. 2.2 Initiator caspases: architecture and activation. (A) Initiator caspases are expressed as single chains, comprising one or two adaptor domain(s) belonging to the DEATH domain family at the N-terminus followed by a catalytic domain, which can be divided into a large and small subunit and a relatively long loop region between the subunits. Although they can be cleaved (as revealed, for example, in their crystal structures) initiator caspases, such as caspase-9, show full activity in their uncleaved forms, which could be due to the long linker loops between subunits. (B) Their activity is regulated by dimerization, instead of by cleavage. Initiator caspases exist as inactive monomers (*top*). Binding to an oligomeric platform, such as the apoptosome in the case of caspase-9, occurs via adaptor domains (such as CARD, caspase recruitment domain) and results in an induced proximity of the catalytic domains of initiator caspases. Recent results suggest that this leads to dimerization, which allows for the formation of a productive active site as shown here in the structure of cleaved, dimeric caspase-9 (*bottom*). Interestingly, only one of the two sites adopts the active form in the crystal structure of caspase-9. PDB entry: caspase-9, 1JXQ

and the K_d for dimerization in buffers in the physiologic range is more than 50 µM in vitro.[31] The zymogen therefore must exist as a monomer under normal physiologic conditions in vivo (Fig. 2.2). Following formation of the apoptosome, and uncovering of the caspase-9-binding site on Apaf-1, the zymogen can associate with the activator complex.

Two hypotheses have been put forward for the activation of caspase-9. The first was an "allosteric model" that postulated the activation of a monomer directly by the apoptosome.[32] The second, the "induced proximity model," postulated that the apoptosome provides a platform for caspase-9 dimerization.[33] The contrasting hypotheses have been reviewed extensively, including recent revisions of the models.[34, 35]

Significantly, unlike the executioner caspases 3 and 7, pro-caspase-9 does not need to be cleaved in the linker region to become active[30, 36] (Fig. 2.2A). Not only is cleavage unnecessary, but also it is insufficient to produce an active enzyme. Instead, caspase-9 is activated by small-scale rearrangements of surface loops that define the substrate cleft and catalytic residues.[29] In the induced proximity model, this is achieved by dimerization of caspase-9 monomers within the apoptosome,[37] with the dimer interface providing surfaces compatible with catalytic organization of the active site (Fig. 2.2B). Consistent with the induced proximity model is the finding that activation of caspase-9 by the apoptosome has bimolecular kinetics, and that a hybrid containing the catalytic domain of caspase-8 tagged onto the recruitment domain of caspase-9 is also activated by the apoptosome.[31] It is tempting to speculate that a similar dimerization mechanism activates the caspase-8 zymogen to trigger the extrinsic pathway, especially since clustering of adaptors is critical for caspase-8 activation.[22–24] Finally, we note that the induced proximity activation of apical caspases by dimerization may also explain the requirement for apoptosome-like structures, known as inflammasomes, to activate inflammatory caspase zymogens, reviewed in Refs.[28, 38]

2.1.3 Executioner Caspases: Activation by Cleavage

Once an apical caspase has become active ensuing activation of the executioners is more easily explained. At their cytosolic concentration in human cells, the caspase-3 and caspase-7 zymogens are already dimers, but they are not active (Fig. 2.3). Cleavage within their respective linker segments is required for activation.[39, 40] Caspase-6 is not as widely studied as caspase-3 and caspase-7, but is classified as an executioner caspase based on its lack of a long pro-domain and its cleavage downstream of the initiators. The crystal structures of zymogen caspase-7, active caspase-7, and inhibitor-bound caspase-7 serve as models with which to rationalize the apparent conflict between the cleavage mechanism for executioner caspase activation and the dimerization mechanism for apical caspase activation.[39, 40, 57] When cleaved and uncleaved caspase-7 structures are compared, a similar reordering of catalytic and substrate binding residues occurs as seen in caspase-9, so the fundamental mechanism of zymogen activation is equivalent (Fig. 2.3B). Only the driving forces are distinct. Most importantly, the linker segment of pro-caspase-7 blocks ordering of the catalytic residues, and requires cleavage to allow a productive active site. The new N- and C-terminal sequences so generated aid in active site stabilization. The property that allows the very different driving forces of dimerization and cleavage to converge on the same activation mechanism seems to be the unusual mobility of the residues that together constitute the caspase active site, which are mainly placed on flexible loops and not ordered secondary structure.

Progress in understanding caspase structures and mechanisms now allows us to answer the question of why the executioner caspase zymogens are dimeric whereas the apical caspase zymogens are monomeric at physiologic concentrations.

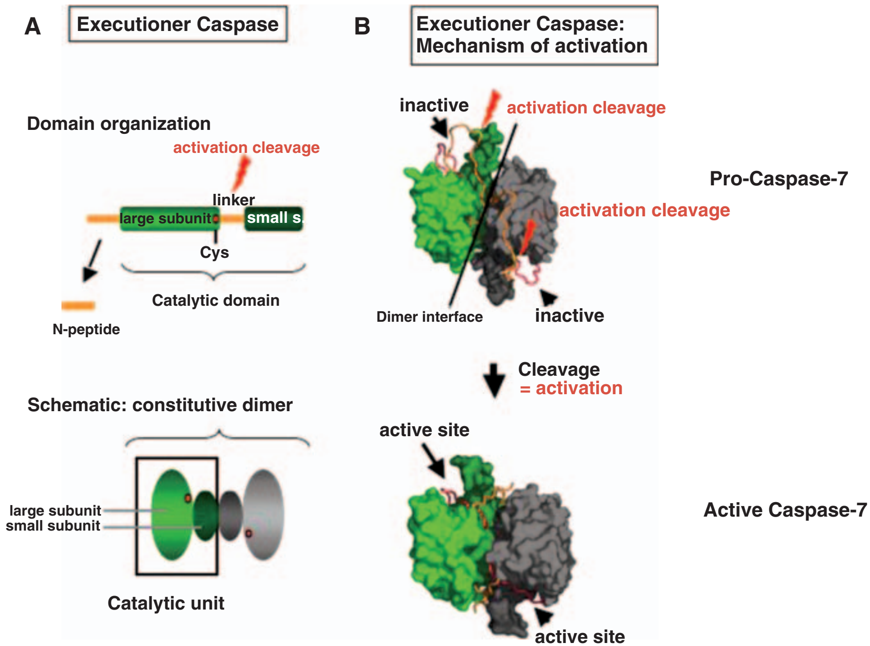

Fig. 2.3 Executioner caspases: architecture and activation. (A) Activation of executioner caspases. Caspases are initially expressed as single-chain proteins that undergo an activation cleavage. An executioner caspase is typically cleaved twice, ultimately leading to the release of a short N-terminal peptide. The actual activation cleavage divides the catalytic unit into a large and small subunit. The position of the active-site cysteine residue is indicated in red. Bottom: schematic illustrating that executioner caspases are constitutive dimers of two catalytic units. (B) Surface rendering of an executioner caspase (caspase-7) preactivation and postactivation cleavage. The same color code as in panel A is used and important loop regions are displayed as ribbons. Cleavage releases strains on surface loops (red and orange) and the chains rearrange. The newly formed termini of large and small subunit (orange) interact with each other across the other catalytic unit, and with the red loops to nicely align the substrate-binding pockets at the bottom of the active-site cleft. This results in a highly active caspase (*bottom*). PDB entries: caspase-7, 1F1J; pro-caspase-7, 1GQF

Much of the reason for this lies the relatively weak hydrophobic character of the dimer interface in caspase-8 and caspase-9, strongly contrasting with a more hydrophobic nature of the dimer interface in caspase-3 and caspase-7. Specifically, the K_d for caspase-3 dimerization is less than 50 nM,[41] which is more than three orders of magnitude tighter than that for caspase-8 ~~50 μM).[42] Interestingly, caspase-3, like apical caspases, can also be activated by experimentally induced proximity employing hybrids that possess engineered dimerization domains (see, e.g., Mallet et al.[43]). In this context, the dimerization domains, which must be introduced as multiple tandem copies, likely recruit high local concentrations of preformed pro-caspase-3

dimers leading to proteolytic activation *in trans* of the type seen in high-level expression in *E. coli*.

2.2 IAPs: Caspase Inhibitors

The best-characterized endogenous caspase inhibitor is the X-linked IAP (XIAP), a member of the IAP family, also known as BIRC proteins.[44] The family is broadly distributed and, as the name implies, the founding members are capable of blocking apoptosis, having initially been identified in baculoviruses, reviewed by Verhagen et al.[45] Eight distinct IAPs have been identified in humans, and despite initial reports, it seems now that only XIAP is capable of directly inhibiting caspases,[46] having been found by multiple research groups to be a potent but restricted inhibitor targeting caspases 3, 7, and 9, reviewed by Salvesen and Duckett[44] and Deveraux and Reed.[47] IAPs have functions in addition to caspase inhibition because they have been found in organisms such as yeast which neither contain caspases nor undergo apoptosis.[48]

XIAP contains three baculovirus IAP repeat (BIR) domains, which represent the defining characteristic of the family. Currently, there is no known function for BIR1, but the second BIR domain (BIR2) of XIAP specifically target caspases 3 and 7 ($K_i \approx 0.1$–$1\,nM$), and the third BIR domain (BIR3) specifically target caspase 9 ($K_i \approx 10\,nM$). This led to the general assumption that the BIR domain itself was important for caspase inhibition. Surprisingly, the structures of BIR2 in complex with caspase-3 and caspase-7 have revealed that the BIR domain has a secondary role in the inhibitory mechanism, and that the main inhibitory contacts are made by the flexible region preceding the BIR domain.[49–51] Interestingly, the mechanism of inhibition of caspase-9 by the BIR3 domain requires cleavage in the inter subunit linker to generate the new sequence NH_2-ATPF.[36] In part, this explains the cleavage of caspase-9 during apoptosis, which as described above is not required for its activation. Paradoxically, it is required for its inactivation by XIAP. Another surprise was in store for researchers when the structure of the BIR3–caspase-9 complex was solved.[52] Here, there was no interaction of the BIR3 domain with the active site, but instead the BIR was found associated with the dimer interface of caspase-9. Essentially, BIR3 had monomerized the caspase thus reversing the activation mechanism.

Together, the structures of BIR2 bound to the executioner caspase-3 and caspase-7, and BIR3 bound to the apical caspase-9 complete our understanding of caspase activation, at least at the structural level. Each domain has found a solution matched to the special properties of their targets. BIR2 binds to active caspase-3 and caspase-7 in their dimeric active forms, with a very specific and somewhat unusual geometry blocking the catalytic site. BIR3 subverts the dimer/monomer transition of caspase-9, and is thus totally selective for this protease. The BIR domains of XIAP represent extraordinary mechanisms that are unique in the field of protease inhibitors, achieving tight binding and stringent specificity.

3 Conclusions

Stemming from the original observation that executioner caspases are activated by proteolysis, but that apical caspases are not, has come from the current understanding of the controls placed on caspase activity. So far as we know, caspase zymogens reside mainly as soluble cytosolic proteins. Upon ligation of death receptors, or formation of the apoptosome, apical caspase zymogen monomers are recruited to their cognate activation complexes where they are activated, most likely by proximity-induced dimerization. Thus, the first protease in each pathway gains catalytic activity. Following this, the zymogens of executioner caspases are activated by a direct proteolytic attack of the apical caspases. The executioners now orchestrate the demise of the cell by cleaving a large number of cellular proteins. There is no appropriate evidence to suggest why two steps are required for apoptosis. For example, in *C. elegans* a single apoptotic caspase, CED3, seems to be able to orchestrate apoptosis on its own. Possibly, advanced animals incorporated the executioner caspases as a mechanism to provide additional regulation, or to allow the apical apoptotic caspases to function in additional, non-death, roles. Indeed, the proposed non-death roles of apoptotic caspases, reviewed by Lamkanfi et al.,[53] provides a fertile field of investigation now that the fundamental mechanisms of caspase activation have been elucidated.

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Chapter 3
The Mitochondrial Death Pathway

Anas Chalah and Roya Khosravi-Far*

Abstract Mitochondria have long been known to be critical for cell survival due to their role in energy metabolism. However, not until the mid-1990s did it become evident that mitochondria are also active participants in programmed cell death (PCD). This chapter focuses mainly on the role the mitochondria in mammalian cell death and cancer progression and therapy.

Keywords apoptosis, death receptors, mitochondria, bid, membranes, phospholipases, cardiolipin

1 Introduction

Apoptosis, or programmed cell death (PCD), is an evolutionarily conserved mechanism for the selective removal of aging, damaged or otherwise unwanted cells (Abe et al., 2000; Degli Esposti, 1999; Lawen, 2003; Ozoren and El-Deiry, 2003; Peter and Krammer, 1998; Strasser et al., 2000; Thorburn, 2004). It is an essential component of many normal physiological processes such as embryogenesis, normal tissue development, and the immune response (Vaux and Korsmeyer, 1999). Thus, regulation of apoptosis is critical for tissue homeostasis and its deregulation can lead to a variety of pathological conditions including carcinogenesis and chemoresistance (Burns and El-Deiry, 2003; Daniel et al., 2001; Green and Evan, 2002; Ozoren and El-Deiry, 2003; Sheikh and Huang, 2004; Thompson, 1995; Zornig et al., 2001).

Apoptosis is mediated primarily through the activation of specific proteases called caspases (cysteinyl, aspartate-specific proteases) (Algeciras-Schimnich et al., 2002;

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Ozoren and El-Deiry, 2003; Salvesen and Dixit, 1997; Stegh and Peter, 2001; Thorburn, 2004). Caspases are effectors of cell suicide and cleave multiple substrates, leading to biochemical and morphological changes that are characteristic of apoptotic cells(Abe et al., 2000; Strasser et al., 2000). These alterations include: mitochondrial outer membrane permeabilization; cell membrane remodeling and blebbing; exposure of phosphatidylserine (PS) at the external surface of the cell; cell shrinkage with cytoskeletal rearrangements; nuclear condensation; and DNA fragmentation (Ashkenazi and Dixit, 1999; Green and Evan, 2002; Lawen, 2003; Peter and Krammer, 2003; Schulze-Osthoff et al., 1998; Thorburn, 2004). These morphological changes culminate in the formation of apoptotic bodies that are normally eliminated by phagocytosis (Geske and Gerschenson, 2001; Wallach, 1997). In mammalian systems, the extrinsic death receptor pathway and the intrinsic mitochondrial pathway are the two major signaling systems that result in the activation of the executioner/effector caspases and the consequent demise of the cell (Abe et al., 2000; Ozoren and El-Deiry, 2003; Peter and Krammer, 2003; Strasser et al., 2000; Thorburn, 2004). In many cell types, including cancer cells, activation of the extrinsic pathway also engages the mitochondrial pathway for full execution of cell death (Jaattela, 2004; Khosravi-Far and Esposti, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Thorburn, 2004). Thus, many apoptotic signals merge at the mitochondria, and thus mitochondria have been termed “gatekeepers” of the apoptotic machinery (Jaattela, 2004; Khosravi-Far and Esposti, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Thorburn, 2004).

As gatekeepers, the proteins comprising the intrinsic mitochondrial pathway are the major mediators of the cytotoxic effects of many chemotherapeutic agents and radiation therapy (Brenner et al., 2003; Costantini et al., 2000; Debatin et al., 2002; Hersey and Zhang, 2003). Cancer cells often evade this apoptosis and develop chemoresistance and radioresistance. Indeed, disruption of the mitochondrial apoptotic machinery has been observed in many tumors (Daniel et al., 2001; Morisaki and Katano, 2003). It is also likely that disruption of the mitochondrial machinery or mutations in the mitochondrial DNA could play a role in cancer initiation. Because of the central role of mitochondria in these processes, various components of the mitochondrial machinery can be targets for novel therapeutic strategies.

2 The Mitochondrial Pathway of Apoptosis

Mitochondria are thought to be the primary organelles involved in mediating most apoptotic pathways in mammalian cells (Green and Kroemer, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Ravagnan et al., 2002; Sorice et al., 2004; Zamzami and Kroemer, 2001). Mitochondria are engaged via the intrinsic pathway of cell death, which can be initiated by a variety of stress stimuli, including ultraviolet (UV) radiation, γ-irradiation, heat, DNA damage, the actions of some oncoproteins and tumor suppressor genes (i.e., P53), viral virulence factors, and most chemotherapeutic agents (Fig. 3.1) (Kroemer, 2003). These diverse forms

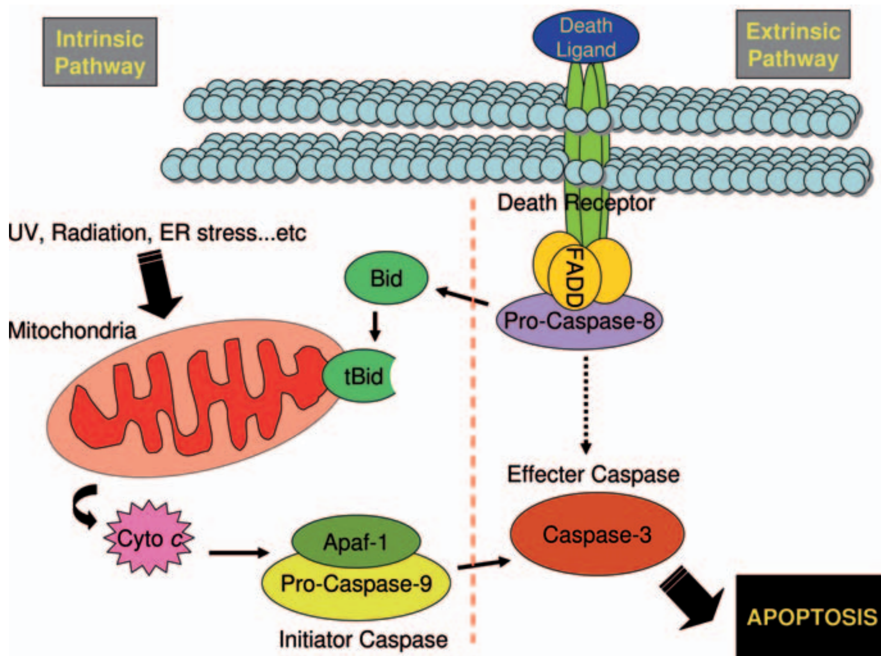

Fig. 3.1 Schematic representation of the intrinsic and extrinsic apoptotic pathways

of stress are sensed by multiple cytosolic or intraorganellar molecules. Transduction of these signals to the mitochondria ultimately results in alterations of the outer mitochondrial membrane (OM) (Esposti et al., 2003; Green and Kroemer, 2004; Kuwana et al., 2002; Newmeyer and Ferguson-Miller, 2003; Zamzami and Kroemer, 2001). These changes in the OM then lead to increased permeability to proteins that normally reside between the OM and the inner mitochondrial membrane (IM), enabling these proteins to escape the mitochondria and diffuse into the cytosol.

The mitochondrial pathway of apoptosis can also be activated in response to death ligands. In a majority of cells (type II cells), including tumor cells, extracellular death signals engage the mitochondria in a way that is equivalent to the intrinsic pathway (Abe et al., 2000; Algeciras-Schimnich et al., 2002; Ozoren and El-Deiry, 2002; Peter and Krammer, 1998). In these cells, signals originating from the death ligand-induced activation of caspase-8 and caspase-10 bifurcate into two arms, one of which directly engages mitochondria via a sequence of events causing activation of the effector caspases (i.e., caspase-3). The second arm promotes the cleavage of noncaspase substrates, such as Bid, inducing changes in the mitochondrial OM and the release of apoptogenic factors and activation of caspase-9, which then cooperates with the less-efficient activation of caspase-8 in these cells.

3 The Release of Proapoptotic Factors

Mitochondria contain and release many soluble proteins that are involved in the apoptotic cascade (Fig. 3.2) (Daniel et al., 2001; Debatin et al., 2002; Green and Kroemer, 2004; Reed, 2004). The variety of mitochondrial proteins participating in this pathway indicates the pivotal role of these organelles in determining cellular fates. Bcl-2 family members control apoptosis by regulating the permeabilization of the mitochondrial membrane (Chao and Korsmeyer, 1998; Cory et al., 2003; Daniel et al., 2001). The release of mitochondrial proteins, including cytochrome *c*, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspases (Smac/Diablo), high-temperature requirement A2 (HtrA2/Omi), and endonuclease G, is believed to play a pivotal role in inducing PCD (Martinou and Green, 2001; Zamzami and Kroemer, 2001).

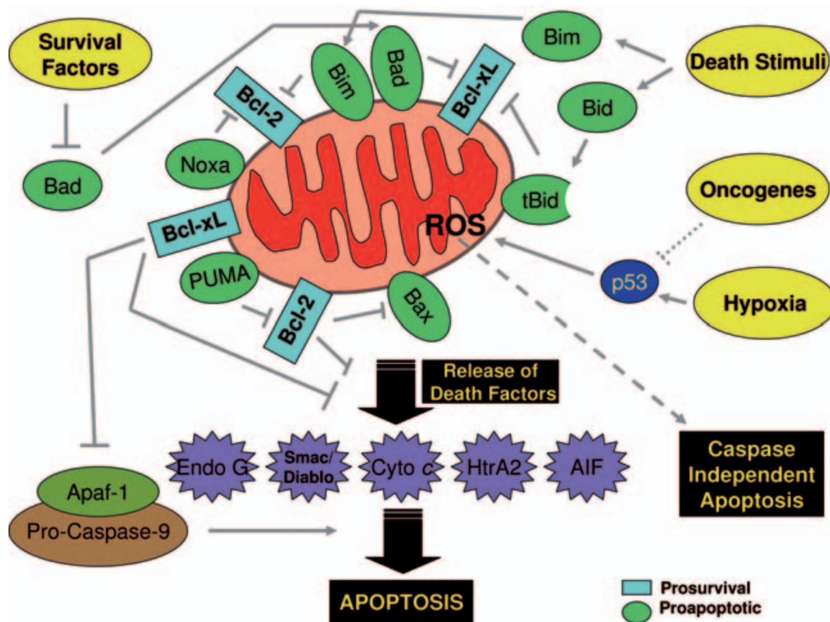

Fig. 3.2 Mitochondrial membrane permeabilization is regulated by an elegant balance of opposing actions of proapoptotic and antiapoptotic Bcl-2 family members. Bax, Bad, and Bak promote the release of cytochrome *c* and AIF through the formation of transmembrane channels across the mitochondrial outer membrane, while Bcl-2 and Bcl-X_L delay this release and abort the apoptotic response, leading to cell survival. Besides the release of mitochondrial proapoptotic components, the loss of mitochondrial membrane integrity results in the loss of many essential biochemical cellular functions such as ATP synthesis and results in the generation of reactive oxygen species (ROS). The increased levels of ROS are directly linked to the oxidation of lipids, proteins, and nucleic acids

4 Cytochrome *C*

Cytochrome *c* (Cyt *c*), a small (13 kDa) nuclear encoded mitochondrial protein, was the first protein identified as being released from mitochondria upon apoptosis. It is considered a key regulator of apoptosis because once it is released from the mitochondrial intermembrane space (IMS), the cell is irreversibly committed to death (Green and Evan, 2002; Kluck et al., 1997; Zhivotovsky et al., 1998a; Zhivotovsky et al., 1998b) and Cyt *c* is synthesized in the cytosol and translocates to the mitochondria as an unfolded apoprotein through the TOM (translocase in the OMM) complex (Diekert et al., 2001). The driving force for translocation of apo-Cyt *c* into the IMS appears to be its interaction with the enzyme cytochrome c heme lyase (Dumont et al., 1991; Mayer et al., 1995).

The release of cytochrome *c* to the cytosol is considered among the major steps in the intrinsic death pathway (Kluck et al., 1997; Newmeyer and Ferguson-Miller, 2003; Zhivotovsky et al., 1998a). Once it escapes to the cytosol, it is captured by the apoptosis protease activating factor (APAF-1), a 130 kDa adaptor protein (Soengas et al., 1999; Zou et al., 1999). Prior to binding Cyt *c*, APAF-1 is virtually inactive. Once bound to Cyt *c*, the APAF-1 monomer goes through a cytochrome *c*-induced conformational change that promotes its activation. Further oligomerization occurs, resulting in a cartwheel-shaped heptameric structure containing seven Cyt *c*/APAF-1 complexes. This larger multiprotein complex is termed the apoptosome (Acehan et al., 2002; Adrain et al., 2001; Adrain et al., 1999; Srinivasula et al., 1999). Pro-caspase-9 is recruited to the apoptosome through its CARD domain, promoting its cleavage and converting it to an active protease (Adrain et al., 1999). Consequently, caspase-9 dissociates from the complex and goes on to activate effector caspases (3, 6, and 7) which collectively orchestrate the execution of apoptosis (Slee et al., 1999; Srinivasula et al., 1999; Zou et al., 1999).

5 Apoptosis-Inducing Factor

The precursor of the protein AIF is synthesized in the cytosol and imported into mitochondria (Susin et al., 1999). It contains an N-terminal mitochondrial localization sequence (MLS) which is cleaved upon its mitochondrial translocation to form the mature 57 kDa AIF (Susin et al., 1999). Under apoptosis-inducing conditions, AIF translocates through the permeabilized mitochondrial outer membrane to the cytosol (Cande et al., 2002; Susin et al., 1999). Subsequently, AIF is transported to the nucleus where it induces ATP-independent nuclear chromatin condensation, as well as large-scale DNA fragmentation (Cande et al., 2002; Susin et al., 1999). In contrast to cytochrome *c*, AIF acts in a caspase-independent fashion and does not require the presence of cytosolic factors to induce apoptotic features in the nuclei (Lorenzo et al., 1999; Miramar et al., 2001; Susin et al., 1999; Zamzami and Kroemer, 2001). Moreover, AIF translocation occurs in Apaf-1-null mice which

fail to activate the executioner caspase (Cecconi et al., 1998). However, some studies indicate that crosstalk does occur between AIF and the apoptotic caspase cascade (Cande et al., 2002). For instance, AIF was observed to trigger the release of cytochrome *c* from isolated mitochondria (Susin et al., 1999). Additionally, AIF interacts with heat-shock protein 70 (Hsp70), a known protective factor and inhibitor of Apaf-1-dependent caspase activation (Ravagnan et al., 2002).

6 Smac/Diablo

Second mitochondria-derived activator of caspases (Smac) is a 22 kDa mitochondrial protein also known as direct IAP-associated binding protein with low pI (Diablo). Inhibitors of apoptosis (IAP) family members have the ability to interact and inhibit the enzymatic activity of caspases through their baculovirus inhibitor repeat (BIR) functional motif (Deveraux and Reed, 1999; Miller, 1999). Smac/Diablo was first identified as a mammalian IAP (Srinivasula et al., 1999; Verhagen and Vaux, 2002). Specifically, XIAP, c-IAP1, and c-IAP2 are proapoptotic factors regulated by Smac/Diablo (Ekert et al., 2001; Srinivasula et al., 1999; Verhagen and Vaux, 2002). The Smac/Diablo precursor is synthesized in the cytosol, then imported to the mitochondria where it is cleaved and activated. A mature form of Smac/Diablo is released to the cytosol under apoptotic conditions. Unlike cytochrome *c*, which directly activates APAF-1 and caspase-9, Smac/Diablo binds to the BIR domains of multiple IAP members, antagonizing them and promoting indirect caspase activation (Ekert et al., 2001; Srinivasula et al., 1999; Verhagen and Vaux, 2002). Smac/Diablo and cytochrome *c* were found to be released from the mitochondria at around the same time. Moreover, the release was found to coincide with mitochondrial membrane potential depolarization (Rehm et al., 2003; Springs et al., 2002; Verhagen and Vaux, 2002). However, a recent study presented evidence suggesting that the release of Smac/Diablo may, in fact, depend on the release of cytochrome *c* (Hansen et al., 2006).

7 HtrA2/Omi

HtrA2, also referred to as Omi, is a mitochondrial protein that belongs to the family of serine proteases. This proapoptotic protein is expressed as a 50 kDa precursor that is cleaved at the N-terminal, upon translocation to the mitochondria, to generate the active 36 kDa protein (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; Verhagen and Vaux, 2002). Similar to cytochrome *c* and Smac/Diablo, mature HtrA2/Omi localizes to the IMS (Hegde et al., 2002; Suzuki et al., 2004). Its release to the cytosol is stimulated by apoptotic triggers. Upon its release, HtrA2/Omi binds directly to the BIR domain of IAPs and inhibits their caspase-inhibitory activity (Suzuki et al., 2001). The first four N-terminal amino acids of the mature HtrA2 protein (AVPS) constitute the IAP-binding motif.

In addition to the proapoptotic effect of IAP binding and inhibition, Omi/HtrA2 appears to utilize its serine protease activity to induce an IAP inhibition-independent, caspase-independent apoptosis (Hegde et al., 2002; Suzuki et al., 2001). Recently, it was reported that the proapoptotic serine protease activity of HtrA2/Omi also plays a significant role in antagonizing IAPs. The observed HtrA2 cleavage of c-IAP produced significant caspase activation and sensitized cells to apoptosis (Yang et al., 2006).

8 Endonuclease G

As with most mitochondrial proteins, Endonuclease G is expressed as a precursor in the cytosol. Upon its translocation to the mitochondria, the 33 kDa protein is cleaved to a 28 kDa mature form (Cote and Ruiz-Carrillo, 1993). During apoptosis, endonuclease G is released from the mitochondrial IMS and translocates to the nucleus, where it causes oligonucleosomal DNA fragmentation (Li et al., 2001; van Loo et al., 2001). Endonuclease G release appears to be dependent on caspase activation downstream of mitochondria (Arnoult et al., 2003). Interestingly, endonuclease G-induced DNA degradation was observed to be caspase-independent (Li et al., 2001; Susin et al., 1999), suggesting an important role for endonuclease G in bringing about caspase-independent cell death.

9 Mitochondrial Proteins and Caspase Activation

Among the various proteins that leak out of mitochondria, a few, such as cytochrome *c*, play a major role in promoting caspase activation. (Kluck et al., 1999; Saelens et al., 2004) These apoptogenic factors are released in a hierarchical manner during cell death. Upon activation of the intrinsic pathway, cytochrome *c*, Htr2A/Omi and Smac/Diablo are released first, with similar kinetics (Saelens et al., 2004). The subsequent release of AIF and endonuclease G (Arnoult et al., 2003; Penninger and Kroemer, 2003) is associated with more severe damage to both the outer and inner membranes. Notably, cytochrome *c* has been shown to be directly involved in the mediation of cell death, as it is indispensable for the activation of Apaf-1 and subsequent formation of the apoptosome (Arnoult et al., 2003).

The apoptosome itself is a platform for recruiting and facilitating the autocatalytic activation of pro-caspase-9, the apical caspase of the intrinsic pathway of apoptosis (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Chinnaiyan, 1999; Hill et al., 2003; Salvesen and Renatus, 2002; Shi, 2002). The activation of caspase-9 leads to the local accumulation of zymogens, promoting an autocatalytic process of downstream caspase activation (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Chinnaiyan, 1999; Hill et al., 2003; Salvesen and Renatus, 2002; Shi, 2002). However, the apoptosome requires additional

regulatory factors, including Smac/Diablo, for full activation of the caspase cascade. Smac/Diablo interacts with several IAPs to release them from their inhibitory interaction with pro-caspase-9 and other caspases (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Shi, 2002). Smac/Diablo is also present in the mitochondria, where it is directly attached to the OM and is released upon alterations in the OM permeability (Cain et al., 2002; Saelens et al., 2004).

10 Mechanisms of Mitochondrial Protein Release

The exact mechanism by which mitochondrial proapoptotic components are released from the IMS is a matter of a long and ongoing debate. Currently, two general mechanisms are considered: nonspecific and specific release (Lim et al., 2001). The opening of the permeability transition pore (PTP) located in the mitochondrial IMS is proposed as the first possible mechanism. The permeability pore is comprised of three proteins: cyclophilin D, adenine nucleotide translocator (ANT), and voltage-dependent anion channel (VDAC), a matrix, an inner membrane, and an outer membrane protein, respectively (Crompton, 1999). The opening of the PTP triggers many processes, including (A) loss of the proton gradient produced by the electron transport machinery; (B) leakage of cellular water into the mitochondrial matrix, resulting in the gradual swelling of the IMS and the rupturing of the inflexible OM (Green and Kroemer, 2004); and (C) leakage of apoptotic factors from the IMS into the cytoplasm, which begins the cascade of proteolytic activities leading ultimately to nuclear damage and cell death (Brenner et al., 2000; Dejean et al., 2006; Kroemer, 2003; Marzo et al., 1998a; Marzo et al., 1998b). This mechanism represents a nonspecific release mode for proapoptotic mitochondrial mediators. However, the physical outer membrane disruption theory fails to explain the release of proapoptotic factors such as cytochrome *c* and AIF in the absence of any loss of outer membrane structural integrity (Dejean et al., 2006).

The second suggested mode of release involves the opening of large outer membrane channels that would allow cytochrome *c* and other IMS proteins to move into the cytosol. In contrast with the other scenarios, this model would leave the outer membrane largely intact. A benefit of this model is that there is no need for the mitochondrial matrix to swell. This better fits with the evidence that mitochondrial morphology remains the same in most cell death in vivo. Several outer membrane channels, including the VDAC and mitochondrial apoptosis-induced channel (MAC), have been targeted as possible specific regulators of mitochondrial release. Both provide aqueous pathways through the hydrophobic environment of the mitochondrial membrane.

VDAC is a 30 kDa highly conserved voltage-dependent, ion-selective, mitochondrial OM protein. The OM is densely packed with VDAC proteins which form barrel structures that enclose 3 nm internal diameter channels. VDAC can switch between two functional states, open and partially open. The "open" state is defined by large conductance and anion selectivity, while the "partially open" state is

defined by lower conductance (about half that of the fully open state) and cation selectivity. The voltage-dependent change between these two states is widely attributed to structural rearrangements that lead to changes of size and charge distributions within the channel (Colombini et al., 1996; Mangan and Colombini, 1987; Thomas et al., 1993).

MAC was first identified in 2001. It is a mitochondrial outer membrane channel that, according to some reports, forms at early stages of the intrinsic apoptotic pathway (Dejean et al., 2006; Guo et al., 2004). Alternatively, other studies have reported the formation of MAC at late stages of the extrinsic apoptotic pathway (Guihard et al., 2004). MAC was found to be slightly cation-selective, and unlike VDAC, voltage-independent (Dejean et al., 2005; Guo et al., 2004). MAC activity was found to be induced by apoptosis and regulated by Bax, a proapoptotic Bcl-2 family protein. Bax translocation to the mitochondria was linked to MAC formation and cytochrome *c* release (Antonsson et al., 1997; Dejean et al., 2006; Guo et al., 2004; Saito et al., 2000; Schendel et al., 1997). Bax oligomerization is proposed to form MAC channels (Cheng et al., 2001; Dejean et al., 2006; Wei et al., 2001). The pore diameter of the MAC channel was measured to be ~4 nm, which is proposed to allow for the release of the ~3 nm diameter cytochrome *c* (Pavlov et al., 2001).

11 The Bcl-2 Family of Proteins and Regulation of the Mitochondrial Pathway to Cell Death

The process of mitochondrial release of proapoptotic factors such as cytochrome *c* is elegantly regulated through members of the Bcl-2 family (Fig. 3.2) (Antonsson et al., 1997; Cory et al., 2003; Danial and Korsmeyer, 2004; Green and Kroemer, 2004; Schendel et al., 1997). In mammals, the antiapoptotic members of this family include Bcl-2, Bcl-X_L, and Bcl-W, while the proapoptotic members include Bax, Bak, Bad, Bik, Bim, and Bid. The proapoptotic family members are further classified based on domain sequence homology into two groups: one that contains multiple BH domains and one that contains only the BH3 domain (Cheng et al., 2001; Fiers et al., 1999; Kuwana and Newmeyer, 2003; Wei et al., 2001). The fate of the cell depends to a great degree on the precious balance of function between these proapoptotic and antiapoptotic Bcl-2 proteins. Studies have shown that Bax, Bad, and Bak promote the release of AIF and cytochrome *c*, while Bcl-2 and Bcl-X_L delay the release and abort the apoptotic response, promoting cell survival (Cory and Adams, 2002; Yang et al., 1997).

It is believed that Bcl-2 family members regulate the apoptotic response by controlling mitochondrial membrane permeabilization (MMP) (Green and Kroemer, 2004). The proapoptotic proteins Bax and Bak have been shown to contribute to the formation of transmembrane channels across the mitochondrial OM, leading to the escape of AIFs (Dejean et al., 2005; Korsmeyer et al., 2000; Kuwana et al., 2002; Nechushtan et al., 2001; Wei et al., 2001). Bcl-2, Bcl-W, and Bcl-X_L are, on the other hand, believed to prevent pore formation and to inhibit the release

of cytochrome *c* from the mitochondria (Kluck et al., 1997; Yang et al., 1997). Moreover, heterodimerization of Bax or Bad with Bcl-2 or Bcl-X_L is thought to inhibit their protective effect.

Bid is a potent proapoptotic protein that is normally located in the cytosol, but also shuttles through the surfaces of intracellular membranes due to its lipid-interacting capacity. Bid plays an important role in the mitochondrial pathway to apoptosis as it has been identified as the link between the death receptor signal and the release of cytochrome *c*. Activated caspase-8 engages the intrinsic apoptotic pathway through the truncation of Bid (Li et al., 1998; Luo et al., 1998). Upon death signaling, activated caspase-8 cleaves Bid (26 kDa) into two fragments: a C-terminus fragment (15 kDa) and an N-terminus fragment (11 kDa) (Luo et al., 1998). The 15 kDa fragment, which contains the BH3 domain, is termed truncated Bid or tBid. This functional fragment translocates to the mitochondria where it interacts with several proteins through its BH3 domain (Wang et al., 1996). There are two modes of Bid proapoptotic action. (1) In the BH3-dependent mode, Bid interacts with the antiapoptotic Bcl-X_L through its BH3 domain and prevents the formation of the Bcl-X_L/Apaf1 antiapoptotic complex. (2) In the BH3-independent mode, after truncation, Bid is proposed to form selective channels similar to BAX through its structural motifs (Chou et al., 1999; McDonnell et al., 1999). Moreover, tBid has been shown to induce the oligomerization of Bax and Bak, resulting in MAC formation and the subsequent release of proapoptotic cytochrome *c* (Eskes et al., 2000; Wei et al., 2000).

The mitochondrial receptor for caspase-cleaved Bid is thought to be cardiolipin (CL), a mitochondrial lipid (Esposti et al., 2003; Kuwana et al., 2002; Newmeyer and Ferguson-Miller, 2003; Sorice et al., 2004). CL is a glycerophospholipid that is synthesized and localized in the inner membrane of the mitochondria, making it one of its major constituents (Khosravi-Far and Esposti, 2004; McMillin and Dowhan, 2002; Schlame et al., 2000; Wright et al., 2004). This dimeric molecule apparently plays a significant role in controlling the mitochondrial membrane structure and function. Abnormal mitochondrial morphology and function have been observed in cells defective in the CL synthesis mechanism (Ohtsuka et al., 1993). It has been proposed that upon apoptotic stimulation, CL contributes to the apoptotic signal through the recruitment of cytosolic proteins such as tBid to the mitochondrial membrane. Additionally, it is thought that CL is involved in altering MMP, leading to the subsequent release of proapoptotic factors (Lutter et al., 2000).

12 Mitochondria and Oxidative Stress

Mitochondria are the sites of aerobic respiration. Energy is generated in mitochondria through the process of ATP synthesis via the oxidative phosphorylation pathway. This process, however, also results in the formation of single unpaired electrons, leading to reactive oxygen species (ROS). ROS such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-), and hydroxyl radicals (OH) are highly

reactive molecules generated and eliminated in a balanced process in normal cells. In particular, free radicals (superoxides) are byproducts of ATP generation by the mitochondrial respiratory chain (Andreyev et al., 2005; Beyer, 1992; Raha and Robinson, 2000). Cellular energy is usually liberated from ATP molecules through the removal of single phosphate-oxygen groups, producing adenosine diphosphate (ADP). ADP is recycled in the mitochondria where it is recharged through oxidative processes to reproduce ATP. Since ROS are harmful, the balance between energy supply and energy demand is extremely critical. Any shift in this balance would introduce excess ROS to cells and would result in oxidative stress.

The damaging effect of elevated levels of ROS is thought to be due to the highly reactive free electrons available to form stable chemical bonds. While H_2O_2 is free to escape the mitochondrion, both the superoxide anion and hydroxyl radicals have limited diffusion, and are more likely to contribute to inner membrane damage of mitochondria (Szeto, 2006). Several studies have demonstrated a direct relationship between mitochondrial ROS and the mitochondrial apoptotic pathway. For example, the release of cytochrome *c* to the cytosol has been linked to mitochondrial oxidation (Shidoji et al., 1999). It is believed that the release mechanism might involve the opening of mitochondrial PTPs (Vieira et al., 2001). Several antioxidant compounds, such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), and ubiquinol are naturally present in the cell and act to protect against the effects of ROS (Sies and de Groot, 1992).

13 Mitochondria and Cancer

Given the important roles mitochondria play in cellular energy metabolism, free radical formation and PCD, defects in mitochondrial function are suspected to contribute to the development and progression of cancer and to resistance to therapy (Bettaieb et al., 2003; Brenner et al., 2003; Costantini et al., 2000; Debatin et al., 2002; Hersey and Zhang, 2003; Jaattela, 2004; Kasibhatla and Tseng, 2003; Kim et al., 2004). Defective apoptosis is one of the hallmarks of tumorigenicity and is implicated in multiple stages of tumor progression (Burns and El-Deiry, 2003; Hanahan and Weinberg, 2000; Ozoren and El-Deiry, 2003). Furthermore, the ability of tumor cells to escape apoptosis plays a key role in promoting resistance to conventional chemotherapy and radiation therapy (Abe et al., 2000; Barnhart et al., 2004; Daniel et al., 2001; El-Deiry, 1997; Thompson, 1995; Zornig et al., 2001).

A link between mitochondria and cancer progression was suggested over half a century ago when Warburg reported the role of mitochondria in cellular energy metabolism. This phenomenon was coined the “Warburg effect.” The Warburg effect suggested that the development of an injury to the respiratory machinery is an important event in carcinogenesis (Warburg, 1951). This injury results in compensatory increases in glycolytic ATP production to fulfill the energy needs of tumor cells. Since then, preferential reliance on glycolysis over the oxidative metabolism has been shown to correlate with tumor progression in several types of

cancer (Semenza et al., 2001). Since the initial report of the Warburg effect, a number of cancer-related mitochondrial defects have also been identified (Brenner et al., 2003; Carew and Huang, 2002; Debatin et al., 2002; Jaattela, 2004). These defects include altered expression and activity of respiratory chain subunits and glycolytic enzymes, changes in oxidation of NADH-linked substrates and mutations in mitochondrial DNA. Thus, the differences in energy metabolism between normal cells and cancer cells constitute a biochemical basis for the development of therapeutic strategies that might selectively kill cancer cells in their compromised respiratory state.

Furthermore, dysregulation of members of the Bcl-2 family has been detected in a variety of malignancies, especially hematological cancers. Bcl-2 itself was originally discovered as an oncogene in B cell lymphoma Danial and Korsmeyer, 2004. Additionally, overexpression of Bcl-2 has been detected in AML and non-Hodgkin's lymphomas. Dysregulation of other Bcl-2 family proteins have also been detected in other cancers; for example, increased expression of Mcl-1 has been detected in relapsed AML and multiple myeloma. Increased expression levels and mutations in the promoter of the *mcl-1* gene have also been observed in chronic lymphoblastic leukemias. These studies reiterate that changes to the mitochondrial-associated proteins, mainly members of the Bcl-2 family, are directly involved in tumor progression.

Additionally, there is some evidence that alterations in the mitochondrial DNA could also be involved in cancer progression. Besides hosting hundreds of nuclear encoded proteins, mitochondria have their own DNA that encodes 13 mitochondrial proteins (Schatz, 1995; Singh et al., 1999). Mutations in mtDNA could occur during oxidative phosphorylation involving ROS. Investigations of human bladder, lung, neck, and head primary tumors revealed a high percentage of mtDNA mutation (~50%) in these tumors (Fliss et al., 2000). These observations suggest a link between cancer development and mitochondrial dysfunction; however, they do not present a clear answer to whether mitochondrial DNA mutation is simply a result, or rather the cause, of alterations in PCD.

Mitochondria also play an important role in resistance to chemotherapy and radiation therapy. Since mitochondria are integrators of apoptotic signaling pathways, induction of apoptosis in many cell types leads to the induction of MMP (Brenner et al., 2003; Kroemer, 2003). MMP defines the point of no return in most PCD pathways and is regulated by pre-mitochondrial signal transduction pathways. These pathways involve caspase-dependent and caspase-independent mechanisms, members of the Bcl-2 family of proteins and changes in the composition of mitochondrial membranes (Bettaieb et al., 2003; Brenner et al., 2003; Green and Kroemer, 2004; Kim et al., 2004; Kroemer, 2003; Kuwana et al., 1998; Newmeyer and Ferguson-Miller, 2003; Peter and Krammer, 1998; Ravagnan et al., 2002; Sorice et al., 2004; Waterhouse et al., 2001; Zamzami and Kroemer, 2001). In response to MMP, proapoptotic factors are released into the cytosol to trigger the execution of cell death. This is likely due to the opening of protein channels such as the VDAC. Under pathological conditions, cancer cells escape from apoptosis and/or become resistant to treatment by affecting MMP (Bettaieb et al., 2003; Debatin et al., 2002; Hersey and Zhang, 2003; Kim et al.,

2004). Therefore, overcoming abnormalities in tumor cells that suppress MMP could lead to therapeutic targets by generating a potent proapoptotic stimulus. Additionally, since MMP is an early event in apoptosis, strategies to detect this process can be useful in assessing the response to chemotherapy.

Mutations in mtDNA have been implicated in the cellular response to chemotherapy. For example, Singh et al. (1999) examined the response of a tumor cell line lacking mitochondrial DNA to several anticancer drugs, including adriamycin (a DNA-interacting drug widely used in chemotherapy for its role in binding DNA and stopping the process of replication). Cancer cells lacking mtDNA showed great chemotherapy resistance, indicating an important role of the mitochondrial genome in regulating the cellular response to therapeutic agents. Similar findings were also reported in A549 non-small-cell lung cancer cell lines and their rho0 derivatives in which mitochondrial DNA has been eradicated (Lo et al., 2005). The parental cell line showed increased sensitivity to chemotherapy when compared with the mtDNA-compromised derivative cell line. Notably, the restoration of mtDNA restored chemosensitivity of the resistant cell line (Lo et al., 2005).

14 Targeting Mitochondria in Cancer Therapy

As mitochondria are gatekeepers of apoptotic signals, targeting mitochondria to induce apoptosis of malignant cells is an important therapeutic strategy. In the past several years, extensive research has focused on screening for chemical compounds, small molecules and peptides that could target the mitochondria. Therapeutic tactics have included strategies that involve the Bcl-2 family proteins, activation of PTPs, the respiratory chain, mitochondrial DNA depletion, and selective targeting of ROS-stressed malignant cells, as well as targeting inhibitors of apoptosis such as IAPs (Dias and Bailly, 2005). Targeting the antiapoptotic members of the Bcl-2 family, namely Bcl-2 and Bcl-X_L, and targeting the PTP are among the most studied mechanisms (Dias and Bailly, 2005; O'Neill et al., 2004; Shangary and Johnson, 2003; Walensky, 2006). Targeting of the Bcl-2 family of proteins is discussed in Chapter 8. Here, we will briefly describe strategies for targeting and activation of the PTP.

15 Targeting and Activation of the Permeability Transition Pore

The induction of proapoptotic protein release through increased PTP formation and opening has been explored in the recent years as a possible mechanism for cancer treatment. As a chemotherapeutic approach, this method involves perturbation of the mitochondrial membrane through direct targeting of the components of the

membrane permeability transition pore complex (PTPC) (Brenner et al., 2003; Costantini et al., 2000; Debatin et al., 2002; Fantin and Leder, 2006; Galluzzi et al., 2006; Khosravi-Far and Esposti, 2004; Morisaki and Katano, 2003; Reed, 2004). Additionally, alterations in energy metabolism, such as depletions in ADP and ATP, can also facilitate formation of the PTPC.

In addition to therapeutic strategies that target Bcl-2 family members, several chemotherapeutic agents such as paclitaxel or etopiside have been shown to induce opening of the PTPC, albeit at high concentrations. Additionally, several experimental anticancer agents act directly on the components of the PTPC. For example, the synthetic retinoid CD437, arsenic acid and lonidamine are inhibitors of ANT (Debatin et al., 2002; Fantin and Leder, 2006; Galluzzi et al., 2006). Arsenic acid also inhibits the VDAC. Hexokinase, which is a component of the PTPC and a major player in maintaining the malignant state of transformed cells, is also inhibited by lonidamine (Debatin et al., 2002; Fantin and Leder, 2006; Galluzzi et al., 2006). Additionally, jasmonates are known to act selectively and directly on cancer cell mitochondria in a PTPC-mediated mechanism, resulting in membrane depolarization, swelling, and the release of cytochrome *c* (Rotem et al., 2005) leading to apoptosis of tumor cells. Similarly, lamellarins are another group of anticancer drugs that target mitochondria of cancer cells and induce permeability transition effects (Kluza et al., 2006).

16 Conclusions and Future Prospects

Mitochondria are the power generators of the cell due to their involvement in glucose metabolism, and they are "gatekeepers" of the cell involved in integrating apoptotic signals in majority of cells. Because tumor cells rely on glycolysis and since evasion of apoptosis is one of the hallmarks of cancer, mitochondria therefore play a central role in cancer cell biology. The intrinsic and extrinsic death pathways leading to changes in mitochondrial permeability; the components of the PTPC, including members of the Bcl-2 family; apoptogenic factors and their regulators, and mutations in mtDNA have been studied extensively in the past for their contributions to cancer progression or resistance to therapy. These constitute an extensive list of targets that could induce apoptosis, some with possible specificity for cancer cells. Therapeutic agents against many of these targets, including Bcl-2 family members and components of the PTP, are currently at various stages in the development pipeline. The ultimate goal of these studies is to generate novel mitotoxic agents that can selectively induce apoptosis of cancer cells and reduce the possibility of resistance.

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Chapter 4
Apoptotic Pathways in Tumor Progression and Therapy

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Abstract Apoptosis is a cell suicide program that plays a critical role in development and tissue homeostasis. The ability of cancer cells to evade this programmed cell death (PCD) is a major characteristic that enables their uncontrolled growth. The efficiency of chemotherapy in killing such cells depends on the successful induction of apoptosis, since defects in apoptosis signaling are a major cause of drug resistance. Over the past decades, much progress has been made in our understanding of apoptotic signaling pathways and their dysregulation in cancer progression and therapy. These advances have provided new molecular targets for proapoptotic cancer therapies that have recently been used in drug development. While most of those therapies are still at the preclinical stage, some of them have shown much promise in the clinic. Here, we review our current knowledge of apoptosis regulation in cancer progression and therapy, as well as the new molecular targeted molecules that are being developed to reinstate cancer cell death.

Keywords apoptosis, cancer, therapy, inhibitors, signal transduction, oncogene, intrinsic, extrinsic

1 Introduction

Apoptosis, is an evolutionarily conserved mechanism for the selective removal of unwanted cells (Abe et al., 2000b; Degli Esposti, 1999; Lawen, 2003; Ozoren and El-Deiry, 2003; Peter and Krammer, 1998; Strasser et al., 2000; Thorburn, 2004). Regulation of apoptosis is critical for tissue homeostasis, therefore, its deregulation can lead to a variety of pathological conditions, including cancer. For this reason,

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inhibition of apoptosis or the promotion of resistance to apoptosis contributes to carcinogenesis and chemoresistance (Burns and el-Deiry, 2003; Daniel et al., 2001; Green and Evan, 2002; Ozoren and El-Deiry, 2003; Sheikh and Huang, 2004; Thompson, 1995; Zornig et al., 2001).

Apoptosis is primarily mediated through the activation of specific proteases called caspases (cysteinyl, aspartate-specific proteases) (Algeciras-Schimnich et al., 2002; Ozoren and El-Deiry, 2003; Salvesen and Dixit, 1997; Stegh and Peter, 2001; Thorburn, 2004). Caspases, which are effectors of PCD, cleave multiple substrates, leading to biochemical and morphological changes that are characteristic of suicidal cells (Abe et al., 2000b; Bouillet et al., 2000). Cells undergoing apoptosis undergo cell membrane remodeling and blebbing; the exposure of phosphatidylserine (PS) at the external surface of the cell; cell shrinkage with cytoskeletal rearrangements; nuclear condensation; and DNA fragmentation (Ashkenazi and Dixit, 1999; Green and Evan, 2002; Lawen, 2003; Peter and Krammer, 2003; Schulze-Osthoff et al., 1998; Thorburn, 2004). These morphological changes culminate in the formation of apoptotic bodies that are normally eliminated by phagocytosis (Geske and Gerschenson, 2001; Wallach, 1997).

In this chapter, we introduce the major apoptotic machinery and discuss some recent insights into the involvement of apoptosis in cancer progression, cancer therapy, and resistance to therapy.

2 Apoptotic Machinery

In mammals, the two major signaling systems that result in the activation of caspases and the consequent induction of apoptosis are the *extrinsic* death receptor pathway and the *intrinsic* mitochondrial pathway (Fig. 4.1) (Abe et al., 2000b; Ozoren and El-Deiry, 2003; Peter and Krammer, 2003; Strasser et al., 2000; Thorburn, 2004). In the past few years, increasing evidence indicates that the death receptor and mitochondrial pathways are not isolated systems. Instead, significant cross talks and “biofeedbacks” regulate the apoptotic machinery (Abe et al., 2000b; Li and Yuan, 1999; Reed, 2000; Zornig et al., 2001).

2.1 The Death Receptor Pathway of Apoptosis

The extrinsic apoptotic pathway is activated upon the binding of cytokine ligands (i.e., FasL, tumor necrosis factor [TNF], and TNF-related apoptosis-inducing ligand [TRAIL]) to members of the TNFα receptor superfamily, which are usually called the death receptors (i.e., Fas, also called CD95/Apo-1; TNF receptors; and TRAIL receptors) (Abe et al., 2000b; Ashkenazi and Dixit, 1999; Ozoren and El-Deiry, 2003; Peter and Krammer, 2003; Schulze-Osthoff et al., 1998; Thorburn, 2004). Death receptors contain an intracellular globular interaction domain known

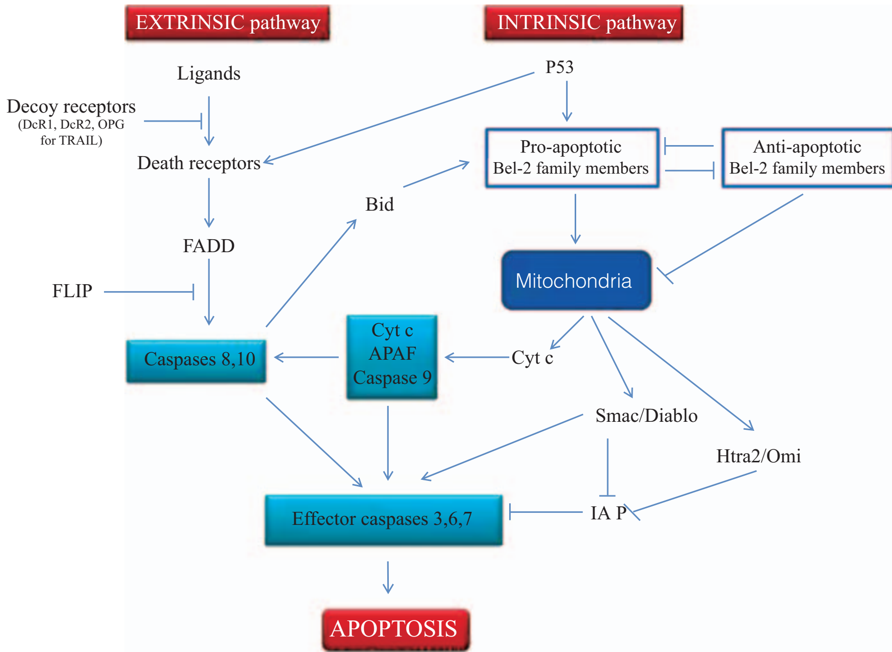

Fig. 4.1 Extrinsic and Intrinsic Apoptotic Machinery

as a death domain (DD). Upon ligand binding to their extracellular domains, death receptors aggregate at the cell surface and possibly form trimes. This results in the recruitment of adaptor molecules to the aggregated intracellular domains of the receptors. The Fas-associated death domain (FADD) is one of the major adaptors to be recruited to the death receptors. FADD possesses a DD that interacts either directly with the DD of death receptors, or indirectly through another adaptor molecule, TNF receptor-associated death domain (TRADD). FADD also contains a second protein interaction domain, known as the death effector domain (DED). This DED domain interacts with the DED of the weakly active zymogen pro-caspase-8, to form an intracellular multiprotein complex known as the death-inducing signaling complex (DISC) (Abe et al., 2000a; Ashkenazi and Dixit, 1998; Boatright et al., 2003; Cory and Adams, 2002; Wallach et al., 1999). Once formed, the DISC promotes the proximity-induced processing of caspase-8, which then proceeds to be further activated via an autoproteolysis mechanism (Salvesen and Dixit, 1999; Yang et al., 1998). Active caspase-8 subsequently activates executioner/effector caspases, such as caspase-3, leading to cell execution via degradation of the nucleus and other intracellular structures (Ashkenazi and Dixit, 1998; Cohen, 1997; Peter and Krammer, 2003; Scaffidi et al., 1998). This direct activation of caspase-dependent cell execution is thought to occur in certain cell types, including thymocytes, that

are classified as type I cells (Boatright et al., 2003; Ozoren and El-Deiry, 2002; Scaffidi et al., 1998). These cells are able to efficiently activate caspase-8 so that it can cleave and consequently activate its primary targets, the executioner caspases including caspase-3. This simplified pathway of type I cells plays an important role in the immune response that is involved in the deletion of transformed cells (Hickman, 2002; Zornig et al., 2001) and resembles the linear pathway of developmental cell death established in genetic studies of *Caenorhabditis elegans* (Horvitz, 1999; Vaux, 2002). Nonetheless, PCD in *C. elegans* is distinct in that Bcl-2/Ced-9 is unable to block caspase activation following death receptor stimulation in type I cells (Peter and Krammer, 2003; Scaffidi et al., 1999).

2.2 The Mitochondrial Pathway of Apoptosis

Mitochondria are thought to be the central organelles involved in mediating most apoptotic pathways in mammalian cells (Green and Kroemer, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Ravagnan et al., 2002; Sorice et al., 2004; Zamzami and Kroemer, 2001). Mitochondria are engaged via the intrinsic pathway of cell death, which can be initiated by a variety of stress stimuli, including ultraviolet (UV) radiation, γ-irradiation, heat, DNA damage, the actions of some oncoproteins and tumor suppressor genes, viral virulence factors, and most chemotherapeutic agents (Kroemer, 2003). These diverse forms of stress are sensed or decoded by multiple cytosolic or intraorganellar molecules which then transduce the signals to mitochondria, resulting in alterations in the permeability of the outer mitochondrial membrane (OM) (Esposti et al., 2003; Green and Kroemer, 2004; Kuwana et al., 2002; Newmeyer and Ferguson-Miller, 2003; Zamzami and Kroemer, 2001). This leads to increased permeability to apoptotic proteins that are normally trapped between the OM and the inner mitochondrial membrane (IM), thus enabling these proteins to escape the mitochondria and diffuse into the cytosol.

The release of apoptotic factors leads to apoptosome-mediated activation of caspases (Fig. 4.1). The apoptosome works like a large platform for recruiting and facilitating the self-activation of the apical caspase of the intrinsic pathway of apoptosis, pro-caspase-9 (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Chinnaiyan, 1999; Hill et al., 2003; Salvesen and Renatus, 2002; Shi, 2002). The apoptosome promotes the local accumulation of zymogens that initiate an autocatalytic activation of caspase-9 in a manner similar to the activation of caspase-8 at the DISC (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Chinnaiyan, 1999; Hill et al., 2003; Salvesen and Renatus, 2002; Shi, 2002). The apoptosome, however, requires additional regulatory factors to fully activate the caspase cascade. These factors include Smac/Diablo, a protein that interacts with several inhibitor of apoptosis proteins (IAPs) and displaces them from their inhibitory interaction with pro-caspase-9 and other caspases.(Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Shi, 2002).

The mitochondrial pathway can also be activated in response to death ligands. In type II cells, selective cleavage of Bid by caspase-8 has been found to connect upstream signals from the DISC to the mitochondria (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). Furthermore, genetic ablation of Bid reduces Fas-induced hepatotoxicity and mitochondrial damage (Zinkel et al., 2003). The caspase-cleaved form of Bid, tBid, migrates to the OM, where it cooperates with other Bcl-2 family proteins, such as Bak or Bax, to induce the release of mitochondrial proteins into the cytosol (Wei et al., 2001b).

3 Oncogene-Induced Evasion of Apoptosis: A Mechanism for Tumor Progression

It has become clear that a fundamental property of cancer cells is their ability to evade the apoptotic cellular death program (Hanahan and Weinberg, 2000). This not only promotes their unchecked growth, but also suggests a mechanism whereby they can be controlled. Investigating the mechanisms underlying this resistance of tumor cells to apoptosis remains of significant interest, since a desired goal of anticancer therapies is to selectively unleash the apoptotic potential of tumor cells.

In the normal cellular context, proliferation and death programs are tightly linked. Given this, cells harboring a single oncogenic mutation driving proliferation undergo a protective growth inhibitory response, appropriately resulting in apoptosis of the pre-neoplastic cell. In contrast, such as in the progression of tumors, oncogenes overcome these protective cellular responses by taking advantage of cooperating, additional mutations in apoptosis signaling molecules, resulting in the abnormal proliferation and survival/antiapoptosis of the tumor cell (Lowe et al., 2004). In a classic example, overexpression of the wild-type c-Myc oncoprotein can induce apoptosis and sensitize cells towards a host of apoptotic stimuli in certain cell types (Pelengaris et al., 2002). However, several events, including inactivation of p53, overexpression of Bcl-2, and loss of Bim are able to cooperate with Myc in inducing tumorigenesis (Pelengaris et al., 2002). In another strategy for tumorigenicity, fusion proteins such as Bcr-Abl can simultaneously activate multiple pathways, including those involved in cellular proliferation and in the promotion of survival and suppression of apoptosis.

3.1 Myc

C-Myc is a proto-oncogene first identified as the cellular homologue of the oncogene found in the avian myelocytomatosis retrovirus (Gonda et al., 1982). The other two *Myc* genes in mammals are *MYCN* and *L-Myc*. Myc, which is a transcription factor, can both activate and repress target genes. Recent estimates suggest that *c-Myc* could regulate as many as 15% of genes in genomes from flies to humans

(Fernandez et al., 2003; Orian et al., 2003). These target genes are involved in diverse functions including cell proliferation, differentiation, cell adhesion, metabolism, DNA repair, and apoptosis (Dang, 1999; Oster et al., 2002). Myc expression and its activity in normal cells are tightly regulated. However, Myc overexpression has been found in up to 50% of all human cancers (Alitalo and Schwab, 1986; Pompetti et al., 1996). Myc is thought to contribute to tumorigenesis through unrestrained cellular growth and proliferation and also exerts its effects on cellular processes such as cellular adhesion, angiogenesis and genomic instability (Calvisi et al., 2004; Felsher and Bishop, 1999; Ingvarsson, 1990; Knies-Bamforth et al., 2004). In addition to its established roles in promoting cellular growth and proliferation, Myc was also found to be an inducer of apoptosis (Evan and Vousden, 2001; Evan et al., 1992). It has been reported that Myc potentiates apoptosis through both p53-dependent and p53-independent mechanisms (Sakamuro et al., 1995). Even though the mechanisms by which Myc protein drives such disparate functions are still not well understood, it has been suggested that the ability of Myc to sensitize cells to apoptosis could be an intrinsic property of cells. Abrogation of this proapoptotic property profoundly contributes to cancer progression (Sakamuro et al., 1995). Some principles have also emerged from studies in cell culture and animal models to explain how Myc can promote cancer growth while acting as an inducer of apoptosis.

Modes of Myc dysregulation include chromosomal translocation and amplification, activation of upstream growth stimulatory signaling cascades, and increased protein stability (Oster et al., 2002). One of the important cellular processes caused by Myc dysregulation is genomic instability, which is prone to additional genomic mutations. Thus, activation of other oncogenes may follow in response to Myc deregulation. The antiapoptotic functions of some oncogenes can overcome the proapoptotic function of Myc. For example, in a conditional transgenic model of Myc-induced breast adenocarcinomas (Arvanitis and Felsher, 2006; Boxer et al., 2004; D'Cruz et al., 2001; Hutchinson and Muller, 2000), Myc inactivation results in tumor regression in about 50% of the tumors. Many of the tumors that initially regress subsequently relapse. Half of the tumors that do not regress and those that later relapse have active mutations in K-Ras or H-Ras. In those mice, mutant Ras appears to facilitate the ability of tumors to become independent of Myc.

Myc cooperation with other oncogenes is another important mechanism by which Myc promotes tumorigenesis. In mice, when the *C-Myc* transgene is coupled to the immunoglobulin heavy chain μ-enhancer, it leads to B-cell-specific overexpression of the *C-Myc* gene and development of lymphomas (Adams et al., 1985; Harris et al., 1988). This Eμ-Myc mouse is a model for the human disease Burkitt's lymphoma, where a reciprocal chromosomal translocation to the immunoglobulin locus leads to inappropriate expression of Myc in the B-cell compartment. The lymphomas that develop in the mouse model are consistently clonal, indicating that additional mutations are necessary to produce tumors. However, mice doubly transgenic for Eμ-Myc and Eμ-BCL2 mutations display a marked decrease in latency of disease, developing a leukemia of early progenitor cells (Strasser et al., 1990), rather than the lymphoma that develops with Eμ-Myc alone (Harris et al., 1988).

Studies in both cell culture and transgenic mice have shown that enforced Bcl2 expression was capable of blocking Myc-induced apoptosis and left the proliferation functions of Myc intact (Bissonnette et al., 1992; Fanidi et al., 1992; Letai et al., 2004).

Notably, the specific consequences of Myc inactivation appear to depend both on the type of cancer cells and the constellation of genetic events unique to a given tumor. Studies in conditional transgenic mouse model systems have shown that Myc inactivation results in the proliferation arrest, differentiation and/or apoptosis of tumor cells (Arvanitis and Felsher, 2006). Additionally, recent reports have suggested that targeted inactivation of Myc is a potential approach to cancer therapy, if used in conjunction with other anticancer treatments (Arvanitis and Felsher, 2006). To date, however, no drugs that target Myc have been identified for the treatment of humans with cancer (Arvanitis and Felsher, 2006).

3.2 Signaling Pathways Activated by Bcr-Abl and the Suppression of Apoptosis

The Bcr-Abl fusion protein activates multiple signaling pathways that lead to proliferation, reduced dependence on growth factors, apoptosis, and abnormal interactions with the extracellular matrix and stroma. Recent research suggests that one key mechanism by which Bcr-Abl facilitates the expansion of myeloid cells involves the suppression of apoptosis. Notably, the primary consequence of tyrosine kinase inhibition with imatinib in Bcr-Abl-transformed cells is the induction of apoptosis (Druker et al., 1996; Gambacorti-Passerini et al., 1997). Additionally, in growth factor-dependent hematopoietic cells, Bcr-Abl induces the survival and proliferation of cells that would otherwise undergo apoptotic cell death in response to growth factor withdrawal (Bedi et al., 1994). Furthermore, antisense oligonucleotide-mediated inhibition of Bcr-Abl expression in these transformed cells results in apoptosis without altering their cell cycle (Bedi et al., 1994). It has also been demonstrated that Bcr-Abl-positive cells are highly resistant to various apoptotic stimuli and become sensitized to drug treatment upon antisense inhibition of Bcr-Abl (McGahon et al., 1994). Additional evidence for the antiapoptotic effects of Bcr-Abl comes from experiments with temperature-sensitive Bcr-Abl kinase mutants, in which induction of Bcr-Abl kinase activity at the permissive temperature led to a significant decrease in apoptosis in the absence of growth factors (Carlesso et al., 1994; Kabarowski et al., 1994). In fact, studies in primary cells have revealed that chronic myelogenous leukemia (CML) progenitors show a normal proliferative response to growth factors and do not have a greater proliferative potential than normal progenitors (Emanuel et al., 1991). Furthermore, in the absence of serum and growth factors, neither normal nor CML progenitors proliferated, yet the latter maintained higher cell viability (Bedi et al., 1994).

As a result of its elevated tyrosine kinase activity, the Bcr-Abl fusion protein activates several signaling pathways, including Ras (Sawyers et al., 1995), PI3-K/Akt (Skorski et al., 1997; Varticovski et al., 1991), Stat (Carlesso et al., 1996; Ilaria and

Van Etten, 1996; Shuai et al., 1996), and NF-κB (Reuther et al., 1998) some of which may be crucial for its leukemogenic activity. In accordance with the ability of Bcr-Abl to substitute for the requirement of cytokines, many of these pathways are also activated by hematopoietic cytokines upon binding to their respective cytokine receptors. A functional consequence of the activation of these pathways involves changes in the activity and gene expression of key molecules, which have a direct impact on cellular survival, growth, and behavior. In particular, the Ras (Sawyers et al., 1995), PI3-K/Akt (Varticovski et al., 1991), Stat (Nieborowska-Skorska et al., 1999; Sillaber et al., 2000), NF-κB (Reuther et al., 1998), and FOXO (Ghaffari et al., 2003) pathways are capable of transmitting antiapoptotic signals, which could promote the evasion of Bcr-Abl-transformed cells from apoptosis. Thus, determining which of these antiapoptotic signals plays a role in Bcr-Abl-mediated evasion of apoptosis and promotion of leukemogenesis is of interest, especially since the cross talk between, and potential cooperation among, these pathways may be important in mediating the leukemogenic effects of Bcr-Abl.

4 Chemotherapeutic Drugs and Conventional Radiation-Induced Apoptosis in Tumor Cells

Aberrant cell proliferation, a major hallmark of cancer, has been exploited for anti-cancer drug development. Most existing chemotherapeutic drugs interfere with DNA synthesis and cell division, thereby preferentially killing rapidly dividing cells such as cancer cells (Schulze-Bergkamen and Krammer, 2004). These drugs include such diverse groups as antimetabolites, genotoxic/DNA-damaging agents (alkylating and intercalating agents, topoisomerase inhibitors) and mitotic inhibitors (vinca alkaloids and taxanes) (Luqmani, 2005). It is now well established that these cyto-toxic agents exert their antitumor activity mainly through induction of apoptosis and that defects in apoptotic pathways can lead to treatment failure (Johnstone et al., 2002; Kaufmann and Earnshaw, 2000; Lowe and Lin, 2000; Mesner et al., 1997).

Apoptosis induced by chemotherapeutic drugs primarily involves the mito-chondrial apoptotic pathway and, in some cases, the death receptor pathway and upregulation of death receptors and/or ligands (Bucur et al., 2006; Pommier et al., 2004). The relative contribution of each pathway to drug-induced apoptosis may depend on the cytotoxic drug, dose, kinetics, and cell type ((Fulda et al., 2001b), reviewed in (Debatin and Krammer, 2004)).

4.1 DNA-Damaging Agents and Induction of Apoptosis

Chemotherapeutic drugs damage DNA either directly or indirectly (antimetabolites) and subsequently initiate a DNA-damage response through both p53-dependent and p53-independent mechanisms (Waxman and Schwartz, 2003). Irradiation mainly induces direct DNA damage, but can also act indirectly, one example being

the modulation of the epigenetic effectors in distant bystander tissue in vivo. X-ray exposure to one part of the animal body induces DNA strand breaks and causes an increase in levels of Rad51 in unexposed bystander tissue (Koturbash et al., 2006).

Drug- and radiation-induced DNA damage is first sensed by DNA-binding factors such as Rad17-RFC and the Rad9-1-1 supercomplex, BRCA1 and the Ku subunit of DNA-PK. The DNA damage signal is then transduced by activation of the PI3K family members DNA-PK, ATM, and ATR which, in turn, phosphorylate effector kinases such as the Ser/Thr kinases Chk1 and Chk2 and the tyrosine kinase c-Abl. These activated kinases then phosphorylate their downstream targets including the transcription factors p53, p73, and E2F, resulting in the transactivation of numerous genes involved in DNA repair, cell cycle arrest, and apoptosis.

The tumor suppressor p53 plays a key role in cellular response to stress and DNA damage (Meek, 2004) and has been implicated frequently in drug-induced apoptosis (Blagosklonny, 2002). Following DNA damage, p53 is induced by phosphorylation via ATM and Chk2. Phosphorylation of p53 not only enhances its DNA binding and transcriptional activity, but also stabilizes the protein by inhibiting its MDM2-mediated ubiquitination and its subsequent proteasomal degradation. The resulting increase in protein stability ultimately enhances the transcription of p53 target genes. p53 was shown to activate the mitochondrial apoptotic pathway by upregulating proapoptotic genes such as Bax, Bid, Noxa, and Puma, and down-regulating antiapoptotic proteins such as Bcl-2 and Mcl-1 (Michalak et al., 2005; Schuler and Green, 2005; Yu and Zhang, 2005). In addition, p53 can activate the extrinsic pathway by upregulating death receptors such as Fas, DR4, and DR5, although this pathway alone seems insufficient to induce apoptosis in some cancer cells (Reinke and Lozano, 1997). Recent evidence also suggests that p53 exerts proapoptotic functions independent of transcription, by translocating to the mitochondrion (Erster and Moll, 2005; Marchenko et al., 2000) and binding to Bcl-2 (Mihara et al., 2003; Tomita et al., 2006) and Bcl-XL (Mihara et al., 2003; Xu et al., 2006).

DNA-damaging agents can also induce apoptosis through p53-independent pathways involving, for instance, the transcription factor E2F (Lin et al., 2001). E2F exerts important proapoptotic activity in p53-deficient cells through transactivation of proapoptotic genes such as *Apaf-1* (Furukawa et al., 2002; Moroni et al., 2001), the caspase proenzymes (Nahle et al., 2002), *p73* (Irwin et al., 2003; Seelan et al., 2002; Stiewe and Putzer, 2000; Wang and Ki, 2001), and through repression of *Mcl-1* (Croxton et al., 2002). In certain cell types, radiation treatment, when used alone, may activate death receptors to execute the apoptotic program (Gong and Almasan, 2000). Finally, DNA-damaging drugs can engage a stress response via the stress-activated protein kinase/JNK pathway to activate the AP-1 and NF-κB-dependent transcription of *FasL* (Herr and Debatin, 2001; Kasibhatla et al., 1998).

4.2 Targeting the Apoptotic Machinery Directly

Targeting Bcl-2 family of proteins, death receptors, IAPs, caspases, and p53 are discussed in Chapter 8.

4.3 Microtubule Inhibitors and Induction of Apoptosis

Like DNA-damaging agents, microtubule inhibitors also lead to the phosphorylation and stabilization of p53 as a mechanism for drug-induced apoptosis (Blagosklonny, 2002; Wang et al., 1999). However, in MCF-7 breast cancer cells, inactivation of p53 does not affect cellular sensitivity to paclitaxel killing. In those cells, p53 may act as a survival factor by blocking them in the G2/M phase, rather than serving as an apoptotic inducer. By contrast, the transcription factor FOXO3a has been shown to upregulate the proapoptotic Bcl-2 family member, Bim, and contribute to paclitaxel-induced cell death in MCF7 cells (Sunters et al., 2003). Similarly, another FOXO family member, FOXO1, has been implicated in drug-induced apoptosis through the transcriptional activation of the TNF-R1-associated protein TRADD (Rokudai et al., 2002).

4.4 Anticancer Therapeutics and Other Forms of Cell Death

In addition to classical apoptosis, anticancer drugs sometimes trigger autophagic and necrotic modes of cell death (Gozuacik and Kimchi, 2004; Kim et al., 2006; Kondo et al., 2005; Nelson and White, 2004). For instance, tamoxifen induces autophagic cell death in MCF-7 cells (Bursch et al., 1996). Similarly, the alkylating agent temozolomide kills malignant glioma cells through autophagy rather than apoptosis (Kanzawa et al., 2004). Some reports also indicate that paclitaxel and vinblastine induce both autophagic and apoptotic cell death (Broker et al., 2004; Hirsimaki and Hirsimaki, 1984). Necrotic cell death (Proskuryakov et al., 2003) has also been observed in vitro in resistant human ovarian carcinoma cells exposed to HPMA copolymer-bound doxorubicin (Demoy et al., 2000) and in vivo in p53/Bcl-2-deficient mice treated with DNA-alkylating agents (Zong et al., 2004). These alternative modes of cell death have only recently been identified and their respective importance and possible cross talk in drug cytotoxic action remain to be further defined. These forms of cell death together with mitotic catastrophe are further discussed in Chapter 3.

5 Mechanisms of Radiation and Drug Resistance

Drug resistance can be classified as nononcogenic (impaired drug-target interaction) and oncogenic (deregulation of apoptosis and the cell cycle). Principal mechanisms of nononcogenic resistance include increased drug membrane export involving the PgP protein product of the MDR gene; decreased drug activation; increased drug degradation; enhanced DNA repair; and mutations of drug targets (Longley and Johnston, 2005; Luqmani, 2005). In oncogenic resistance, the drug interacts with its target, but downstream pathways of apoptosis and the cell cycle are altered

(Longley and Johnston, 2005; Luqmani, 2005). Intrinsic or acquired oncogenic resistance can result from multiple mechanisms, as outlined below.

5.1 Prosurvival Signaling (Mitogenic Kinases and NF-κB)

Mitogenic protein tyrosine kinases play a major role in drug resistance through their regulation of antiapoptotic signaling pathways (Blume-Jensen and Hunter, 2001). These include, for instance, members of the EGFR and Ras families, Bcr-Abl, and Akt. Overexpression of EGFR and Her-2 has been reported to increase resistance to chemotherapeutic drugs (Chevallier et al., 2004; Knuefermann et al., 2003; Mendelsohn and Fan, 1997; Nagane et al., 1998; Pegram et al., 1997). Activated Ras family members have also been shown to decrease cells' sensitivity to cytotoxic agents (Fan et al., 1997; Jansen et al., 1997). For example, several reports suggest that expression of Ras oncoproteins can contribute to cisplatin resistance by reducing drug uptake and increasing the degree of DNA repair (Dempke et al., 2000; Levy et al., 1994). Similarly, Bcr-Abl-expressing hematopoietic cell lines and various patient-derived CML cell lines are highly resistant to apoptotic induction by chemotherapy (Aichberger et al., 2005; Bedi et al., 1994; Cortez et al., 1996; Gesbert and Griffin, 2000; Keeshan et al., 2001; McGahon et al., 1994; Ray et al., 2004; Skorski, 2002; Underhill-Day et al., 2006).

The PI3K/Akt pathway, at the crossroads of multiple signaling networks, has been shown to be overactivated by upstream mitogenic kinases and oncogenic mutations in a wide range of tumors (Osaki et al., 2004). A number of studies have established that overexpression or activation of Akt increases chemoresistance both in cell lines (Page et al., 2000; Pommier et al., 2004) and in vivo (Kim et al., 2005; Martelli et al., 2005; McCormick, 2004; Wendel et al., 2004). Accordingly, inhibition of the PI3K/Akt pathway enhances the cytotoxic effects of a variety of chemotherapeutic agents (Hennessy et al., 2005; Nguyen et al., 2004; Nicholson et al., 2003; O'Gorman et al., 2000; Toretsky et al., 1999). The PTEN tumor suppressor is frequently mutated in human tumors. Loss of PTEN is associated with constitutive survival signaling through the PI3K/Akt pathway. Adenovirus-mediated expression of PTEN completely suppressed Akt activation in various cancer cell lines, such as the LNCaP prostate cancer cell line, and enhanced apoptosis induced by a broad range of apoptotic stimuli, including the chemotherapeutic agents mitoxantrone and etoposide, and death receptor-mediated treatments such as TRAIL, TNF-α, and agonistic antibodies against Fas (Yuan and Whang, 2002).

Finally, tumors with constitutive NF-κB activity are highly resistant to cytotoxic drugs (Arlt and Schafer, 2002; Baldwin, 2001). Accordingly, inhibition of NF-κB dramatically increases the sensitivity of these tumors to drugs by downregulation of antiapoptotic proteins (Nakanishi and Toi, 2005). Moreover, treatment with diverse cytotoxic drugs (including 5-FU, doxorubicin, paclitaxel, and cisplatin) can activate NF-κB, thereby blunting the ability of chemotherapy to induce cell death (Chuang et al., 2002).

5.2 Loss of p53 Function

Loss of p53 function is frequently encountered in human tumors and plays a critical role in resistance to chemotherapeutic drugs and conventional radiation (Levine et al., 2004; Lowe et al., 1994; Weller, 1998). Mechanisms responsible for p53 dysfunction include mutations or allelic loss in the p53 gene; upregulation of p53 inhibitors such as Mdm2; silencing of key p53 coactivators such as ARF; and altered upstream or downstream signaling (Vogelstein et al., 2000). For instance, lymphomas from mice deficient in p53 are markedly resistant to chemotherapy both in vitro and in vivo (Schmitt et al., 1999). p53 expression is predictive for response to chemotherapy in non-small-cell lung cancers (NSCLC) (Harada et al., 2003). Moreover, p53 mutations have been correlated with resistance to doxorubicin treatment and early relapse in patients with breast carcinomas (Aas et al., 1996). Cancers that retain wild-type p53 are more likely to respond to chemotherapy than other tumor types. However, many types of wt p53 tumors with defective apoptotic machinery do not undergo apoptosis despite genotoxic stress (Blagosklonny, 2001; Gudas et al., 1996).

5.3 Defective Apoptotic Machinery

5.3.1 Defective Mitochondrial Activation

The Bcl-2 protein family plays a pivotal role in the regulation of the mitochondrial apoptotic pathway and, consequently, its members serve as major regulators of tumor sensitivity to drugs (Kirkin et al., 2004; Kostanova-Poliakova and Sabova, 2005; Pommier et al., 2004). Overexpression of antiapoptotic Bcl-2 members such as Bcl-2, Bcl-XL, and Mcl-1, or deficiency of the proapoptotic members Bak and Bax, has been associated with drug resistance in cell lines, mouse models, and patients (Kirkin et al., 2004; Kostanova-Poliakova and Sabova, 2005; Pommier et al., 2004). Indeed, overexpression of Bcl-2 (Dole et al., 1994; Kamesaki et al., 1993; Miyashita and Reed, 1992; Walton et al., 1993) or Bcl-XL (Amundson et al., 2000) prevents apoptosis induced by most chemotherapeutic drugs in vitro. Some evidence also indicates similar effects with Mcl-1 overexpression (Song et al., 2005; Zhou et al., 1997). In concordance with overexpression data, downregulation of Bcl-XL or Bcl-2 has been shown to sensitize cancer cells to DNA damage-induced apoptosis. Fibroblasts deficient in both Bak and Bax are resistant to apoptosis induced by various agents (Wei et al., 2001a). While Bak deficiency renders Jurkat cells resistant to staurosporin, bleomycin, and cisplatin (Wang et al., 2001), loss of Bax expression is associated with acquired resistance to oxaliplatin (Gourdier et al., 2002) or resistance to 5-FU (Zhang et al., 2000) in colon carcinoma cell lines. Clinically, high expression of antiapoptotic Bcl-2 family members (Reed, 1996) and loss or inactivation of Bax (Ionov et al., 2000; Tai et al., 1998) has been

correlated with poor response to chemotherapy in some types of malignancy, but not all kinds of tumors.

5.3.2 Impaired Activation of the Death Receptor Pathway

Alterations in activation of the death receptor pathway are also implicated in chemoresistance. For instance, downregulation of Fas/CD95 in lymphoid and solid tumors is often associated with resistance to drug-induced cell death (Debatin and Krammer, 2004; Friesen et al., 1997; Fulda et al., 1998a; Fulda et al., 1998b). Direct downstream signaling molecules such as FADD and c-FLIP are also involved. Of note, absence or low expression levels of FADD in acute myeloid leukemia cells predicts resistance to chemotherapy and poor outcome (Tourneur et al., 2004). c-FLIP silencing dramatically sensitizes colorectal cancer cells to the chemotherapeutic agents 5-fluorouracil, oxaliplatin, and irinotecan (Longley et al., 2006). In addition, decoy receptors seem to be also implicated in resistance of cancer cells to different treatments, like Apo2L/TRAIL. This ligand has five receptors, two of which have cytoplasmic DDs (DR4 and DR5) and three of which act as "decoys" (DcR1, DcR2, and osteoprotegerin [OPG]). DcR1 and OPG lack a cytosolic region and DcR2 has a truncated, nonfunctional cytoplasmic DD (Almasan and Ashkenazi, 2003).

5.3.3 Deregulation of Caspase Activation

Both the death receptor and mitochondrial pathways lead to the activation of caspases, the final effectors of apoptotic cell death. Deregulation in the expression of caspases or their regulators (Apaf-1 and IAPs) has been observed in tumors. Although caspase mutations occur at low frequency, caspase expression and function appears to be impaired frequently by epigenetic mechanisms in cancer cells (Teitz et al., 2000). Caspase-8 expression was found to be inactivated by hypermethylation in varied resistant tumors, including childhood neuroblastoma, Ewing and malignant brain tumors and melanoma (Teitz et al., 2000). Importantly, restoration of caspase-8 expression by gene transfer or by demethylation treatment sensitizes resistant tumor cells to drug-induced apoptosis (Fulda et al., 2001a; Teitz et al., 2001). Downregulation of caspase-3 has been proposed as a possible mechanism for breast cancer chemoresistance. Doxorubicin-induced apoptosis was restored by reconstitution of caspase-3 expression in caspase-3-deficient MCF-7 breast cancer cells (Devarajan et al., 2002).

Inhibition of caspase activity by members of the IAP family is also involved in chemotherapy resistance in some tumors. The X-linked inhibitor of apoptosis protein (XIAP) is a factor in chemoresistance of human androgen-insensitive DU145 prostate cancer cells, as its inhibition induces apoptosis and enhances sensitivity to chemotherapy (Amantana et al., 2004). Overexpression of many IAPs has been reported in multidrug-resistant HL-60 leukemia cells (Notarbartolo et al.,

2002). Survivin expression has been shown to inhibit paclitaxel-induced apoptosis in HeLa cells (Giodini et al., 2002) and to correlate with paclitaxel resistance in ovarian cancer (Zaffaroni et al., 2002). As a final example, loss of *Apaf-1* has been associated with chemoresistance in melanoma cells (Soengas et al., 2001).

6 Strategies to Overcome Chemotherapeutic Resistance

Conventional drugs target cancer cells preferentially, but not exclusively. As a result, they also kill high-proliferating normal cells from bone marrow and the gut, causing unwanted side effects. Moreover, the efficacy of therapy is limited by innate or acquired resistance to such agents. New targeted cancer therapies, though, aim at using drugs that interfere with specific defects of cancer cells to improve selectivity. Current therapies include the use of monoclonal antibodies, small molecules, RNAi, and adenovirus-based gene therapy. These methods are currently being developed and studied for use alone or in combination with conventional drugs to overcome resistance (Fesik, 2005). The major proapoptotic targeted therapies are outlined below.

6.1 Inhibition of Mitotic Kinases (RTK, Ras, Akt, and mTOR)

Targeting the mitotic kinases that are involved in the survival of cancer cells has become a potential strategy for the induction of apoptosis either as a single treatment or in combination with traditional therapies. Two major approaches are being considered for targeting these kinases: small-molecule inhibitors and blocking monoclonal antibodies.

Tyrosine kinase inhibitors have been designed to compete with and prevent the binding of ATP to the tyrosine kinase domain. One of the greatest advances in molecular targeted therapy in cancer involves the treatment of CML with a small-molecule inhibitor of the Bcr-Abl oncogenic kinase called imatinib-mesylate (Gleevec) (Deininger et al., 2005; Druker et al., 1996). Imatinib leads to unprecedented responses in the chronic phase, with 80% of newly diagnosed patients achieving complete hematological remission. Imatinib has been shown to eradicate Bcr-Abl-positive leukemia cells through the induction of apoptotic (le Coutre et al., 1999) or nonapoptotic caspase-independent cell death (Okada et al., 2004). Since imatinib also inhibits other kinases such as c-Kit and PDGFR, its application has been broadened to other types of cancer such as c-Kit-positive gastrointestinal stromal tumors (GIST) (Dagher et al., 2002). Other successful examples of targeted small molecules include two EGFR/HER1 tyrosine kinase inhibitors, gefitinib (Iressa), and erlotinib (Tarceva), both of which have recently been approved by the Food and Drug Administration (FDA) for the treatment of NSCLC (De Marinis et al., 2006).

Humanized monoclonal antibodies targeting the EGFR superfamily have also been developed to bind the extracellular domain of these receptors competitively and thus prevent tyrosine kinase activation. Trastuzumab (herceptin), a monoclonal antibody against the extracellular domain of Her-2, is a prime example (Emens, 2005). The drug, which is approved for the treatment of breast cancers overexpressing Her-2, is best used in combination with paclitaxel for first-line therapy, but may also be used as a single agent as second- and third-line therapy.

As Ras mutations have been found in a great majority of carcinomas, targeting of Ras or downstream effector pathways of Ras has been of great interest (Khosravi-Far et al., 1998; Khosravi-Far and Der, 1994; Wennerberg et al., 2005). Farnesyl transferase inhibitors have potently inhibited Ras in preclinical studies, but have exhibited rather disappointing results in clinics so far (Appels et al., 2005). Chemical inhibitors of the PI3K/Akt pathway have a potential use as suppressors of tumor growth and inducers of apoptosis (Hennessy et al., 2005). Although inhibition of the PI3K family members has been shown to inhibit growth of both cancer cells in vitro and tumors in animal models, these compounds so far lack selectivity. By contrast, rapamycin and its analogues, which inhibit the Akt downstream substrate mTOR, slow the growth of tumors in animal models without displaying significant toxicity (Morgensztern and McLeod, 2005) (Dudkin et al., 2001; Eng et al., 1984). These compounds are currently in clinical trials for the treatment of breast, colon, and lung cancers.

6.2 *Targeting of Transcription Factors*

Several transcription factors, including p53, members of the FOXO superfamily, and NF-κB, are involved in drug-induced cellular response and have therefore emerged as attractive targets for new apoptosis-inducing therapies (Kim et al., 2003). Restoring p53 activity in tumor cells has a therapeutic potential because p53 loss or dysfunction in many tumors is a major cause of drug resistance. Different approaches to restore p53 function include gene transfer of wt p53, chemical restoration of wt p53 activity, and inhibition of Mdm2–p53 interaction (Blagosklonny, 2002). Many clinical trials employing wt p53 gene transfer are ongoing in different types of p53-deficient cancers. p53 activity can also be restored by small molecules that modify mutant p53 back to wt (Bykov et al., 2003; Foster et al., 1999). CP-31398, a styrylquinazoline, restores a wt DNA-binding conformation to mutant p53 and is capable of suppressing tumor growth in vitro and in vivo (Luu et al., 2002). Blocking the interaction of Mdm2–p53 in order to inhibit p53 degradation has also been considered as a valuable strategy for cancer therapy. A small molecule inhibiting the p53 pocket of Mdm2 (IC50 = 100 nM) was recently discovered within a series of *cis*-imidazoline analogues called the nutlins (Vassilev et al., 2004). Dose-dependent antiproliferative and cytotoxic activities of nutlins were shown to be dependent on the p53 status of tumor cell lines. Nutlins inhibited the growth of tumors in xenograft models without causing

significant toxicities (Vassilev et al., 2004). These results emphasize that small molecule inhibitors of Mdm2 could be valuable anticancer agents, especially for tumors retaining wt p53 but overexpressing Mdm2.

Recently, Hu et al. (2004) demonstrated that FOXO3a is inactivated by IKKβ in two thirds of breast cancer patients studied and that the presence of active FOXO3a correlates with improved patient survival. Additionally, FOXO3a has also been shown to be involved in paclitaxel-induced apoptosis in MCF-7 breast cancer cells. Notably, the FOXO family of transcription factors have been shown to regulate expression of proapoptotic genes such as Fas (Suhara et al., 2002), TRAIL (Ghaffari et al., 2003; Modur et al., 2002), and Bim (Gilley et al., 2003; Stahl et al., 2002). Taken together, these studies suggest that downregulation of FOXO transcription factors may be a key mechanism in tumorigenesis. As a proof of concept, chemical library screening identified a series of compounds that could target FOXO1 to the nucleus and that restored the induction of apoptosis in PTEN-null cells (Kau et al., 2003; Wang and El-Deiry, 2004).

Finally, an NF-κB inductive response to cytotoxic drugs can be targeted through its physiological inhibitor, IκB. Indeed, adenovirus-based inhibition of NF-κB elicited by gene delivery of an IκB superrepressor abrogates chemoresistance in some types of tumors such as androgen-independent prostate cancer cells and in glioma-derived cell lines (Orlowski and Baldwin, 2002). Targeting the IKK kinases that phosphorylate and promote the proteasomal degradation of IκB could be another approach, which is all the more appealing since FOXO3a is also regulated by IKKs in breast cancer cells. Moreover, conditional knockout of IKKβ in intestinal epithelial cells impedes irradiation-induced NF-κB activation and promotes the activation of p53 and apoptosis in those cells (Egan et al., 2004). Thus, as suggested by Finnberg and El-Deiry (2004), direct activation of FOXO3a, inhibition of NF-κB, and indirect activation of p53 by targeting IKKs could be an effective multifaceted anticancer therapy to inhibit cellular proliferation and promote cell death by multiple signaling pathways.

6.3 Direct Targeting of the Apoptotic Machinery

6.3.1 Activators of the Intrinsic/Mitochondrial Pathway

The mitochondria, as a major cell death checkpoint, constitute a prominent target for new anticancer therapies. The mitochondrial pathway can be selectively targeted by gene delivery of proapoptotic proteins such as Apaf-1 (Perkins et al., 2000) or Bax (Kagawa et al., 2000; Kaliberov et al., 2002). Alternatively, overexpressed antiapoptotic proteins such as Bcl-2, Bcl-XL, and XIAP can be downregulated. An antisense oligo against BCl-2, oblimersen, sensitizes patient-derived malignant melanoma cells to apoptosis induced by dacarbazine (Jansen et al., 2000) and has been recently approved by FDA for use in combination with this drug in advanced melanoma (Kim et al., 2004; Klasa et al., 2002). Phase II/III clinical trials are being

carried out to assess the benefits of oblimersen in combination with conventional drugs in Acute myelogenous leukemia (AML) and Non-small Cell Lung Cancer (NSCLC). Varied designer ligands (peptidomimetics or organic small molecules) that bind to the BH3-binding pocket of BCL-2 and Bcl-XL have been shown to induce apoptosis in vitro (Yin et al., 2005; Degterev et al., 2001; Enyedy et al., 2001; Kutzki et al., 2002; Tzung et al., 2001; Walensky et al., 2004; Wang et al., 2000). To date, a stapled BH3 peptide has been reported to inhibit the growth of leukemia xenografts (Walensky et al., 2004) and a small-molecule inhibitor of the Bcl-2 family members, ABT-737 (Abbot Laboratories), has been shown to induce regression of solid tumors in vivo (Oltersdorf et al., 2005). Moreover, a dual Bcl-2/BclXL antagonist (GX15-070, GeminX Biotechnology) entered clinical trials last year.

Inhibition of XIAP and other IAPs is another mechanism considered to induce apoptosis in cancer cells (Huang et al., 2004; Schimmer et al., 2006). Knockdown of XIAP by antisense oligos or RNAi induces apoptosis in cancer cells (Adams, 2003; Lima et al., 2004; McManus et al., 2004). Peptidic and nonpeptidic inhibitors of XIAPs have also been reported (Huang et al., 2004; Schimmer et al., 2006). In particular, cell-permeable Smac peptidomimetics that inhibit IAPs potently induce caspase activation and apoptosis in cancer cells and inhibit tumor growth in xenograft mouse models (Fulda et al., 2002).

Finally, drugs which act directly on mitochondrial components are also being developed to enforce cell death in tumor cells in which upstream apoptotic pathways are disabled (reviewed in Dias and Bailly, 2005; Bouchier-Hayes et al., 2005; Costantini et al., 2000; Debatin et al., 2002). Betulinic acid, a natural pentacyclic triterpenoid which acts via the permeabilization transition pore, has been shown to exert antitumor effects against neuroblastodermal and malignant head and neck tumors irrespective of their p53 status (Fulda and Debatin, 2000; Pisha et al., 1995).

6.3.2 Activators of the Extrinsic/Death Receptors Pathway

There is significant interest in targeting the extrinsic pathway to circumvent drug resistance, since chemorefractory cells tend to have dysfunctional p53 and defects in their intrinsic pathway. Death receptor ligands such as Fas, TNF, and TRAIL can be strong inducers of apoptosis in tumor cells in vitro. Among these ligands, TRAIL emerges as the most promising antitumor agent due to its lack of toxicity (Abe et al., 2000b; Yagita et al., 2004). Unlike Fas and TNF, recombinant TRAIL induces tumor regression in preclinical models with little toxicity to normal tissues (Ashkenazi et al., 1999; Walczak et al., 1999) and is currently in phase I clinical trials for the treatment of solid tumors. Agonistic antibodies against DR4 and DR5 also induce apoptosis in cancer cells, but not in normal cells and slow the growth of tumors in xenograft tumor models with no apparent systemic toxicity (Yagita et al., 2004). Phases I and II clinical trials have been initiated for an agonistic antibody targeting DR4 and phase II clinical trials are ongoing for an antibody that targets DR5.

6.4 General Inhibitors (Proteasome, Hsp90, and HDAC)

Targeting more general cellular components such as the 26S proteasome (Adams, 2004), the molecular chaperone protein Hsp90 (Whitesell and Lindquist, 2005), and histone deacetylases (HDAC) (Minucci and Pelicci, 2006; Yoo and Jones, 2006) has led to some surprising success in specific anticancer therapy. These therapies, while not intended to induce apoptosis, preferentially kill cancer cells by exploiting their greater dependence on the targeted cellular processes than their normal counterparts.

The proteasome inhibitor Velcade has been approved for treatment of multiple myeloma and is under evaluation as a single agent or in combination chemotherapy for the treatment of other hematopoietic and solid cancers (Adams and Kauffman, 2004). Preclinical studies demonstrate that proteasome inhibition by Velcade potentiates the activity of other cancer therapeutics, in part by downregulating chemoresistance pathways such as NF-κB and by inducing proapoptotic proteins such as p53 or FOXO3a (Fujita et al., 2005; Ghaffari et al., 2003).

Hsp90 is a molecular chaperone protein required for the stability and function of multiple mutated chimeric and overexpressed signaling proteins. Hsp90 inhibitors have shown promising antitumor activity in preclinical model systems (Banerji et al., 2005) and a 17-AAG compound has reached phase II clinical trials (Heath et al., 2005).

HDAC inhibitors are novel anticancer agents in clinical development that target the family of HDAC enzymes responsible for deacetylating core nucleosomal histones and other proteins. The precise mechanisms resulting in the antiproliferative biological effects of these agents are not fully understood. Nevertheless, a phase I clinical trial of suberoylanilide hydroxamic acid (SAHA) has shown that it is well tolerated, and has antitumor activity in both solid and hematological tumors (Kelly et al., 2005).

6.5 Combined Treatment as a Strategy to Overcome Resistance to Conventional Radiation and Chemotherapeutic Drugs

Most chemotherapeutic agents utilize the apoptotic pathway to induce cancer cell death, as does radiation. To overcome resistance to apoptosis, combination therapies involving two or more treatments can be used. The efficiency is usually highest when these treatments act on different signaling pathways. Targeting apoptosis through both the intrinsic and extrinsic pathways has been shown to be a good strategy. For example, joint activation of the intrinsic pathway (via the Bcl-2 family of proteins) and the extrinsic pathway (using different ligands like Apo2L/TRAIL) has a synergistic effect in prostate cancer cell lines (Almasan and Ashkenazi, 2003). Also, resistance to death receptor-induced cell death (as in resistance to Apo2L/TRAIL treatment) can be overcome by using a variety of therapeutic strategies,

like the activation of the intrinsic pathway, inhibition of survival factors, metabolic inhibition (blocking protein synthesis), proteasome inhibition (bortezomib), and others (Bucur et al., 2006).

6.5.1 Conclusions and Future Directions

Apoptosis and its deregulation in cancer has been an intensive field of research over the past decades. A deeper understanding of the mechanisms involved in evasion of cancer cells from apoptosis, and its link to drug resistance, has enabled the recent development of molecular targeted proapoptotic therapies. These therapies have produced significant results in cancer treatment and, in the case of Gleevec in CML, have even exceeded expectations. However, as for conventional drugs, resistance has subsequently emerged, even to single targeted agents. To avoid resistance, combination therapies involving both an apoptosis inducer and a conventional drug appear to be the best approach to date, but different strategies can be also used.

When apoptosis is impaired, resistance can often be overcome by targeting both the extrinsic and intrinsic pathways of apoptosis. In addition, alternative modes of cell death, such as autophagy, mitotic catastrophe, or necrosis, might be activated. Involvement of these different types of cell death in drug-induced cytotoxicity raises the possibility of using these newly identified cellular pathways instead to treat chemoresistant cancers. A deeper understanding of these alternative modes of cell death and identification of the interplay and molecular switches between apoptosis–autophagy and necrosis might provide new therapeutic targets for cancer therapy.

Finally, it is becoming clearer that tumor cells are not homogeneous and that neither most conventional drugs nor even targeted agents such as Gleevec can eliminate the cancer stem cells from which the disease arises (Bhatia et al., 2003). Relapses could occur in part due to the failure of current therapies to target this specific and original cancer cell population. Therefore, future directions of cancer research must better decipher the different modes of cell death and their potential application in attacking cancer stem cells.

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Chapter 5
Therapeutic Targeting of Death Pathways in Cancer

Mechanisms for Activating Cell Death in Cancer Cells

Ting-Ting Tan and Eileen White*

Abstract Defects in apoptosis that evolve during the course of cancer progression not only provide cancer cells with intrinsic survival advantage, but also provide inherent resistance to chemotherapeutic agents. Thus, modulation of apoptosis by targeting components of the apoptotic machinery and its regulators to restore apoptotic function is a rational approach for treating cancer. With our increasing knowledge of the mechanisms of apoptosis regulation and of how apoptosis is disabled in cancer cells, numerous novel approaches targeting apoptotic pathways can now be exploited for cancer therapy. While most of these therapies are still in preclinical development, some have shown considerable promise and progressed into the clinic. This chapter summarizes the current knowledge of the apoptotic pathways and provides a selective review on the development of drugs that target the apoptotic machinery.

Keywords apoptosis, chemotherapy, targeted therapy, BCL-2 family, death receptors, signal transduction inhibitors

1 Introduction

Inactivation of apoptosis is selected for in cancer, endowing cells with intrinsic survival advantage and the capacity to evade surveillance by the immune system. Furthermore, killing of cancer cells by currently used cytotoxic therapies, including chemotherapy, γ-irradiation, and immunotherapy largely depends on activation or

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reactivation of the apoptosis program. Accordingly, failure to engage in apoptosis produces resistance to treatment. Defective apoptosis can allow genetically unstable and damaged cells to avoid elimination, further facilitating tumor progression (Nelson et al., 2004) and treatment failure (Johnstone et al., 2002). Advances in the understanding of the molecular mechanisms of apoptosis have laid the foundation for discovery of new drugs targeting various components of the apoptotic pathway to increase the effectiveness of cancer treatment. Several new approaches are being investigated that include gene therapy, small molecule peptide mimetics, antibodies, kinase inhibitors, and proteasome inhibitors to target specific apoptosis regulators. Knowing how apoptosis is regulated, identifying the key components that control the apoptotic response in cancer cells, and how common mutations found in human tumors alter apoptotic signaling, has provided a rational approach to cancer therapy.

2 Apoptosis Signaling Pathways

Apoptosis is a stringently regulated, evolutionarily conserved mechanism of cell death that is considered a critical regulatory process for development and for maintaining a homeostatic balance between cell survival and cell death. Disruption of apoptosis contributes to the pathogenesis of a wide variety of diseases. Too much cell death can contribute to degenerative disorders, whereas too little cell death leads to autoimmunity and cancer (Cory and Adams, 2002; Danial and Korsmeyer, 2004). Two alternative pathways can initiate apoptosis: one is mediated by death receptors on the cell surface, and is referred to as the "extrinsic pathway"; and the other is referred to as " intrinsic pathway" and involves the BCL-2 family proteins that regulate mitochondrial function. Ultimately, the two pathways converge on downstream effector cysteine aspartyl-specific proteases (caspases), activation of which leads to the biochemical and morphological changes that are characteristic of apoptosis (Shi, 2002). Caspase activation results in a collapse of cellular ultrastructure and function through internal proteolytic digestion, which is evident as dismantling of the cytoskeleton, metabolic dysfunction, and genomic fragmentation. In the end, the condensed cell corpse is engulfed by nearby cells in tissues and eliminated without inflammation (Wyllie, 1980).

2.1 The Intrinsic Pathway

The BCL-2 family members serve as key regulators of the intrinsic apoptotic pathway that signals through mitochondria (Fig. 5.1). About 20 BCL-2 family members in mammals fall into three interacting groups that share at least one of four relatively conserved BCL-2 homology domains (BH1–4). Multidomain antiapoptotic BCL-2 and its homologues (e.g., BCL-x_L BCL-w, BFL-1/A1, MCL-1, and adenoviral homolog E1B19K) act predominantly to inhibit apoptosis (Cuconati and White,

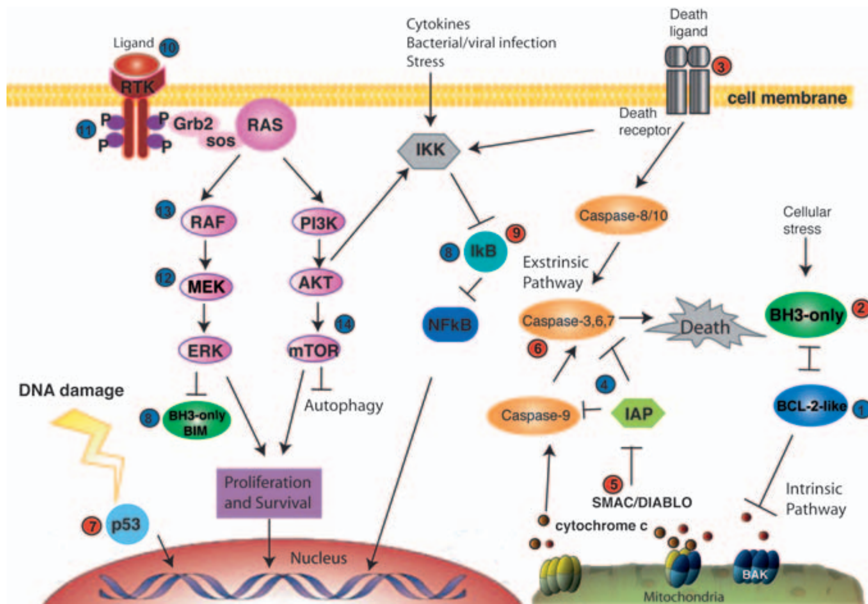

Fig. 5.1 Schematic representation of the major apoptotic pathway components and the acting points of agents that target the regulators of apoptosis. Apoptosis occur through two main pathways: the extrinsic and intrinsic pathways. Both pathways converge on activation of caspases that culminate in cell death. Extracellular signals via cytokines and growth factors are central to cell survival. Loss of p53 and hyperactivation of survival pathways are commonly found in cancer cells to deregulate cell cycle control and interfere with apoptotic signaling. Comprehensive knowledge of these pathways provides a variety of options for targeted therapy. Summarized here are major acting points of targeted agents indicated as numbered and colored circles. Red solid circles represent activation and blue solid circles represent inhibition of the target/pathway. The numbers stand for different classes of drugs: (1) represents antisense oligonucleotides or small-molecule inhibitors targeting antiapoptotic BCL-2-like proteins; (2) BH3 mimetics; (3) soluble death receptor ligands or agonistic antibodies against death receptors; (4) IAP inhibitors; (5) SMAC mimetics; (6) caspase activators; (7) p53 activators; (8) proteasome inhibitors; (9) IκB stabilizers; (10) antireceptor tyrosine kinase antibodies; (11) tyrosine kinase inhibitors; (12) MEK kinase inhibitors; (13) RAF kinase inhibitors; and (14) mTOR inhibitors

2002; Danial and Korsmeyer, 2004). The other two groups instead are proapoptotic. One of these proapoptotic groups comprises the multidomain proteins represented by BAX, BAK, and BOK that share BH1–3 with BCL-2. These three conserved regions in multidomain BCL-2 family members form a hydrophobic surface groove for binding of either a putative transmembrane helical domain at the carboxyl terminus, or a BH3 (Fesik, 2000; Suzuki et al., 2000). The other proapoptotic group is comprised of the BH3-only proteins (e.g., BAD, BID, BIM, HRK, PUMA, NOXA, and NBK/BIK) that are the most apical regulators of this intrinsic death signaling (Gelinas and White, 2005; Willis and Adams, 2005). BH3-only proteins typically initiate the apoptotic activity of the BCL-2 family in response to diverse cytotoxic stimuli. The BH3 is an amphipathic α-helix that serves as a binding motif for interaction with the hydropho-

bic groove on either multidomain antiapoptotic or proapoptotic BCL-2 proteins. Systematic study of the binding of BH3-only proteins to BCL-2 antiapoptotic proteins has shown that certain BH3-only proteins target specific subsets of the prosurvival proteins (Letai et al., 2002; Chen et al., 2005; Kuwana et al., 2005; Willis et al., 2005). BIM, PUMA, and tBID bind avidly to all five antiapoptotic proteins and demonstrate potent killing. In contrast, BAD and BMF bind preferentially to BCL-2, BCL-x_L, and BCL-w, whereas NOXA binds preferentially to MCL-1 and BFL-1/A1. Although BH3-only proteins with restricted targets can be less-potent inducers of apoptosis, BAD and NOXA with complementary affinity to antiapoptotic BCL-2 family members cooperate to induce substantial cell death (Chen et al., 2005). Although BH3-only proteins cannot initiate apoptosis in the absence of BAX and BAK (Cheng et al., 2001; Zong et al., 2001), it remains unresolved whether they activate BAX and BAK directly or indirectly. A direct binding model has been proposed for BAX activation where binding of BH3-only sensitizers (e.g., BAD or BIK) to BCL-2 displaces the normally sequestered BH3-only activators (e.g., tBID or BIM), releasing tBID or BIM to trigger BAX oligomerization (Letai et al., 2002). Evidence suggests a displacement model for the activation of BAK whereby MCL-1 and BCL-x_L sequester BAK, and that the binding of BH3-only proteins such as NOXA to MCL-1 displaces BAK as an apoptosis-activating step (Cuconati et al., 2003; Gelinas and White, 2005; Willis and Adams, 2005; Willis et al., 2005).

BH3-only proteins initiate apoptosis in response to a wide range of damage and stress, including DNA damage, deregulated growth, survival factor deficiency, hypoxia, anoikis, and Ca^{+2} overload (Adams, 2003; Cory and Adams, 2005; Willis and Adams, 2005). Although these diverse apoptotic stimuli activate different upstream components in the apoptotic signaling pathway, in most cells, these signals are transduced to and converge on mitochondria and cause permeabilization of outer mitochondrial membrane causing the release of cytochrome c and other apoptogenic proteins (e.g., SMAC/DIABLO). Cytochrome *c* release promotes the formation of the apoptosome, a large protein complex that contains cytochrome c, apoptotic protease-activating factor 1 (APAF1) and caspase-9 (Li et al., 1997; Zou et al., 1997). Apoptosome formation triggers activation of caspase-9, which further cleaves and activates the effector caspase-3, resulting in selective destruction of subcellular structures and organelles, and of the genome (Earnshaw et al., 1999).

2.2 *The Extrinsic Pathway*

Activation of the extrinsic apoptotic pathway is initiated by ligand-mediated activation of cell surface death receptors (DRs). It plays an important role in immune surveillance of transformed or virus-infected cells and in the removal of self-reactive lymphocytes. Death receptors form a subgroup of the tumor necrosis factor (TNF) receptor superfamily that includes TNF-R1, CD95 (also called APO-1 or FAS), DR3 (APO-2), DR4 (TNF-related apoptosis-inducing ligand receptor 1

[TRAIL R1]), DR5 (TRAIL R2), and DR6 (Zapata et al., 2001). Upon ligand binding, the death receptors interact via their intracellular motif called the death domain (DD) with the DD of adapter proteins such as FAS-associated death domain (FADD). These adapter proteins also contain a second protein interaction motif, the death effector domain (DED), that facilitates binding to a corresponding DED in the amino-terminal prodomains of initiator caspase-8 (or in some cases, its relative caspase-10) to form the death-inducing signaling complex (DISC) (Wallach et al., 1999). DISC formation activates caspase-8, which subsequently cleaves and activates caspase-3, resulting in further cleavage of cellular targets.

In many cells, however, DISC formation mediated caspase-3 activation is insufficient to complete the cell death program, and death receptor signaling must be amplified by engagement of the mitochondria-mediated cell death pathway through the caspase-8-mediated cleavage of the BH3-only protein BID (Fig. 5.1). Once cleaved, truncated BID translocates to mitochondria, where it can activate BAX and BAK and induce the release of cytochrome *c* and SMAC/DIABLO serving to amplify apoptosis signaling (Danial and Korsmeyer, 2004).

The intrinsic and extrinsic apoptotic pathways converge on downstream effector caspases that implement cell elimination. The caspase family forms the engine of apoptosis and is divided into two major groups (Fischer et al., 2003; Fuentes-Prior and Salvesen, 2004). The subset of caspases that cleave selected substrates to produce the typical alteration associated with apoptosis are known as executioner caspases, which in mammals are caspase-3, caspase-6, and caspase-7. Executioner caspases are activated by apical initiator caspases, including caspase-8, caspase-9, and caspase-10. Effector caspases are targets of suppression by an endogenous family of antiapoptotic proteins called inhibitor of apoptosis proteins (IAPs). The IAP family, characterized by one or more baculovirus IAP repeat (BIR) domains, includes X-linked IAP (XIAP), c-IAP1, c-IAP2, Survivin, Livin (ML-IAP), ILP2, and Apollon. Different BIR domains are responsible for suppression of specific caspases. Structural studies have revealed that the BIR3 of XIAP is responsible for binding and inhibition of caspase-9 and that a region adjacent to BIR2 is the major determinant for inhibition of caspase-3 and caspase-7. ML-IAP contains a single BIR and inhibits caspase-9 but not caspase-3 and caspase-7. BIRs are sometimes accompanied by really interesting new gene (RING) and ubiquitin-conjugating enzyme domains, which are associated with the ability to target them and other proteins for proteasome degradation. SMAC/DIABLO is an IAP antagonist that is released into the cytoplasm upon mitochondrial permeabilization. SMAC/DIABLO binds to IAPs in a manner similar to caspases, thereby promoting apoptosis by liberating caspases from IAPs (Fesik and Shi, 2001). The amino-terminal tetrapeptide motif of SMAC/DIABLO is responsible for binding to BIR domains of IAPs (Wu et al., 2000). Evidence suggests that apoptosis requires or is facilitated by coordinate inhibition of IAPs by SMAC/DIABLO and activation of the apoptosome by cytochrome *c*.

3 Targeting the Apoptotic Machinery

3.1 BCL-2 Family Proteins as Targets

Proteins of the BCL-2 family are crucial checkpoints of the intrinsic mitochondrial death pathway. Overexpression of antiapoptotic BCL-2 family proteins such as BCL-2, BCL-x_L, BFL-1/A1, or MCL-1 has been observed in various malignancies and can confound cancer treatment (Reed and Pellecchia, 2005). Reduction of expression levels of antiapoptotic BCL-2 family genes is considered to potentially contribute to the proapoptotic effects of some novel anticancer agents, such as retinoids, histone deacetylase inhibitors, and peroxisome-proliferator-activated receptor-γ (PPARγ)-modulating drugs (Reed and Pellecchia, 2005). Drug design targeting antiapoptotic BCL-2 family members has been focused on three strategies inducing mRNA degradation with antisense oligonucleotides, BH3-domain peptido-mimetics, and synthetic small molecule drugs interfering directly with BCL-2 family member protein function (Fig. 5.1).

Currently, oblimersen sodium (G3139, Genasense; Genta, Inc., Berkeley Heights, New Jersey) is the only nucleic acid-based inhibitor of BCL-2 and its antiapoptotic relatives to enter clinical trials. Oblimersen is a DNA-based synthetic 18-mer antisense oligonucleotide to BCL-2 and has been reported to induce RNaseH-mediated degradation of BCL-2 mRNA (Fig. 5.1). A completed phase 3 clinical trial of oblimersen for advanced melanoma in combination with dacarbazine demonstrated slowed disease progression, but failed to extend the survival time (Fischer and Schulze-Osthoff, 2005). Several phase 3 clinical trials of oblimersen in combination with conventional chemotherapy involving patients with other tumors are still being evaluated. Antisense directed against BCL-x_L also displays proapoptotic effects in cancer cells (Fennell et al., 2001). Bispecific BCL-2/BCL-x_L-suppressing antisense oligonucleotides under preclinical studies may optimize efficacy because simultaneous overexpression of multiple antiapoptotic members of the BCL-2 family may occur in malignant cells (Del Bufalo et al., 2003). An MCL-1 antisense compound has recently demonstrated efficacy in a sarcoma xenograft model (Thallinger et al., 2004). Efficient delivery of these agents to tumor cells remains as a potential limitation of this approach.

An alternative means to interfere with BCL-2 antiapoptotic proteins is direct inhibition by modified BH3 peptides or small molecules (Fig. 5.1). A hydrocarbon-stapled BID BH3 peptide engineered to be helical, protease resistant, and cell permeable, potently induces apoptosis in Jurkat T leukemia cells and slows the growth of a transplanted leukemia (Walensky et al., 2004). A BAD-BH3 peptide preferably kills BCL-x_L and BCL-2 overexpressing Jurkat cells, and a BAX BH3 peptide is slightly more effective in BCL-2 overexpressing cells, suggesting that the efficacy of BH3 peptides might depend on the affinity of a certain BH3 domain for a limited set of antiapoptotic BCL-2 proteins (Shangary and Johnson, 2002).

Small-molecule inhibitors of BCL-2 or related antiapoptotic relatives have recently been identified through high-throughput screening of chemical libraries

for the ability to dock onto the BH3 pocket of antiapoptotic BCL-2 family proteins, negating their prosurvival activity. The most advanced among these is the natural product gossypol, and its semisynthetic analogs with less toxicity, which are undergoing late steps of preclinical and clinical testing (Qiu et al., 2002). Chelerythrine, identified as an inhibitor of BCL-x_L/BAK-BH3 interaction from a natural compound library, induces apoptosis effectively in BCL-2 or BCL-x_L overexpressing cells (Chan et al. 2003). Others include BH3I-1 and BH3I-2, identified by screening using a BH3 peptide displacement assay, and HA14–1 and antimycin analogs, identified by computational modeling (Reed and Pellecchia, 2005). Most promising is ABT737 (Abbott, Abbott Park, Illinois), a synthetic small molecule developed by NMR-guided, structure-based drug design (Oltersdorf et al., 2005) that exhibits very high affinity for the hydrophobic pocket of BCL-2, BCL-x_L, and BCL-w (Cory and Adams, 2005). ABT737 significantly sensitizes many tumors to cytotoxic agents and is effective as a single agent against certain lymphomas and solid tumors, inducing tumor regression in xenograft models. The BAD-like selectivity of ABT737 suggests that tumor resistance to ABT737 could result from high-level expression of MCL-1 or BFL-1/A1, and small molecules that target these proteins should synergize with ABT737 (Cory and Adams, 2005). A chapter by Moore and Letai will provide more detail on rationale designs of therapeutics that target BCL-2 family of proteins.

3.2 Targeting Death Receptors

Most chemotherapeutic agents and radiation therapy induce apoptosis in cancer cells primarily by engagement of the mitochondrial apoptosis machinery. Accordingly, chemorefractory tumor cells often evolve defects in their intrinsic apoptotic pathway. By directly activating the caspase cascade, death receptor-mediated apoptosis, in contrast, can bypass the mitochondria and thereby sensitize resistant tumor cells to conventional chemotherapeutic agents or ionizing radiation (Fig. 5.1).

Despite the selective antitumor activity, the proinflammatory actions of TNF preclude its systemic administration in cancer therapy. Nonetheless, because TNF destroys tumor-associated blood vessels by apoptosis and improves vascular permeability to cytotoxic drugs, local application of TNF has been exploited for cancer therapy. Low-dose TNF was shown to improve penetration of doxorubicin for the treatment of melanoma and lymphoma (Curnis et al., 2000). Isolated limb perfusion of high-dose TNF combined with chemotherapeutic drugs demonstrated significant synergistic effect in treatment of locally advanced melanomas and sarcomas (Eggermont and ten Hagen, 2001).

CD95L and TRAIL are expressed on cytolytic T cells, natural killer cells, and other immune cells and play an important role in eradication of virus-infected and transformed cells (Locksley et al., 2001). Unlike TNF, these death ligands do not induce concomitant NF-κB activation (Karin and Lin, 2002). Unfortunately, severe

hepatotoxicity precluded the systemic administration of CD95 ligand (Ogasawara et al., 1993). Most promising is TRAIL and agonistic antibodies that bind TRAIL receptors, which selectively kill tumor cells in mouse xenograft models without harming normal tissues (Ashkenazi et al., 1999). Genentech (South San Francisco, California) and Amgen (Thousand Oaks, California) have initiated phase 1 clinical trials with soluble TRAIL. Human Genome Sciences, Inc. (Rockville, Maryland) has recently completed phase 1 clinical trials with an agonistic monoclonal antibody against TRAIL-R1 (HGS ETR1) and advanced to phase 2 clinical trials for the treatment of a variety of cancers, such as non-small-cell lung cancer (NSCLC), colorectal carcinoma, and non-Hodgkins lymphoma. So far, patients had little toxicity (Le, 2004; Georgakis et al., 2005; Pukac et al., 2005). Humphreys and Halpern will discuss targeting of TRAIL receptors for cancer therapy in Chapter 7.

3.3 Therapeutic Inactivation of IAPs

IAPs inhibit executioner caspases activated by extrinsic or intrinsic pathways. XIAP, cIAP1, cIAP2, ML-IAP, and Survivin are upregulated in many tumors including leukemias and neuroblastomas, and have been correlated with adverse prognosis (Salvesen and Duckett, 2002). Targeted therapy attacking XIAP is currently under preclinical and clinical investigation and includes XIAP antisense, XIAP antagonists that specifically target BIR2 domain of XIAP, and SMAC-peptide and nonpeptide mimetics.

Antisense molecules targeting XIAP have been shown to sensitize a variety of tumor cell lines to radiotherapy and chemotherapy. Second-generation oligonucleotides, comprising DNA/RNA hybrid backbones with improved pharmacokinetics and reduced toxicity are in phase 1 clinical trials in patients with solid tumors (Fischer and Schulze-Osthoff, 2005). XIAP antagonists that target BIR2 and displace caspase-3 were identified by an enzyme derepression assay where XIAP-mediated suppression of caspase-3 is overcome by chemical compounds (Wu et al., 2003; Schimmer et al., 2004). These compounds display proapoptotic effects in tumor cell lines through a BCL-2/BCL-x_L-independent pathway (Wang et al., 2004).

Proapoptotic SMAC/DIABLO is released from mitochondria during the apoptotic process and relieves inhibition of caspase-3, caspase-7, and caspase-9 by IAPs. The four N-terminal residues (AVPI) of SMAC/DIABLO recognize a surface groove on BIR3 of XIAP normally occupied by processed caspase-9, thereby dislodging caspase-9 from the XIAP-inhibitory complex (Shiozaki et al., 2003). A series of SMAC peptido-mimetics consisting of 4, 5, or 7 amino acids of the amino-terminus of SMAC/DIABLO fused to a carrier peptide for intracellular delivery (cell-permeable SMAC peptides), overcome resistance of cancer cells with high levels of XIAP expression to apoptosis, and enhance the activity of conventional anticancer drugs in vitro and in vivo (Fulda et al., 2002; Guo et al., 2002; Yang et al., 2003; Sun et al., 2005). A series of nonpeptidic small-molecule

XIAP antagonists are being developed to improve proteolytic stability, cell permeability, and pharmacokinetics (Li et al., 2004; Nikolovska-Coleska et al., 2004; Oost et al., 2004; Park et al., 2005). These potent mimetics represent a novel class of anticancer drugs particularly useful in combination chemotherapy.

Survivin is an IAP member that plays a major role in both cell division and apoptosis. Survivin is highly expressed in cancer cells and is implicated in tumor resistance to radiotherapy and chemotherapy (Altieri, 2003). In addition, Survivin deficiency results in abnormal spindle formation and mitotic catastrophe independent of p53 and BCL-2 (Okada and Mak, 2004). Several preclinical studies demonstrated that inhibition of Survivin by antisense oligonucleotides, ribozymes, small interfering RNAs, dominant negative mutants, and cyclin-dependent kinase inhibitors was able to promote spontaneous apoptosis in tumor cells and to enhance the efficacy of conventional treatments including chemotherapy, radiotherapy, and immunotherapy (Zaffaroni et al., 2005). The high level and specificity of Survivin expression in cancer cells make it an attractive target for anticancer drug discovery.

3.4 Caspase Activators

Selective activation of caspases might be a valuable strategy for cancer therapy. Several approaches to trigger caspase activation in tumor cells are presently being developed. Inducible caspases have been engineered by fusing them to chemical dimerization domains. After delivery of these chimeric, regulatable caspases by adenoviral gene transfer, they can be activated to trigger apoptosis in tumor cells by cell permeable dimerization drugs (Shariat et al., 2001). Tumor-specific delivery is also achieved by fusing caspases with antibodies against receptors that are overexpressed in human cancers. For instance, caspase-3 linked to a anti-HER2 antibody is internalized via endocytosis by HER2 overexpressing tumors (Xu et al., 2004). In addition, high-throughput drug screening has identified a series of small molecule caspase activators, which have been shown to induce apoptosis in multiple cancer cell lines including prostate, breast, colorectal, lung cancer (Jiang et al., 2003; Nguyen and Wells, 2003). How specificity to tumor cells will be achieved in this case is not yet clear.

3.5 Modulation of the p53 Tumor Suppressor

The p53 pathway is composed of a network of genes and their products that respond to stresses, which disturb the fidelity of DNA replication and cell division (Balint and Vousden, 2001). Loss of p53 leads to genomic instability,

impaired cell cycle regulation, and inhibition of apoptosis. p53 mutation and thereby inactivation are found in more than 50% of human cancers, and lack of functional p53 may render tumor cells resistant to apoptosis induced by chemotherapy and radiotherapy. In Chapter 10, El-Diery describes the regulation of programmed cell death by p53.

Three main therapeutic strategies are currently in development that target the p53 pathway (Fig. 5.1). First is reconstitution of wild type p53 in cancer cells by introduction of exogenous p53 with viral vectors. The most commonly used viral p53 delivery mechanism is the use of an adenoviral vector carrying the wild-type p53 gene. Due to the low efficiency of gene delivery of gene therapy vectors and hepatotoxicity associated with systemic applications, current clinical trials evaluate the efficacy of Ad-p53 through intratumoral injection in advanced solid tumors. Ad-p53 gene therapy alone failed to demonstrate beneficial effects in patients and new trials in combination with chemotherapy or radiotherapy are being investigated (Khuri et al., 2000).

Second is reactivation of mutant p53 to the wild-type form to induce apoptosis. Several compounds have been identified by screening or rational design with the capability of restoring the transcriptional function of mutant p53 and thereby apoptosis. These p53 reactivators include PRIMA-1 (Bykov et al., 2002), CP-31398 (Foster et al., 1999), and CDB3 (Friedler et al., 2002). As p53-mediated apoptosis is induced by transcriptional upregulation of the BH3-only proteins PUMA and NOXA, and the majority of human tumors have mutant p53, this approach has enormous potential for success.

Third, is the interruption of the regulatory interaction between p53 and MDM2 to prevent p53 degradation by the E3 ligase activity of MDM2. Nutlins and RITA increase p53 levels by binding to the p53 pocket for MDM2 interaction thereby inhibiting tumor growth in mice (Issaeva et al., 2004; Vassilev et al., 2004). However, restoration of p53 function in tumors with a lower frequency of p53 mutations maybe counterproductive as certain tumors may adapt normal p53 activation to achieve cell cycle arrest and DNA damage repair inflicted by chemotherapy and/or radiotherapy (Scott et al., 2003; Stoklosa et al., 2004).

4 Targeting Survival Signaling Pathways

Another set of targeted therapies aims to inhibit survival signaling pathways that are regulated by cytokines, hormones, and growth factors. In cancer cells, key components of these pathways are altered by oncogene activation or loss of tumor suppressor gene function, resulting in deregulated cell proliferation, inhibition of apoptosis, and enhanced angiogenesis. Strategies targeting survival signaling include neutralization of ligands, inhibition of receptors, and inhibition of cytoplasmic secondary messengers.

4.1 The NF-κB Pathway

Growth factors, cytokines such as interleukin-1 and TNF, hormones and other signals activate NF-κB by phosphorylation of inhibitor of κB (IκB), which has been linked to enhancement of both survival and tumorigenesis (see Chapter 11). Substantial evidence indicates that NF-κB plays an important role in tumorigenesis. Tumor suppressor genes such as CYLD and ING4 have been shown to negatively regulate NF-κB (Brummelkamp et al., 2003; Garkavtsev, 2004), and NF-κB activation is implicated in the increased incidence of cancer associated with inflammatory diseases (Greten et al., 2004; Pikarsky et al., 2004). Constitutive activation of NF-κB observed in tumors contributes to chemoresistance, perhaps by blocking apoptosis through direct transcriptional induction of expression of antiapoptotic proteins such as BCL-x_L, BFL-1/A1, or IAP1/2 and XIAP and/or repression of proapoptotic p53 (Nakanishi and Toi, 2005). Several anticancer agents stimulate NF-κB, such as taxanes, vinca alkaloids, and topoisomerase inhibitors, which can potentially lead to chemoresistance (Nakanishi and Toi, 2005). Thus, the possibility of increasing the efficacy of anticancer drugs by inactivation of NF-κB makes this pathway an attractive chemotherapeutic target (Fig. 5.1).

BAY11-7082 and BAY11-7085 inhibit IκB phosphorylation and stabilize IκB, allowing it to sequester NF-κB in the cytoplasm in an inactivated state. In preclinical studies, these two drugs sensitized tumor cells to conventional chemotherapy agents. For example, the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) increases the NF-κB transcriptional activity through the enhanced nuclear translocation of the p65 subunit, which may diminish its effectiveness as a cancer therapeutic. However, inhibition of NF-κB by BAY11-7085 coordinately administrated with SAHA increases cell death in NSCLC cell lines (Rundall et al., 2004). BAY11-7085 also increases the efficacy of cisplatin and paclitaxel in an in vivo ovarian cancer model (Mabuchi et al., 2004a; Mabuchi et al., 2004b).

Inhibition of NF-κB activity is considered as one of the major mechanisms of proteasome inhibitors, a novel class of anticancer drugs. IκB is polyubiquitinated upon phosphorylation by the IκB kinase, IKK, which then targets it for degradation by the 26S proteasome. Proteasome inhibitors thereby induce IκB accumulation, which retains NF-κB in the cytoplasm and prevents transcriptional activation of target genes. The proteasome inhibitor bortezomib (Velcade, PS-341; Millennium, Cambridge, Massachusetts) (also see Chapter 12) exhibits antitumor activity against a wide range of malignancies either as a single agent or combined with conventional chemotherapeutic drugs, and has been approved by US Food and Drug Administration (FDA) for the treatment of relapsed or refractory multiple myeloma (Rajkumar et al., 2005).

4.2 Therapeutic Modulation of the BH3-Only Protein BIM

While proteasome inhibitors may work in some situations as NFκB inhibitors, other targets for the anticancer activity of proteasome inhibitors include cell cycle regulatory proteins, p53-mediated apoptosis, unfolded protein response pathway,

intrinsic and extrinsic apoptosis pathway. For example, the H-ras/MAP kinase pathway suppresses apoptosis induced by the proapoptotic BH3-only protein BIM in response to taxanes (paclitaxel) by phosphorylating BIM and targeting BIM for degradation in proteasomes. The proteasome inhibitor bortezomib restores BIM induction and apoptosis, abrogating resistance to paclitaxel conferred by H-ras, promoting BIM-dependent tumor regression. This suggests the potential benefits of combinatorial chemotherapy of bortezomib and paclitaxel preferentially in tumors with MAP kinase activation (Fig. 5.1) (Tan et al., 2005). The newly developed orally bioactive proteasome inhibitor NPI-0052 induces apoptosis in multiple myeloma cells resistant to conventional chemotherapeutic drugs and bortezomib, with less toxicity as it is mechanistically distinct from bortezomib (Chauhan et al., 2005) (also see Chapter 12). More drugs of this class, whether the ultimate therapeutic target is NF-κB, BIM, or yet another protein, are likely to be entering the clinic in the near future.

4.2.1 Tyrosine Kinase Inhibitors

Tyrosine kinases (TKs), particularly receptor tyrosine kinases (RTKs), are key factors in the promotion of cancer cell survival, and as such represent an attractive therapeutic target for cancer therapy (Fig. 5.1). Deregulated TK activity can cause increased cell proliferation, reduced apoptosis, invasion and angiogenesis. Small-molecule inhibitors of TKs compete with the ATP-binding site of the catalytic domain of oncogenic TKs and thereby prevent their activation. This has been the basis for the success in the treatment of chronic myelogenous leukemia (CML), where development of the inhibitor of the oncogenic BCR-ABL TK fusion protein, imatinib mesylate (Gleevec, STI571; Novartis, Basel, Switzerland), has produced dramatic clinical responses (see also Chapter 4). Over 90% of CML patients carry the Philadelphia chromosome, a translocation between chromosomes 9 and 22 that generates the *bcr-abl* oncogene (Faderl et al., 1999). The constitutively activated BCR-ABL kinase leads to growth factor independence and apoptosis resistance by activation of RAS-MAP kinase and Janus activating kinases-signal transducers and activators of transcription (JAK-STAT) pathways (Yamauchi et al., 1998). BCR-ABL also activates the antiapoptotic PI3K/AKT pathway, increases BCL-2, and suppresses BIM expression (Kuribara et al., 2004; Essafi et al., 2005). Imatinib produces major hematologic and cytogenetic responses in 65–90% of CML patients after failed interferon-α therapy and in 80–90% of newly diagnosed and untreated patients (Kantarjian et al., 2002a; Kantarjian et al., 2002b). However, most patients experienced relapse after treatment discontinuance and a significant number of newly diagnosed patients start out resistant (Druker, 2004). The most common resistance mechanism involves mutations that affect the conformation of the BCR-ABL kinase domain and prevent binding to imatinib (Gorre et al., 2001). Second-generation kinase inhibitors retain activity against almost all imatinib-resistant mutants and are currently under early clinical evaluation (Shah et al., 2004).

Combination therapy of imatinib with other therapeutics is also being investigated to overcome resistance. Imatinib also targets the receptor c-KIT and platelet-derived growth factor receptor (PDGFR). Gastrointestinal stromal tumors (GISTs), where mutated c-KIT is implicated in the pathogenesis, also show significant responses to imatinib (Debiec-Rychter et al., 2004).

A member of the epidermal growth factor receptor (EGFR) family, Her-2/*neu* is overexpressed in 20–30% of malignant breast tumors. A recombinant humanized monoclonal antibody targeted to the extracellular domain of the Her-2/*neu* receptor, trastuzumab (Herceptin; Genentech, Inc., San Francisco, California) has demonstrated overall tumor response rates between 15% and 26% in the metastatic setting. In combination with chemotherapy, trastuzumab produces prolonged disease-free and overall survival when compared to standard chemotherapeutic treatment regimens. Trastuzumab induces induction of G1 arrest of cell cycle progression and apoptosis and is now part of the treatment of choice for Her-2-positive breast cancers (Emens, 2005).

The EGFR is overexpressed in a variety of tumors, including tumors of the breast, lung, ovaries, and kidney, and thus a rational target for cancer therapy (Jones et al., 2005). Presently, two classes of EGFR antagonists are in phase 2 and 3 trials: anti-EGFR monoclonal antibodies and TK inhibitors. Cetuximab (Erbitux; ImClone Systems, Inc., New York), the most established monoclonal antibody, is approved for use as a single agent or in combination with irinotecan in patients with metastatic colorectal cancer (Wong, 2005). Erlotinib (Tarceva; OSI, Long Island, New York), an orally available selective inhibitor of the EGFR (ErbB1) TK, has received FDA approval for the treatment of patients with locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen (Comis, 2005). Erlotinib is the only EGFR TK inhibitor that showed survival improvement in NSCLC patients in a randomized phase 3 clinical trial (Perez-Soler, 2004). When administered in combination with gemcitabine, erlotinib also significantly improved survival in patients with advanced or metastatic pancreatic cancer in a phase 3 trial (Thomas and Grandis, 2004). Gefitinib (Iressa; AstraZeneca, Wilmington, Delaware), is another EGFR TK inhibitor that is approved for refractory NSCLC. In the subset of patients with specific EFGR TK domain mutations, the response rate to gefitinib was high, suggesting that screening for these mutations in lung cancers to identify patients that respond is advisable (Lynch et al., 2004; Sordella et al., 2004).

4.2.2 RAS-MAP Kinase Pathway

The RAS-RAF-MEK-ERK pathway represents a common downstream pathway for several key RTKs such as EGFR, PDGFR, and VEGFR, which are frequently mutated or overexpressed in human malignancies and thus is a logical therapeutic target (Fig. 5.1). Constitutive activation of the MAP kinase pathway not only promotes tumor cell proliferation, but may also interfere with apoptosis. Activation of

the MEK-ERK cascade upregulates antiapoptotic proteins BCL-2, BCL-X_L, and MCL-1 (Liu et al., 1999; Leu et al., 2000; Jost et al., 2001), and promotes survival by phosphorylating BCL-2 and blocking its degradation in proteasomes (Dimmeler et al., 1999) and by phosphorylating BIM and accelerating its proteasomal degradation (Ley et al., 2003; Luciano et al., 2003; Tan et al., 2005).

Two novel MEK inhibitors CI-1040 (PD 184352) and PD 0325901 are in clinical trials (Pfizer, Inc., New York). A phase 2 study testing the MEK inhibitor CI-1040 in NSCLC, breast, colorectal, and pancreatic cancers was performed with negative results (Rinehart et al., 2004). The second-generation agent PD 0325901 with better bioavailability and increased potency is currently in clinical trials. In addition, MEK inhibitors suppress the expression of several antiapoptotic players, thus lowering the apoptotic threshold and have shown striking synergistic effects with conventional chemotherapy. For example, MEK blockade sensitizes leukemic cells to classical cytotoxics including nucleoside analogs, microtubule-targeted drugs, and γ-irradiation (Milella et al., 2005).

Although no mutations in A-RAF or C-RAF have been found in human cancers, B-RAF is mutated and constitutively activated in 70% of melanomas and other cancer types (ovarian, thyroid, colon, lung) with a moderate to high frequency, suggesting its implication in cancer development (Wan et al., 2004). Sorafenib (Nexavar, BAY43-9006; Bayer, West Haven, Connecticut and Onyx, Emeryville, California) is one of the most promising agents of the class of RAF kinase inhibitors and has shown significant efficacy and minimal toxicity both as a single agent and in combination with standard chemotherapies in renal cell, hepatocellular, colorectal, ovarian, and breast cancers in phase 1 and 2 studies (Thompson and Lyons, 2005). Sorafenib can also indirectly inhibit several important TKs including VEGFR-2, VEGFR-3 Flt-3, and c-Kit that are upstream of RAF, which contributes to its antiproliferative, antiapoptotic, and antiangiogenic properties (Wilhelm et al., 2004).

4.2.3 PI3KAKT/mTOR Pathway

The PI3K/AKT/mTOR pathway regulates cell proliferation and cell survival and is commonly found aberrantly activated in a variety of tumors due to amplification of the PI3KC gene encoding for the p110α catalytic subunit of PI3K, gene amplification of AKT, and loss of PTEN tumor suppressor function (Morgensztern and McLeod, 2005). AKT promotes cell survival by inhibiting BAD, caspase-9 and FORKHEAD, and activating several antiapoptotic proteins including IKK (Downward, 2004). A downstream effector in the PI3K pathway is the protein kinase mTOR, which is inhibited by tuberous sclerosis complex (TSC1/2). Activation of AKT results in phosphorylation of TSC2 which disrupts TSC1/2 complex, leading to derepression of mTOR (Inoki et al., 2002). mTOR promotes cell proliferation by regulating translation initiation, mediated by activation of the 40S ribosomal protein p70S6 kinase (S6K1) and inactivation of 4E-binding protein (4E-BP1). The increase in the translation of a subset of mRNAs produces proteins

that are required for G1/S phase cell cycle progression (Hay and Sonenberg, 2004). Rapamycin, a natural mTOR inhibitor, was not developed as an anticancer drug due to poor solubility and instability. Rapamycin analogs, including CCI-779 (Wyeth-Ayerst, Princeton, New Jersey), RAD001 (Novartis, Basel, Switzerland), and AP23573 (Ariad, Cambridge, Massachusetts), have improved pharmacokinetics and are currently under clinical evaluation for cancer treatment (Fig. 5.1). While RAD001 and AP23573 are in the early stage of phase 1 clinical trials, CCI-779 has completed phase 1 and 2 studies with good tolerance and impressive response rate in patients with renal cell carcinoma (RCC), breast, lung and neuroendocrine tumors, which has led to phase 3 studies in patients with RCC and breast cancer (Morgensztern and McLeod, 2005). Identification of biomarkers to predict tumor sensitivity and the synergy between CCI-779 and standard chemotherapy, hormone or growth factor inhibitors are also being investigated (Vignot et al., 2005).

5 Targeting Pathways for Alternate Forms of Cell Death in Cancer Therapy

Although apoptosis represents the predominant mechanism by which cancer cells are eliminated, other modes of cell death, such as necrosis, autophagy, and mitotic catastrophe are also considered as cell death response to cytotoxic therapies. What determines the form of cell death induced by a particular anticancer agent depends on the cell type, the genotype of the cell, the type of cellular damage that the drug induces, the dose of the agent used, as well as the microenvironment. Thus, a better understanding of these diverse modes of cell death in cancer therapy may lead to new approaches to overcome drug resistance.

Necrosis refers to cell death characterized by cell swelling and rupture in response to profound damage or a physical insult, that subsequently releases its intracellular components into the surrounding tissue. A major consequence of this is the activation of an inflammatory response and thereby immune surveillance. Chronic inflammation is thought to promote tumor formation and progression, which is the basis for current efforts to use nonsteroidal anti-inflammatory agents for chemoprevention (Balkwill et al., 2005). DNA-alkylating agents cause necrotic cell death, which is equally effective in cells with and without apoptotic defects, and is independent of p53 or BCL-2 family proteins (Zong et al., 2004). Interestingly, alkylating agents selectively target cells using aerobic glycolysis, as is characteristic of many cancer cells, but not normal cells that use mitochondrial substrates for oxidative phosphorylation (Zong et al., 2004). Induction of necrosis has also been reported with arsenic trioxide, which triggers a regulated form of caspase-independent necrotic cell death (Scholz et al., 2005). Thus, stimulation of necrotic cell death may be an alternative in cancer cells with a defective apoptotic response, but may be coupled to inhibition of inflammation.

Autophagy is also an ordered cellular process where cell compartmentalizes to form autophagic vacuoles in cytoplasm and digests itself (Klionsky and Emr,

2000). It is a bulk protein degradation system that is essential for normal cell activity and survival when nutrients are scarce. Recent studies have linked defective autophagy to tumor development. Loss of *beclin1*, the mammalian ortholog of the yeast autophagy gene *apg6* that is monoallelically deleted in many human tumors, correlates with reduced autophagy and promotes tumorigenesis in mice (Qu et al., 2003; Yue et al., 2003). It is likely that activation of the AKT and mTOR signaling pathway contributes to malignant transformation by simultaneous inhibition of autophagy and apoptosis. Autophagic cell death is reportedly activated in cancer cells in response to various chemotherapeutic drugs, such as paclitaxel, vinblastine, and rapamycin, as well as to irradiation (Kim, 2005), although the clinical significance of autophagy in cancer therapy is unclear. If autophagy functions to promote survival of cancer cells by enabling catabolism, then autophagy inhibitors may be therapeutically useful. Alternatively, if autophagic cell death is a significant mechanism of cancer cell elimination, then inhibition of mTOR and activation of autophagy may be therapeutically beneficial.

Finally, driving cells past mitotic checkpoints and into aberrant mitoses that lead to death by mitotic catastrophe has recently attracted interest as a means to kill tumor cells independently of a defective apoptotic response (Castedo et al., 2004). Inhibiting normal mitosis in tumor cells can result in death due to mitotic failure and many current antimicrotubule drugs already in use in the clinic may induce death this way, and others that directly target regulation of mitosis are in development.

6 Future Prospects

Defective apoptosis is essential in tumor development and renders cancer cells refractory to chemotherapeutic agents. The identification of genes and gene products that regulate apoptosis at different molecular levels, along with an increased knowledge about their mechanisms of action provides a variety of therapeutic options for rational drug design targeting apoptosis.

In targeted anticancer drug development, high-throughput screening of chemical libraries, along with modification by structural biology and combinatorial chemistry to generate potent drugs with highly specific targets and favorable pharmacology has replaced the previous random screening. However, the sheer number of targets requires the development of a rapid, efficient preclinical and clinical screening system to eliminate ineffective agents with the minimal cost. In the preclinical setting to provide the proof of concept in vivo, it requires judicious application of the most appropriately genetically defined animal models of human cancers based on the proposed target of the drugs. In clinical trials, careful patient selection based on genetic information of the tumors being treated and the therapeutic target of the drug being tested is considered critical. The hypothesis with this approach is that those tumors in which the targeted apoptotic pathway or survival pathway is critical will be more susceptible to the therapeutic agents. The successful application of TK

inhibitor imatinib mesylate in CML patients with Philadelphia chromosome supports this hypothesis. Similarly, the recent identification of point mutations in the EGFR gene in tumors from patients responding to gefitinib and their absence in nonresponders provides a means for patient selection. These findings illustrate the importance of matching the therapy to the tumor as a form of personalized medicine.

Cancer cells may be more dependent on apoptosis suppression because of oncogene activation, deregulated cell cycle control, and environmental stress. Improved understanding of how cancer cells interfere with apoptotic pathways in contrast with normal cells is required for selectively killing cancer cells without affecting normal tissues. The conventional chemotherapy and radiotherapy will remain the mainstay in cancer treatment, however, specific apoptosis-targeted drugs will tip the balance in favor of death, thereby sensitizing tumor cells to lower doses of chemotherapy and reducing side effects. Thus, molecularly targeted drugs will be evaluated in select patient populations as a platform for combinatorial chemotherapy to achieve optimal synergistic effect.

Future targeted cancer therapy will be characterized by individualized treatment, matching the genetic lesions in tumors to the optimal agents. However, tumor cells represent a heterogeneous and constantly evolving population where multiple apoptosis resistance mechanisms may be involved or a single cell may have acquired mutations paralyzing more than one apoptotic pathway. Thus, for targeted therapy to work successfully, drugs that target common apoptotic pathways will be needed and alternatively, combinatorial chemotherapy will be more effective to achieve maximal efficacy. Because of the complex nature of cancer, analysis of clinical samples using genomic and proteomic arrays is necessary to study the impact of targeted drugs on apoptosis signaling molecules and to correlate the genotype of tumors with the therapeutic outcome of particular treatment regimens, thereby providing the basis for targeted, personalized cancer therapy.

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Chapter 6
Overcoming Resistance to Apoptosis in Cancer Therapy

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Keywords apoptosis, resistance, oncogene, melanoma

1 Introduction

A fundamental characteristic of cancer cells is suppression of apoptosis and increased cell survival.[1,2] These properties, when combined with deregulated cell proliferation, are the basic requirements for development of cancer. Increased deregulated cell proliferation by itself paradoxically may trigger cell death pathways which prevent outgrowth of the cancer cell unless the cell death pathways are inhibited.[3] Another consequence of the latter may be resistance to treatments that depend on induction of apoptosis in the cancer cell. These widely held concepts have given rise to intense study of the antiapoptotic mechanisms generated in different cancer cells that are driven by different oncogenic stimuli and how these mechanisms may operate against different therapies used against cancers. The mechanisms by which different therapies induce apoptosis are in turn poorly understood and answers to both questions are needed in development of effective treatment approaches. In the following sections, we review recent information about regulation of apoptosis, how oncogenes interact with apoptotic pathways, and some of the therapeutic opportunities that are developing as a consequence of this information. Emphasis is given to studies on melanoma as a model system in these developments.

2 Recent Concepts About Regulation of Apoptosis

Although apoptosis is traditionally described in terms of intrinsic and extrinsic pathways in most instances, apoptosis induced by oncogenes proceeds via the mitochondrial "intrinsic" pathway. Much is known about this pathway and in particular the proteins involved in regulation of the pathway.

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2.1 Bcl-2 Family Proteins in Regulation of Apoptosis

Apoptosis via the mitochondrial pathway is regulated by the Bcl-2 family of proteins which share at least one conserved Bcl-2 homology (BH) domain. The prosurvival Bcl-2 proteins share four such domains and act to protect intracellular membranes associated with mitochondria, nuclei, and endoplasmic reticulum. The proapoptotic Bax and Bak proteins have three BH domains and are located in the cytosol (Bax) and mitochondrial outer membrane (Bak). They are essential for apoptosis to proceed and mice lacking both genes have a number of developmental abnormalities.[1] Similarly, apoptosis of cancer cells induced by several chemotherapy agents is dependent on Bax.[4–6]

Once activated, Bax and Bak oligomerize and insert into the outer mitochondrial membrane and thereby cause the release of several factors from mitochondria that can trigger apoptosis. These include cytochrome-c, Smac/DIABLO, Omi, apoptosis-inducing factor (AIF), and endonuclease G. These factors are located in the membrane or intermembranous space between the outer and inner mitochondrial membranes. Two models have been proposed to explain the release of these proteins during apoptosis. In one model, an autonomous channel formed by Bax or Bak is formed and this allows the release of the factors from the intermembrane space.[2] Another model depends on specific interaction of Bax or Bcl-2 with components of the permeability transition pore (PTP), which exists at sites of contact between outer and inner mitochondrial membranes. This results in opening of the PTP, swelling of the mitochondrial matrix, and rupture of the outer mitochondrial membrane.[7]

2.1.1 Bcl-2 Sensor Proteins

The discovery of a third group of Bcl-2 proteins which share a single BH3 domain has had a major influence on concepts regarding initiation of apoptosis.[8,9] They are regarded as sensors of damage to cells and different members respond to a diverse array of damaging agents by activating the Bax/Bak proteins to damage mitochondria. Two of the members, Bid and Bim, may be able to directly cause changes in Bax and Bak, which result in their oligomerization and insertion into mitochondria.[9] The other members, such as Bad, Noxa, and P53-upregulated modulator of apoptosis (PUMA), appear to function by binding to and neutralizing the antiapoptotic proteins. In addition, they may displace other BH3 proteins such as Bid, Bim, and p53, which have the ability to activate Bax and Bak.[10]

Bid appears to mediate apoptosis induced by tumor necrosis factor (TNF) family ligands and by granzyme B from cytotoxic T lymphocyte (CTL). Bid is cleaved by caspase-8 at Asp59 into tBid or by granzyme B at Asp75 into active (gtBid) form.[11] tBid is able to cause oligomerization of cytosolic Bax or Bak associated with mitochondria which facilitates binding of the Bax/Bak oligomers to the outer mitochondrial membrane and release of aptogenic proteins as referred to above.

Several sensor proteins appear to be located in the cytoskeleton of cells. BimEL, BimL, and BimS are the main splice variants of Bim. BimEL is a relatively weak inducer of apoptosis and is degraded in proteasomes after phosphorylation by ERK1/2 on Serine 65. BimL and BimS appear to be the main inducers of apoptosis.[12,13] BimL (and BimEL) is associated with microtubules by attachment to the Dynein motor complex and is released from this by agents such as the vinca alkaloids and taxols. Once released into the cytosol, Bim binds to prosurvival Bcl-2 proteins (Bcl-2, Bcl-XL, and Mcl-1) and may also bind to and activate Bax similar to that proposed for interaction of tBid and Bax. In some hematopoietic cell types Bim is located predominantly with Bcl-2 antiapoptotic proteins on mitochondria. Bak may be activated simply by releasing it from the antiapoptotic Mcl-1 and Bcl-XL proteins (not Bcl-2) due to competitive binding of BH3 (Bim) proteins to antiapoptotic proteins.[9] Bmf is associated with the Actin Myosin V motor complex[14] and is released by anoikis (cell detachment) and appears to have more restricted binding to the antiapoptotic proteins Bcl-2 and Bcl-XL.[15]

Agents which damage DNA and upregulate p53 result in p53-dependent upregulation of several BH3 proteins, Bad, Noxa, and PUMA (see also Chapter 3). Noxa appears to bind predominantly to the antiapoptotic protein Mcl-1 and competitively inhibits binding of Bak to Mcl-1. This results in release of Bak, allowing it to oligomerize and bind to the outer mitochondrial membrane. PUMA is also believed to mediate its effects by binding to the antiapoptotic proteins Bcl-XL (and Bcl-2) and thereby cause the release of proapoptotic proteins bound to them. One of the proteins so released may be p53 itself, which may be able to induce mitochondrial permeabilization directly[16] or by binding to Bak on the outer mitochondrial membrane and thereby induce apoptosis.[17] This nontranscriptional role of p53 is dependent on it being transported into the cytosol[18] Noxa has a more restricted specificity to the antiapoptotic proteins Mcl-1 and A1.[19] In addition to transcriptional regulation by p53, Noxa may be increased by inhibition of proteasome degradation[20] and by a gamma-secretase tripeptide inhibitor.[21]

Another transcription target of p53 is a relatively little studied protein called PIDD, which is believed to combine with an adaptor protein, RAIDD, and form a complex with caspase-2 called the PIDDOSOME.[22] Caspase 2 is an initiator caspase that appears to act upstream of mitochondria. Substrates may include Bid[23] and PKCδ, as well as proteins in the Golgi apoptosis complex and cytoskeleton. It may have direct effects on mitochondria and cause release of aptogenic proteins.[24] Caspase 2 may also be activated by casein kinase 2 and sensitize cells to TNF-related apoptosis-inducing ligand (TRAIL) by processing of caspase 8.[25]

2.2 Inhibitor of Apoptosis Proteins

Apoptosis is also regulated by another family of proteins referred to as inhibitor of apoptosis proteins (IAPs).[26–28] These include IAP 1 and 2, XIAP, ML-IAP, and Survivin. In general, they bind to caspases and prevent their activation (caspase-9)

or inhibit their effector function (caspases 3 and 7). As discussed elsewhere, they also have other roles as E3 ligases and in ubiquination of proteins for degradation by proteasomes.[29] Binding of IAPs to caspases is competitively inhibited by Smac/DIABLO and OMI released from mitochondria and this allows effector caspases to induce apoptosis. This mechanism was shown to be the principal pathway in TRAIL-induced apoptosis of melanoma.[30]

3 Oncogenes and Apoptosis

3.1 Drivers of Cell Proliferation

Several transcription factors appear to be key players in cell proliferation, such as E2F, which is under the control of the Retinoblastoma protein (Rb), and c-Myc, which targets a number of proteins involved in cell division. Transition from G1 to S phase is regulated mainly by cyclin D, CDK4/6 complexes which phosphorylate Rb proteins and thereby activate E2F1–3 transcription of proteins involved in cell division. c-Myc is believed to play an essential role in this process by increasing cyclin D1/CK4/6 levels and suppression of CDK inhibitors such as p27.[31] Regulatory control of the G1/S transition is believed to vary widely between different tissues and different cancers. In melanoma the Ras and P1(3)K pathways appear to be key drivers of cell division in response to a number of mitogenic factors acting on Tyrosine kinase and G protein-coupled receptors. Activating mutations of BRAF are relatively common in melanoma[32,33] and naevi,[34] and have focused attention on this particular pathway. There is some evidence that loss of inhibitors of the cell cycle such as P16 may differentiate melanoma from naevi.[35] There may also be subsets of melanoma that are particularly dependent on constitutive activation of this pathway, e.g., melanoma in skin without signs of chronic sun damage were more likely (81%) to have activating mutations of N-RAS or BRAF than melanoma in chronic sun-damaged skin. The latter had increases in gene copies for Cyclin D1 (CCND1) and CDK4[36] but whether this is caused by elevated c-Myc levels, as discussed above, is not known. Immunohistological studies on nodular melanoma also found correlations with nuclear staining for Rb, Cyclin D1 and high mitotic rate measured by Ki-67 staining.[37]

3.2 Oncogene Pathways and Apoptosis

The transcription factors E2F and c-Myc target a number of genes involved in initiation of apoptosis. Most important of these is the transcription factor p53, which in turn targets genes for proteins such as PUMA, Noxa, PIDD, Bid, Bax, and Apaf-1 (see also Chapter 10). As discussed above, p53 itself may have a direct nontranscriptional role

in inducing apoptosis.[9] Oncogenes may also upregulate p53 via the alternate reading frame (ARF) of the CDKN2A gene and thereby increase p53 levels by inhibition of HDM2, which ubiquinates and degrades p53. c-Myc may also be more important in induction of ARF than E2F and in induction of Bim, which mediates p53-independent apoptosis.

Given the evidence that oncogenes may also drive apoptosis, the survival of cancer cells implies that cancer cells that have been selected by outgrowth of apoptosis-resistant cells. Rb is inactivated (and E2F thereby activated) in melanoma by high cyclin D/CDK4 levels associated with extracellular stimuli or mutated signal pathway intermediates such as BRAF. This is complemented in some cells by mutated CDKN2a genes and low p16 protein levels which normally would inhibit the CDKs. In normal cells the increased levels of E2F would induce proteins associated with apoptosis, but it is speculated that Rb may selectively dissociate from E2F promoter regions involved in cell cycle regulation, but may not dissociate from those inducing apoptosis.[38]

Mutations in CDKN2a are present in approximately 20% of patients with familial melanoma, but are uncommon in sporadic melanoma which account for over 90% of melanoma cases.[39] Mutations in the p53 gene is also uncommon in melanoma compared to some other cancers,[40,41] but were reported to be higher in melanoma from sun-exposed sites.[42] Protein levels of p53 appear to be elevated in 18–40% of melanoma.[40,43,44] The reasons for the elevated p53 levels are not clear. p53 in some melanoma appeared functionally inactive and could not induce cell cycle arrest.[45] This question is of much interest in view of reports of splice variants which may act like dominant negatives to inhibit the function of wild type p53.[46]

These studies on oncogenic pathways do not adequately explain a number of changes in cancer cells, e.g., in melanoma, contrary to expectations, the antiapoptotic protein Bcl-2 was reduced in progressive forms of the disease, whereas Bcl-XL and Mcl-1 increased in thick primary melanoma and in metastases.[47] The basis for elevation of Mcl-1 in melanoma is not clear. Activation of signal transducer and transcription activator 3 (Stat 3) by Src kinases in melanoma cells was reported to upregulate both Mcl-1 and Bcl-XL[48] and Stat 3 was regarded as a critical transcriptional activator of Mcl-1, Bcl-XL, and survivin.[49] Activation of Akt was also held responsible for upregulation of Mcl-1 levels in Cholangiocarcinoma cells.[50] Akt is frequently activated in melanoma and may therefore in part be responsible for elevation of Mcl-1. Mcl-1 levels were downregulated by the multikinase BRAF inhibitor BAY 43-9006 (Sorafenib), but this was apparently due to increased proteasome degradation.[51] Mcl-1 was reported to be ubiquinated by a specific Mcl-1 ubiquitin ligase E3 (Mule),[52] which also targets p53.[19,53]

The decrease in Bcl-2 expression in metastatic melanoma is also hard to explain. The microphthalmia-associated transcription factor (MITF) appears to be a key factor in its regulation. MITF in turn is regulated through the receptor c-kit and is believed to be responsible for differentiation and survival of melanocytes.[54] C-kit is downregulated in melanoma cells[55] and this may play some role via decreased activation of MITF in the decreased levels of Bcl-2. Another transcription factor regulating Bcl-2 and c-kit is activator protein 2 (AP-2).[56] This was shown to be lost in

progression of melanoma and loss of AP-2 was associated with short overall and relapse-free survival.[57] AP-2 proteins were reported to bind with p53 to p53 target genes such as p21 and so act as a tumor suppressor.[58] It is not clear why AP-2 is lost in melanoma but AP-2 appears essential for development of neural crest lineages.[56]

4 Signal Pathways Involved in Resistance of Cancer Cells to Cell Death

The above-mentioned studies suggest that activation of signal pathways may be all important in driving both cell division and resistance to apoptosis. Some of the principal pathways are described as follows.

4.1 The ERK1/2 Kinase Pathway in Inhibition of Apoptosis

The RAS, RAF, MEK ERK1/2 pathway has received particular attention in melanoma.[59] In previous studies on melanoma cell lines, we found that activation of this pathway was a common cause of resistance to apoptosis.[60] Similar results were found in studies on other cancers.[61] Activation of MAPK (ERK1/2) was detected more frequently in primary melanoma than in naevi, and activation of ERK was higher in thick melanoma and subcutaneous metastases.[62] Introduction of activated MAPK kinase into melanocytes resulted in tumorigenesis in nude mice.[63]

As discussed earlier, a high proportion of melanoma has activating mutations (such as the V600E) in BRAF downstream of Ras.[32] A smaller proportion has activating mutations in Ras that were exclusively seen in melanoma without BRAF mutations.[33] These findings suggested this pathway may be responsible for induction of melanoma, but this idea was tempered by the finding that benign naevi also frequently had BRAF mutations.[34] Further insights into growth arrest of naevi was the finding that expression of the p16 protein was high in naevi and may account for growth arrest of naevus cells despite activation of the ERK1/2 pathway. p16 expression was not uniform and other senescence-inducing factors were thought to be involved.[35] One study suggested that melanoma with BRAF mutations were more sensitive to MEK inhibitors[64] but this was not the finding in studies by Zhang et al.[60] Clinical responses to the BRAF inhibitor BAY 43-9006 (Sorafenib) also did not correlate with BRAF mutation in the melanoma.[65]

Apart from activating mutations of BRAF and NRAS, the RAS, RAF, and ERK pathways are activated by a number of external factors such as β3 integrin/adhesion interactions[66,67] and autocrine growth factors acting through receptor tyrosine kinases such as c-kit, IL-6, insulin growth factor, basic fibroblast growth factor (bFGF), hepatocyte growth factor.[59] Factors acting on G protein-coupled receptors such as MSH also activate adenylate cyclase and thereby RAS.[59]

Several target proteins in the apoptosis pathway are phosphorylated by the ERK1/2 kinases. BimEL is phosphorylated directly by ERK1/2 on Serine69 and possibly two other sites.[68] This promotes proteasomal degradation of BimEL and may prevent interactions with Bax. In both cases the effect is to limit apoptosis mediated by BimEL. It is not clear whether BimL is phosphorylated by ERK1/2.[68]

The ERK1/2 pathway has also been implicated in transcriptional upregulation of Mcl-1 by the transcription factor ElK-1[69] and of Bcl-2 and Bcl-XL.[61] Bcl-2 is known to be regulated by the microphthalmic transcription factor (MITF).[54] MITF, however, may be suppressed by activation of ERK perhaps due to degradation of the protein.[70] ERK phosphorylates and stabilizes c-Myc, which in turn induces cyclin D1 and cell proliferation.[2]

4.2 Inhibition of Apoptosis by Akt Signaling

An equally important cell survival signal pathway appears to be the Akt/PKB pathway. This is initiated by tyrosine kinase and G protein-coupled receptor activation of phosphoinositide-3-kinase PI(3)K, which in turn phosphorylates phosphatidylinositol biphosphate (PIP2) to PIP3. This causes translocation of PIP3 to the cell membrane and phosphorylation of Akt by phosphoinositide dependent kinase-1 (PDK-1) on threonine 308 and on Serine 473 in the hydrophobic tail by the rictor–m TOR complex.[71,72] Akt consists of three family members; Akt, Akt2, and Akt3. The latter appears to be preferentially upregulated in melanoma.[71]

Akt is constitutively activated in many melanoma cells[73] and is able to suppress apoptosis via a number of mechanisms. These include phosphorylation of forkhead transcription factors, which regulate several proapoptotic proteins such as Bim and Fas ligand. The phosphorylated forkhead proteins are trapped in the cytosol and cannot enter the nucleus. Akt also phosphorylates and inactivates several proapoptotic proteins such as Bad and caspase 9.[71,74,] Importantly, it activates I Kappa B Kinase (IKK) and thereby activates the transcription factor NF-κB, leading to transcription of several antiapoptotic proteins such as Bcl-XL, AI, and XIAP.[75]

The main factors involved in upregulation of Akt in melanoma are not clearly defined, but may result from growth factor stimulation of surface receptors as proposed for the RAS, RAF, MEK, and ERK pathway. Activating mutations of proteins in this pathway have not been described in melanoma, but have been in colon carcinoma (PDK1, ATK2, and PAK4).[76] Another possibility is that the downregulatory mechanisms in this pathway are abnormal, e.g., there has been much interest in PTEN status in melanoma as this phosphatase inactivates PIP3. Abnormalities in PTEN appear, however, to be a low-frequency event[77] and would not account for activation of this pathway in the majority of melanoma.

4.3 The Protein Kinase C Pathway in Apoptosis

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases comprising at least 11 isoforms that play fundamental roles in signal transduction pathways that regulate cellular proliferation, differentiation, and apoptosis.[78,79] Activation of PKC by phorbol esters (PMA) has been shown to have variable effects on apoptosis.[80–86] In particular, activation of PKCδ seemed proapoptotic,[80–82] whereas activation of PKCε and PKCα was antiapoptotic.[83–86] Activation of PKC has been reported to abrogate Fas-induced apoptosis through inhibition of death-inducing signaling complex formation by blocking Fas-associated death domain (FADD) recruitment and thus caspase-8 activation.[87–89] A similar mechanism has also been implicated in protection of HeLa cells from TRAIL-induced apoptosis.[90] Moreover, inhibition of TRAIL-induced apoptosis by PKC activation was suggested to occur at the level of proteolytic cleavage of caspase-8 or downstream of caspase-8-mediated Bid cleavage.[89,91]

The expression levels of PKCε may play an important role in determining sensitivity of melanoma to apoptosis induced by TRAIL.[92] This was supported by studies using an adenovirus vector expression systems to express PKCε in the PKCε-deficient melanoma cells, which reversed the potentiating effect of PMA on TRAIL-induced apoptosis whereas expression of a dominant-negative PKCε in PKCε-expressing Mel-RM cells reversed the protective effect of PMA on TRAIL-induced apoptosis. In contrast, PKCδ in melanoma cells increased TRAIL-induced apoptosis. Hence, activation of PKC by TRAIL may provide positive or negative regulation of sensitivity of cells to TRAIL-induced apoptosis depending on the levels of these two PKC isoforms. Activation of PKC was found to regulate TRAIL-induced apoptosis of melanoma by modulating Bax activation and did not cause significant changes in the expression levels of TRAIL death receptors, alterations in activation of caspase-8, or cleavage of Bid. The protective effect of PKCε was found in part to be associated with activation of ERK1/2 induced by TRAIL[60] as inhibition of ERK1/2 by the MAPK kinase-specific inhibitor partially reversed the protective effect on melanoma cells. In addition, activation of ERK1/2 was downstream of PKC as inhibition of PKC blocked TRAIL-induced activation of ERK1/2. These results suggest that measurement of the relative amounts of PKC isoforms may help define melanoma that is sensitive or resistant to treatment.

4.4 The Jun NH2 Kinase Pathway

The Jun NH2 kinase (JNK) pathway may be activated by TNF or TRAIL receptor-associated factors (TRAFs) or by environmental and genotoxic stresses such as ultraviolet (UV) or gamma radiation. The JNK proteins are coded for by three separate genes and these give rise to approximately ten different splice variants. All JNK are able to phosphorylate c-Jun and thereby upregulate activator protein-1 (AP-1)-dependent genes. One of the genes so regulated is Bim.[68] In addition, JNK

has important posttranscriptional effects on Bim, which include phosphorylation of the Bim motif binding to the Dynein motor complex of microtubules and thereby release of Bim into the cytosol.[93] In addition, JNK may phosphorylate Bcl-2 and Bcl-XL[94] and inhibit their ability to bind to the BH3-only sensor proteins such as Bim, PUMA, and human protein harakiri (HRK). It may also phosphorylate 14-3-3 proteins in the cytosol and promote translocation of Bax to mitochondria.[95] These proapoptotic effects of JNK may be inhibited by the Akt pathway.[96]

Activation of JNK by TRAIL was reported to occur predominantly via TRAIL-R2 rather than –R1 death receptors[97] and to involve FADD and caspase activation.[98] Subsequent studies showed that activation of JNK was dependent on formation of a secondary complex of FADD, TRAF2, RIP1, and IKK.[99] TRAIL may therefore mediate some of its apoptotic effects via the JNK Bim pathway. This secondary complex is also responsible for activation of NF-κB which exerts antiapoptotic effects by upregulation of antiapoptotic proteins Bcl-2, Bcl-XL, and A1, as well as the IAP proteins, some of which bind to and inhibit TRAF2.[100]

Inhibitors of JNK have attracted much attention, particularly in treatment of neurological diseases.[101] SP600125 is a direct inhibitor of JNK and has been used to treat arthritis in animal models. CEP-1347 acts to inhibit MAP kinases upstream of JNK. Peptide inhibitors that inhibit substrate-binding sites or regulatory regions have also been studied with some success.[101]

5 Therapeutic Opportunities

The rational development of treatments against cancer would ideally be based on the known oncogenic pathways involved and the resistance mechanisms which prevent oncogene-induced apoptosis. In practice, several factors act against implementing such an idealized approach. Principal among these is the heterogeneity of most solid cancers so that treatments focused on any particular pathway or against particular targets may only be effective against 10–20% of patients with particular cancers. The second limitation is the state of ignorance surrounding particular mechanisms involved in resistance to cell death.

5.1 Understanding how Commonly used Agents Kill Cancer Cells and Resistance Mechanisms Against them

In the case of melanoma with apparently normal p53 pathways, it should be a simple matter of using DNA-damaging agents such as Cisplatin or Doxorubicin to activate p53 and thereby the apoptotic pathway to cell death discussed earlier. In practice, melanoma shows low response rates to Doxorubicin and Cisplatin. In the case of Cisplatin, cell death, when it occurs, may be more related to necrosis induced by activation of poly (ADP-ribose) polymerase (PARP) in DNA repair and

consumption of ATP[102] as described by others.[103] In studies on melanoma cell lines several proapoptotic BH3 proteins (PUMA, Noxa, and Bim) appeared constitutively upregulated, but there was no evidence that this adversely affected the cell lines. The mechanism involved in resistance to apoptosis of these cells remains uncertain. Inhibition of ERK1/2 and Akt pathways increased apoptosis in the lines but other factors were clearly involved.

p53-independent initiators such as BimEL were also detectable in melanoma. Agents targeting microtubules such as the Taxols and Vinca alkaloids are postulated to release BimEL and possibly BimL from the microtubules. Nevertheless, studies on Doxetaxel showed a wide variation in susceptibility to Doxetaxel-induced apoptosis. ERK1/2 inhibitors potentiated apoptosis induced by Doxetaxel strongly in some melanoma cell lines and this was in proportion to activation of ERK1/2 by Doxetaxel. In contrast, there was a good correlation between activation of JNK and induction of apoptosis. Hence, in the case of Doxetaxel the relative activation of these two pathways appears to largely determine the overall degree of apoptosis[103a]. These results provide further support for the use of inhibitors of the RAF/MEK/ERK pathway in combination with Taxols. Similar results were seen with Vincristine, but the mechanism of induction of apoptosis differed from that of Doxetaxel[144]. Whether results from such studies can be utilized to define responsive tumor subgroups remains unknown. It is encouraging however to think that further insights into the mechanisms of induction and resistance to commonly used agents may help to define responsive tumor subsets.

Apart from more intelligent use of existing agents, studies over the past few years have generated a number of new agents designed to overcome resistance to apoptosis. These are summarized as follows.

5.2 Therapeutic Approaches Targeting Signal Pathways

5.2.1 The RAS, RAF, MEK, and ERK Pathway

Several inhibitors of this pathway have been produced, such as the Onyx/Bayer 43-9006 agent (Sorafenib)[104] and the Pfizer compound CI-1040.[105] In phase II studies with Sorafenib as a single agent there was only one response in 34 patients, but when given in combination with Carboplatin and Paclitaxel there were 20 partial responses and 26 with stable disease in 54 patients. Response rates in 23 previously untreated patients was 48%.[65] These results are now being tested in a randomized trial in previously untreated patients (ECOG trial 2603) and in previously treated patients (Onyx/Bayer [117118 protocol]).

RAS is upstream of RAF and requires a farnesyl group to be attached for membrane anchorage. It may therefore be possible to inhibit the pathway with inhibitors of farnesyl transferase. These have shown antitumor activity in

preclinical studies[106,107] and sensitized human melanoma cells to Cisplatin[108] but further evaluation is needed[108a]. Recent studies suggest that the MEK inhibitor, VO126, induced by upregulation of Bim and PUMA and down regulation of Mcl-1 (Wang et al).

5.2.2 Inhibitors of the Akt Pathway

Relatively few studies have been carried out with inhibitors of this pathway. PX-866 is a specific inhibitor of PI3K which was shown to have single agent activity and to enhance chemotherapy and radiation in preclinical studies.[109] Heat shock protein 90 (HSP90) is a chaperone for a number of signal proteins, including Akt and RAF. A geldanamycin derivative (17AAG) was shown to deplete Akt and cyclin D1 in melanoma lines.[110,111] Phase I studies have been conducted in patients with advanced malignancies and phase II studies on melanoma patients in the Memorial Sloan Kettering Institute are in progress. A more soluble preparation, referred to as KOS-953, is about to enter clinical trials (Kosan Biosciences, Inc.). A nonpeptide small-molecule compound API-59-OME was shown to inhibit Akt activity in ovarian carcinoma lines, but not a wide range of other kinases. Studies were in vitro.[112]

CCI-779, a rapamycin analogue, was tested in 33 patients with melanoma. Only one partial response was seen[113] but studies in combination with apoptosis-inducing agents may be needed. Rapamycin was found to inhibit activation of NF-κB by Doxorubicin but the mechanism of action appeared independent of P13K.[114] Specific inhibitors of NF-κB activation do not appear to have been clinically evaluated, but proteasome inhibitors such as PS-341/Bortezomib have been thought to act by inhibiting activation of NF-κB and account for its effects in potentiating chemotherapy[115] and radiotherapy[116] (also see Chapter 12). Nevertheless, proteasome inhibitors affect a wide range of apoptosis regulators. One study in fact found no effect on NF-κB activity, but instead apoptosis appeared to be due to upregulation of Noxa.[20] A number of agents inhibit NF-κB activation in vitro, such as Curcumin,[117] but are yet to be tested in vivo.

5.2.3 Protein Kinase C Inhibitors

PKC as a target for anticancer drugs has been recognized for some time. Bryostatin is an activator of PKC that has been evaluated in phase II trials in melanoma. No responses were seen when used as a single agent.[118,119] Aprinocarsen is an antisense reagent against the PKCα isoform that was tested in patients with ovarian carcinoma. But it had no activity as a single agent.[120] Some of the difficulty in evaluating such agents is the diverse functions of different PKC isoforms and cross talk with other signal pathways.[121] As noted earlier, TRAIL appears to activate the ERK1/2 pathway via PKC activation. Similarly, PKC may activate the JNK pathway in the presence of the receptor for casein kinase 1 (CK1).[122] These two pathways may have opposing effects on apoptosis and illustrate the potential difficulty in targeting PKC in treatment.

5.3 Histone Deacetylase Inhibitors

Histone deacetylase (HDAC) inhibitors may directly induce apoptosis of cancer cells, e.g., by activation of Bim or by a number of other mechanisms, as reviewed elsewhere.[123,124] Some drugs in this class, however, appear to have relatively weak direct cytotoxic effects, but may synergize with other agents such as TRAIL to markedly enhance apoptosis.[123,102] These drugs are discussed further in a Chapter 13.

5.4 Activating the Extrinsic Pathway

The agents discussed earlier are also applicable to attempts to treat melanoma by agents such as TRAIL or Fas Ligand. These pathways have several additional obstacles that may need to be overcome. Principal among these is the low or absent death receptor expression on many melanoma, particularly on fresh isolates.[125] The main death receptor for TRAIL, TRAIL-R2 (DR5), was shown to be transcriptionally regulated by p53 and non-p53-dependent mechanisms as reviewed elsewhere[126] and also discussed in Chapter 10. In melanoma mRNA for the death receptors appeared at normal levels and nontranscriptional events appeared more important in regulation.[127] It was shown in TRAIL-resistant colon carcinoma that TRAIL-R1 appeared located in the Golgi and treatment with tunicamycin resulted in upregulation of TRAIL-R1.[128] Similarly, tunicamycin was shown to upregulate TRAIL-R2 in prostate carcinoma cells.[129] These findings have been reproduced in cultured melanoma cells[143]. Further studies are needed to investigate their clinical applicability.

5.5 Agents Targeting Antiapoptotic Proteins

Arguably, some of the most exciting new agents are those being developed against the antiapoptotic Bcl-2 proteins (also see Chapter 8) and IAPs. A list of these is given in Table 6.1. Evaluation of most of these is at an early stage and only one at this stage has gone through to phase III clinical trials. This was the antisense molecule against Bcl-2. This particular trial did not reach its primary end point of an effect on overall survival when all patients were included but did so when only patients with normal lactic dehydrogenase (LDH) levels were included in the analysis. The trial has been criticized on several grounds, but clear benefit was seen in some patients. It is hoped that experience gained from this trial will be used to plan future trials. In particular, antisense agents against Mcl-1 would appear an attractive target in melanoma.

Table 6.1 Agents against anti-apoptotic proteins

Target/action	Drug	Study reference
Anti-apoptotic Bcl-2 proteins		
BH3 mimics	ABT-737 (Abbot)	130
	GX015–070, BL-193	131
	Gossypol	132
Bcl-2 antisense	Oblimersen (Genta)	133, 134
Bcl-XL antisense		135–137
Mcl-1 antisense		135, 138
Inhibitor of apoptosis proteins		
Smac/DIABLO mimics	IDN-13389 (Idun Pharmaceuticals)	139–142

6 Conclusion

The widely held view that the oncogenic process involves deregulated cell division as well as resistance to apoptosis has been useful in focusing attention on how cancer cells evade cell death induced by the many therapeutic agents available to treat cancers. Part of the evolution of this concept is the realization that the selection pressures acting against cancer cells generate a variety of defects in the cell death pathways. These selection pressures include apoptotic pathways generated by oncogenes, neighboring cells, or the immune system. One striking conclusion is that information about these resistance mechanisms is still very limited even in particular cancer types such as melanoma. In the case of killing by the immune system through TRAIL, individual cell lines can be identified with a variety of defects in the apoptotic pathway such as absence of caspase-8, loss of Bid, or the death receptors. Downregulation of death receptors seems a more general cause of resistance to TRAIL that may be a worthwhile target in therapy.

Inactivating mutations in the p53 pathway are well known and common in many cancer types, but we suspect this particular pathway may also be inactivated by other as yet poorly characterized mechanisms. Whether it will become the focus of new therapies is uncertain. Activating mutations in signal pathway intermediaries appears common, as discussed earlier, and perhaps provides the best therapeutic options with agents targeting such pathways. Experience with BAY-43-9006 (Sorafenib, Nexator) however indicates that it has a number of unwanted toxicities such as skin rashes, diarrhea, hypertension, and hand-foot syndrome. Much remains to be learnt about the basis of these toxicities and whether other agents may have different toxicity profiles.

Therapeutic agents also become part of the selective process acting to generate resistant cancer cells. It is well known that cancer, which recurs after treatment with chemotherapy often have increased growth rates and metastatic potential. The taxols also appear to activate antiapoptotic pathways such as the ERK1/2 MAP kinases. It is therefore quite possible that such agents will select cancer cells where

this pathway is dominant over proapoptotic mechanisms. Such insights should translate quickly into new protocols and provide optimism that agents for control of cancer may already be at hand provided we know how to use them and which cancers to use them against.

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Chapter 7
Trail Receptors: Targets for Cancer Therapy

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Abstract A human tumor cell's ability to avoid the normal regulatory mechanisms of cell growth, division, and death are the hallmarks of transformation and cancer. Numerous novel therapeutic agents currently in preclinical or clinical evaluation aim to revive the normal regulation or evade these regulatory defects and induce growth arrest and cell death. One of the cell death pathways that has garnered significant interest, as a potential target for therapeutic intervention, is the programmed cell death pathway regulated by the tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL-RS). Receptor agonist molecules including forms of the native ligand and monoclonal antibodies are being developed and tested as therapeutics in the treatment of human cancer.

Keywords apoptosis, monoclonal antibody, agonist, TRAIL, TRAIL receptor

1 Introduction

This review will focus on the tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) signaling pathway and the therapeutic agents currently in development that activate this cell death pathway as a treatment for cancer. The TRAIL receptors are an attractive therapeutic target because of their relatively restricted expression on tumor cells, their capacity, when activated, to induce cell death in a spectrum of human tumor cells and their ability to act in concert with various chemotherapeutic agents to promote tumor cell death. TRAIL agonists, including various forms of the ligand and agonist antibodies, have demonstrated significant antitumor activity in preclinical studies across a spectrum of different

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human tumors. Recently, some of these agents have begun evaluation in the clinical setting. In addition, emerging molecular therapies are being developed to act on specific key regulatory molecules in the TRAIL-R apoptosis pathway to complement the action of TRAIL-R agonists. The combination of receptor agonist activation and attenuation of the anti-apoptotic threshold with targeted molecular therapy holds promise as a rational approach to cancer treatment.

2 Trail Receptor Signaling

The ability to induce programmed cell death is mediated in all eukaryotic cells through distinct signaling pathways that are responsive to both external and internal inputs. A spectrum of sources can induce programmed cell death, including secreted and membrane-bound proteins, DNA damage, radiation, Ca^{+2} stress, viral and oncogenic transformation, and serum and growth factor starvation.

The active induction of cell death is effected through a family of related cell surface proteins and their cognate ligands. One of these death-inducing ligands, TRAIL, can instigate cell death through a cell surface receptor-mediated catalytic activation of a series of cysteine proteases, leading to cleavage of key cellular structural and signaling components. Binding to either of the competent TRAIL-R's by ligand or antibody agonists can activate this protease cascade through two distinct but intersecting pathways; an extrinsic receptor-mediated pathway and an intrinsic pathway associated with the mitochondria. TRAIL binds to five cognate cell surface receptors, but only two of these receptors TRAIL-R1 and TRAIL-R2, are death receptors that have the ability to transmit a complete death signal. TRAIL-R1 (DR4, TNFSFR10a) and TRAIL-R2 (DR5, TNFSF10b) are members of the TNF receptor superfamily (TNFRSF). Only these two receptors possess the capability to competently transmit a TRAIL death signal. The other members of this family capable of binding to the ligand, TRAIL; DcR1, DcR2, and osteoprotegerin, lack a required cytoplasmic signaling domain, known as a death domain (DD) (Table 7.1).

TRAIL-Rs exist as a functional homotrimeric subunit. Members of the TNFSFR can form and function as heterotrimers. The TRAIL-Rs have been identified in a heteromeric structure in cells transfected with TRAIL-R1 and TRAIL-R2 expression constructs. It is unclear whether this is a physiologically relevant formation as this heterotrimer has not been isolated in immunoprecipitation experiments from nontransfected cells. (Kischkel et al., 2000; Schneider et al., 1997). Although initial reports suggested that ligand is required for receptor trimerization, studies of

Table 7.1 TRAIL receptors

Receptor	TNFSF	Other names	Death domain
TRAIL-R1	10A	DR4, Apo2	Complete
TRAIL-R2	10B	DR5, TRICK, KILLER	Complete
TRAIL-R3	10C	Decoy receptor 1	None
TRAIL-R4	10D	Decoy receptor 1	Truncated
Osteoprotegerin	11B	OPG, OCIF, TR1	None

TNFR1 and Fas have demonstrated the presence of a preligand association domain (PLAD) that is required for ligand-independent trimerization. Interestingly, the PLAD domains interactions are very specific and only permit homotrimeric formations (Chan et al., 2000). This data suggests that TRAIL-R1 and TRAIL-R2 only form homotrimers. However, a recent report suggested that the TRAIL-binding decoy receptor, DcR1, may regulate TRAIL-R2 activity by forming a heterocomplex through the PLAD (Clancy et al., 2005). A common structural feature present in all TNFSF receptors is a series of extracellular cysteine-rich domains (CRD). The number of these domains can vary between different TNFSFRs from 1 to 6. Each CRD domain is defined by six highly conserved cysteines that form three intrachain disulfide bridges. TRAIL-R1 and TRAIL-R2 possess three such CRD repeats that contain seven intrachain disulfide bridges (Hymowitz et al., 1999; Locksley et al., 2001; Marsters et al., 1992; Mongkolsapaya et al., 1999). The TRAIL- Rs also possess a structural feature that is unique to death-inducing receptors in the TNFSFR. Each of the receptors in this class possesses a short (65–80 aa) cytoplasmic protein–protein domain that is required for interaction with a key adaptor protein that is required for transmission of the death signal. Consequently, this structure is known as the DD. Seven members of the TNFSFR, including TRAIL-R1 and TRAIL-R2, possess DD. (Igney and Krammer, 2002)

3 Trail Receptor Expression

Two of the most intriguing and attractive features of the TRAIL-Rs are that TRAIL-R1 and TRAIL-R2 are proapoptotic and that these two receptors are expressed on many types of tumor cells. These features make the proapoptotic TRAIL-Rs an extremely appealing target for the generation of therapeutic agents.

Surface expression of TRAIL receptors has been reported for both normal (Atkins et al., 2002; Dorr et al., 2002; Jo et al., 2000; Leverkus et al., 2000b; Mundt et al., 2003) and tumor cells (Arts et al., 2004; Ashkenazi et al., 1999; Bouralexis et al., 2004; Clodi et al., 2000; Cuello et al., 2001; Frank et al., 1999; Frese et al., 2002; Ibrahim et al., 2001; Mitsiades et al., 2000; Odoux et al., 2002; Shin et al., 2001; Song et al., 2003a; van Geelen et al., 2003; Vignati et al., 2002). Weak but detectable TRAIL-R1 and TRAIL-R2 expression has been identified by flow cytometry on the surface of a limited number of normal (diploid) cell types, including hepatocytes, keratinocytes, astrocytes, and osteoblasts (Atkins et al., 2002; Dorr et al., 2002; Jo et al., 2000; Leverkus et al., 2000a; Mundt et al., 2003). However, a broad spectrum of tumor cell types has been identified with variable levels of TRAIL-R1 and /or TRAIL-R2 including some examples of relatively high expression. Cells isolated from primary tumors of the lung (Odoux et al., 2002), blood (Cappellini et al., 2005; Clodi et al., 2000), skin (Song et al., 2003a), bone (Bouralexis et al., 2004), and the brain (Ciusani et al., 2005) have detectable cell surface expression of TRAIL-R1 and TRAIL-R2 by flow cytometry. Likewise, human tumor cell lines derived from carcinomas of the colon (van Geelen et al.,

2003), breast (Ashkenazi et al., 1999), ovary (Cuello et al., 2001; Vignati et al., 2002), thyroid (Mitsiades et al., 2000), lung (Frese et al., 2002), pancreas (Ibrahim et al., 2001), and liver (Griffith et al., 1998), as well as from melanomas (Song et al., 2003a), sarcomas (Bouralexis et al., 2003), and tumors of the brain (Song et al., 2003b), have variable and high-level FACS-detectable TRAIL-R1 and TRAIL-R2. In many tumor cell lines where resistance to TRAIL-R agonism was observed, the relevance of cell surface expression of the TRAIL-Rs was complicated by the fact that receptor levels did not have a role in regulating response. A clear relationship between receptor expression level and potential for activation of apoptosis through proapoptotic TRAIL-Rs has not been established. However, evaluation of receptor expression in a tissue context is desirable in understanding more about the TRAIL-Rs as targets of systemic therapies.

Antibody reagents specific for linear peptides of the C-terminal, intracellular portion of TRAIL-R1 and TRAIL-R2 have been utilized in studies of TRAIL-R distribution in tissues (Arts et al., 2004; Koornstra et al., 2003; Reesink-Peters et al., 2005; Spierings et al., 2003; Spierings et al., 2004). Arts et al. demonstrated that most ovarian tumors expressed one or both proapoptotic TRAIL receptors, and that TRAIL-R2 expression was increased after chemotherapy in paired samples collected pre-therapy and post-therapy. Likewise, Koornstra et al. highlighted that expression of these death receptors was increased in colon tumors vs normal colon, and that both TRAIL-R1 and TRAIL-R2 were detected on all adenomas and carcinomas evaluated. In parallel, Spierings et al. (2003) evaluated a large panel of stage III non-small-cell lung (NSCL) tumors ($n = 87$) and related the staining to available clinical outcome data. In this study, TRAIL-R1 was identified on essentially all specimens (99%), with staining often strongest at the basal cell layers in tumors with squamous differentiation. TRAIL-R2 was also identified on the majority of the specimens (82%); interestingly, TRAIL-R2 expression was correlated with increased risk of death (odds ratio 5.76). A second study by Spierings et al. (2004) evaluated distribution of TRAIL and TRAIL-RS on normal tissues from humans and chimpanzees. In this study, as with the tumor panels, there was fairly widespread labeling of tissues evaluated for both TRAIL-R1 and TRAIL-R2, but staining patterns were similar across the two species. Finally, a recent study by Reesink-Peters et al. evaluated the distribution of TRAIL-Rs and markers of proliferation and apoptosis in cervical neoplasia. TRAIL-R1 and TRAIL-R2 were each identified in >80% of the specimens evaluated, with slightly more staining for TRAIL-R2; however, there was no correlation of TRAIL-Rs to either proliferation or ongoing apoptosis in these specimens.

Interestingly, in several of the studies listed above, staining was often restricted to the cytoplasmic compartment; therefore, it is unclear whether this distribution is relevant to the potential activity of therapeutics that target the extracellular portion of the receptor. It should also be noted that the peptides used for immunization to produce these polyclonal antibodies include considerable homology between the published TRAIL-R1 and TRAIL-R2 sequences. Although these antibody reagents perform well for specific recognition of the linear peptide in a western blot, it may be difficult to demonstrate highly specific staining in an immunohistochemical

assay format where the receptor protein has not been denatured and stabilized as a linear peptide target.

Others have reported tissue distribution of the proapoptotic TRAIL-Rs using monoclonal antibody reagents raised against the extracellular domains of these TRAIL-Rs, including use on formalin-fixed tissues (Daniels et al., 2005), frozen sections (Strater et al., 2002a), or a fluorescence-based method of quantitative tissue staining (McCarthy et al., 2005). Daniels et al. reported widespread staining of TRAIL-R1 and TRAIL-R2 in both tumor and normal tissues, with tumors staining more intensely than the adjacent normal tissue, but noted that the staining was often patchy in breast carcinomas, and that there was much less staining than expected on lymphoid tumors. Strater reported widespread TRAIL-R1 and TRAIL-R2 staining in tumors of the colon, but reported also that there was a positive correlation between TRAIL-R1 expression and survival. In contrast, McCarthy et al. identified a strong negative correlation between TRAIL-R2 expression and survival in breast cancer, with TRAIL-R2 expression associated with increased node-positive tumors. The TRAIL-R2 specific monoclonal antibody described in these studies can also be used for flow cytometry applications for determination of surface receptor levels, but is not currently recommended by the manufacturer for immunohistochemical studies in tissue specimens.

In evaluating the distribution of potential targets for agonist TRAIL receptor antibodies, it was considered critical to focus efforts specifically on detection of the extracellular portion of these receptors in order to understand the distribution of the part of the receptor recognized by TRAIL-R agonists. Antibodies have been developed to TRAIL-R1 and TRAIL-R2, respectively and the specificity of these antibodies has been tested by western blotting, flow cytometry, and immunohistochemistry utilizing fixed and embedded cell pellets and xenografts. These antibodies have been utilized for development of sensitive and specific immunohistochemical tests for TRAIL-R1 and TRAIL-R2 in formalin-fixed tissue specimens as described (Roach et al., 2004). To evaluate TRAIL-R distribution using these tests, approximately 270 tumor and normal tissue specimens have been evaluated. A summary of the expanded results is presented in Figs. 7.1–7.3.

After screening several proprietary and commercially available antibodies selected for specificity to TRAIL-R1 or TRAIL-R2, we concluded that, for both TRAIL-R1 and TRAIL-R2, rabbit polyclonal antibodies represented the best option for developing these tests (Roach et al., 2004). These antibodies performed well on formalin-fixed paraffin-embedded tissues and had minimal background staining. Importantly, staining of sectioned cell pellets or xenografts of cell lines was consistent with receptor expression levels identified by other methods such as flow cytometry, using different TRAIL-R antibodies, and TaqMan to quantitate RNA levels ((Roach et al., 2004) and hierarchical genetic search [HGS] data not shown).

The mean staining scores were determined for the 10 tumor types for which there were at least 10 evaluable specimens, and are presented in Figs. 7.1 and 7.2. Like the other studies reported, we identified stronger staining for both TRAIL-R1 and TRAIL-R2 in tumor specimens overall than in normal tissues. In addition, for most tumor types, although granular to diffuse cytoplasmic staining was noted,

Fig. 7.1 General tumor survey for TRAIL-R1: Tumor types with at least 10 samples. Immunohistochemical score for TRAIL-R1 staining, using a subjective scale of 0–4+, is indicated for 10 general tumor types for which at least 10 specimens were evaluated. Circles indicate individual specimen scores, and the mean score is indicated by a bar for each tumor type. Abbreviations in tumor type: carcinoma (Ca), non-small-cell (NSC)

distinct membrane staining was also observed and was often the stronger pattern. We identified general patterns of relatively high staining in colorectal carcinomas and relatively little staining in most lymphomas, as has been reported by others (Daniels et al., 2005; Koornstra et al., 2003). However, we identified less staining overall, in both tumor and normal tissues, than in most other reports in the literature. Instead of >80% staining for each receptor in tumor tissues, an average of 43% of tumors scored at least a 2+ for TRAIL-R1 ($N = 235$), and an average of 64% of tumors scored at least a 2+ for TRAIL-R2 ($N = 227$). These scores were based upon both distribution and intensity of receptor staining (Fig. 7.3). It was noted that there was often heterogeneous staining of tumor cells within individual specimens, as well as occasionally between specimens from a single individual. There was more staining identified for TRAIL-R2 than for TRAIL-R1 in both normal and tumor tissues using this method. This was in agreement with other nonclinical data suggesting more widespread distribution of TRAIL-R2 than TRAIL-R1 on several human tumor cell lines.

Importantly, the potential clinical relevance of TRAIL receptor expression has yet to be determined. Agonist monoclonal antibodies specific for TRAIL receptors can cause decreases in cell line viability even in cell lines with very low surface TRAIL receptor as determined by flow cytometry (HGS data not shown). It is possible

Fig. 7.2 General tumor survey for TRAIL-R2: Tumor types with at least 10 samples. Immunohistochemical score for TRAIL-R2 staining, using a subjective scale of 0–4+, is indicated for 10 general tumor types for which at least 10 specimens were evaluated. Triangles indicate individual specimen scores, and the mean score is indicated by a bar for each tumor type. Abbreviations in tumor type: carcinoma (Ca), non-small-cell (NSC)

that immunostaining methodology on formalin-fixed tissues will not be sensitive enough to detect functionally meaningful levels of the extracellular portion of the receptor, which would be targeted by TRAIL-R agonists. However, this possibility cannot be fully evaluated in the absence of clinical outcome data. Therefore, the immunohistochemical staining of tumor tissue specimens from subjects enrolled in the ongoing clinical trial programs represents a critical next step in the development of these tests. In addition, further studies of the downstream signaling pathways will likely contribute to our understanding of the appropriate application of TRAIL-R agonist therapies.

4 The Extrinsic Pathway and the Death-Inducing Signaling Complex

There are two essential pathways for TRAIL-mediated cell death (see also Chapter 3): one "extrinsic," which is dependent on the signal from the ligand and activation of a TRAIL-R protein complex, which leads directly to cell death; the other,

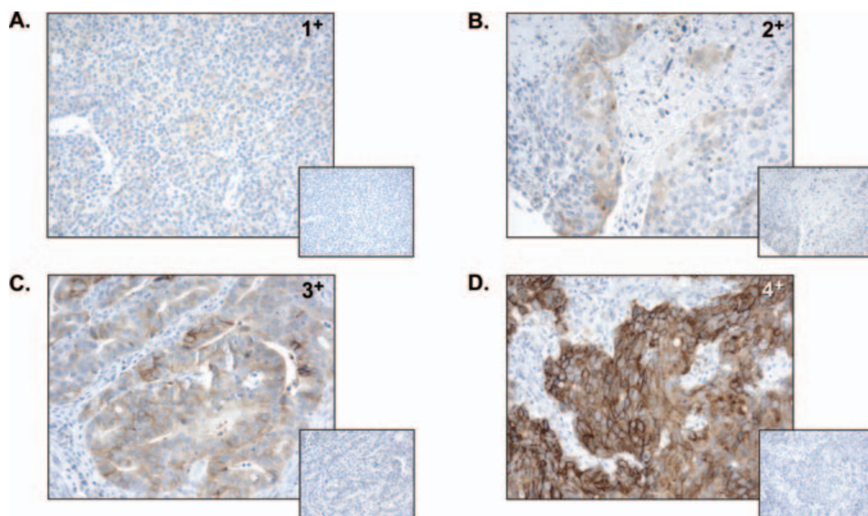

Fig. 7.3 Examples of IHC scoring scale for TRAIL-receptors. Panels A–D indicate examples of the 0–4+ scale used to evaluate TRAIL-R1 and TRAIL-R2 staining, and illustrate some of the typical patterns observed. Panel A, illustrating 1+ staining, has weak, but widespread, staining (non-Hodgkin's lymphoma); panel B, illustrating 2+ staining, has focally stronger staining of the tumor population (cervical carcinoma); panel C, illustrating 3+ staining, has widespread staining with variability in intensity (gastric carcinoma); and panel D, illustrating 4+ staining, considered exceptional, has uniformly strong staining of the tumor cell population, highlighting membrane areas and excluding nuclei (colon carcinoma). The smaller image includes the same field stained with a non-specific IgG as a control (scored 0). All photomicrographs were taken using a 20× objective

"intrinsic," which is activated by TRAIL binding to the TRAIL-Rs, but initiation of cell death is mediated through the mitochondria.

The extrinsic pathway of TRAIL-R cell death mediated through the formation of a ligand–receptor complex. Each DD on a TRAIL-R molecule interacts with a similar DD on a cytoplasmic adaptor protein, Fas-associating protein with a death domain (FADD). FADD acts as a bridge between the ligand–receptor complex and the receptor proximal caspase-8, through the death effector domain (DED). Transfection of dominant negative forms of FADD, or wild-type TRAIL-R1 or TRAIL-R2 into cells lacking FADD, blocks apoptosis demonstrating that FADD is a critical component of TRAIL-R signaling. Recently, it has been shown that the C-terminal tails of TRAIL-R1 and TRAIL-R2 are required for efficient FADD binding, caspase cleavage, and TRAIL-dependent apoptosis. (Ashkenazi, 2002; Bodmer et al., 2000a; Kuang et al., 2000; Luschen et al., 2000; Muhlenbeck et al., 1998; Thomas et al., 2004a; Yeh et al., 1998). The multiprotein complex of ligand, death receptor, adaptor, and protease is known as the death-inducing signaling complex (DISC). This signaling structure is unique amongst cell surface receptor signaling pathways for its threefold symmetry. The formation of this complex is a critical regulatory event in the process of apoptosis. Inactive caspase-8 molecules

are recruited into the DISC by FADD and are cleaved into active proteases through an unknown mechanism. It has been suggested in the “induced proximity” model that inactive initiator caspases brought into close proximity during DISC formation, promotes mutual cleavage and activation. (Boatright et al., 2003; Boatright and Salvesen, 2003; Muzio et al., 1998; Salvesen and Dixit, 1999). Autocleavage and activation of the receptor-associated caspase-8 leads to its release from the DISC and formation of heterodimeric active subunits. The initiator caspase is now able to target the “effector caspases” 3, 6, and 7. These terminal caspases, once activated, cleave key structural and signaling components of the cell and begin the physical destruction of apoptosis. This relatively short signaling cascade emphasizes the potential for rapid induction of cell death. Various apoptosis assays have demonstrated cellular and molecular changes associated with apoptosis appearing within 30 min after TRAIL-R engagement (Houghton, 1999; Walczak and Sprick, 2001). Importantly, the TRAIL-R pathway can activate cell death independently of p53, a primary target for apoptosis regulation by tumor cells (Galligan et al., 2005; Igney and Krammer, 2002; Wang and El-Deiry, 2003). Interestingly, p53 regulates expression of TRAIL-R2, suggesting p53 can increase sensitivity to TRAIL-R agonists in response to other apoptotic stimuli (Sheikh and Fornace, 2000).

5 The Intrinsic Pathway

The bridge from the DISC to the intrinsic pathway is formed through an intervening catalytic event. One of the cytoplasmic targets for the TRAIL-R-activated initiator caspases is the cytoplasmic protein Bid. Bid is a member of the Bcl-2 family of proteins responsible for regulating the mitochondrial pathway of apoptosis. In addition, the intrinsic pathway is also activated through several molecular monitors of cellular health such as p53 and AKT. In response to apoptotic stimuli from various metabolic and structural insults, including DNA damage, serum starvation and radiation, there is a loss of mitochondrial membrane integrity that precipitates the activation of another initiator caspase, caspase-9, and subsequently the effector caspases. Here, at the mitochondria the two pathways of apoptosis intersect emphasizing the importance of the regulation of this intersection.

The cleavage of Bid by caspase-8 creates a truncated form of Bid (tBid) that can translocate to the mitochondrial membrane (Srivastava, 2001). Bid is thought to form a heteromeric complex with other apoptosis-promoting molecules, Bax and Bak (Luo et al., 1998; Wei et al., 2000). Bax is liberated from its complex with the antiapoptotic protein Bcl-2 in response to apoptotic stimuli. This translocation of Bax or Bid to the mitochondrial membrane disrupts membrane integrity and induces release of cytochrome *c* and the formation of a protein complex known as the apoptosome (Adams and Cory, 2002). The apoptosome is comprised of cytochrome c, pro-caspase-9 and a scaffolding protein, apoptotic protease activating factor (APAF1). APAF1 forms a heptamer after binding cytochrome *c* and recruits several molecules of pro-caspase-9 through reciprocal caspase recruitment domains

(CARD) present in APAF1 and caspase-9 (Pan et al., 1998). This recruitment and oligomerization leads to caspase-9 activation and suggests again a role for the induced proximity model of caspase activation. Deletion of APAF1 demonstrates its necessary role in caspase-9 activation (Yoshida et al., 1998). Active caspase-9 can now cleave and activate the executioner caspase-3, caspase-6, and caspase-7.

The intrinsic pathway can be activated independent of the TRAIL-R pathway through other signals such as those transmitted by p53. One of the dominant mechanisms of chemotherapeutic resistance in cancer cells is the gene deletion or acquisition of inactivating mutations in TP53. Conversely, the ability of the TRAIL-R pathway to bypass the loss or inactivation of p53, via Bid cleavage, and still induce apoptosis through the mitochondria is one of the distinct advantages of targeting the TRAIL-Rs. Therefore, there are two pathways, extrinsic and intrinsic, for activation and execution of the TRAIL-R-mediated signals that lead to cell death.

6 Regulation of Death Signaling

Not surprisingly, given the activation of the death signal and its resulting dire consequences for the cell, the apoptotic pathway is highly regulated at several key points. Importantly, tumor cells have exploited these normal regulatory check points through acquired or induced modifications to attenuate the activity of caspases, alter the formation or composition of the DISC, or alter the interaction of intrinsic apoptosis regulatory proteins Bcl-2 and Bax, or their family members (Igney and Krammer, 2002).

FLICE-like inhibitory protein (FLIP) is a dominant negative form of caspase-8 that competes with caspase-8 for binding in the DISC. FLIP plays an important role in regulating sensitivity to TRAIL signaling (Griffith et al., 1998). Chemotherapy or FLIP siRNA can modify FLIP levels in tumors and promote TRAIL-induced apoptosis (Chawla-Sarkar et al., 2004; Galligan et al., 2005; Kang et al., 2005; Song et al., 2003a; Xiao et al., 2005). Interestingly, in support of the data that FLIP receptor complexes exist prior to ligand binding, a peptide sequence at the COOH terminus of FLIP (L) and TRAIL-R2 interact preventing FADD binding to TRAIL-R2. Upon ligand binding, FLIP is dislodged and a competent DISC is formed (Jin et al., 2004). The intimate interaction of FLIP with the receptor makes it an attractive target for pharmacologic intervention (Roth and Reed, 2004).

All of the apoptotic caspases described are regulated not only by a requirement for death receptor- or mitochondrial-mediated cleavage, but also by endogenous inhibitory proteins as well. These caspase-inhibitory proteins contain a protein interaction domain that classifies them as inhibitor of apoptosis proteins (IAPs). Their baculovirus IAP repeat (BIR) domains are zinc-binding folds that play a role in forming binding grooves for the active caspase. Once bound within the groove, caspase-9 cannot self-activate. Several members of this family are overexpressed in tumors (Igney and Krammer, 2002). Interestingly, the protein SMAC/DIABLO

Table 7.2 Tumor modifications of the extrinsic and intrinsic pathways

Location	Target	Modification and consequence	References
Upstream of mitochondria	AKT	AKT constitutive activity promotes Bad phosphorylation	Bortul et al. (2003); Cenni et al. (2004); Chen et al. (2001); Whang et al. (2004)
Upstream of mitochondria	PTEN	Loss of PTEN yields an inability to dephosphorylate AKT	Deocampo et al. (2003); Nesterov et al. (2001)
Upstream of mitochondria	Bcl-2	Overexpression, blocks apoptosis	Nencioni et al. (2005)
Upstream of mitochondria	Bcl-XL	Overexpression, blocks apoptosis	Dole et al. (1995); Foreman et al. (1996); Nagane et al. (1998)
Upstream of mitochondria	Mcl-1	Overexpressed in AML, blocks apoptosis	Kaufmann et al. (1998); Taniai et al. (2004); Yu et al. (2005)
Downstream of mitochondria	Survivin	Overexpressed in neuroblastoma blocks apoptosis	Adida et al. (2000); Kim et al. (2005); Wang et al. (2005); Yamaguchi et al. (2005b)
Downstream of mitochondria	cIAP2	Gene rearranged in MALT	Dierlamm et al. (1999)
Downstream of mitochondria	ML-IAP	Overexpressed in melanoma	Vucic et al. (2000)
Downstream of mitochondria	APAF1	Loss of APAF1 Blocks Caspase 9	Soengas et al. (2001); Soengas et al. (2006)
Downstream of mitochondria	XAF	Binds to XIAP	Leaman et al. (2002)
Receptor complex	Caspase-8	Methylation of gene represses expression, blocks cytoplasmic apoptosis signal	Ashley et al. (2005); Poulaki et al. (2005); van Noesel et al. (2003); Zuzak et al. (2002)
Receptor complex	TRAIL-R1, TRAIL-R2	Point mutations and genetic deletion	Fisher et al. (2001); Kuraoka et al. (2005); McDonald et al. (2001); Ozoren et al. (2000); Pai et al. (1998); Wolf et al. (2006)
Receptor complex	Decoy receptors, DcR1, DcR2	Occasional elevated expression	Meng et al. (2000)
Upstream of mitochondria	Bax	Inactivating mutation prevents apoptosis	Rampino et al. (1997); Zhang et al. (2000); Ionov et al. (2000)
Upstream of mitochondria	c-Myc	Represses FLIP expression	Ricci et al. (2004)
Downstream of mitochondria	SMAC/ Diablo	Reduced release of SMAC/Diablo	Zhang and Fang (2005)

is released from the mitochondria and antagonizes the binding of caspase-9 to the IAP family member, X-linked IAP (XIAP), thereby promoting apoptosis (Ng and Bonavida, 2002). A summary of the known modifications of proteins involved in regulating apoptosis found in tumor cells is described in Table 7.2.

7 Agonists of the Trail-R Apoptotic Pathway

7.1 Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Members of the tumor necrosis factor (TNF) superfamily have demonstrated the ability to induce apoptosis in virally and oncogenically transformed cells, human tumor cell lines and activated lymphocytes, NK, and monocytes (TRAIL, TNFα, TNFβ, and FASL). The ability to induce cell death, 14 other TNF ligands possess a diverse array of immunomodulatory and growth-stimulatory capabilities, including stimulation and proliferation of B-cells (BLyS) and T-cells (CD40L, LIGHT, OX40L, and 4–1-BBL) and regulation of bone metabolism (RANKL) (reviewed in Locksley et al. (2001) and Ashkenazi (2002)). TRAIL is a type II membrane-bound protein which exists as a self-assembling homotrimeric molecule that possesses apoptotic activity in a membrane bound or soluble form. The membrane form can be cleaved from the cell surface by an extracellular cysteine protease (Lawrence et al., 2001; Mariani and Krammer, 1998). TRAIL exists as a trimer in solution and requires elemental Zn+2 and a cysteine residue to coordinate and properly organize the trimeric structure. (Hymowitz et al., 2000) TRAIL that is generated in the absence of zinc permits the formation of cysteine disulfide bonds that result in an asymmetric molecule, which is less stable and insoluble in solution (Ashkenazi, 2002; Lawrence et al., 2001). Crystal studies of the ligand bound to TRAIL-R2 have revealed that the inverted pyramid-shaped trimeric ligand binds in the pocket between three receptor molecules (Hymowitz et al., 2000; Mongkolsapaya et al., 1999).

Cell surface expression of the ligand TRAIL has been observed on a variety of immune cells including IL-15- or IL-2-activated NK cells, virally infected T-cells, interferon gamma-activated monocytes, and dendritic cells, as well as CD4+ and CD3+ T-cells. TRAIL can confer tumoricidal activity to monocytes and NK cells and plays a role in immune surveillance against tumor development (Kayagaki et al., 1999a, b; Mariani and Krammer, 1998; Nieda et al., 2001; Takeda et al., 2002). Recently, a "window of TRAIL sensitivity" was observed in CD34 erythroid progenitor cells that is promoted initially by the expression of TRAIL-Rs and then inhibited by intercellular expression of Bcl-2 (Mirandola et al., 2006a). TRAIL has also been detected on the surface of colonic epithelium (Strater et al., 2002b). The soluble and membrane-bound form of TRAIL-induced apoptosis in a wide variety of human tumor cells both in vitro and in vivo without affecting the viability of normal cells.

Several forms of recombinant TRAIL have been generated to evaluate the ligand in preclinical studies. Histidine-tagged (Pitti et al., 1996), leucine zipper (Walczak et al., 1999), Flag-tagged (Bodmer et al., 2000b; Schneider and Tschopp, 2000), and Zn+2-stabilized versions (Ashkenazi and Dixit, 1999; Kelley et al., 2001) have all been generated and tested for activity against tumor and normal cells in preclinical studies. These different forms of the ligand have

displayed a spectrum of antitumor activity in human cell lines in vitro, in xenograft models and primary tissues transplanted into nude mice. TRAIL, either alone or in combination with chemotherapeutic agents, has demonstrated apoptosis activity in tumor cell lines derived from a broad array of human tumors including colon, brain, uterus, ovary, liver, breast, prostate, kidney, liver, lung, thyroid, and blood (Asakuma et al., 2003; Ashkenazi et al., 1999; Bouralexis et al., 2003, 2004; Chen et al., 2003; El-Zawahry et al., 2005; Jazirehi et al., 2001; Jeon et al., 2003; Keane et al., 1999; Kelly et al., 2002; LeBlanc and Ashkenazi, 2003; Miao et al., 2003; Mitsiades et al., 2001a; Muhlethaler-Mottet et al., 2004; Nagane et al., 2001; Naka et al., 2002; Ohtsuka et al., 2003; Pitti et al., 1996; Secchiero et al., 2002; Singh et al., 2003; Srivastava, 2001). TRAIL can overcome chemoresistance or radioresistance when administered in combination with chemotherapy in adriamycin-resistant myeloma, radio-resistant lymphoma, and taxane- and platinum-insensitive breast and osteosarcoma cell lines (Belka et al., 2001; Clayer et al., 2001; Cuello et al., 2001; Evdokiou et al., 2002; Frese et al., 2002; Jazirehi et al., 2001; Johnston et al., 2003; Keane et al., 1999; Liu et al., 2001; Mitsiades et al., 2001b; Nagane et al., 2000, 2001; Voelkel-Johnson, 2003).

While the epitope-tagged forms of the ligand assisted the isolation and purification of the recombinant protein, and in many instances enhanced the activity of TRAIL, they also enhanced the toxicity on normal cells. HIS-tagged, leucine-zipper or Flag-tagged antibody cross-linked forms of TRAIL-induced apoptosis in normal hepatocytes in vitro(Jo et al., 2000; Lawrence et al., 2001). Conflicting results were obtained when no apoptosis was observed with soluble TRAIL administered to normal primary cells from the lung, bone, liver, endothelium, breast, brain, and kidney (Ashkenazi et al., 1999). Safety studies of Zn2+-stabilized TRAIL administered in short-term treatment of mouse, monkey, and chimpanzees showed no detectable toxicities (Lawrence et al., 2001). Additional studies with soluble TRAIL were performed in chimeric mice whose livers were reconstituted with human hepatocytes. Repeated injection of soluble nontagged form of TRAIL did not generate any hepatotoxicity (Hao et al., 2004). These conflicting results suggested that nonphysiologically or inappropriately aggregated forms of TRAIL can be toxic. Whereas a soluble, correctly organized Zn2+-stabilized TRAIL was not toxic. It is important to note that there is a role for native TRAIL in response to inflammation or infection. Acute bacterial or viral infection of the liver or pancreas or in mouse models of hepatitis or pancreatitis TRAIL can induce apoptosis (Mundt et al., 2003; Hasel et al., 2003). Membrane-bound TRAIL has been shown to induce liver damage in adenoviral-transfected hepatocytes in vivo (Ichikawa et al., 2001). These types of responses coincide with the predicted role for TRAIL in mediating an immune surveillance response to acute bacterial- or viral-induced infection or inflammation.

The substantial preclinical antitumor data observed with the ligand implied that TRAIL-R agonism could potentially yield significant clinical antitumor activity. In fact, a recombinant form of the TRAIL ligand is currently is phase 1 clinical development. Nonetheless, this optimism should be tempered with the knowledge that certain versions of the TRAIL ligand, albeit in nonphysiological forms, did induce

severe cytotoxicity of normal cells. Therefore, clinical development should be prudently conducted with awareness toward potential indicators of toxicity.

7.2 Antibodies

A spectrum of mouse and human monoclonal and polyclonal antibodies has demonstrated the ability to agonize the TRAIL-Rs and induce death in tumor cells. They have proven to be valuable tools to explore mechanism of action, define chemotherapeutic combinations agents that enhance apoptosis, and describe functional differences between the TRAIL-R1 and TRAIL-R2 pathways. Importantly, human monoclonal antibodies selected for high-affinity binding and maximal agonism have been advanced into clinical development as therapeutic cancer agents.

Experiments using antibodies, which target the TRAIL-Rs, revealed that only TRAIL-R1 and TRAIL-R2 were capable of inducing apoptosis and not the decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) (Griffith et al., 1999). TRAIL-R2 specific antibodies when cross-linked, generated a distinct activation of NF-κB, apoptosis, and Jun NH2 kinase (JNK) activation compared to the NF-κB activation and apoptosis induced by cross-linking of TRAIL-R1 antibodies (Muhlenbeck et al., 2000). Mouse monoclonal antibodies against TRAIL-R1 were potent agonists in vivo but minimally active in vitro. In vitro activity was enhanced by secondary cross-linking antibodies, presumably through multimerization of receptor complexes. These antibodies, however, were very active against human xenografts when administered in vivo (Chuntharapai et al., 2001; Griffith et al., 1998). This result suggested that the mouse contributed a cross-linking function possibly through Fc receptors on immune cells. However, the use of agonist antibodies of immunoglobin isotypes that preferentially bind to Fc receptors or have the ability to fix complement were not significantly more active in vivo suggesting that this is not the mechanism that enhances these antibodies in vivo.

Receptor-specific antibodies selected for high-affinity binding and their TRAIL-R agonism have been identified and generated from phage display libraries, hybridomas, and transgenic mice containing human immunoglobin genes (Dobson et al., 2002; Ichikawa et al., 2001; Motoki et al., 2005; Pukac et al., 2005).

TRA-8, an agonist mouse monoclonal antibody to the human TRAIL-R2, was generated by immunization of mice with the extracellular domain of human TRAIL-R2 fused to the Fc portion of human IgG_1. TRA-8 bound to TRAIL-R2 specifically induced apoptosis in human T-cell leukemia, B-cell lymphoma, and glioma lines, and enhanced antitumor activity in combination with chemotherapeutic agents including Adriamycin (doxorubicin hydrochloride) and cisplatin. In TRA-8-resistant glioma lines sensitivity was restored after overexpression of Bax mediated by adenoviral transfer. Importantly, TRA-8 was also tested against hepatocytes in vitro and did not display any evidence of apoptosis (Choi et al., 2002; Ichikawa et al., 2001; Kaliberov et al., 2004; Ohtsuka et al., 2003).

Human Genome Sciences in collaboration with Cambridge Antibody Technology generated a series of fully human monoclonal antibodies, which target TRAIL-R1 or TRAIL-R2. The most active of these candidates were selected for evaluation in preclinical studies and are now advancing through clinical development. HGS-ETR1 (mapatumumab) an antibody specifically targeting TRAIL-R1, demonstrated potent in vitro apoptotic activity against human tumor cell lines derived from colon, lung, pancreas, ovary, uterus, renal, and hematologic malignancies. This in vitro activity was achieved in the absence of cross-linking agents. HGS-ETR1 enhanced the cytotoxicity of chemotherapeutic agents (camptothecin, cisplatin, carboplatin, or 5-fluorouracil) even in tumor cell lines that were not sensitive to HGS-ETR1 alone. In preestablished colon, NSCL, and renal xenografts, HGS-ETR1 treatment resulted in rapid tumor regression or repression of tumor growth. Addition of chemotherapeutic agents like topotecan, 5-fluorouracil, and irinotecan in colon xenograft models enhanced antitumor efficacy and in some models a synergistic antitumor activity was observed (Pukac et al., 2005).

Phase 1 trials of HGS-ETR1 have been conducted in advanced solid tumor patients and have demonstrated the safety and tolerability of single agent HGS-ETR1 up to 20 mg/kg. Single agent phase 2 studies were conducted in colorectal cancer, non-small-cell lung carcinoma (NSCLC), and non-Hodgkin's lymphoma (NHL). While stable disease was the best response observed in the two solid tumor studies, objective responses, including one complete response, were observed in the NHL study. Further, phase 1b studies have demonstrated that HGS-ETR1 can be safely administered in combination with standard doses of chemotherapy agents, such as carboplatin and paclitaxel. Additional phase 2 studies are planned to assess the activity of HGS-ETR1 in combination with chemotherapy.

HGS-ETR2 (lexatumumab), a fully human antibody identified via screening of phage display libraries for high-affinity, single-chain antibodies to TRAIL-R2, has been evaluated in similar human tumor cell lines for apoptotic activity. HGS-ETR2 produced potent apoptotic activity in a spectrum of human tumor cell lines including NSCL, colon, renal, and ovarian in the absence of cross-linking agents. (Alderson et al., 2003; Humphreys et al., 2003; Johnson et al., 2003, 2004). HGS-ETR2 has demonstrated the ability to enhance the activity of chemotherapeutic agents from various classes including taxanes and platinums (Georgakis et al., 2003; Humphreys et al., 2003; Johnson et al., 2004; Zeng et al., 2006). HGS-ETR2 induced cell death in two human RCC cell lines and nine human primary RCC cell cultures. This in vitro effect was enhanced with addition of a cross-linking antibody. In a renal xenograft model using primary renal carcinoma tumor cells HGS-ETR2 was able to induce tumor regression (Zeng et al., 2006). HGS-ETR1 and HGS-ETR2 were effective in cell lines from multiple myeloma, acute lymphoblastic leukemia (ALL), NHL, and chronic myelogenous leukemia and in primary hematological tumor cells from NHL, chronic lymphocytic leukemia, and multiple myeloma patients (Georgakis et al., 2003; Johnson et al., 2003). Phase 1 trials of HGS-ETR2 have been conducted in advanced solid tumor patients. This agent has demonstrated that it can be safely and repetitively administered up to 10 mg/kg. The results of the phase 1 studies support

the additional study of HGS-ETR2 in phase 2 trials to evaluate its potential for use in the treatment of cancer.

Another TRAIL-R2 mAb (HGS-TR2J, KMTR2) was identified in collaboration between Human Genome Sciences and Kirin Brewery, Inc. This agonist antibody, derived from transchromosomal mice expressing human Ig locus, showed in vitro and in vivo activity against human tumor cell lines. Importantly, HGS-TR2J generated significant apoptotic activity without cross-linking and was active in many human tumor cell lines. It was also shown that ligation of HGS-TR2J to cell surface receptors induced clustering of TRAIL-R2 (Motoki et al., 2005). HGS-TR2J is currently in phase 1 clinical development.

7.3 Agonist Signaling

Receptor oligomerization is potentially a key event in TRAIL-R signaling. In vitro and in vivo experiments have shown that cross-linking TRAIL-R agonists, including various forms of the recombinant ligand and antibodies, altered antitumor activity. Antibodies, because of their bivalent binding, have the potential to oligomerize receptor molecules, which could lead to activation of TRAIL-R signaling, DISC formation and cell death. The recombinant ligand, generated in several forms that permitted cross-linking or aggregation, demonstrated potent antiapoptotic activity. Additionally, chemotherapeutic treatment has been able to induce TRAIL-R1 and TRAIL-R2 receptor aggregation and enhance apoptosis (Bergeron et al., 2004; Delmas et al., 2004). Experiments have shown that cross-linking an agonist, including the ligand and antibodies, can improve apoptosis in vitro. Even in those experiments where cross-linking was required for activity in vitro, agonists were readily effective in vivo without cross-linking. In addition, some agonists can achieve maximal apoptosis activity without any enhancement from in vitro cross-linking. This conflicting data suggests several possible mechanisms of killing by TRAIL-R agonists.

Conceivably, both cross-linking-dependent and cross-linking-independent mechanisms may exist for TRAIL-R agonists. Where cross-linking is involved in vivo this function may be provided by the host through immune cells that can cross-link IgG molecules, i.e., Fc receptors. Alternatively, in the absence of cross-linking a TRAIL-R agonist could bind to the trimerized receptor and induce a conformational change similar to the alteration that is theorized to occur with the native ligand. Conformational change in the receptor could expose relevant binding domains on FADD and induce the apoptotic cascade. In fact, the ability to expose different protein-binding domains of FADD has been observed with TRAIL-R agonist antibodies (Thomas et al., 2004b). The ability to cross-link cell surface receptors with antibodies induces capping and increases agonistic activity that has been shown in other signaling systems including those within the TNFSFR. (Cremesti et al., 2001; Liu et al., 2003; Ludwig et al., 2003; Miller et al., 2003). While the precise nature of the interaction between TRAIL-R agonists and the formation of the DISC

remains to be determined, their ability to activate this pathway and induce tumor cell death has been proven in preclinical studies and is being validated in the clinical setting.

8 Agents Targeting the Apoptosis Pathway

The availability of human tumor cell lines that are refractory to TRAIL-R agonism has allowed exploration of potential mechanisms of resistance (Igney and Krammer, 2002; Wang and El-Deiry, 2003). Both extrinsic and intrinsic regulatory proteins have been blamed for this resistance, including FLIP (Griffith et al., 1998; Kim et al., 2000; Leverkus et al., 2000a), XIAP, survivin (Kim et al., 2004) Bcl-2 (Fulda et al., 2002a), and Bax (Deng et al., 2002; He et al., 2003; Kandasamy et al., 2003; LeBlanc et al., 2002). Genetic alterations have been identified in TRAIL-R1 and TRAIL-R2 in NSCLC, colon cancer, head and neck cancer, and lymphoma. Some of these modifications induced a loss of apoptotic signaling. Unfortunately, their role in TRAIL resistance in the clinic has not been validated. (Arai et al., 1998; Fisher et al., 2001; Jeng and Hsu, 2002; Lee et al., 1999; Ozoren et al., 2000; Pai et al., 1998; Wolf et al., 2006; Wu et al., 2000). Changes in the level of cell surface receptor expression, caspase-8/FLIP ratio and loss of caspase-8 have all been discovered as mechanisms of resistance to TRAIL-R agonism (Poulaki et al., 2005; Van Geelen et al., 2004; Wachter et al., 2004).

Consequently, many strategies have been evaluated for their ability to enhance sensitivity or maximize responsiveness to TRAIL-R agonism. Early obvious strategies involved combining standard, approved chemotherapeutic agents with TRAIL-R agonists. Chemotherapy agents or radiation improved response in breast, colorectal, and NSCLC cell lines that displayed resistance to TRAIL-R agonism (Adams and Cory, 2002; Ganten et al., 2005; Kondo et al., 2006; Wendt et al., 2005; Zhang et al., 2005). The use of chemotherapy agents modified levels of specific molecules including TRAIL-R1, TRAIL-R2, FLIP, XIAP, or the proapoptotic protein Bad and restored TRAIL-R responsiveness (Fesik, 2005; Galligan et al., 2005; Mirandola et al., 2006b; Xiao et al., 2005; Yamaguchi et al., 2005a). Other strategies have targeted specific molecules known to regulate the pathway at important catalytic or survival signaling steps. For example, many new compounds have targeted the ubiquitous, antiapoptotic protein Bcl-2, or related family members, through antisense or small molecules (Chawla-Sarkar et al., 2004; Sinicrope et al., 2004; Zhu et al., 2005a) (Table 7.3) (also see Chapter 8). Oblimersen sodium (Bcl-2 antisense) as a single agent or in combination with chemotherapy has shown some clinical activity. (Marcucci et al., 2005; O'Brien et al., 2005; Tolcher et al., 2005). Many strategies are focused on the elimination or reduction of inhibitors that block activation of the initiator caspase-8 and caspase-9, namely XIAP, survivin, and FLIP. Small-molecule and antisense techniques have yielded promising results in preclinical models. FLIP, survivin, and XIAP inhibitors in combination with TRAIL-R agonists have significantly enhanced apoptosis across

Table 7.3 Apoptosis therapeutics in development

Compound	Type	Target	Institute/company	Status
HGS-ETR1	Human agonist mAb	TRAIL-R1	Human Genome Sciences	Ph2
HGS-ETR2	Human agonist mAb	TRAIL-R2	Human Genome Sciences	Ph1
HGS-TR2J	Human agonist mAb	TRAIL-R2	Human Genome Sciences	Ph1
TRA-8	Agonist mAb TRA-8	TRAIL-R2	Sankyo	Preclinical
APO2L/TRAIL-PRO1762	Recombinant TRAIL ligand	TRAIL-R1 TRAIL-R2	Amgen/Genentech	Ph1
Genasense (oblimersen sodium)	Antisense	Bcl-2	Genta	Ph2/3
GX15–070	Small molecule	Bcl-2	GeminX	Ph1
AT101	Small molecule	Bcl-2	Ascenta	Ph1/2
ApoGossypol	Small molecule	Bcl-2	Burnham Institute/NCI	Preclinical
EGCG	Small molecule	Bcl-2	Mayo Clinic	Preclinical
ABT-737	Small molecule	Bcl-2	Abbott/Idun	Preclinical
HA14–1	Small molecule	Bcl-2	Raylight	Preclinical
CDDO	Triterpenoid	FLIP	Reata Discovery/ Dartmouth	Preclinical
ISIS 2181308	Antisense	Survivin	Isis/Lilly	Ph1
AG35156	Antisense	XIAP	Aegera	Ph1
Not defined	SMAC mimetic peptide	XIAP	Joyant Pharmaceuticals	Preclinical
Not defined	SMAC mimetic peptide	XIAP	Tetralogics	Preclinical

various cancer cell lines. (Amantana et al., 2004; Chawla-Sarkar et al., 2004; McManus et al., 2004; Ou et al., 2005; Wang et al., 2005; Yamaguchi et al., 2005a, b). There are other compounds that mimic the action of the mitochondrially released XIAP inhibitor, SMAC/DIABLO (Bockbrader et al., 2005; Fulda et al., 2002b; Li et al., 2004; Pei et al., 2004; Roa et al., 2003). There are examples of single-agent activity in tumor cell lines and xenografts for many of these targeted therapies. More importantly, where they have been evaluated, the apoptosis activity of these agents shows a dramatic enhancement in combination with TRAIL-R agonists. These data demonstrate that the use of TRAIL-R agonists and compounds that lower hurdles for active apoptosis signaling may be potent therapeutic agents and importantly active in TRAIL insensitive cells.

Another avenue that has generated encouraging results has come from the use of agents with less direct action on TRAIL-R signaling. The proteosome inhibitor, bortezomib, has broad-ranging effects on receptor expression, upregulation of proapoptotic proteins such as Bik and Bim, and TRAIL production (also see Chapter 12). Bortezomib has also shown activity in combination with the agonist antibodies HGS-ETR1 and HGS-ETR2 in hematological cell lines and primary cells from NHL and CLL patients. (Georgakis et al., 2005; Lashinger et al., 2005; Matta and Chaudhary, 2005; Nencioni et al., 2005; Nikrad et al., 2005; Papageorgiou et al., 2004; Sayers and Murphy, 2006; Zhang et al., 2004; Zhu et al., 2005b). Histone

deacetylase (HDAC) inhibitors have demonstrated significant antitumor activity in combination with TRAIL-R agonists. Effects with HDAC inhibitors include changes in TRAIL-R2 expression, decreasing levels of Bcl-2 and FLIP, and increasing the proapoptotic protein Bik. Some early HDAC inhibitors are now progressing through clinical trial development and show early signs of activity (Ganten et al., 2005; Guo et al., 2004; Kelly and Marks, 2005; Kelly et al., 2005; Marks et al., 2004; Yoshida et al., 2005; Zhu et al., 2005b) (also see Chapter 13).

The use of these apoptosis-promoting compounds as single agents or in combination with standard chemotherapy has, in those agents being advanced into clinical development, shown signs of biological activity. These strategies directly targeting the apoptosis pathway are exploiting the potential that they will confer greater effectiveness to chemotherapy. Alternatively, the elimination or obstruction of antiapoptotic molecules may lower the threshold for induction of apoptosis when used in combination with a TRAIL-R agonist. This combination strategy of TRAIL-R agonists and proapoptotic-targeted therapy has the potential to significantly enhance antitumor activity and eliminate the need for nonspecific chemotherapeutic agents that elicit toxic side effects. While a broad range of exciting preclinical data has verified the activity of this amalgamation, a combinatorial apoptotic strategy needs to be validated in a clinical setting.

9 Conclusion

Targeting the TRAIL-R pathway with therapeutic agents provides an opportunity to induce apoptosis selectively in tumor cells. In preclinical studies the use of TRAIL-R agonists like recombinant TRAIL ligand or monoclonal antibodies have demonstrated significant, potent antitumor activity and have enhanced chemotherapeutic agent activity in a spectrum of human tumor cell lines and xenografts. Several human monoclonal antibodies and a recombinant TRAIL ligand have advanced through preclinical evaluation and are now in clinical development. Hopefully, other novel agents that target the apoptotic pathway will enter and advance successfully through the clinical arena, strengthening, and diversifying the armamentarium against the tumor cell.

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Chapter 8
Rational Design of Therapeutics Targeting the BCL-2 Family

Are Some Cancer Cells Primed for Death but Waiting for a Final Push?

Victoria Del Gaizo Moore and Anthony Letai*

Abstract A mechanism for circumventing apoptosis prevalent in many cancer cells is the overexpression of antiapoptotic BCL-2 family members. Upregulated expression of BCL-2 may be required to permit ongoing death signaling without a cellular response. Therefore, antagonizing BCL-2 function may cause death in many cancer cells. The selection for expression of BCL-2 or other antiapoptotic proteins during oncogenesis may derive from these proteins' ability to bind and sequester proapoptotic BH3-only proteins. This situation may be advantageous from a therapeutic viewpoint because cancer cells may be distinguished from normal cells by being primed with death signals. There are several strategies currently under investigation that may lead to improved treatment of many cancers by taking advantage of these differences.

Keywords apoptosis, BCL-2, BH3, therapeutics, peptide

1 The BCL-2 Family of Proteins

The BCL-2 family of proteins plays a critical role in controlling death via the intrinsic, or mitochondrial, programmed cell death pathway. BCL-2, the namesake of the family, was identified at the breakpoint of the t(14;18) translocation common to follicular lymphoma (1–3). More than 85% of follicular lymphomas contain a chromosomal translocation involving the fusion of the *bcl-2* gene at 18q21 to the immunoglobulin heavy chain locus on 14q32 (4). This translocation places the *BCL-2* gene under the control of the immunoglobulin heavy chain elements. Thus, overexpression of BCL-2 protein is driven in B-cells possessing the t(14;18). BCL-2 was credentialed as an oncogene when it was shown that overexpression was linked to the

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induction of lymphoma in mice (5, 6). Until the discovery of BCL-2, only oncogenes that increased cell proliferation, like *myc, ras*, and *src* had been described. BCL-2's discovery and characterization opened a new class of oncogenes: inhibitors of cell death. The last 20 years have seen the discovery of a family of proteins related to BCL-2 by structural homology and by participation in control over the mitochondrial apoptotic pathway.

BCL-2 proteins largely interact at the mitochondria, the nexus of events that irreversibly commit a cell to programmed cell death via the intrinsic pathway. Some BCL-2 proteins are localized to the mitochondria even during normal cellular conditions while many have other subcellular locations. For example, BAK resides as a monomer at the mitochondrial outer membrane as well as the endoplamic reticulum (7). Prior to activation, BAX exists as a monomer, either in the cytosol or loosely attached to the mitochondrial outer membrane. When activated, however, BAX undergoes alkali-stable insertion into the mitochondrial membrane. BCL-2 itself is found not only at mitochondria, but also at the endoplasmic reticulum where it is implicated in calcium homeostasis (8). Many BCL-2 family members have identified roles outside of control of apoptosis, and it is likely that BCL-2 family members are important in other aspects of cellular homeostasis. The extra-apoptotic functions of BCL-2 family members remain an area of active investigation (9–11).

BCL-2 family members can be divided into three broad groups: antiapoptotic, multidomain proapoptotic, and BH3-only proapoptotic proteins (Fig. 8.1). Antiapoptotic proteins include BCL-2, MCL-1, BCL-X_L, BCL-w, and BFL-1, all of which have the ability to oppose cell death. These antiapoptotic proteins possess sequence homology in four alpha-helical BCL-2 homology or BH regions. Multidomain proapoptotic proteins, including BAX and BAK, promote the progression of cell death and share homology in the BH1–3 regions. BH3-only proapoptotic proteins also promote cell death but, as their name implies, have only a BH3 domain in common. The BH3 domains contain an amphipathic α-helix that is necessary for the proapoptotic function of BH3-only proteins. However, this pro-death function requires interaction with multidomain BAX or BAK (12–14).

Upon cellular stress such as oncogene activation, uncontrolled proliferation, DNA damage, or growth factor withdrawal, BH3-only proteins become functionally upregulated via transcriptional or posttranslational means (15, 16). Proapoptotic BH3-only proteins may be further categorized as "activators" or "sensitizers" (17)

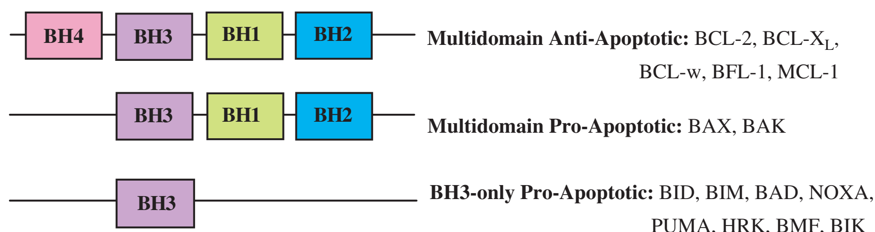

Fig. 8.1 Three classes of the BCL-2 family of proteins. BH3 domains are coded by color

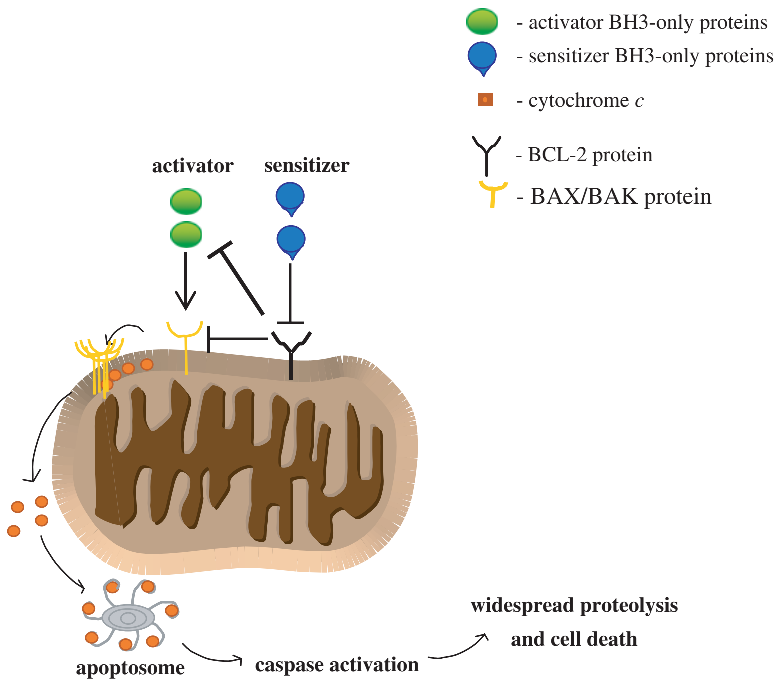

Fig. 8.2 BCL-2 family "activators" vs "sensitizer." BH3 domain-only activators, such as BID or BIM, interact with BAX or BAK to induce their activation, leading to MOMP, caspase activation, and apoptosis. BCL-2 may also bind and sequester BID or BIM, preventing activation of BAX or BAK. Sensitizers binding to BCL-2 may either block activators from binding or displace them from BCL-2

(Fig. 8.2). "Activator" BH3-only proteins, such as BID or BIM, interact with BAX or BAK, inducing an allosteric change. Subsequently, activated BAX or BAK can oligomerize. Oligomerized BAX or BAK, perhaps in complex with other proteins, induce mitochondria outer membrane permeablization (MOMP) (14, 18–22). Permeablization allows certain mitochondrial factors such as cytochrome *c*, Smac/Diablo, and AIF, to be released into the cytosol (23–28). Once in the cytosol, cytochrome *c* forms a holoenzyme complex with caspase-9 and APAF-1, called the apoptosome, which cleaves procaspase-3, into an active protease (29). Widespread proteolysis ensues, leading to cellular dysfunction and death. Consequently, MOMP can be considered the step at which commitment to cell death occcurs. Notably, there are recent studies that suggest that a key proapoptotic function of p53 is mediated by its ability to act as an activator (30–33).

While antiapoptotic proteins like BCL-2 and MCL-1 have been shown to directly interact with multidomain BAX and BAK, their interaction with BH3-only proteins may be more important to their antiapoptotic function (13, 34). The BH1–3 domains of BCL-2 form a hydrophobic cleft where the BH3 domain of multidomain and BH3-only proteins can bind. BCL-2 binding of BID or BIM causes

sequestration of these activator proteins, thereby preventing interaction and activation of BAX and BAK and thereby preventing MOMP (13, 17). Not all BH3-only proteins, however, are able to activate BAX or BAK. BH3-only proteins that do not activate BAX or BAK, including BAD, BIK, BMF, NOXA, and PUMA, we classify as "sensitizers" (17, 35). In contrast to activators that can activate BAX and BAK, these BH3 domains exert their proapoptotic function by binding to antiapoptotic BCL-2 proteins. In so doing, they compete with the binding of activators, either preventing activator binding, or displacing activators from BCL-2. In the presence of sensitizers, displaced activator BH3-only proteins are freed from antiapoptotic proteins to activate BAX and BAK and induce MOMP (17, 35, 36). While antiapoptotic proteins apparently share the common function of inhibiting apoptosis by sequestering activator BH3-only proteins, their binding pockets are nonetheless distinct. This is most clearly shown by the fact that each antiapoptotic protein has a distinct pattern of interaction with the range of sensitizer BH3 domains (35–37).

In addition to the intrinsic or mitochondrial pathway, apoptosis also can be initiated through the death receptor-mediated, or extrinsic, pathway. The extrinsic pathway is triggered when ligands, such as TNF, Fas ligand, or TRAIL, are bound by cell surface death receptors that cause changes in the intracellular domains of these receptors, resulting in assembly of a so-called death-inducing signaling complex (DISC) reviewed in (16). Activation of the initiator caspase-8 activation results, leading to activation of downstream effector caspases. In some systems, linkage to the intrinsic apoptotic pathway is accomplished by caspase-8 cleavage of the activator BH3-only protein BID, which can then trigger BAX or BAK oligomerization and MOMP (38, 39). Even though initiation of the intrinsic and extrinsic pathways is different, both converge at the activation of downstream effector caspase-3 and caspase-7.

2 The Link Between BCL-2 and Cancer

While elevated BCL-2 levels as a result of the t(14;18) translocation involving the *BCL-2* gene occurs in 80–90% of follicular non-Hodgkins lymphomas, aberrant expression of antiapoptotic expression has been implicated in many other cancers (4, 40, 41). 20–55% of diffuse large cell lymphomas have elevated BCL-2, either due to t(14;18) translocations, gene amplification, or other mechanisms, which may correlate with decreased patient survival (42–44). Many other cancers exhibit high levels of BCL-2 protein in the absence of a t(14;18); the mechanism of upregulated BCL-2 remains obscure in most of these instances. Examples include 70% of breast cancer (45, 46), 30–60% prostate cancer (47), and 90% of colorectal cancer cases (41, 48, 49). Chronic lymphoid leukemia (CLL) is largely considered a disease of failed apoptosis (50–52), but usually not due to t(14;18) (53). Nonetheless, the majority of CLL cells express high levels of BCL-2 (54). Recently, a more common chromosomal aberration, deletion, or translocation of 13q14.3, was implicated in

elevated BCL-2 in CLL (55). Changes affecting region 13q14.3 downregulated two microRNAs (miRNA) *mir-15A* and *mir-16-1*, and occurred in >50% of all CLL cases. miRNAs are a class of genes involved in tumorigenesis that produce short, single-stranded RNAs that bind to specific mRNA sequences and either prevent the translation of the mRNA or hasten degradation of the mRNA, thereby lowering the levels of the corresponding protein (56, 57). Expression of *mir-15A* and *mir-16-1* inversely correlates to BCL-2 expression in CLL samples and both negatively regulate BCL-2 levels (58, 59).

Expression of other antiapoptotic proteins has been detected in many cancers, including BFL-1 in diffuse large-cell lymphoma (60), MCL-1 in myeloma (61), and BCL-X_L in lung adenocarcinoma (62). Both BCL-2 and MCL-1 have been implicated as important contributors to melanoma development and maintenance (63–65). The oncogenic Epstein-Barr virus (EBV) and human herpes virus-8 (HHV-8; also known as Kaposi sarcoma herpes virus) encode BCL-2 homologs that oppose cell death from multiple stimuli, analogous to BCL-2 (66, 67). EBV has been implicated in the causation of HIV-related lymphoma, Burkitt lymphoma, nasopharyngeal carcinoma, and posttransplantation lymphomas, and HHV-8 in the causation of Kaposi sarcoma, Castleman disease, and body cavity lymphomas. The evolutionary selection for BCL-2 homologs in these viruses suggests that blocking the intrinsic pathway to programmed cell death is important in viral infection, and perhaps also for oncogenesis.

Multiple myeloma (MM) cells have been shown to express BCL-2, BCL-X_L, and MCL-1. Clinical and in vitro data suggest important roles for these proteins in MM cell survival as well as clinical resistance to therapy (68, 69). Despite the lack of chromosomal translocations, protein expression of each of these antiapoptotic proteins has been observed in clinical isolates (68–70). Antisense oligonucleotides (ASO) have been used with MM cells to determine if BCL-2, BCL-X_L. or MCL-1 expression is critical for the survival of these cancer cells, with mixed results (61, 71).

It has been hypothesized that oncogenesis requires an apoptotic defect (72, 73). One apparent strategy for apoptotic escape exploited by certain cancer cells is the overexpression of antiapoptotic BCL-2 family members. These proteins can bind and sequester activator BH3-only death signals likely initiated by cancer phenotypes including genomic instability, oncogene activation, and inappropriate cell contact. Therefore, in cancer cells that adopt such a strategy it seems likely that much of the antiapoptotic proteins will be "primed" with activator BH3-only proteins (Fig. 8.3). Primed cells are rendered exquisitely sensitive to mimetics of the sensitizer BH3 domains, which function as selective antagonists of BCL-2 and other antiapoptotic proteins (17, 35, 74) (Fig. 8.4). Certain, though probably not all, normal tissues may lack this priming, as they do not violate the rules of normal cellular behavior that provoke death signals in many cancer cells. Thus, the possibility exists of targeted intervention to exploit the therapeutic window between "primed" cancer cells and "unprimed" normal cells by antagonizing BCL-2 family antiapoptotic protein function.

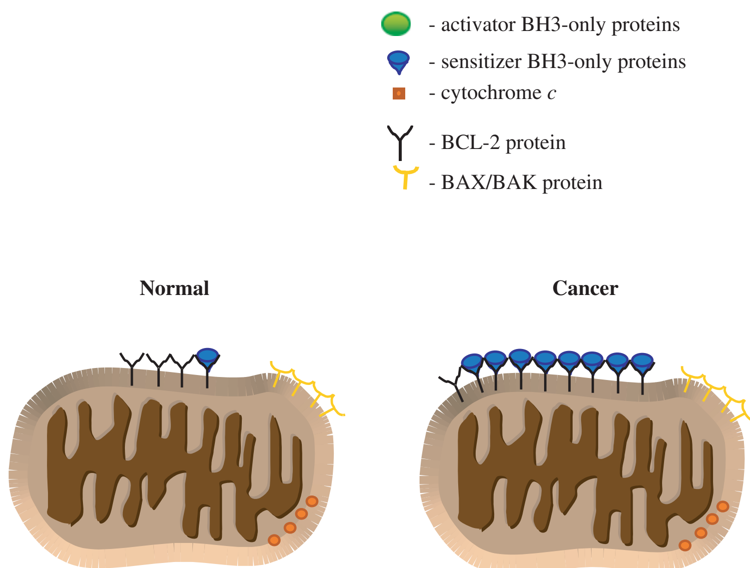

Fig. 8.3 Idealized cartoon representation of a normal mitochondrion compared to a cancer mitochondrion. Though they express more BCL-2 than the normal mitochondrion, the cancer mitochondrion has less antiapoptotic reserve due to significant priming by activator BH3-only proteins

3 Therapeutic Strategies Targeting Antiapoptotic BCL-2 Family Members

Efforts have begun to target the expression of antiapoptotic BCL-2 family members. One strategy is to downregulate antiapoptotic genes by ASO. An 18-mer phosphorothioated oligonucleotide directed against the first six codons of the human BCL-2 open reading frame, called Oblimersen or Genasense, was introduced by Genta, Inc. and has advanced through clinical trials (75, 76). Side effects have been tolerable, generally limited to thrombocytopenia, fatigue, back pain, weight loss, and dehydration (77). However, efficacy has been difficult to demonstrate. For example, treatment of metastatic melanoma with dacarbazine and oblimersen in a randomized phase III study showed no significant benefit in overall survival compared with dacarbazine alone (274 vs 238 days, $P = 0.18$). Even though significant benefit in progression free survival was observed (74 vs 49 days, $P = 0.0003$), overall survival was the primary end point, thus an FDA panel declared that clinical benefit was not demonstrated. In a phase III trial of myeloma, oblimersen plus high-dose dexamethasone was compared with dexamethasone alone; this trial also failed to meet its primary end point, time to disease progression. Furthermore, response to oblimersen in another myeloma trial did

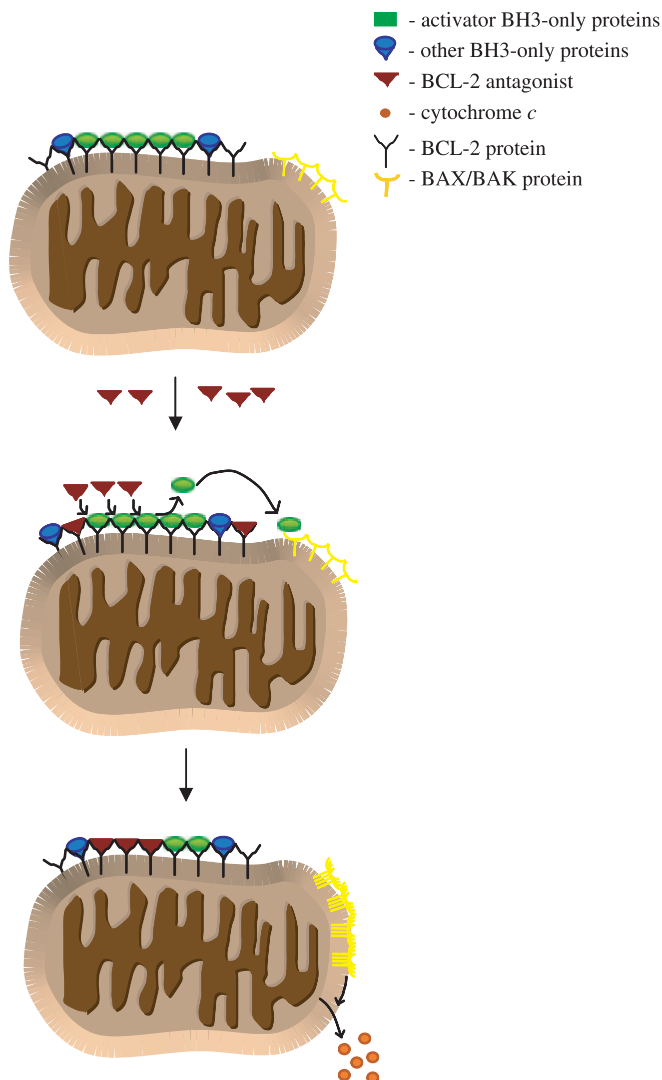

Fig. 8.4 Model of BCL-2 antagonist inducing death in a "primed" cancer mitochondrion. Cancer mitochondria have activator proteins like BIM sequestered by BCL-2 on the outer membrane. Upon addition of a BCL-2 antagonist, BIM is displaced and BCL-2 becomes occupied by the antagonist. Freed BIM then interacts with BAX or BAK, causing oligomerization and leading to cytochrome c release, MOMP, and apoptosis

not correlate with reduced BCL-2 protein levels, which provokes the question of whether oblimersen has significant off-target activity. In general, ASO has been a somewhat disappointing strategy for targeting BCL-2. The cellular effects of the lowering of BCL-2 levels by antisense oligonucelotides may not only provoke

undesirable coregulation of other BCL-2 family members but decreasing the mRNA is likely very different from functional antagonism of the protein (78). Furthermore, BCL-2 protein levels tend to be in the 10–50% range, which is unlikely to have a widespread cellular effect. Finally, oblimersen contains 2 CpG dinucleotides which may well produce many off-target effects on the immune system (78). While some of these off-target effects may be beneficial, others may well limit its maximum tolerated dose.

4 Delivery of Therapeutic Compounds into Cells

Delivery of drugs and therapeutic compounds is limited by the ability to penetrate the cell membrane. Compounds cross membranes either by passive processes or by mechanisms involving active participation of membrane components. In general, water, small hydrophilic molecules, and molecules <200 Da (79) passively diffuse through membranes. Therefore, most drugs need to be either small and water soluble, or polar enough for absorption into the body yet lipophilic enough to promote passage through the nonpolar lipid bilayer (80). This narrow range of physical characteristics limits the success of many compounds. Additionally, the degree of ionization of the compound, the circulation to the site of absorption, and its concentration can affect a compound's ability to reach its site of action. Even if a compound is able to circumvent passive passage across membranes by interacting with membrane receptors, there are still stringent criteria that must be met. No matter how a drug enters cell, once inside its effects can be terminated by metabolism or excretion. An additional difficulty is that the compound not only has to cross into cells rapidly and efficiently, but it then needs to make its way through the cellular milieu, which is full of proteases and other proteins, and eventually travel to the desired subcellular location to be effective.

While peptides based on sensitizer BH3-domains have been demonstrated to function as selective inhibitors of antiapoptotic proteins, unmodified BH3 domain peptides are cell impermeant (81). One strategy to augment cell entry is use of protein transduction domains (PTD) (82). PTDs are generally small (~10–20 amino acids) peptide sequences enriched for positively charged amino acids that rapidly and efficiently cross cell membranes. When fused to larger molecules, they have been shown to transport into cells a wide variety of cargo along with such large proteins (83), liposomes (84), and even metallic beads (85). The transduction process is not receptor mediated and is temperature independent, making it unlikely that endocytosis or transporter mechanisms are involved (86–88); however, the exact mechanism is not known.

To facilitate cell internalization, BH3 peptides have been linked with PTD such as a poly-D-arginine or Antennapedia internalization sequence tags (17, 26, 89, 90). N-terminal poly-D- arginine octomer (r8) linkage to BH3 peptides from BAD or BID have been shown to kill a human leukemia cell line that expresses BCL-2, while r8BIDBH3 double point mutant did not. Furthermore, r8BADBH3

peptide caused no apoptosis on its own but when added with the r8BIDBH3 peptide increased apoptosis, suggesting that the moiety did indeed facilitate internalization and that an intact BH3 domain was necessary for killing (17). In a separate study, a 27-amino acid peptide derived from the BH3 domain of BAD was linked to decanoic acid (26).

Decanoic acid allows cell permeablization by a different mechanism than PTDs, which may involve activation of phospholipase C which causes intracellular stores of calcium to be released followed by contraction of calmodulin-dependent actin filaments (91). The BAD-decanoic acid compound, called, cpm-1285, but not a peptide bearing a point mutation at a residue necessary for BH3 function, induced apoptosis in a BCL-2-expressing human myeloid leukemia line, HL-60. Furthermore, immunodeficient mice injected with HL-60 cells survived longer when treated with cpm-1285. However, these studies do not conclusively demonstrate the mechanism of action of the peptide derivatives, and the cytotoxic effects could be independent of direct interaction with BCL-2 family members. Such off-target toxicity was demonstrated by Schimmer and coworkers where linking the BH3 domain of BAD to the Antennapedia internalization sequence had considerable off-target toxicity (89). Their compound was toxic to a wide variety of cells, including yeast, wherein BCL-2 family members have yet to be identified. Others have demonstrated that BH3 peptides derived from BAX and BCL-2 linked to an Antennapedia internalization sequence induce MOMP and apoptosis, but overexpression of either BCL-2 or BCL-X_L did not rescue the cells from apoptosis (92). All of these effects may be due to a nonspecific membrane disruption rather than to interaction with the BCL-2 family pathway. For example, the Antennapedia internalization sequence is mainly a positively charged amphipathic α-helix that could interact and disrupt the negatively charged mitochondrial membranes independent of BCL-2 family protein interaction, in a manner similar to certain natural antibiotics (93–95). Therefore, nonspecific killing due to intrinsic biophysical properties of these internalization moieties make interpretation of cell killing by linked, some tagged BH3 peptides difficult. Further pharmaceutical development of such molecules would require considerable attention to reducing this toxicity.

The α-helix of BH3 peptides is vital for their function, but in aqueous solution the α-helical conformation can be less than 25%. Attempts have therefore been made to improve peptide function by stabilizing α-helicity. Small improvements have been gained by grafting a BAK BH3 domain to a helix-stabilizing miniprotein (96) or synthesizing BH3 peptide analogs with covalent molecular bridges, which improved affinity for BCL-2 or stabilized the α-helical conformation (97). Perhaps the most striking example of the potential of α-helix stabilization was provided by a BID BH3 peptide stabilized by an all-hydrocarbon "staple" (81). Not only did this modification enhance α-helicity, but it also increased affinity for BCL-2, cell entry, protease resistance, as well as leukemia cell line toxicity in vitro and in vivo. Mice bearing leukemia cell line xenografts demonstrated statistically significant survival improvement after 6 days and normal tissues appeared unaffected as measured by histological analysis. Since the molecule was modeled after a BID BH3 domain previously shown to be an activator (17), the compound was able to directly induce

cytochrome *c* release in a BAK-dependent fashion in vitro. Even though the compound did not behave as a selective BCL-2 antagonist but rather an activator, it was still able to exploit an apparent therapeutic window between the tumor xenograft and the normal tissues. It remains to be seen whether an analogous sensitizer BH3-based compounds would provide an even greater therapeutic window.

5 Cell-Permeant Small Molecules

Cell-permeant small molecules that bind to antiapoptotic BCL-2 family members have been identified through structure-based computer screening. One molecule isolated was able to displace the BAK BH3 peptide from BCL-2 with an IC_{50} of 1–14 μM. Since the *K*d for the BAK BH3 peptide is approximately 200 nM, it is reasonable to surmise that the *K*d for binding of these molecules to BCL-2 may be significantly higher. Another molecule identified was toxic to four cell lines tested at concentrations of 10–20 μM and toxicity correlated with BCL-2 expression levels (98, 99). Screens of chemical libraries have also been used. Out of 16,320 screened, Degterev et al. identified two molecules that disrupt a BCL-X_L/BAK BH3 complex, both which had toxicity in the 10–90 μM range in a leukemia cell line (100). A screen of a library of natural products allowed the isolation of Tetrocarcin A, which is derived from *Actinomyces*, identified for its ability to counteract BCL-2 protection of anti-Fas/cycloheximide-treated HeLa cells at concentrations in the micromolar range (101). Antimycin A, an antimicrobial agent with antitumor properties in experimental systems and a known inhibitor of electron transport at mitochondrial respiratory chain complex III, was identified from a screen for inhibitors of mitochondrial respiration in mammalian cells (102). Further characterization demonstrated that antimycin A interacts with BCL-2 and BCL-X_L, and that increasing cellular levels of BCL-X_L correlated with increasing toxicity. Nuclear magnetic resonance (NMR) spectroscopy used to investigate natural products found certain polyphenols from green tea extracts were able to bind to BCL-X_L (103). In addition, these compounds displaced a BH3 domain from BCL-X_L and BCL-2 in the submicromolar range. Another screen of a small library of natural products identified two molecules, purpurogallin and gossypol, both of which resemble human BAD and inhibit binding of a BH3 domain to BCL-X_L (104). While chemical modification of purpurogallin did not lower the IC_{50} of peptide displacement of the parental compound, a racemic mixture of the (+) and (−) isomers of gossypol displaced the BH3 peptide with an IC_{50} of 0.5 μM. Molecular modeling suggested that removal of two aldehyde groups from gossypol might reduce steric hindrance in binding the hydrophobic pocket of BCL-X_L, however this modification actually decreased the binding to BCL-2 family members (105).

Small molecules that enter cells and bind the hydrophobic pocket of BCL-2 analogously to sensitizer BH3 peptides are currently in clinical development. The biotechnology company Gemin X has isolated a compound (GX01) that has been reported to bind BCL-2 and BCL-X_L and displace BH3 domains from their binding

pockets (106). GX01 was identified from a high-throughput screen of chemical libraries and is in phase I clinical trials in both chronic lymphocytic leukemia (at the University of California, San Diego [UCSD]) and solid tumors (at Georgetown University). Ascenta Therapeutics has an orally administered gossypol derivative in an ongoing phase I cancer trial.

Using a strategy of combining high-throughput screening with interactive modulation of chemical structure based on NMR, Abbott Laboratories has developed compounds reported to displace BH3 domains from BCL-2, BCL-X_L, and BCL-w with an IC_{50} of not more than 1 nM (74). One lead molecule, ABT-737, is a BAD-like sensitizer that can antagonize BCL-2 protection but cannot directly cause activation of BAX/BAK. ABT-737 was reported to have significant activity in primary CLL cells and mouse xenograft models of lung cancer and lymphoma. When injected into mice ABT-737 was well tolerated with minimal side effects in noncancerous tissues except for a reduction in platelets and lymphocytes. Furthermore, ABT-737 enhanced the cytotoxicity of paclitaxel against a cancerous cell line where single-agent activity was not achieved. Other preclinical studies have shown that the toxicity of ABT-737 is due to selective antagonism of BCL-2 in cells that require BCL-2 for survival (35). Given its high affinity for BCL-2, the data that support its function via its designed mechanism, and its effectiveness across several different cancer types in vitro, ABT-737 seems to be a promising lead compound, although clinical trials are yet to begin.

6 Conclusions

Our current understanding of the mechanisms by which BCL-2 family members control commitment to cell death gives good theoretical backing to strategies aimed at manipulating this system for clinical benefit. Certain cancers in which antiapoptotic BCL-2 is overexpressed and activator BH3-only proteins are upregulated may be "primed" for death, needing only a modest, targeted biochemical nudge for final execution of apoptosis. Small molecules designed to antagonize BCL-2 and related antiapoptotic proteins appear to be useful tools to generate this targeted signal. As the binding clefts among proteins like BCL-2, MCL-1, and BFL-1 are demonstrably distinct, it may be possible to design molecules which selectively antagonize individual proteins. Whether such "narrow spectrum" antagonists will be better cancer therapeutics than "broad spectrum" antagonists that might target the entire antiapoptotic group remains to be seen. Experimental evidence suggests that the state of protein–protein interactions among BCL-2 family members within cancer cells is different from those within normal cells. Therefore, even if an antagonizing compound entered all cells, induction of apoptosis might selectively be triggered within cancer cells. The promise of these molecules as anticancer therapeutics will soon be tested as clinical trials of compounds targeting BCL-2 are currently underway. It is exciting to witness the emergence of a potentially new class of anticancer drugs, those specifically designed to unleash the latent apoptotic potential within cancer cells.

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Chapter 9
Autophagy and Tumor Suppression

Recent Advances in Understanding the Link between Autophagic Cell Death Pathways and Tumor Development

Shani Bialik and Adi Kimchi*

Abstract Autophagy is a process by which the cell recycles its components through self-consumption of cellular organelles and bulk cytoplasm. In times of stress, it serves to generate much needed nutrients. When overactivated, however, the orderly destruction of organelles can lead to cell death. At times, autophagic cell death is used as an alternative to apoptosis to eliminate unwanted, damaged, or transformed cells. Consistent with this, tumorigenesis is associated with a down-regulation in autophagy, and genes that mediate the execution of the process have been shown to be tumor suppressors. At the same time, basal autophagy has been harnessed by some tumor cells as a survival mechanism to protect against ischemia and signals that induce apoptosis. Thus, the relationship between autophagy and tumor development is complex. Here, we discuss the basic machinery of mammalian autophagy and its regulators, with specific emphasis on those genes that have been linked to cancer. Research supporting the divergent nature of autophagy in both tumor suppression and tumor progression is presented. We conclude with a survey of recent approaches to treating cancer with strategies that modulate autophagy.

Keywords autophagy, programmed cell death, tumor suppressor, DAPk, Beclin 1, mTOR

1 Introduction

It is now an accepted dogma that cancer can develop from the imbalance of cells which results from disruptions in cell death. This realization gave impetus to analyze the molecular, cellular, and genetic mechanisms of programmed cell death, in particular apoptosis. However, apoptosis is not the only means by which a cell can die in a programmed, regulated manner. Different cell death morphologies were long

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observed in tissue (e.g., Schweichel and Merker, 1973), however, it is only within the past few years that these death processes were more precisely classified and their molecular aspects deciphered. Of these alternate death pathways, autophagic cell death, also referred to as type 2 cell death, has recently been characterized in more detail (see, e.g., Gozuacik and Kimchi, 2004). Autophagic cell death results from the self-consumption of cellular organelles from within by means of the basic cellular autophagy machinery. This involves the de novo formation of double membrane- or multimembrane-enclosed vesicles called autophagosomes that elongate and surround portions of cytosol, including organelles such as mitochondria and endoplasmic reticulum (ER) (Fig. 9.1). The mature autophagosome eventually fuses with the lysosome, forming an autolysosome, in which its contents are degraded by lysosomal enzymes. Autophagic cell death can be accompanied by membrane blebbing and partial chromatin condensation, yet DNA fragmentation and caspase activation do not have an active part in the process.

One salient question that has emerged from the recent studies on autophagic cell death is whether autophagy suppresses tumorigenesis, as does its better known counterpart, apoptosis. Although it seems obvious that any block in any cell death pathway would promote cancer growth, for autophagy the question is not so simple. Unlike apoptosis, autophagy has homeostatic functions as a catabolic process by which cellular components are recycled. During times of cell stress, such as starvation, autophagic degradation of cellular organelles and proteins provides the cell with essential nutrients and biochemical building blocks that are not available through external supply or de novo biosynthesis. Autophagy can also be used to remove damaged organelles, such as depolarized mitochondria, which, rather than killing the cell, prevents further damage and release of proapoptotic factors, thereby blocking cellular demise. In these scenarios, autophagy serves a prosurvival role. There are in fact, many examples in which inhibition of autophagy enhances cell death (see below for details). However, other scenarios clearly indicate that beyond some unknown threshold, too much self-eating and destruction of cellular contents can be lethal and contribute to cell death. Furthermore, several death-inducing stimuli

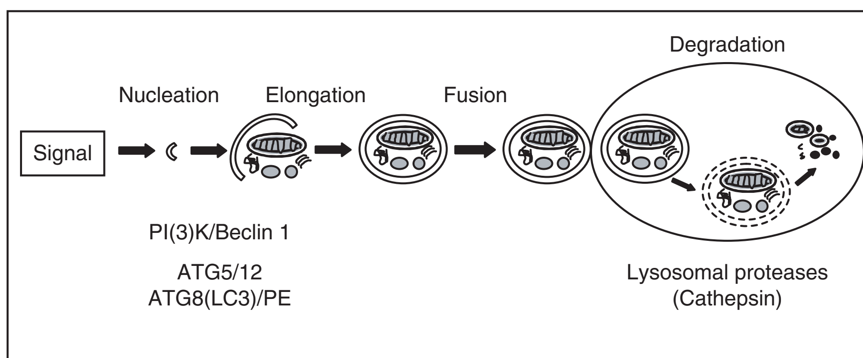

Fig. 9.1 Stages of autophagosome formation and the protein complexes that regulate them

have been shown to induce characteristics of autophagy, in addition to, or instead of, apoptosis. There is still some debate in the literature whether these signs of autophagy are causative to cell death, or merely accompany it, and may actually reflect a futile attempt at rescuing the cell. The recent identification of several mammalian autophagic genes has enabled researchers to elegantly block the autophagic pathway through genetic knockout and RNA interference (RNAi)-based knockdown experiments. Results of these studies has indicated that in certain circumstances (i.e., depending on the type of stimulus and the genetic makeup of the cell), autophagy, does in fact, contribute to the death of the cell. This may be one reason why several genes which regulate and/or execute autophagy have been implicated as tumor suppressors, in much the same way that apoptotic genes have been so characterized. These include Beclin 1 and DAP-kinase. In addition, several prominent oncogenes and tumor suppressor genes more commonly known to play a role in apoptotic signaling, such as p53, PI(3)K, PTEN, Bcl-2, and p19ARF, have now been shown to regulate autophagy.

This chapter will briefly present a summary of what is known about the molecular machinery that mediates and regulates mammalian autophagy, with particular emphasis on the components that have been linked to tumorigenesis. It will describe research indicating the contribution of autophagy to both cell survival and cell death pathways. Furthermore, it will also explore the possibility of harnessing autophagy as a means of destroying tumor cells.

2 The Molecular Basis of Autophagy

2.1 The Basic Machinery of Autophagosome Formation

Much of the known molecular mechanisms that control and/or execute autophagy were originally deciphered in yeast, although, recently, many of the relevant mammalian orthologues were identified (Tsukada and Ohsumi, 1993; Thumm et al., 1994 Harding et al., 1995). The yeast genes, now referred to by common consensus as the *ATG* genes (autophagy-related genes), encode 27 proteins that are necessary for the various stages of autophagic vesicle formation, fusion to the lysosome, and degradation of autophagosome contents (Fig. 9.1) (Klionsky et al., 2003). Prominent among these are several proteins that form a complex with the class III phosphatidylinositol 3-kinase (PI(3)K) Vps34, to produce phosphatidylinositol 3-phosphate (PI3-P), a lipid signaling molecule that is critical in the early stages of autophagosome nucleation (Petiot et al., 2000). Vps34 forms a complex with, and is regulated by, ATG6 (Beclin 1 in mammalians) and myristylated serine kinase Vps15/p150 (Stack et al., 1995). A fourth component of the yeast complex, ATG14, directs the complex to organizing centers of prevacuolar structures known as pre-autophagosomal structures (PAS) (Kim et al., 2002). The mammalian equivalent of ATG14 has yet to be discovered, and the PAS has not been observed in mammalian

cells. Yet, in these cells, the PAS may be mimicked by sites on the trans-Golgi network and the ER to which Beclin 1 localizes, which serve as foci of PI3-P formation (Liang et al., 1998; Kihara et al., 2001; Pattingre et al., 2005). PI3-P is necessary for the nucleation of nascent membranes that will form the autophagosome. The exact mechanism is not yet known, but it presumably involves the docking to these nucleation sites of autophagy-specific proteins containing the domains FYVE (conserved in Fab1, YOTB, Vac1, and EEA1) or PX (Phox homology), which have a high affinity for PI3-P (Gillooly et al., 2001; Wishart et al., 2001). These proteins are predicted to control membrane formation and elongation.

The next stages of autophagic vesicle membrane recruitment and elongation utilize two ubiquitin-like pathways (Fig. 9.2). ATG12, a ubiquitin-like protein, is covalently conjugated to ATG5 in a constitutive manner via the sequential E1-ligase and E2-like activities of ATG7 and ATG10 (Mizushima et al., 1998; Tanida et al., 1999; Shintani et al., 1999). The ATG12/ATG5 dimer binds ATG16, which, through its ability to homo-oligomerize, leads to the formation of larger complexes of 800 kDa in mammals (Mizushima et al., 1999, 2003). This complex associates with the outer membrane of the elongating vesicle until completion of the autophagosome (Mizushima et al., 2001, 2003). The ATG12/ATG5 conjugation system is necessary for the second ubiquitin-like pathway (Mizushima et al., 2001; Suzuki et al., 2001). In this pathway, ATG7 and a second E2-like protein, ATG3, mediate the conjugation of ATG8 (or its mammalian counterpart, microtubule-associated protein 1 light chain 3, or LC3), not to a ubiquitin-like molecule, but rather to the lipid phosphatidylethanolamine (PE) (Ichimura et al., 2000). This is a critical step in the recruitment of lipid molecules for the expansion of the autophagic vesicle. The conjugation occurs via an amide bond formed between the amino

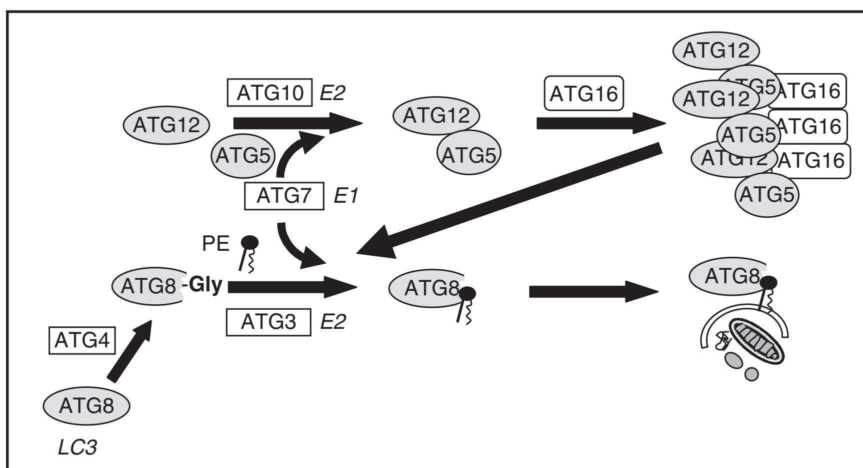

Fig. 9.2 Two ubiquitin-like conjugating systems mediate vesicle nucleation

group of the lipid molecule and the carboxyl-terminal glycine residue of ATG8, which is exposed following the cleavage of ATG8's C-terminus by the ATG4 cysteine protease (Ichimura et al., 2000; Kirisako et al., 2000). Although the proteolysis takes place immediately following translation of the protein, its lipidation occurs only upon stimulation of autophagy, and converts it from a soluble, cytosolic protein of 18 kDa (LC3-I) to a vesicle-associated form that migrates more rapidly on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (LC3-II). These properties of LC-3 have been used extensively as a marker for autophagy in mammalian cells (Kirisako et al., 1999; Kamada et al., 2000).

Once formed, the mature autophagosome fuses with the lysosome, generating the autolysosome. An intact microtubular network is required for this fusion step, at least in some cell types (Webb et al., 2004; Kochl et al., 2006), while actin microfilaments are involved in earlier stages of autophagosome formation (Aplin et al., 1992). Within the autolysosomal compartment, the engulfed organelles and cytosolic proteins are degraded by resident lysosomal enzymes, such as cathepsins.

2.2 *Regulators of Autophagy Signaling*

Many complex signaling pathways regulate the activity of the ATG proteins and autophagosome formation (Fig. 9.3). One prime regulator is target of rapamycin (TOR) kinase, a sensor of growth factor, nutrient, and energy availability, which converts these signals to cell growth and proliferative responses. TOR, whose activity is associated with inhibition of autophagy, is active in nutrient-rich conditions and upon growth factor stimulation. The mammalian TOR (mTOR) is regulated by several survival signals emanating from signaling molecules such as Akt/PKB (Inoki et al., 2002; Hahn-Windgassen et al., 2005), ERK (Ma et al., 2005), RSK1 (Roux et al., 2004), and the small GTP-binding protein, Rheb (Inoki et al., 2003; Fingar and Blenis, 2004). In yeast, TOR phosphorylates ATG13, which reduces its affinity to the ATG1 kinase (Kamada et al., 2000; Scott et al., 2000). ATG1 kinase activity is necessary for autophagy induction, and this activity requires tight association with ATG13. When TOR activity is blocked, such as during nutrient deprivation or upon treatment with rapamycin, ATG13 is rapidly dephosphorylated and binds to and activates ATG1 (Kamada et al., 2000; Abeliovich et al., 2003). The precise function of ATG1 and the mammalian counterpart of this pathway are not yet known.

mTOR is an important determinant of cell survival vs growth. Two critical mTOR substrates are 4E-BP1 and p70S6K. Phosphorylation of 4E-BP1 enhances cap-dependent translation. p70S6K in turn phosphorylates proteins that are involved in transcription, protein synthesis, and RNA splicing (Fingar and Blenis, 2004; Wang et al., 2001). For example, p70S6K phosphorylates eEF-2 kinase, inhibiting its activity (Wang et al., 2001). The active form of eEF-2 kinase, through phosphorylation of the translation factor eEF-2, blocks the elongation phase of translation. Thus, activation of mTOR leads ultimately to derepression of eEF-2

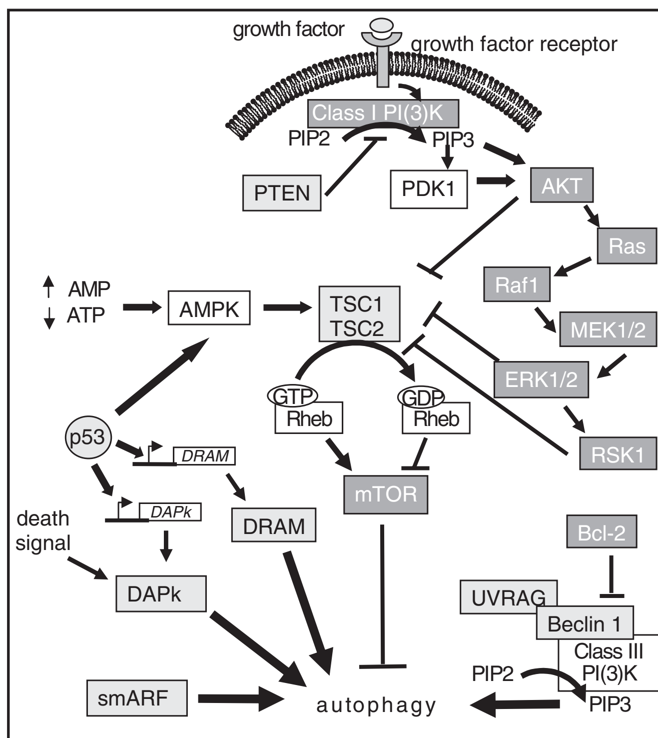

Fig. 9.3 Signaling pathways that regulate mammalian autophagy. Lightly shaded boxes represent tumor suppressor genes and darkly shaded genes are known oncogenes

and the promotion of translation. Under these circumstances, autophagy is repressed. In scenarios when cap-dependent protein synthesis is turned off, such as during amino acid starvation, ER-stress, and viral infection, certain proteins involved in the transcriptional regulation of autophagy-related genes are nevertheless upregulated (Natarajan et al., 2001), and autophagy results.

2.3 Autophagy Regulators Linked to Cancer

The most powerful genetic evidence linking autophagy to cancer development and progression is the emerging notion that genes which positively control autophagy display tumor suppressive functions when assessed in human tumors or in cancer model systems. Examples of such genes include Beclin 1, DAPk, p19ARF (via a novel isoform, SmARF [For short, mitochondrial ARF]), p53, PTEN, and TSC1/2. Conversely, several oncogenes, such as AKT, ERK1/2, and class I PI(3)K, have been shown to antagonize autophagy. This section will discuss these genes and their contributions to the promotion and suppression of autophagy, respectively.

2.3.1 Beclin 1

The cloning of Beclin 1, the human orthologue of yeast ATG6, provided the first link between an autophagic gene and tumor suppression. Beclin 1 was originally identified in a screen for Bcl-2-interacting proteins (Liang et al., 1998). The *Beclin 1* gene showed a high incidence of haploinsufficeincy in numerous breast cancer cell lines, and was downregulated in more than 50% of breast tumors analyzed in one initial study (Liang et al., 1999). In fact, the Beclin 1 monoallelic deletion on chromosome 17q21 is common not only to breast cancer, but also to ovarian and prostate cancers (Aita et al., 1999). Experimental deletion of Beclin 1 in mice confirmed the findings in human tumors; heterozygous knockout mice showed an increase in the preponderance of spontaneous lung cancer, lymphoma, and hepatocellular carcinoma (Qu et al., 2003; Yue et al., 2003). These tumor-suppressive activities of Beclin 1 were attributed to its role as an inducer of autophagy. Expression of Beclin-1 in MCF-7 breast carcinoma cells induced autophagy and blocked tumor formation in nude mice (Liang et al., 1999).

These studies provide strong evidence that autophagy, like apoptosis, is tumor suppressive, and that its downregulation provides tumor cells with a distinct advantage that promotes tumor growth. Furthermore, they may provide an additional functional explanation to Bcl-2's oncogenic properties. Bcl-2 antagonizes Beclin 1's autophagic activity, by binding Beclin 1 and blocking its association with Vps34, thus inhibiting PI(3)K activity (Pattingre et al., 2005). In this manner, overexpression of Bcl-2, a common occurrence in cancer, may lead to tumor growth as a result of its antiapoptotic properties as well as its ability to inhibit autophagy (Pattingre et al., 2005; Cardenas-Aguayo Mdel et al., 2003; Saeki et al., 2000).

A second Beclin 1-binding protein is UVRAG (for UV irradiation resistance-associated gene), which has the opposite effect on the Beclin 1/PI(3)K complex from Bcl-2 (Liang et al., 2006). UVRAG was isolated as part of a multiprotein complex containing Bcl-2, Beclin 1, and PI(3)K. Expression of UVRAG in a colon cancer cell line in which the endogenous gene is downregulated due to a heterozygous mutation in the *UVRAG* gene led to increases in both basal and starvation-induced autophagy. This was dependent on the presence of Beclin 1 and on the ability of UVRAG to interact with Beclin 1. Conversely, UVRAG was necessary for Beclin 1 and starvation-induced autophagy in MCF-7 cells. UVRAG binding to Beclin 1 directly enhanced both the Beclin 1/PI(3)K complex and PI(3)K activity. As a consequence of the increased autophagy, UVRAG expression suppressed proliferation, anchorage-independent growth, and tumor formation in vivo, but paradoxically, did not enhance cell death or otherwise reduce cell number. Thus, UVRAG-mediated autophagy is tumor suppressive, but not simply as a consequence of increased cell death. UVRAG appears to be a bona fide tumor suppressor; it maps to chromosome 11q13, a locus frequently associated with breast and colon cancer, and is found to be monoallelically deleted in multiple human cancer cell lines and tumors.

2.3.2 DAPk and Family Members

Death-associated protein kinase (DAP-kinase, DAPk) was isolated as a gene whose function was necessary for interferon-γ (IFN-γ)-induced death in HeLa cervical cancer cells (Deiss et al., 1995). It was later recognized that IFN-γ induced a caspase-independent death in these cells that bore evidence of autophagy (Inbal et al., 2002). In fact, overexpression of DAPk, as well as its closely related family members, DRP-1 (DAPK2) and ZIPk (DAPk3), can induce autophagosome formation and cell death in numerous cell lines (Inbal et al., 2002; Shani et al., 2004). DRP-1, too, was shown to be necessary for autophagic cell death in MCF-7 cells starved of amino acids or treated with tamoxifen (Inbal et al., 2002). The interest in these kinases further increased once it became apparent that they can be linked to both apoptotic and autophagic cell deaths, suggesting that they may function as molecular switches or integrators of both pathways. Hence, it became important to define the exact cellular settings and the underlying molecular mechanisms which dictate the choice between apoptosis and autophagy when triggered by these kinases.

DAPk is a Ca^{2+}/calmodulin-dependent Ser/Thr protein kinase and the founding member of a family of death-associated kinases, all of which share significant homology within the common kinase domain (for review, see Bialik and Kimchi, 2006). The family members' extra-catalytic domains differ, and reflect their divergent regulation and cellular localizations. While DAPk, and sometimes ZIPk, localize to the actin cytoskeleton, DRP-1 is a soluble protein, which has been found inside autophagosomes upon overexpression (Cohen et al., 1997; Bialik et al., 2004; Inbal et al., 2002; Page et al., 1999; Vetterkind et al., 2005; Komatsu and Ikebe, 2004). Their kinase activity is necessary for both apoptotic and autophagic cell deaths. A wide range of death stimuli have been reported to activate these kinases, and furthermore, require their activities for completion of the death process (Bialik and Kimchi, 2006). The kinases are regulated mostly by posttranslational modifications, including phosphorylation, and DAPk has also been shown to be regulated at the transcriptional level by p53 and TGFβ (Martoriati et al., 2005; and see Bialik and Kimchi, 2006).

Several substrates and downstream pathways have been identified over the years that may explain some of the death-inducing capabilities of these kinases. For example, one substrate common to DAPk, DRP-1, and ZIPk is the regulatory light chain of myosin II, phosphorylation of which mediates membrane blebbing in both apoptotic and autophagic cells (Bialik et al., 2004; Kuo et al., 2003; Vetterkind et al., 2005; Komatsu and Ikebe, 2004; Murata-Hori et al., 2001; Inbal et al., 2002). Another DAPk substrate is syntaxin-1A, a component of the SNARE complex, which mediates docking and fusion of synaptic vesicles with the plasma membrane (Tian et al., 2003). This, combined with the fact that RNAi-based knockdown of both DAPk and DRP-1 blocked clathrin-mediated endocytosis (Pelkmans et al., 2005), indicates a potential role in membrane fusion events that may be related to their ability to induce autophagy.

DAPk has been shown to be a tumor suppressor, whose activities have been directly linked to its ability to promote cell death. It functions at several stages of tumor development. It can block initial cellular transformation by growth-promoting oncogenes, by activating a p53/p19^{ARF}-dependent apoptotic checkpoint (Raveh et al., 2001). Specifically, expression of DAPk in primary mouse embryonic fibroblasts suppressed the oncogenic properties of E2F-1 and c-Myc by inducing caspase-dependent apoptosis, provided that functional p53 and p19^{ARF} were present in these cells. Moreover, DAPk expression led to increases in p53 and p53 transcriptional activity. The apoptotic response to oncogenes was attenuated in DAPk knockout cells, as was the induction of p53 and p19^{ARF} (Raveh et al., 2001). In addition to this role in an early apoptotic checkpoint, DAPk has also been shown to block tumor metastasis. Highly metastatic lung carcinoma cells lacked DAPk expression. Reintroduction of DAPk to these cells at physiological levels resulted in a reduced metastatic activity in mouse models of metastasis compared to the parental clones (Inbal et al., 1997). This was attributed to the ability of DAPk to sensitize the tumor cells to various death stimuli. Significantly, loss of DAPk expression due mainly to promoter methylation, but also to loss of heterozygosity, has been documented in a wide range of tumors, including B- and T-cell malignancies, breast cancer, lung carcinoma, head and neck cancer, gastric cancer, cervical, and prostate cancer (see Bialik and Kimchi (2004) for review, and also supplementary Table 9.1 in Bialik and Kimchi (2006) for details). In fact, DAPk promoter methylation has been used as a diagnostic tool for cancer detection in tumor and blood samples. Furthermore, DAPk loss of expression has been associated in some cases with disease progression and severity, metastatic rates and disease recurrence (Bialik and Kimchi, 2004).

As mentioned earlier, DAPk can modulate both apoptotic and autophagic cell deaths. It is not known the degree by which each of these mechanisms contributes to its tumor suppressive capabilities. This may very well be dependent on cell type and the individual signaling environment present.

2.3.3 The ARF Tumor Suppressor and smARF

The INK4a/ARF locus is commonly deleted in many cancers (Lowe and Sherr, 2003). It encodes two tumor suppressors: the p16^{INK4a} inhibitor of the retinoblastoma gene (*Rb*) and the ARF protein (p14^{ARF} in human and p19^{ARF} in mouse), translated from an alternative leading frame. ARF's tumor suppressive capabilities stem in most part, from its ability to activate p53 by negatively antagonizing its inhibitor, Mdm2. Yet, p53- and Mdm2-independent functions have also been ascribed to ARF. These include inhibition of rRNA processing in the nucleolus by binding of ARF to nucleoplasmin/B23 (Bertwistle et al., 2004; Sugimoto et al., 2003). Recently, it has been reported that the p53-independent effects of ARF on cell death may be attributed to a short, mitochondrial ARF isoform (known as smARF) that is produced from internal translation of both the human and the mouse mRNAs (Reef et al., 2006). This novel isoform lacks the N-terminal domains that mediate nuclear localization and Mdm2 binding. smARF localizes to the mitochondria in a compartment which is resistant to

proteinase K and induces mitochondrial depolarization, without causing cytochrome C release or caspase activation. At the cellular level, smARF expression led to pronounced autophagy and caspase-independent cell death. smARF-induced cell death was partially attenuated by knockdown of Beclin 1 or ATG5, implying that, in this case, autophagy was causative to cell death. smARF is an unstable, short-lived protein that was upregulated by oncogene expression. This suggests that the autophagic function of ARF, mediated by its short form, may serve to counteract hyperproliferative signals generated by oncogenes. The dual nature of ARF, to induce apoptosis via the long p19ARF nucleolar isoform, or autophagy via mitochondrial smARF, may enable a choice of death pathways whose execution will depend on the particular genetic environment. In cases when p53-mediated apoptosis is blocked, as occurs in many tumor cells, smARF may provide a convenient back up plan that ensures cell death and maintains ARF's tumor suppressive function.

2.3.4 Tumor Suppressors and Oncogenes that Regulate the mTOR Pathway

Many of the signaling molecules that regulate the mTOR pathway are known oncogenes or tumor suppressors (see Fig. 9.3). mTOR activity is controlled by Rheb, a small GTPase of the Ras superfamily that activates mTOR in its GTP-bound form (see Sarbassov dos et al., 2005 for review). Rheb's GTPase activity is enhanced by a GTP-activating protein complex comprised of TSC1 and TSC2. Thus, TSC1/TSC2 negatively regulate mTOR by converting active Rheb-GTP to inactive Rheb-GDP. Significantly, TSC1 and TSC2 are tumor suppressors, mutations in which lead to tuberous sclerosis syndrome, a disease manifested by the occurrence of benign tumors in multiple organs, especially in the brain, leading to severe neuropathologies (reviewed in Kwiatkowski and Manning, 2005).

The TSC1/TSC2 complex is regulated by phosphorylation of TSC2 by either AKT, ERK1/2, or RSK, in response to growth factors, or by the AMP kinase (AMPK), which senses energy and nutrient deprivation and is activated by high AMP/ATP ratios. AKT/ERK/RSK-mediated phosphorylation serves to inhibit TSC's activity, and thus activates the mTOR pathway, while phosphorylation by AMPK has the reverse effects, leading to inactivation of mTOR. AKT, ERK1/2 and RSK, and class I PI(3)K, which is an upstream activator of AKT, are all oncogenic and are associated with proliferative growth (reviewed in Samuels and Ericson, 2006). Interestingly, RSK, in addition to its role in activating mTOR through phosphorylation of TSC2, was also recently shown to phosphorylate DAPk on a site known to antagonize its functions (Anjum et al., 2005). This may suggest a second mechanism by which RSK can block autophagy. Conversely, ERK1/2 was shown to activate DAPk through phosphorylation, and DAPk, in turn, suppressed ERK nuclear functions through its sequestration to the cytoplasm (Chen et al., 2005a, b). Whether these inhibitory and activating phosphorylations affect DAPk's autophagic properties has not yet been assessed. Thus, how these events are integrated with ERK and RSK's established roles in suppression of autophagy, through modulation of the mTOR pathway, remains to be seen.

Upstream of the class I PI(3)K lies in the dual protein- and phosphoinositide-phosphatase PTEN, which antagonizes the PI(3)K/AKT pathway by dephosphorylating the second messenger PIP3. PTEN is also a known tumor suppressor, located on chromosome 10q23. It is subject to deletion and/or mutation in numerous cancers (reviewed in Kim and Mak, 2006). Thus, many of the signaling molecules that negatively regulate the mTOR pathway are known tumor suppressors, while activators of the pathway have been described as oncogenes. While these factors have multiple targets and affect many cellular signaling and survival pathways, their modulation of autophagy through regulation of mTOR may contribute to their tumor suppressive and oncogenic tendencies.

2.3.5 p53

The p53 tumor suppressor is mutated in 50% of all human cancers. Its antitumor properties stem from its function as the pivotal controller of cell cycle checkpoints, inducing, as appropriate, cell cycle arrest, cellular senescence, or apoptosis (also see Chapter 10). Now autophagy can be added to its numerous tumor-suppressive functions, as two recent reports have linked p53 to signaling pathways that mediate autophagy.

p53 can modulate autophagy through regulation of the mTOR pathway. Activation of p53 by DNA damage resulting from etoposide treatment, or p53 overexpression, led to inhibition of mTOR and reduced phosphorylation of its downstream substrates (Feng et al., 2005). This was accompanied by induction of autophagy. p53's effects were mediated by TSC1 and TSC2; deletion of TSC1 and TSC2 blocked p53's inactivation of mTOR, as did chemical inhibition of AMPK. AKT, on the other hand, seemed not to be affected by p53. Inactivation of mTOR by p53 may be an important component of its growth-suppressive functions. Upon sensing genotoxic or cytotoxic stresses, such as oncogene activation, DNA damage, or hypoxia, p53's first line of defense is to induce cell cycle arrest to enable repair, or at the very least, to prevent passage of the damage to daughter cells. Through inactivation of mTOR and subsequent suppression of protein synthesis, p53 achieves a halt not only in cell cycle progression, but also in cell growth. At the same time, autophagy is induced. This may provide nutrients and energy to the cell in its time of stress, or, in more extreme circumstances, may join p53's apoptotic responses in eliminating the damaged cell once and for all.

p53 has also been recently linked to autophagy through the upregulation of a novel transcriptional target, DRAM (for damage-regulated autophagy modulator) (Crighton et al., 2006). DRAM has a p53-response element in its promoter, and is induced by p53 expression or DNA-damaging agents. Knockdown experiments indicated that DRAM is necessary for p53-induced death and autophagy. DRAM may be a specific stress-related regulator of autophagy, and not part of the general autophagic machinery. This is based on the observation that while knockdown of ATG5 inhibits clonogenicity even in the absence of any outside signal, knockdown of DRAM enhances clonogenic growth of cells treated with DNA-damaging agents, but has no effect on basal growth of untreated cells. Interestingly, expression of

DRAM alone is not sufficient to induce death, but does lead to enhanced autophagy. DRAM localizes to the lysosomal membrane, yet its exact functional activity is not yet known. Significantly, DRAM has characteristics of a tumor suppressor. It was found to be downregulated at the mRNA level in nearly 50% of primary squamous cell carcinomas, but not in breast tumors. CpG island methylation-mediated suppression of gene expression accounted for 28% of the cases. Suggestively, *DRAM* tended to be lost more frequently from tumors with intact *p53* compared with mutant *p53*, indicating that the two proteins operate in overlapping pathways to suppress tumorigenesis.

These studies suggest several mechanisms by which p53 can induce autophagy: transactivation of DRAM and inhibition of the mTOR pathway through AMPK and TSC1/2. However, these results are recent and rather preliminary, and require further investigation in order to fully understand their contributions to the regulation of autophagy and to p53's tumor suppressive activity. Considering the multifaceted nature of p53, its numerous transcriptional targets, and transcriptional-independent functions, future research is likely to reveal further mechanisms by which p53 modulates the autophagic pathway.

3 The Cell Death vs Cell Survival Paradox: How Does Autophagy Contribute to Malignancy?

It is clear from the abundance of tumor suppressors and oncogenes that serve some role in the autophagic process that autophagy has a strong link to the development of cancer. Yet, the exact nature of this link has been subject to debate. Autophagy for the most part has been shown to suppress tumor growth and cellular transformation, and several mechanisms have been proposed to explain this phenomenon. Yet, there have also been studies showing that autophagy positively contributes to tumorigenesis. This controversy stems from the bifunctionality of autophagy, which can display either a cytoprotective or cytotoxic role, depending on the nature of the stress conditions and the genetic milieu of the cells which are exposed to these stimuli.

Autophagy, as a fundamental process that controls protein and organelle recycling, has been shown to play an essential cellular survival role. This role is particularly apparent in the phenotype of ATG knockout mice. While Beclin 1 heterozygotes developed spontaneous cancer, homozygous deletion of the gene was embryonic lethal (Yue et al., 2003). ATG7 deficiency in the central nervous system resulted in death within 28 weeks of birth. Severe behavioral defects and neuropathies were observed and the accumulation of inclusion bodies containing uncleared ubiquitinated proteins led to the death of cerebral and cerebellar neurons (Komatsu et al., 2006). Similar accumulation of abnormal ubiquitinated proteins and inclusions were observed in mice deficient for ATG5 in neurons, resulting in neurodegeneration and loss of motor function (Hara et al., 2006). Thus, in these scenarios, autophagy as a means for normal clearance of cytosolic proteins is essential for cell survival.

Early studies on autophagy were performed in yeast, where it is activated by limiting nutrient conditions to provide the cell with energy and amino acids through self-catabolism, thereby ensuring cell survival (Huang and Klionsky, 2002). Although unicellular organisms like yeast may more frequently face a changing extracellular environment that requires autophagic adaptation, multicellular organisms at times are also subjected to stresses that induce autophagy. An elegant example of physiologically relevant starvation-induced autophagy was demonstrated in neonatal mice (Kuma et al., 2004). Immediately after birth, neonates undergo an adaptation phase to the loss of placental blood supply until they learn to suckle and receive nutrients through mother's milk. During this initial period of starvation, autophagy is observed to increase in neonatal tissue, peaking at 3–12 h post-birth, and returning to normal low levels within 1–2 days. This autophagic phase is essential for providing nutrients, and inhibition of autophagy at this stage is lethal. ATG5 knockout mice, though born healthy, die within 12 h of birth unless force-fed, due to the lack of the alternate nutrient source (Kuma et al., 2004). Autophagy is also required for the starvation response in adult mice, as demonstrated by the observance of cell swelling, accumulation of abnormal membrane structures, and damaged mitochondria in the liver of starved ATG7 conditional knockout mice (Komatsu et al., 2005).

Other studies focused on starvation-induced autophagy in cell culture models. For example, removal of IL-3 from cultures of Bax/Bak double knockout bone marrow cells resulted in starvation as a result of an impairment in nutrient uptake (Lum et al., 2005). In the absence of an intact apoptotic pathway, cells remained viable for as long as 24 weeks, but failed to proliferate and showed signs of atrophy and autophagy. The phenotype was reversible; restoration of IL-3 growth factor until 12 weeks after its removal led to the resumption of glycolysis, and eventually, to normal cell growth and proliferation rates. Thus, autophagy in this model was not associated with a point of no return. In fact, inhibition of autophagy through the knockdown of ATG5 or ATG7 accelerated cell death to 2–3 days. Likewise, blockage of autophagy through the use of chemical inhibitors or through RNAi-mediated knockdown of Beclin 1, ATG5, ATG10, or ATG12 enhanced the apoptotic cell death of amino acid- and serum-starved HeLa cells (Boya et al., 2005). In all of these examples, autophagy, rather than killing the cell, supports cell survival through the provision of otherwise lacking nutrients. Similarly, in tumors, cells present in the poorly vascularized tumor core are deprived of oxygen and nutrients, and may utilize autophagy-based recycling to offset starvation. In fact, autophagy was observed in the ischemic, unvascularized central portions of tumors derived from epithelial cells that could not undergo apoptosis due to deletion of Bax and Bak (Degenhardt et al., 2006). In such a scenario, autophagy would contribute to cell survival, and hence, tumorigenesis (Cuervo, 2004).

The very definitive and elegant studies presented earlier were utilized as proof by an adamant school of thought that autophagy is not a death-inducing process. However, equally convincing data has recently emerged that questions this one-sided approach, and it is now clear, that in certain cellular settings, autophagy can, in fact, lead to death. Cell death scenarios have long been observed to be

accompanied by signs of autophagy. For example, autophagy was observed in developmental cell death such as that which occurs during insect metamorphosis, limb bud morphogenesis in birds, and palatal closure in mammals (Schweichel and Merker, 1973; Clarke, 1990; Bursch, 2001). As far back as the 1970s, ultrastructural morphologies that were consistent with autophagic cell death were present upon treatment with certain toxins (Schweichel and Merker, 1973). With the rise in popularity of autophagic cell death, many researchers have now specifically examined cell death morphologies for signs of autophagy, and have observed that common death stimuli thought previously to induce apoptosis can also induce autophagy in certain cell types. For example, both apoptosis and autophagy are observed upon lumen formation of acinar MCF-10A cells, and the death ligand TRAIL was shown to regulate the autophagic pathway (Mills et al., 2004). Etoposide, a known inducer of p53-dependent apoptosis, was shown to induce autophagy in a p53-dependent manner in primary mouse embryo fibroblasts (MEFs) (Feng et al., 2005). Ionizing radiation, inhibition of platelet-derived growth factor (PDGF) signaling, and arsenic trioxide induced autophagy, but not apoptosis, in malignant glioma cells (Kanzawa et al., 2003; Takeuchi et al., 2005a, b; Ito et al., 2005). Autophagy accompanied apoptosis in response to treatment of bovine mammary gland epithelial cells with TGFβ, as a model for mammary gland involution (Gajewska et al., 2005). It was also observed in MCF-7 cells treated with novel analogs of paclitaxel (Gorka et al., 2005) and in gastric cancer and glioma cell lines exposed to oncogenic Ras (Chi et al., 1999).

These basically correlative studies were further supported by results from directed intervention experiments which definitively established a causal relationship between autophagy and subsequent cellular demise in certain scenarios. This latter approach included the use of chemical inhibitors of autophagy, such as 3-methyladenine (3-MA) and wortmannin, which block PI(3)K activity, and more elegantly, specific genetic inhibition of autophagy through the use of RNAi-mediated knockdown of autophagy regulators. For example, anti-estrogen treatment of MCF-7 cells led to death which was accompanied by autophagic vesicle accumulation and was blocked by 3-MA (Bursch et al., 1996). Autophagy was evident upon nerve growth factor deprivation of primary sympathetic neurons, and again, death was blocked by 3-MA (Xue et al., 1999). Treatment of Bax/Bak double knockout fibroblasts with etoposide, thapsigargin, or staurosporine killed the cells despite the absence of apoptosis, and autophagy was evident. Death was attenuated upon addition of 3-MA, ATG5 RNAi, and in Beclin 1 -/- cells, indicating that autophagy contributed to death when apoptosis was blocked (Shimizu et al., 2004). Autophagy was the cause of cell death upon amino acid starvation of PC12 cells, since 3-MA, but not caspase inhibitors, blocked cell death. smARF-induced cell death was likewise shown to be a result of autophagy, as RNAi to Beclin 1 and ATG5 blocked death (Reef et al., 2006). TNFα induced autophagy in Ewing sarcoma cells in the absence of NF-κB signaling, which activates the mTOR pathway. In this system, autophagy enhanced apoptotic cell death, since apoptotic morphologies were inhibited when autophagy was blocked through knockdown of Beclin 1 and ATG7 (Djavaheri-Mergny et al., 2006). A similar phenomenon was observed upon treatment

of T-lymphoblast cell lines with TNFα (Jia et al., 1997). Macrophage cell death, triggered by lipopolysaccharides (LPS) in the presence of zVAD, showed evidence of autophagy and was blocked by chemical autophagy inhibitors and by RNAi to Beclin 1 (Xu et al., 2006). Likewise, autophagy was activated upon inhibition of caspases in L929 mouse fibroblast cells and U937 monocytes (Yu et al., 2004). Cell death, caused by the accumulation of ROS and the selective autophagy-mediated degradation of catalase, was attenuated by knockdown of LC3 and ATG7 (Yu et al., 2006). Furthermore, the involvement of several known death genes in the induction of autophagy, such as DAPk and DRP1 (Inbal et al., 2002), and BNIP3, a BH3-only member of the Bcl-2 family (Vande Velde et al., 2000), is further proof that autophagy promotes death.

In addition to inducing cell death and the removal of unwanted cells, autophagy may suppress tumor growth by other means. Even in circumstances when autophagy does not lead to cell death, it is counterproductive to cell growth. Cancer cells often show lower rates of autophagy and long-lived protein turnover compared to non-transformed cells (e.g., Gunn et al., 1977; Gronostajski and Pardee, 1984; Knecht et al., 1984; Kisen et al., 1993; Toth et al., 2002). Highly proliferative cells require a general increase in protein synthesis to keep up with the high demand for cell mass that must be divided among the ever-increasing number of daughter cells. Blocking the degradation of long-lived proteins through downregulation of autophagy helps favor the balance towards increased cell mass, thus providing a selective advantage to highly proliferative cells (Ng and Huang, 2005). Basal autophagy also serves to eliminate damaged organelles such as depolarized mitochondria that are a source of genotoxic free radicals. In the absence of this important scavenger mechanism, DNA mutations that may lead to cellular transformation can accumulate more readily. Thus, in this manner too, the lack of autophagy promotes tumorigenesis.

4 Autophagy as a Target for Therapeutic Intervention

In light of the data presented earlier, autophagy presents itself as a target for therapeutic intervention in the treatment of malignancies. In fact, several studies have demonstrated that use of chemotherapeutic drugs induces autophagic cell death, in addition to apoptosis, and even in cells that are resistant to apoptosis. For example, the vitamin D analog EB1089 used in cancer treatment induces autophagic cell death in MCF-7 cells, which leads to caspase-independent nuclear apoptosis (Hoyer-Hansen et al., 2005). The proteasome inhibitor MG132 kills PC3 prostate cancer cells by means of a caspase-dependent apoptotic pathway, and at the same time, leads to the upregulation of several autophagic genes. In fact, the cell death response is attenuated by addition of 3-MA. Autophagy was likewise responsible for the toxicity observed in non-small-cell lung cancer cells upon treatment with the rare earth element, neodymium oxide (Chen et al., 2005a, b). An innovative therapy involving a conditionally replicating adenovirus that targets telomerase-positive

cancer cells was shown to kill malignant glioma, cervical cancer, and prostate cancer cells by means of autophagy, most likely through downregulation of mTOR signaling (Ito et al., 2006). The adenovirus treatment also slowed the growth of subcutaneous gliomas in nude mice and prolonged survival of the mice. Both a rapid apoptotic response and a slower autophagic one were observed upon treatment of MCF-7 cells with camptothecin (Lamparska-Przybysz et al., 2005). When apoptotic death was blocked, however, through disruption of Bax and Bid function, autophagy increased. Thus, here, autophagy serves as a backup to the disabled apoptotic pathway. In a similar vein, migrating glioblastoma multiform cells that were refractory to apoptosis-inducing drugs were induced to die via autophagy. The AKT/mTOR signaling pathway was constitutively active in these cells, providing a significant survival advantage. However, inhibition of this pathway with drugs such as temozolomide stimulated autophagy and cell death (Lefranc and Kiss, 2006). Temozolomide was also shown to induce autophagy, but not apoptosis, in malignant glioma cell lines, leading to cytotoxicity (Kanzawa et al., 2004). Histone deacetylase inhibitors, such as butyrate and suberoylanilide hydroxamic acid, can also trigger autophagic cell death in cells that have lost the ability to undergo apoptosis (Shao et al., 2004).

As stated earlier, however, autophagy can also act as a prosurvival pathway in certain cell environments and can thwart the induction of apoptosis. This may be especially true in hypoxic regions of tumors, where autophagy may serve as the only means to provide nutrients and energy to the starved tumor cell. Furthermore, one study of carcinogen-induced pancreatic cancer showed that rates of autophagy, although lower in advanced adenocarcinoma, actually were increased in early-stage premalignant nodules and adenomas (Toth et al., 2002). In these cases, drug treatments that affect autophagy may have opposing effects than those described earlier. For example, sulforaphane induced pronounced autophagy in prostate cancer cell lines, inhibition of which led to the rapid induction of apoptosis (Herman-Antosiewicz et al., 2006). Likewise, inhibition of autophagy enhanced the apoptotic response and, specifically, cytochrome C release, of the colon cancer cell line HT-29 to sulindac sulfide (Bauvy et al., 2001). Crotoxin, a neurotoxin derived from the venom of a South American rattlesnake, induced both apoptosis and autophagy in chronic myeloid leukemia cell lines (Yan et al., 2006). While caspase inhibition blocked cell death, inhibition of autophagy enhanced it. The authors of these papers concluded that the autophagic response of these cells to the chemotherapeutic agent served to suppress the apoptotic response. Similarly, ionizing radiation induced autophagy in breast, prostate, and colon cancer cells, and in malignant glioma cells, yet its inhibition sensitized cells to radiotherapy (Paglin et al., 2001; Ito et al., 2005). These results are consistent with the hypothesis that autophagy actually blocks the damaging affects of radiation by eliminating damaged organelles before they induce apoptosis. Altogether, it appears that effective treatment of malignancies in these specific cases would entail the combinatorial use of chemotherapeutic drugs that induce apoptosis and inhibit autophagy.

Despite the logic behind this approach, one should be wary of drawing conclusions based on responses of cancer cells in culture. An important recent paper addressed

this limitation by assessing the effects of manipulations of the cell death programs, not just on tumor cells in culture, but also in the intact tumor in vivo (Degenhardt et al., 2006). In fact, the authors come to the opposite conclusion of those studies performed exclusively in vitro. Metabolic stress induced by ischemia was utilized to kill epithelial cells. Through genetic manipulation, immortalized baby mouse kidney epithelial cells (iBMK) that were defective in apoptosis, autophagy, or both, were generated. Cells exposed to metabolic stress died primarily by a rapid apoptotic cell death (within 24–72 h). When apoptosis was blocked (i.e., by Bax/Bak deficiency or Bcl-2/X_L expression), autophagy was apparent, but enabled cell survival rather than death. Autophagic cells survived for long periods of time in culture, with normal proliferation rates initially, but eventually stopped dividing and moving, and exhibited signs of cell condensation. These defects, although resembling a death phenotype, were reversible upon restoration of oxygen and nutrients. When autophagy, too, was deficient (i.e., AKT expression or reduction in Beclin 1), cells exhibited a slow, inefficient death defined as necrosis. Significantly, the most aggressive, fastest-growing solid tumors developed from those cells that were deficient in both apoptosis and autophagy. These tumors contained large necrotic areas in ischemic regions, with macrophage infiltration and induction of an innate immune response. Necrotic tumors are known to be particularly aggressive, possibly due to proliferative signals generated by infiltrating immune cells, which encourage cell growth and angiogenesis in regions of the tumor that border the necrotic area.

Based on this study, it seems that effective treatment of solid tumors should involve strategies that encourage both apoptosis and autophagy. The latter could include inhibitors of the mTOR/AKT signaling pathways such as rapamycin and PI(3)K inhibitors (Takeuchi et al., 2005a, b). The benefit of triggering autophagy is somewhat of a paradox, as here, in contrast to the examples cited in the beginning of this section, autophagy enhanced cell survival. Yet, the survival of nonproliferative, dormant cells is preferable to the induction of necrosis, which has severe repercussions on overall tumor growth. Furthermore, treatments that activate autophagy may overcome the safe threshold under which autophagy promotes survival, and actually drive the cells towards autophagic cell death, providing an additional advantage to this strategy.

5 Conclusions and Future Perspectives

The dual nature of autophagy, and the dichotomy created by its contradictory effects on tumorigenesis, translates itself into a debate on whether one should be inhibiting or activating autophagy to treat cancer. More studies on the effects of manipulations of the autophagic program, in conjunction with the apoptotic pathway, on tumor growth in vivo are necessary. The role that autophagy plays, as either a cell survival mechanism or a cell death inducer, may be cancer-type specific, i.e., influenced by the genetic makeup of the corresponding tumor cells and the nature

of the external stresses to which they are exposed. As a consequence, strategies for treatment will require a tumor-by-tumor genetic and environmental analysis. In order to accomplish such an analysis, it is necessary to acquire a complete understanding of autophagy, its molecular regulation, and its cellular effects. In the past several years, the field of mammalian autophagy has advanced in leaps and bounds; hopefully, the continued advances will translate to concrete clinical benefits in the near future.

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Chapter 10
Regulation of Programmed Cell Death by the P53 Pathway

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Abstract The p53 pathway is targeted for inactivation in most human cancers either directly or indirectly, highlighting its critical function as a tumor suppressor gene. p53 is normally activated by cellular stress and mediates a growth-suppressive response that involves cell cycle arrest and apoptosis. In the case of cell cycle arrest, p21 appears sufficient to block cell cycle progression out of G1 until repair has occurred or the cellular stress has been resolved. The p53-dependent apoptotic response is more complex and involves transcriptional activation of multiple proapoptotic target genes, tissue, and signal specificity, as well as additional events that are less well understood. In this chapter, we summarize the apoptosis pathway regulated by p53 and include some open questions in this field.

Keywords p53, apoptosis, transcription, TRAIL receptors, p53-dependent cell death.

1 Introduction

The p53 pathway is inactivated in most human tumors. It is inactivated directly as a result of mutations, with substitution mutations being common, indirectly by binding to viral or cellular proteins, or as a consequence of alterations in proteins regulating its functions (Vogelstein et al., 2000). p53 function is usually switched off, although when the cells get exposed to stress such as DNA damage induced by ionizing radiation or ultraviolet rays, activation of oncogenic signaling, hypoxia, or nucleotide depletion, p53 is accumulated in the nucleus in a tetrameric form (Bode and Dong, 2004). Upon activation, p53 mediates a growth-suppressive effect on cells by blocking the cell cycle or it can lead the cells to undergo programmed cell

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death primarily by binding to particular DNA sequences and activating transcription of specific genes (El-Deiry, 2003).

Programmed cell death, frequently referred to as apoptosis, is induced by either intracellular or extracellular stimuli. In addition to the toxic stresses mentioned earlier, serum deprivation, ligand–receptor interactions between FAS ligand (FasL)–FAS/APO1, tumor necrosis factor (TNF)–TNF receptors, and TRAIL–TRAIL receptors will also induce apoptosis (Ozoren and El-Deiry, 2003). In CD95-mediated apoptosis, there are two cell-type-specific signaling pathways, so-called type I and type II pathways (Scaffidi et al., 1998). In the type I (extrinsic) pathway, caspase-8 activation is sufficient to kill cells as a direct consequence of death receptor ligation with subsequent activation of effector caspase-3, caspase-6, and caspase-7. This death is independent of the mitochondria and is not blocked by overexpression of Bcl-2 or treatment of cells by a caspase-9 inhibitor. On the other hand, the type II (intrinsic) pathway amplifies a cell membrane-initiated death signal via the mitochondria and this form of death can be blocked by Bcl-2 or treatment of cells by a caspase-9 inhibitor.

p53 regulates these classical cell death pathways (Fig. 10.1) by either upregulating proapoptotic genes or by associating with proapoptotic genes in a transcription-independent manner. Understanding of apoptosis is very important as its dysregulation leads to variety of human diseases including cancer, autoimmune diseases, and neurodegenerative disorders. Greater insight into the pathways of apoptosis and their deregulation in disease in fundamental to understanding pathophysiology and to developing novel therapeutic agents.

2 Stabilization and Activation of P53

p53 is normally maintained at low levels in unstressed mammalian cells. The amount of p53 is determined by the rate of its degradation rather than its transcription, as blocking of its interaction with its main negative regulator Mdm2 (also known as HDM2) is sufficient to induce accumulation of the protein in cells (Michael and Oren, 2003; Vassilev et al., 2004). The primary structure of the p53 cDNA can be subdivided into three functional domains. The N-terminal region consists of a transactivation and Src homology 3-like domain, as well as a proline-rich domain. The central core consists primarily of the DNA-binding domain, where contains hot spots for various missense mutations found in human tumors. Several of the hot spots represent contact points between p53 protein and its DNA-response element. The C-terminal domain contains a nuclear localization signal, a nuclear export signal, and a tetramerization domain. The C-terminus provides a regulatory domain whose conformation and acetylation state may impact on p53 DNA binding and transactivation activity.

Mdm2 inactivates p53 by binding to its N-terminal transactivation domain to inhibit its transcriptional activity and by ligating ubiquitin at its C-terminal lysines thereby ultimately targeting p53 for proteasome-mediated degradation

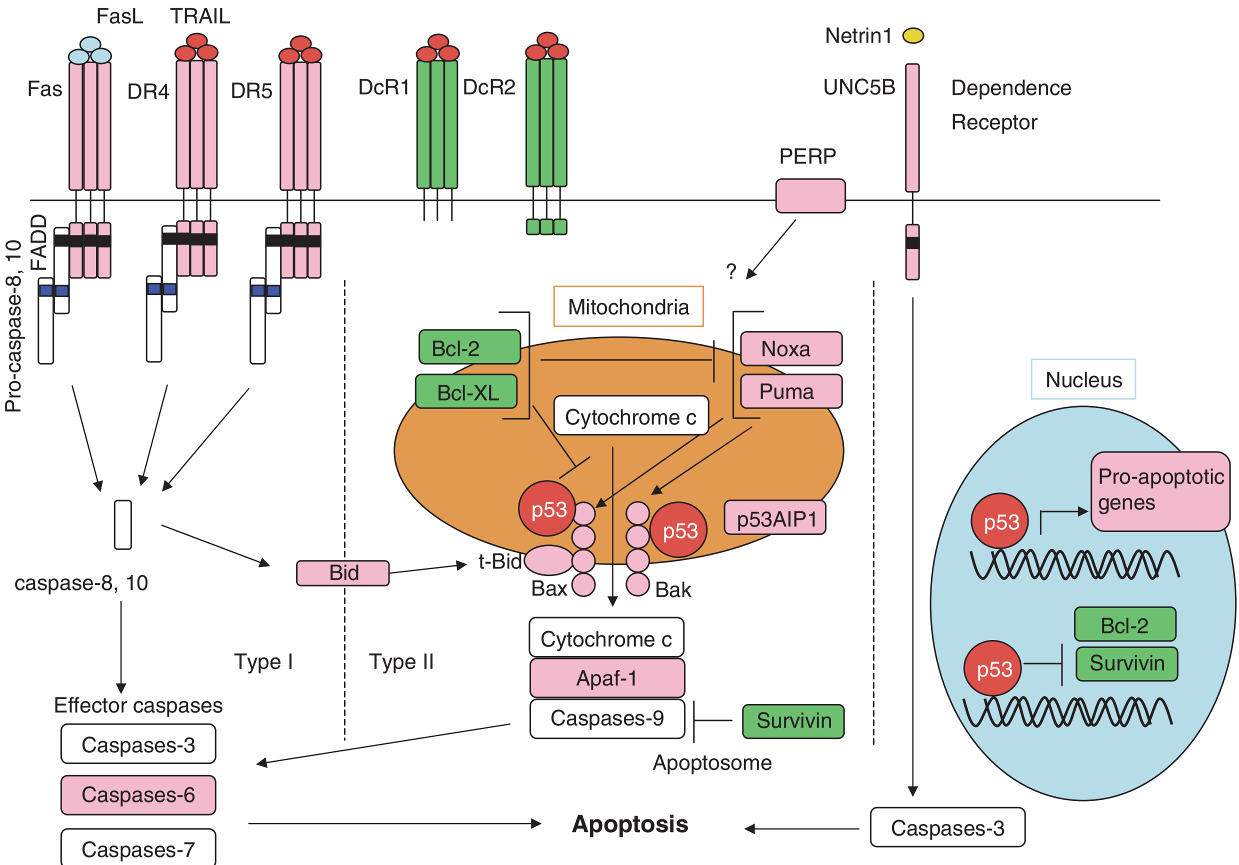

Fig. 10.1 Apoptotic pathways regulated by p53. Please see text for details of the genes, pathways, and mechanisms involved in cell death regulation and the complex signaling networks governed by p53 activity

(Rodriguez et al., 2000). Recent work by the laboratory of Wei Gu has documented monoubiquitination of p53 by Mdm2 leading to nuclear export, polyubiquitination, and degradation. As phosphorylation of N-terminal serines (particularly serine 20) blocks the interaction of p53 with Mdm2 and acetylation of C-terminal lysines prevent p53 ubiquitination by Mdm2 and subsequent degradation, these phosphorylations and acetylations can stabilize and activate p53. DNA damage induced by ionizing radiation and ultraviolet light induce p53 phosphorylation by a number of protein kinases such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), casein kinases, checkpoint kinase 1 (CHK1), checkpoint kinase 2 (CHK2), DNA-dependent protein kinase (DNA-PK), extracellular signal-related kinase (ERK), homeodomain-interacting protein kinase 2 (HIPK2), c-JUN NH_2-terminal kinase (JNK), and p38 kinase in a stimulant/kinase/phosphorylation-site-specific manner (Bode and Dong, 2004). It has also been reported and is well known that phosphorylation of serine 46 is associated with apoptosis induced by p53AIP1 (Oda et al., 2000a) and that exogenous expression of p53 mutant that has defect in serine 46 shows resistance to apoptosis (Ichwan et al., 2006). p300/CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF) acetylate lysines located at carboxyl terminus of p53. Further more, Mdm2 mediates PCAF ubiquitination and degradation (Jin et al., 2004), and can inhibit acetylation of p53 by CBP or p300 (Ito et al., 2001).

Another pathway to stabilize p53 is distinct from the first two mechanisms that involve posttranslational modification of the protein, and rather acts to inhibit the activity of the negative regulator Mdm2. This pathway is activated by oncogenic signals, for example, from Ras and Myc that in turn activate $p14^{ARF}$ leading to inactivation of Mdm2 resulting in p53 activation (Lowe and Sherr, 2003).

3 Type I Pathway

Type I pathway is initiated by ligand binding to its cognate death receptors. Overall eight receptors possessing death domains (DD) have been identified and all belong to the TNF family of receptors, including TNF-R1, Fas (CD95, APO-1), DR3, TRAIL-R1 (DR4), TRAIL-R2 (KILLER/DR5), DR6, $p75^{NTR}$, and EDAR (Ozoren and El-Deiry, 2003). Fas has a secretory decoy receptor (DcR3) that lacks a transmembrane domain. There are two decoy receptors for TRAIL, which lack a DD and these are known as TRAIL-R3 (DcR1, TRID) and TRAIL-R4 (DcR2, TRUNDD). These decoy receptors act as negative regulators of the death pathway. Till date, Fas (Muller et al., 1998), DR4 (Liu et al., 2004), KILLER/DR5 (Takimoto and El-Deiry, 2000), TRID (Ruiz de Almodovar et al., 2004), and TRUNDD (Liu et al., 2005) are reported to contain p53-specific binding sequences in intron 1 and are transcriptionally regulated by p53.

p53 target proteins Fas, DR4, and KILLER/DR5 contain cysteine-rich extracellular domains that bind their cognate ligands and intracellular portions consisting of approximately 80 amino acid DDs that transduce apoptosis-inducing signals.

As death ligands such as FasL or TRAIL exist in a homotrimeric form, binding to their respective receptors leads to receptor trimerization. The ligand/receptor interaction triggers formation of the death-inducing signaling complex (DISC) which contains Fas-activating DD (FADD) and initiator caspases, pro-caspase-8 or pro-caspase-10. FADD is the adaptor protein which links receptors and pro-caspases through its two distinct domains, a DD that binds with the DD of the receptors and a death effector domain (DED) which binds with the DED of pro-caspase-8. Association of pro-caspase-8 with the DISC generates a p20 fragment from the caspase by cleavage and further processing leads to a p10 fragment for its full activation (Medema et al., 1997). This mature caspase can cleave downstream effector caspase-3, caspase-6, and caspase-7 (Riedl and Shi, 2004). The extrinsic pathway can cross talk with the intrinsic pathway via BID. When BID is cleaved by caspase-8, truncated BID is myristoylated, translocates to mitochondria, releases proapoptotic proteins, and further activates death signaling and execution events (Zha et al., 2000).

3.1 Fas (APO-1, CD95)

The Fas receptor is a type I membrane protein expressed abundantly in various tissues. The *Fas* gene is located on human chromosome 10q24.1 and on chromosome 11 in mice (Nagata, 1999). The human and mouse *Fas* genes contain a p53 DNA-binding site in intron 1 and through this site the expression of the *Fas* gene can be transcriptionally upregulated by p53 (Muller et al., 1998; Munsch et al., 2000). Fas is also transcriptionally regulated by Sp1 and NF-κB (Chan et al., 1999; Xiao et al., 2001). The FasL–Fas interaction plays an important role in immune homeostasis, especially maintaining immune privilege in the eye and testis. As tumor cells can evade the host immune surveillance system by overexpressing FasL and can induce apoptosis in the T-cells responsible for the immune response, a phenomenon known as Fas counterattack, an understanding of the Fas-mediated apoptotic pathway is important for understanding tumor biology, It has been reported that CMT93 colon carcinoma cell downregulation of FasL has no effect on cell growth in vitro, but results in reduced tumorigenicity in vivo, possibly by the mechanism of loss of the Fas counterattack (Ryan et al., 2005).

Fas is transcriptionally upregulated by 5-FU and mediates apoptosis in a p53-dependent manner in MCF7 and HCT116 cells (Longley et al., 2004). It has also been reported that p53 relocalizes Fas to the cell surface (Bennett et al., 1998), providing a role of p53 in the Fas apoptotic pathway independent of its transcriptional activity. Wild-type p53 transduction in p53-mutant non-small-cell lung carcinoma cells induces Fas expression and the cells become susceptible to cytotoxic T lymphocyte-mediated killing (Thiery et al., 2005). There are certain p53 mutants, which can induce cell cycle arrest, but not apoptosis, so-called discriminatory mutants. Munsch et al. reported that discriminatory mutants Pro-175 and Ala-143 have activity to induce Fas transcription, but not apoptosis, suggesting upregulation of Fas is not enough to induce apoptosis in some circumstances (Munsch et al.,

2000). Furthermore, Fas does not appear to be required for p53-dependent apoptosis in response to DNA damage by irradiation (Fuchs et al., 1997).

3.2 Trail Receptors

In humans, there are four homologous TRAIL receptors including DR4, KILLER/DR5, TRID, and TRUNDD, as well as a fifth soluble receptor osteoprotegerin (Wang and El-Deiry, 2003a). The extracellular cysteine-rich domains of DR4, KILLER/DR5, TRID, and TRUNDD are 52–69% identical to each other and the DD of DR4 and KILLER/DR5 are 64% identical to each other (Ozoren and El-Deiry, 2003). As these genes are clustered on human chromosome 8p21–22, they might have arisen from a common ancestral gene (Degli-Esposti et al., 1997a). TRAIL seems to be promising for cancer therapeutics as many cancer cells are sensitive to TRAIL while normal cells are not (Wang and El-Deiry, 2003a).

3.2.1 DR4

DR4 protein is a 445 amino acid-containing type I transmembrane receptor, which is generated through the cleavage of a signal sequence of 23 amino acids from a primary protein (Pan et al., 1997a). The protein has three cysteine-rich repeats in the extracellular domain. The DD of DR4 is 30 and 19% identical with that of TNF-R1 and Fas, respectively. It has been reported that nucleotide substitutions in the extracellular domain of DR4 were correlated with increased risk of lung, and head and neck cancers (Fisher et al., 2001). Somatic mutations of DR4 have been found in non-Hodgkin's lymphoma (Lee et al., 2001), breast cancers (Shin et al., 2001), and osteosarcoma (Dechant et al., 2004). The DR4 expression level is known to be the one of the determinants to TRAIL sensitivity in many cancer cell lines (Kim et al., 2000), and homozygous deletion of the *DR4* gene has been reported in the FaDu nasopharyngeal cancer cell line and this is associated with TRAIL resistance (Ozoren et al., 2000). Approximately 20% of the normal population carries the polymorphic DR4 variant that contains adenine to guanine alteration in the DD (K441R). When a DR4 K441R expressing plasmid was transfected into human cells, it acted as a dominant negative TRAIL receptor resulting in decreased sensitivity to TRAIL (Kim et al., 2000). From these observations, DR4 seems to be a major factor determining TRAIL sensitivity. p53 overexpression by adenovirus-p53 induces upregulation of DR4 and DR5 resulting in increased apoptosis by TRAIL treatment in myeloma cells (Liu et al., 2001). As, wild-type p53 is not required for TRAIL sensitivity in many cancer cell lines (Kim et al., 2000), it is the open question that to what extent p53 is involved in DR4-mediated apoptosis.

3.2.2 KILLER/DR5

KILLER/DR5 is a 411 amino acid containing protein that includes a 51 amino acid signal peptide sequence. Other than p53, a recent study demonstrated that NF-κB can also upregulate KILLER/DR5 transcription in the presence of p53 (Shetty et al., 2005). Germline or somatic mutations of the *KILLER/DR5* gene have been reported in head and neck cancer (Pai et al., 1998), non-Hodgkin's lymphoma (Lee et al., 2001), and breast cancer (Shin et al., 2001).

Compared to DR4, many studies were conducted to elucidate the role of KILLER/DR5 in the p53 pathway, as it was first found as a DNA damage-inducible p53-regulated gene in doxorubicin-treated cell lines (Wu et al., 1997). Comparison of the apoptotic response of p53+/+ and p53−/− mice after ionizing radiation is a good in vivo model to study DNA damage-induced p53-dependent apoptosis in the context of studying p53 target gene tissue specificity. While thymus, spleen, and small intestine underwent p53-dependent apoptosis in the mouse model, among p21, E124/PIG8, Bax, Fas, and KILLER/DR5, KILLER/DR5 was the only upregulated gene after γ-irradiation in a p53-dependent manner to induce apoptosis in the spleen and small intestine, implicating a critical role of KILLER/DR5 in the radiation response (Burns et al., 2001). Recent results using DR5 knockout mice further support the importance of DR5 in the p53 pathway (Finnberg et al., 2005). DR5-null mice showed a slightly larger thymus than wild-type mice. As DR5 is the only known TRAIL receptor in mice, the results suggest that negative selection in thymocytes might be in part controlled through the DR5 receptor. In these mice, there was no evidence of spontaneous autoimmune disease as reported in TRAIL knockout mice (Lamhamedi-Cherradi et al., 2003). E1A stabilizes p53 and transactivates its target genes. The result that DR5-null mouse embryo fibroblasts (MEFs) expressing E1A did not undergo apoptosis after TRAIL treatment suggests that there are no other TRAIL receptors in mice besides DR5, which can be transactivated by p53 in mice. DR5-null tissues showed reduced amounts of apoptosis compared to wild-type thymus, spleen, Peyer's patches, and the white matter of the brain. However, because gene targeting of DR5 failed to nullify all death in these organs, it is likely that DR5 is only one of the several p53 target genes that are important in this response. In the colon, DR5 wild type and null mice showed approximately the same amount of radiation-induced cell death. However, in the human colon cancer cell line HCT116 silencing of DR5 induces accelerated growth of tumor xenografts (Wang and El-Deiry, 2004a, b) and also DR5 is required for p53-dependent TRAIL sensitivity in mismatch repair deficient Bax−/− HCT116 cells (Wang and El-Deiry, 2003b). From these observations, even in the colon, DR5 is important in apoptosis within the p53 pathway that suppresses tumor formation or progression. Taken together, DR5 seems to play a critical role in DNA damage-induced programmed cell death in the p53 pathway.

3.2.3 TRID/DCR1 and TRUNDD/DCSR2

TRID and TRUNDD contain extracellular cysteine-rich domains. TRID consists of an extracellular TRAIL-binding domain linked to the membrane through a glycosylphosphatidylinositol (GPI) anchor and completely lacks an intracellular domain, whereas TRUNDD contains an intracellular domain that has a truncated DD which can transduce NF-κB signal (Degli-Esposti et al., 1997b). Over the extracellular domain, TRID is 69 and 52% identical with DR4 and DR5, and TRUNDD is 70%, 57%, and 58% identical with TRID, DR5, and DR4, respectively. TRID mRNA is expressed in normal tissues but not in many tumor cells, giving a rationale for TRAIL on its tumor-specific apoptosis-inducing activity (Sheridan et al., 1997; Pan et al., 1997b).

Although these decoy receptors are regulated by p53, little is known about their role in the p53-regulated apoptotic pathway. TRID is overexpressed by genotoxic stress in p53 intact cells and is overexpressed in gastrointestinal tumors (Sheikh et al., 1999). TRUNDD is induced by adenovirus-p53 overexpression and TRUNDD can delay TRAIL-, p53-, and KILLER/DR5-dependent apoptosis in colon cancer cells (Meng et al., 2000). It has also been reported that silencing of TRUNDD enhances doxorubicin-induced apoptosis in HCT116 cells (Liu et al., 2005). It therefore seems that these decoy receptors are forming a negative feedback loop to dampen p53-mediated apoptotic signaling.

4 Type II Pathway

The type II death pathway is evoked through intrinsic stimuli such as DNA damage, cytotoxic drugs, hypoxia, oncogenic signaling, or even extrinsic death receptor signals in type II cells. Mitochondrial factors are crucial in the efficiency of cell death mediated by this pathway. Cytochrome c released from mitochondria assembles a cytosolic caspase-activating complex called apoptosome which consists of Apaf-1, caspase-9, and cytochrome c, while release of Smac/DIABLO and Htra2/Omi inactivate inhibitor of apoptosis proteins (IAPs), the inhibitors of caspases, enhance apoptosis (Danial and Korsmeyer, 2004). Bcl-2 family members are the key components in this process. They are categorized into three groups according to their function and numbers of Bcl-2 homology (BH) domains. The first group includes antiapoptotic members such as Bcl-2, Bcl-X_L, and MCL-1, which contains four BH domains. Their BH1–3 domains are in close spatial proximity and create a hydrophobic pocket, which can mask a BH3 domain of proapoptotic members, blocking their proapoptotic functions (Muchmore et al., 1996; Sattler et al., 1997). Multidomain proapoptotic members of the family are Bax and Bak, which are thought to form a pore in the mitochondrial membrane and release cytochrome c into the cytosol. These two molecules are thought to be required in the type II pathway as cells lacking both Bax and Bak, but not cells lacking one of them are completely resistant to tBid-induced cytochrome c release and apoptosis (Wei et al.,

2001). The last members of the family are BH3-only proapoptotic proteins, which are Bid, Noxa, Puma, Bad, Bik, and Bim. Bid provides the only known connection between the extrinsic and intrinsic pathways, while the others are thought to act upstream of Bax and Bak. Cartron et al. (2004) showed that Bid and Puma specifically bind to the first α-helix of Bax leading to its activation. Bad and Bik cannot directly activate Bax but promote apoptosis by binding to Bcl-2 to inhibit their antiapoptotic functions (Letai et al., 2002).

4.1 Bid

Bid gene is transcriptionally regulated by p53 and contains a functional p53 DNA-binding site in the first large intron (Sax et al., 2002). Bid−/− MEFs are resistant to adriamycin and 5-FU as compared to Bid+/+ MEFs, showing its role as a chemosensitivity determinant (Sax et al., 2002). Recently, Bid was shown to be a sentinel for DNA damage, and it was reported to be phosphorylated by ATM (Zinkel et al., 2005; Kamer et al., 2005). It was also shown that other than the proapoptotic function of the protein, when it is phosphorylated following exposure to low dose of ionizing radiation or the DNA-damaging agent etoposide, Bid can block the cell cycle in the G2 phase. Even though it does not induce cell cycle arrest in the G1/S phase as is brought about by p21, Bid might be one of the regulators that determines cell fate after DNA damage, i.e., whether the cells should live or die.

4.2 Puma and Noxa

Puma (bbc3) and Noxa are p53 target genes belonging to the BH-3 only proteins, contain p53 DNA-binding sequences in the first intron and can induce apoptosis by p53 overexpression or exposure to DNA-damaging stimuli (Oda et al., 2000a; Yu et al., 2001; Nakano and Vousden, 2001; Han et al., 2001). Serum starvation and glucocorticoid treatment also induce Puma and virus infection and interferon induce Noxa expression independent of p53 activation, respectively (Han et al., 2001; Sun and Leaman, 2005). In hematopoietic progenitor cells, Slug represses p53-mediated transcription of Puma in turn protecting the cells from γ-radiation-induced cell death, and it was also found that Slug itself was upregulated by p53 (Wu et al., 2005). Furthermore, it was recently demonstrated that p53 family member p73 transactivates Puma and Noxa expression independent of p53, and the other member delta p63 acts as their repressor inhibiting head and neck tumor cells from apoptosis (Rocco et al., 2006). Noxa and Puma are tightly regulated genes with redundancy in their stimulation and regulation by transcription factors, suggesting their important role in apoptosis.

These p53 targets seem to have tissue specificity. By ionizing radiation, Noxa was expressed in the red pulp, where as Puma was induced in the white pulp of the

spleen in a p53-dependent fashion (Fei et al., 2005). Puma–/– MEFs and Noxa–/– MEFs showed increased resistance in apoptosis induced by etoposide treatment or γ-irradiation. Although single gene, *Puma* or *Noxa*, knockdown could not attain the resistant level to that of p53–/– MEFs, it was suggested that Puma and Noxa have redundancy in inducing apoptosis in these cells (Villunger et al., 2003). On the other hand, Puma knockout nullified nearly all of the cell death attributed to p53 in primary hematopoietic cells and the developing central nervous system in response to γ-radiation or oncogenic signals from c-Myc, i.e., it has indispensable role in apoptosis in these tissues (Jeffers et al., 2003). Yu et al. (2003) reported that targeted deletion of *Puma* gene in HCT116 cells completely blocked apoptosis induced by p53 overexpression, adriamycin exposure, or a hypoxic environment (Yu et al., 2003). Another important notion of the study is that in the presence of p21, cellular stresses lead to cell cycle arrest, whereas deprivation of p21 by gene targeting results in enhanced apoptosis induced by the same stimuli. From such observations, it has been proposed that cell fate between cell cycle arrest and apoptosis is determined by the balance between p21 and Puma. Another study showed Noxa and Bax doubly knocked out MEFs were more resistant to apoptosis induced by adriamycin or oncogenic signals as compared to single knockouts of these genes (Shibue et al., 2003). It was suggested from the result that Noxa and Bax carry out different functions in the apoptosis pathway.

4.3 Bax and Bak

The gene-encoding BAX is a transcriptional target of p53 (Miyashita and Reed, 1995). BAK has also been reported to be upregulated by p53 (Pearson et al., 2000; Pohl et al., 1999). Bax and Bak appear to have some overlapping roles in apoptosis, as either thymocytes from Bak–/– or Bax–/– null mice do not show radiation-induced apoptosis, although thymocytes from Bak and Bax double-knockout mice show resistance to γ-radiation or etoposide treatment (Lindsten et al., 2000). Furthermore, Bak and Bax doubly deficient MEFs show resistance to multiple intrinsic death-inducing stimuli such as staurosporine, ultraviolet radiation, and growth factor deprivation (Wei et al., 2001). Bax–/– HCT116 cells are resistant to TRAIL treatment, but etoposide and camptothecin treatment of the cells restores their sensitivity to TRAIL by upregulating Bak and DR5 (LeBlanc et al., 2002). However, recent studies with DR5 or Bak knockdown suggests that this restored sensitivity relies more on DR5 upregulation and a conversion of cells from type II to type I signaling, in the case of TRAIL and chemotherapy treatment (Wang et al., 2003b).

Recent studies revealed that p53 may have a transcription-independent activity in the mitochondrial death pathway involving Bak and Bax. After DNA damage induced by irradiation or chemotherapeutic agents, p53 has been reported to translocate to the mitochondria and to activate either Bax- (Chipuk et al., 2005) or Bak- (Leu et al., 2004) dependent mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c into the cytosol. However, translocation of

p53 to the mitochondria is not sufficient to induce cell death (Essmann et al., 2005), as p53 is sequestered by Bcl-X_L at mitochondria and its activity to induce MOMP is blocked (Mihara et al., 2003; Chipuk et al., 2005). Puma has been reported to act on the complex of p53–Bcl-X_L thereby releasing p53 from Bcl-X_L to allow for the MOMP-inducing activity (Chipuk et al., 2005). However, whether the p53–Bcl-X_L or p53–Bcl2 complexes act as positive or negative regulators of cytochrome c release is still under study (Mihara et al., 2003; Chipuk et al., 2005; Tomita et al., 2006).

4.4 P53AIP1

The *p53AIP1* gene is induced following severe DNA damage associated with p53 ser-46 phosphorylation and localization of p53AIP1 at mitochondria (Oda et al., 2000b). Phosphorylation of p53 and subsequent p53AIP1 induction is also regulated by the p53-inducible protein p53DINP1 (Okamura et al., 2001). p53AIP1 has been reported to have potential to release cytochrome c from mitochondria into the cytosol and to induce apoptosis, although its precise mechanism and relation with other apoptotic factors is not clarified yet.

4.5 Apaf-1

The *Apaf-1* gene is a transcriptional target of p53 and it is also transcriptionally induced by E2F (Moroni et al., 2001). The study comparing p53−/− and wild-type mice showed that Apaf-1 expression was p53-dependent in the spleen and heart (Ho et al., 2003). Apaf-1−/− MEFs were resistant to p53-dependent cell death-induced by oncogenic Myc and Ras signaling (Soengas et al., 1999). Apaf-1 was found to be silenced in metastatic melanomas by hypermethylation and restoration of Apaf-1 expression led to efficient caspase-9 activation and adriamycin-induced cell death (Soengas et al., 2001), supporting its role as a chemosensitivity determinant.

5 Dependency Receptor Pathway

There is unique apoptotic pathway called dependency receptor pathway. In the absence of ligand, expression-dependent receptors induce apoptosis, whereas binding of cognate ligands to their receptors blocks apoptosis and this apoptotic pathway seems to be independent of mitochondria (Arakawa, 2004; Bredesen et al., 2004). Examples for the receptor/ligand are $p75^{NTR}$/neurotrophin, UNC5B (p53RDL1)/Netrin-1, and deleted in colorectal cancer (DCC)/Netrin-1. These receptors are involved in axon guidance during neuronal development and among these receptors

UNC5B was shown to be a p53 transcriptional target, which is implicated in p53-dependent apoptosis (Tanikawa et al., 2003). Loss of DCC has been reported in many cancers, and binding of Netrin-1 to UNC5B has been reported to repress the p53 target genes *Bax* and *p21*. This newly found pathway might be also involved in p53-related tumorigenesis.

6 PERP

The *PERP* gene is transcriptionally upregulated by p53 (Attardi et al., 2000) as well as p63 (Ihrie et al., 2005). It is a membrane protein involved in apoptosis induced by p53 overexpression and Bcl-2 reduces the cell death, suggesting that the mitochondria are involved in its signaling (Attardi et al., 2000). PERP localizes specifically to desmosomes, adhesion junctions important for tissue integrity. Numerous structural defects in desmosomes are observed in skin of PERP−/− mice (Ihrie et al., 2005). It was recently reported that PERP-null mice are not tumor-prone as compared to wild-type mice (Ihrie et al., 2006). As p53-null mice are tumor prone, whereas single knockout of the other p53 targets such as Puma, Bak, or Bax do not produce tumor-prone mice, the observation does not imply that PERP is not important in the p53 apoptotic pathway.

7 PIGs

The PIGs are “p53-induced genes,” identified by transducing p53 into the human colorectal cancer line DLD-1 that undergoes apoptosis in response to p53 expression (Polyak et al., 1997). As many of these genes were capable of producing or responding to reactive oxygen species, the importance of reactive oxygen species in the p53 pathway was suggested. One of the PIGs, EI24/PIG8, has also been identified as the gene upregulated by etoposide treatment in murine NIH3T3 cells (Lehar et al., 1996). It was recently shown that EI24/PIG8 colocalizes at the endoplasmic reticulum with Bcl-2 and loss of EI24/PIG8 is positively related with invasiveness of breast cancers (Zhao et al., 2005).

8 Caspase-6

Caspases, the cysteine proteases that cleave after an aspartate residue in their substrate, are the central components of the apoptotic pathway. They are usually divided into two classes, the initiator caspase-2, caspase-8, caspase-9, and caspase-10, and the effectors, caspase-3, caspase-6, and caspase-7 (Riedl and Shi, 2004). Caspase-6 is a transcriptional target of p53 in the apoptotic response (MacLachlan

and El-Deiry, 2002). Caspase-1 is also a p53 transcriptional target, although it is involved in inflammatory response rather than apoptotic pathway (Gupta et al., 2001). p53 seems to have potential to activate caspase-6 and sensitize cells to chemotherapeutic drugs leading them to apoptosis by the mechanism other than its transcriptional upregulation (MacLachlan and El-Deiry, 2002). We have also previously reported that caspase-10 is directly induced by p53.

9 P53-Dependent Apoptosis Under Hypoxic Conditions

Solid tumors acquire regions of hypoxia as a result of insufficient blood supply. Cells containing wild-type but not mutant p53 undergo apoptosis in hypoxic regions (Graeber et al., 1996), leading to a powerful selection pressure to promote tumor progression and therapeutic resistance (Harris, 2002). p53 shows an altered behavior under hypoxia. Under hypoxia, p53 does accumulate in cells, although it does not upregulate most of the known p53 target genes such as *p21, Bax, GADD45, DR5*, or *Puma* (Koumenis et al., 2001; Fei et al., 2004). We have recently identified that Bnip3L is playing a role in apoptosis during hypoxia in some human tumor cell lines (Fei et al., 2004). Bnip3L was found to be a direct transcriptional target of p53 as well as hypoxia-inducible factor 1 (HIF1). p53-dependent apoptosis during hypoxia was reduced after knocking down Bnip3L. Furthermore, nontumorigenic U2OS cells were converted into a tumorigenic state in mouse xenograft experiments following stable Bnip3L knockdown.

10 Transcriptional Repression of Antiapoptotic Genes

IAPs and Bcl2 block apoptosis by inhibiting caspase activation and MOMP. In addition to transcriptional activation activity, p53 exerts its apoptosis-promoting effects by repressing antiapoptotic gene transactivation (Murphy et al., 1999; Wu et al., 2001; Hoffman et al., 2002). Its mechanism appears to involve association of p53 with histone deacetylases (HDACs) and its interaction is mediated by corepressor mSin3a. DNA damage induces the p53–mSin3a interaction and targets HDACs to the promoters of the p53-repressed genes, where HDACs deacetylate histones and create a chromatin environment that is unfavorable for transcription.

11 P53 as a Therapeutic Target

A number of strategies have been developed to target p53 in cancer therapy. For about half of human cancers, which possess wild-type p53, the Mdm2–p53 interaction could be a major target to prevent p53 from degradation. Nutlin-3 is an example of

a small molecule that specifically disrupts the p53–Mdm2 interaction. It was recently demonstrated that administration of Nutlin-3 suppressed xenograft growth in a dose-dependent manner (Tovar et al., 2006). As Mdm2 downregulation and subsequent p53 upregulation is reported to bring lymphocytopenia as a side effect in hypomorphic Mdm2 mice, further study may help to compare its benefit to disadvantage or advantages over standard chemotherapy.

Histone deacetylase inhibitors (HDACIs) have been shown to exert various antitumor effects and they are presently in clinical trials (see Chapter 13). p53 is one of the targets of HDACIs, as HDACIs inhibit deacetylation of the C-terminal lysines and induce apoptosis in gastric cancer (Terui et al., 2003) and prostate cancer cells (Roy et al., 2005). It has also been demonstrated that HDACIs enhance the tumoricidal effects of p53 adenovirally transferred gene therapy (Takimoto et al., 2005).

The status of an intact p53 pathway positively correlates with the response to the majority of chemotherapeutic drugs, most, although not all, of them being DNA-damaging agents (Weinstein et al., 1997; O'Connor et al., 1997). However, there are some clinically useful agents such as the antimitotic agent taxol, which was found to be more effective in tumor cells with mutant p53 (Weinstein et al., 1997). In this context, we have identified the Polo-like kinase family member serum-inducible kinase (Snk/Plk2) as a p53 target and its silencing by siRNA leads to mitotic catastrophe after taxol treatment, suggesting p53-dependent activation of Snk/Plk prevents mitotic catastrophe following spindle damage (Burns et al., 2003).

Much effort has been devoted to overcome mutant p53 by small molecules that can restore the wild-type functions to mutant p53. CP-31398, a strylquinazoline, was identified from a screen of the library containing more than 10,000 synthetic compounds (Foster et al., 1999). The molecule not only promotes the stability of wild-type p53, but also allows mutant p53 to maintain an active conformation, enabling transcription and subsequent tumor growth suppression. CP-31398 can cause either cell cycle arrest or cell death in tumor cell lines carrying mutant p53, and combination of CP-3198 with chemotherapy or TRAIL exhibit synergistic effects enhancing cell killing (Takimoto et al., 2002). It has been shown that stabilization of p53 by CP-31398 involves a mechanism targeting blockade of ubiquitination of p53 and its further degradation (Wang et al., 2003). Neither phosphorylation of p53 at serine 15, 20, or interaction between Mdm2 was inhibited by CP-31398, highlighting a unique mechanism by which it can activate p53. PRIMA-1 also induces apoptosis in tumor cells (Bykov et al., 2002) and it has synergistic effects with chemotherapeutic drugs (Bykov et al., 2005).

A peptide derived from the C-terminus of p53 is known to activate its specific binding to DNA including several p53 DNA contact mutants (Hupp et al., 1995). Several cationic peptides such as TAT and polyArg can penetrate into the cells through a mechanism called macropinocytosis (Wang and El-Deiry, 2004b). Utilizing this technology, Snyder et al. (2004) showed that the C-terminal peptide of p53 fused with TAT induced cell cycle arrest and apoptosis in a peritoneal carcinomatosis model and prolonged survival of the mice.

12 Future Directions

Already a quarter century has passed since the discovery of p53 and we have learned much about its important role as a tumor suppressor gene as well as its complicated network governing programmed cell death. However, there are still important problems left to be solved. There are numerous genes known to be involved in the p53 pathway, but are they all equally important? Which genes are involved in which tissues? No single gene so far can account for p53-mediated apoptosis alone, and it might be possible that there is no such gene. The principle question is that we still do not know how p53 determines cell fate. Progress towards this understanding as well as efforts to develop therapies targeting this p53 pathway and its family members represent important future directions.

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Chapter 11
Regulation of Programmed Cell Death by NF-κB and its Role in Tumorigenesis and Therapy

Yongjun Fan, Jui Dutta, Nupur Gupta, Gaofeng Fan, and Céline Gélinas*

Abstract The Rel/NF-κB transcription factors are key regulators of programmed cell death (PCD). Their activity has significant physiological relevance for normal development and homeostasis in various tissues and important pathological consequences are associated with aberrant NF-κB activity, including hepatocyte apoptosis, neurodegeneration, and cancer. While NF-κB is best characterized for its protective activity in response to proapoptotic stimuli, its role in suppressing programmed necrosis has come to light more recently. NF-κB most commonly antagonizes PCD by activating the expression of antiapoptotic proteins and antioxidant molecules, but it can also promote PCD under certain conditions and in certain cell types. It is therefore important to understand the pathways that control NF-κB activation in different settings and the mechanisms that regulate its anti- vs pro-death activities. Here, we review the role of NF-κB in apoptotic and necrotic PCD, the mechanisms involved, and how its activity in the cell death response impacts cancer development, progression, and therapy. Given the role that NF-κB plays both in tumor cells and in the tumor microenvironment, recent findings underscore the NF-κB signaling pathway as a promising target for cancer prevention and treatment.

Keywords Rel/NF-κB, apoptosis, necrosis, transcription factor, cancer, therapy

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1 Introduction

The Rel/NF-κB family of proteins is comprised of homologous transcription factors that mediate the cellular response to various exogenous or endogenous stimuli including infection, inflammation, stress, or injury (reviewed in Bonizzi and Karin, 2004; Hayden and Ghosh, 2004). This multimember family consists of the vertebrate c-Rel, RelA, RelB, p105/p50 NF-κB1, and p100/p52 NF-κB2 subunits, the viral oncoprotein v-Rel, Xenopus X-Rell, and the Dorsal, Dif, and Relish factors from *Drosophila*. Rel/NF-κB proteins share a highly conserved Rel homology domain (RHD) at their N-terminus that allows them to engage in homodimer or heterodimer formation, enter the nucleus, and bind to consensus GGGRNNYYCC NF-κB DNA sites. It also enables them to associate with inhibitory IκB molecules that act in an autoregulatory feedback fashion to terminate the activation process. The C-terminal domains of NF-κB factors are more divergent across the family and impart transcriptional activation properties to c-Rel, RelA, RelB and v-Rel proteins, or inhibitory properties to p105/NF-κB1 and p100/NF-κB2 that contain ankyrin-repeats akin to those found in IκB proteins.

In resting cells, cytosolic NF-κB dimers are inactive and typically bound to IκB proteins that prevent their nuclear translocation and binding to consensus NF-κB DNA-binding sites. Two distinct NF-κB activation cascades that respond to different stimuli have been documented (Fig. 11.1). The canonical (or classical) NF-κB pathway is activated by proinflammatory and mitogenic stimuli such as cytokines, bacterial lipopolysaccharides (LPS), interleukin-1 (IL-1), and antigens. This pathway commonly converges upon activation of the IκB kinase complex (IKK complex), a large multisubunit entity comprised of the catalytic IKKα and IKKβ subunits and the regulatory subunit IKKγ/NEMO. Phosphorylation of IκBα on serines 32 and 36 targets it for ubiquitination at lysines 21 and 22 by the E3 ligase SCF-βTrCP. Degradation of polyubiquitinated IκBα by the 26S proteasome frees NF-κB dimers, like the classical p50/p65 complex, enabling their entry into the nucleus where they bind to NF-κB DNA sites. This commonly results in the transcriptional activation of genes important for immune and inflammatory responses, cell proliferation, and/or suppression of apoptosis. Among the many genes that NF-κB regulates, transcriptional activation of its inhibitor IκBα generates an autoregulatory feedback loop that terminates the activation process. Consequently, activation of the NF-κB pathway is normally a regulated and transient process that is important for normal innate and adaptive immunity, inflammatory and acute phase responses and for embryonic development, organogenesis, and homeostasis. In contrast, sustained activation of the NF-κB pathway is implicated in a wide variety of pathological conditions including immune system disorders, chronic inflammation, and cancer.

The noncanonical (or alternative) NF-κB signaling cascade is characterized by the tightly regulated processing of the p100/NF-κB2 precursor protein into a mature p52 subunit and is commonly involved in the preferential activation of RelB/p52

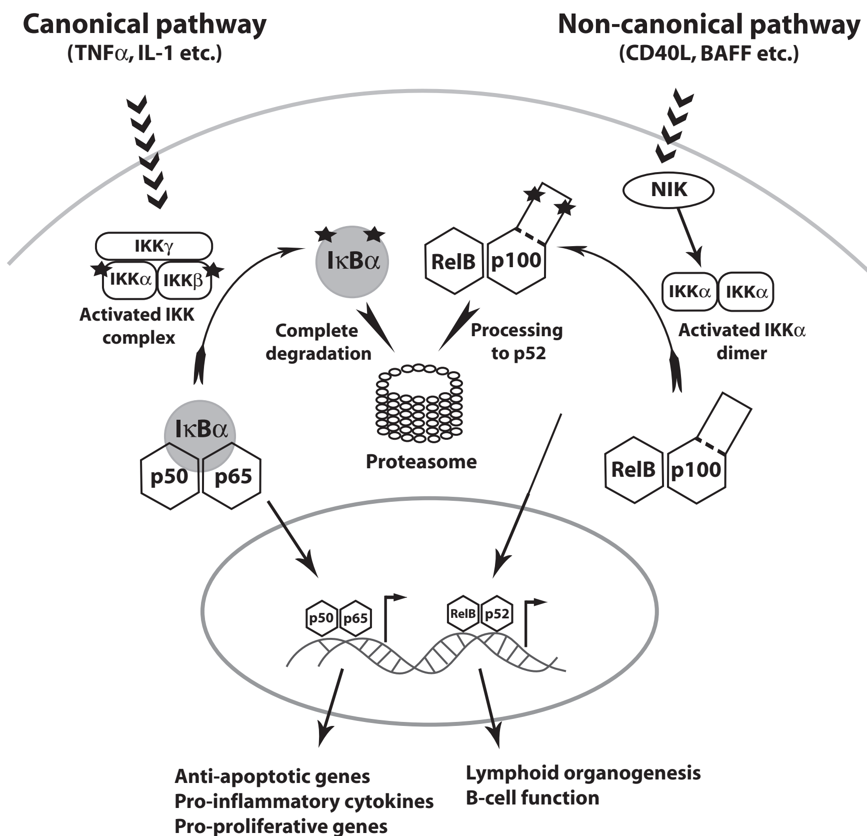

Fig. 11.1 The canonical (classical) and noncanonical (alternative) NF-κB signaling pathways. In the canonical NF-κB pathway, binding of cytokines, LPS, IL-1, or antigen T-cell surface receptors leads to activation of the IKK kinase complex that induces phosphorylation of IκBα and promotes its ubiquitin-dependent degradation via the proteasome. Cytosolic NF-κB dimers (e.g., p50/p65 complexes) are then free to translocate to the nucleus where they bind to consensus NF-κB DNA sites and activate gene expression. This pathway is commonly involved in the activation of antiapoptotic genes, inflammatory cytokines and genes that promote cell proliferation, angiogenesis, and metastasis. The noncanonical NF-κB cascade is activated in response to cell stimulation with BAFF, LTβ, or CD40L and leads to activation of the kinase NIK. NIK phosphorylates IKKα to induce phosphorylation of the C-terminus of p100/NF-κB2. This targets p100 for ubiquitination and partial proteasome-mediated degradation to generate a mature p52/NF-κB2 form. This commonly results in nuclear translocation of RelB/p52 complexes, their binding to NF-κB DNA sites and the activation of gene expression. This pathway is important for lymphoid organogenesis and B-cell function

dimers (Qing and Xiao, 2005; Senftleben et al., 2001; Xiao et al., 2001, 2004); (Fig. 11.1). Activation of this pathway occurs predominantly in B cells stimulated with BAFF, lymphotoxinβ (LTβ), or CD40L and is important for B-cell function and lymphoid organogenesis. In this cascade receptor stimulation leads to activation of the NF-κB-inducing kinase NIK that activates IKKα complexes, independently of

IKKβ and IKKγ/NEMO, to phosphorylate serines 866 and 870 in the C-terminus of p100/NF-κB2 (Liang et al., 2006; Qing et al., 2005). Consequent ubiquitination of p100 by SCF-βTrCP results in the cleavage and partial degradation of p100 to the mature p52 form via the proteasome (Rape and Jentsch, 2004). Some reported that p100/NF-κB2 could also undergo cotranslational processing by the proteasome (Heusch et al., 1999). The functional consequences of NF-κB activation via the canonical or noncanonical pathways are many, but for the purpose of this review, we will focus on those associated with its role in programmed cell death (PCD) via apoptosis or necrosis, and on the mechanisms by which it operates in these different contexts.

2 Role of NF-κB in Apoptosis and Necrosis

NF-κB can protect cells from apoptosis induced by many different death-inducing stimuli including antigen receptor cross-linking in B cells, chemotherapeutic agents, radiation, and the proinflammatory cytokine TNFα, although in some instances it can behave in a proapoptotic manner (Grumont et al., 1998; Owyang et al., 2001; Van Antwerp et al., 1998; Wang et al., 1998; Wu et al., 1996; reviewed in Kucharczak et al., 2003). Studies focusing on the activity of NF-κB in cells treated with TNFα, UV radiation, or chemotherapeutic agents have provided important insights into the mechanisms that underlie its antiapoptotic vs pro-death effects and on those that govern this decision, as reviewed below.

2.1 Choosing between Life and Death Downstream of Activated TNFR1

Detailed analysis of the signaling cascade initiated by TNFα revealed important clues regarding the role that NF-κB plays in the apoptotic response, and recently in necrosis (see Section 2.2). These studies also illustrated that NF-κB plays a crucial role in tipping the balance in favor of survival following Tumor necrosis factor receptor (TNFR) activation.

Although activation of TNF receptor 1 (TNFR1) by TNFα can initiate PCD, TNFα is usually not cytotoxic, as concomitant activation of the NF-κB pathway confers efficient protection. In fact, cell killing by TNFα is seen only under conditions where NF-κB activity is suppressed, or if RNA or protein synthesis is inhibited. Binding of TNFα to TNFR1 triggers trimerization of the receptor and initiates three different cascades that can differentially affect the fate of the cells (Fig. 11.2; Micheau and Tschopp, 2003; reviewed in Jaattela and Tschopp, 2003). The first involves the cooperative recruitment of the adaptor molecule TNFR-associated death domain (TRADD) and the receptor-interacting protein kinase 1 (RIP1), along with TNFR-associated factor 2 (TRAF2). This promotes IKK-dependent activation

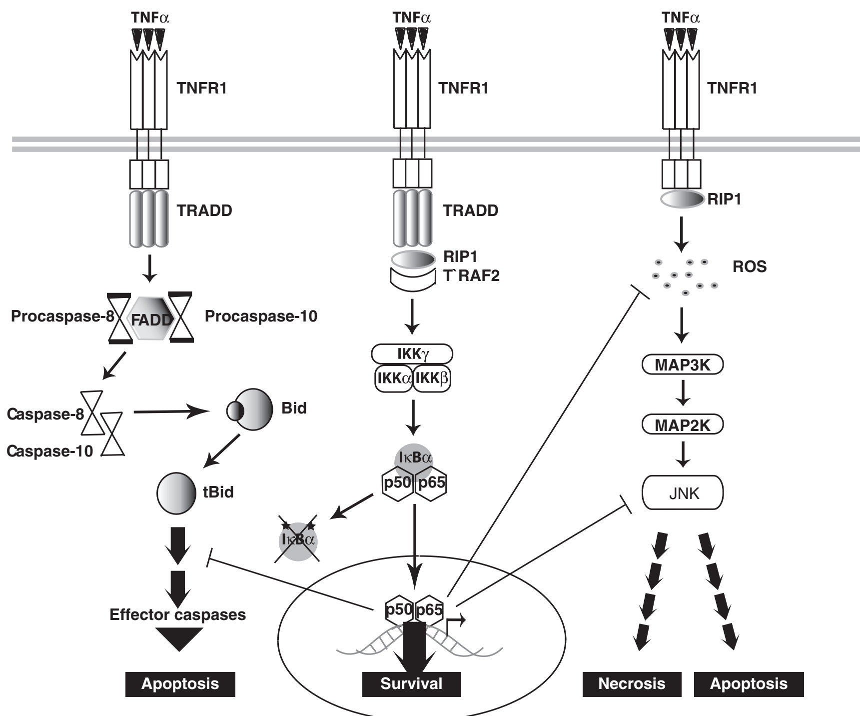

Fig. 11.2 Activation of TNFR1 initiates three different signaling cascades that differentially affect the fate of the cells. Cooperative recruitment of TRADD, RIP1, and TRAF2 to TNFR1 promotes IKK-dependent activation of NF-κB and the activation of antiapoptotic genes, leading to cell survival (*center*). Recruitment of TRADD to TNFR1 in absence of RIP1 engages FADD, caspase-8, and caspase-10 leading to cleavage-mediated activation of Bid into tBid. Translocation of tBid to mitochondria provokes the release of cytochrome C and Smac/Diablo, and activation of effector caspases resulting in apoptosis (*left*). Ligand binding to TNFR1 can also trigger recruitment of RIP1 to the TNFR1 complex in absence of TRADD. This leads to production of reactive oxygen species (ROS), activation of the JNK signaling cascade, and results in PCD via necrosis or apoptosis (*right*). Efficient NF-κB-dependent synthesis of antiapoptotic proteins and antioxidant molecules is thus necessary to block apoptosis and necrosis triggered by TNFα

of NF-κB and that of antiapoptotic proteins like the cellular FLICE/caspase-8 inhibitor protein (c-FLIP). Ubiquitination of TNFR1 and TRADD promotes initiation of a second cascade that engages FLICE-associated death domain (FADD) and caspase-8/FLICE along with caspase-10, that leads to the cleavage-mediated activation of the BH3-only protein Bid into tBid. tBid translocates to mitochondria and associates with the proapoptotic BH1–3 factors Bax and Bak. This provokes the mitochondrial release of cytochrome C and Smac/Diablo, activation of caspase-9, and downstream effector caspases resulting in apoptosis. Thus, efficient NF-κB-dependent synthesis of antiapoptotic proteins like c-FLIP by the first cascade is necessary to block apoptosis induced by the second cascade. Consequently, cells

deficient for NF-κB readily undergo apoptosis in response to TNFα, as do cells in which inhibition of RNA or protein synthesis precludes activation of prosurvival NF-κB target genes (Yeh et al., 2000).

Ligand binding to TNFR1 can also trigger a third cascade that leads to necrotic cell death, a mode of PCD that is morphologically distinct from apoptosis and is independent of caspases. This cascade depends on the recruitment of RIP1 to the TNFR1 complex, in absence of TRADD, on RIP1 kinase activity and its ability to induce production of reactive oxygen species (ROS) and activate the JNK signaling cascade (see Section 2.2; Zheng et al., 2006; reviewed in Jaattela and Tschopp, 2003; Leist and Jaattela, 2001; Papa et al., 2006). While it remains to be determined how cells decide to die by apoptosis vs necrosis, their metabolic state appears to be an important factor in this decision (Edinger and Thompson, 2004). Furthermore, recent evidence that recruitment of RIP1 to TNFR1 precludes engagement of TRADD in this cascade suggests that the joint vs exclusive engagement of these molecules by TNFR1 may also help to determine whether the cell will take the NF-κB survival path, the apoptotic path or will undergo death via necrosis (Zheng et al., 2006). An important distinction between cells dying by apoptosis or by necrosis is that contrary to apoptosis, cells dying by necrosis trigger a strong inflammatory response due to the release of potent proinflammatory factors such as the chromatin-associated HMGB1 protein. HMGB1 binds to the receptor for advanced glycation end products (RAGE), Toll-like receptors TLR2 or TLR4 on macrophages to signal production of proinflammatory cytokines (reviewed in Lotze and Tracey, 2005; Zeh and Lotze, 2005), although some have recently argued that HMGB1 binds preferentially TLR2 and TLR4, but not RAGE as determined by fluorescence resonance energy transfer (FRET) analysis (Park et al., 2006).

2.2 Interplay Between NF-κB and JNK: Additional Insights into NF-κB's Protective Activity Toward Apoptosis and Necrosis

The cross talk between the NF-κB- and JNK-signaling pathways and its impact on the outcome of the cells has been the subject of several excellent reviews (Luo et al., 2005a; Nakano et al., 2006; Papa et al., 2006). Here, we briefly outline how their interplay can result in cell survival, apoptosis, or necrosis.

In addition to promoting activation of NF-κB, binding of TNFα to TNFR1 triggers activation of the MAPK-related stress-activated Jun kinase (JNK), as illustrated in the third cascade (Fig. 11.2). Detailed analyses with cells defective for either JNK or NF-κB outlined a key role for JNK in TNF-induced cell death and showed that the ability of NF-κB to antagonize JNK signaling is an important component of NF-κB's arsenal against the cytotoxic effects of TNFα (reviewed in Luo et al., 2005a; Papa et al., 2004b, 2006; see below). Studies showed that NF-κB is responsible for the transient activation of JNK in response to TNFα, and that suppression of NF-κB activity results in sustained JNK activation, aberrant ROS accumulation, and cell death (De Smaele et al., 2001; Javelaud and Besancon, 2001; reviewed in Papa et al., 2004,

2006; Tang et al., 2001). In turn, ROS can induce sustained JNK activity by inactivating MAPK phosphatases (MKPs), thus allowing TNFα to kill cells in which NF-κB is active (Kamata and Hirata, 1999; Kamata et al., 2005; Sakon et al., 2003). However, this may not be the only mechanism that activates JNK following ROS accumulation, as others showed that ROS activate ASK1/MEKK5 that leads to prolonged JNK activation downstream of TNFR1 (Davis, 2000; Matsuzawa and Ichijo, 2005).

There is still debate in the field regarding the extent to which NF-κB-mediated suppression of JNK signaling blunts apoptosis vs necrosis (Ventura et al., 2004; reviewed in Papa et al., 2006). Differences in the metabolic state of the cells are likely to sway which form of cell death will prevail (Ventura et al., 2004; reviewed in Papa et al., 2006). It was suggested that actively dividing cells that depend on glycolysis are more likely to die by necrosis, whereas quiescent cells that undergo oxidative phosphorylation predominantly die by apoptosis (reviewed in Edinger and Thompson, 2004). Clearly, the interplay between the JNK and NF-κB signaling cascades is an important factor in dictating the fate of the cells be it survival, apoptosis or necrosis.

3 A Role for NF-κB in Autophagy

Autophagy is a form of PCD distinct from apoptosis and necrosis that has come under increasing scrutiny lately, particularly as it relates to cancer (reviewed in Edinger and Thompson, 2004; Hait et al., 2006; Levine and Yuan, 2005) (also see Chapter 9). Cells undergo autophagy in response to nutrient and growth factor deficiency as a temporary means of survival. They do so by undergoing self-digestion under conditions where adequate nutrient supplies are limited, as would be the case for cancer cells lacking an adequate blood supply. However, a prolonged state of autophagy ultimately results in metabolic cell death. The process itself involves assembly of an autophagosome in which a cell's organelles and cytoplasm are swallowed. Its contents are then degraded by lysosomes, which allow salvation of amino acids and fatty acids for energy generation. Although studies are only beginning to explore a possible role of NF-κB in autophagy, its protective activity in ventricular myocytes was recently shown to involve transcriptional repression of the hypoxia-inducible BH3-only protein BNIP3 that was demonstrated to induce autophagy (Baetz et al., 2005; Daido et al., 2004; Kanzawa et al., 2005). Future studies will surely shed more light on this subject and on whether it is implicated in NF-κB-associated cancers.

4 Mechanisms that NF-κB Employs to Suppress PCD

NF-κB utilizes several different means to suppress PCD. NF-κB most commonly suppresses apoptosis by activating the transcription of antiapoptotic genes (reviewed in Kucharczak et al., 2003; Luo et al., 2005b; Papa et al., 2006). Among

them are antiapoptotic Bcl-2 family members Bcl-2, Bcl-x_L, Bfl-1/A1, and NR13 that antagonize the activity of proapoptotic Bcl-2 family proteins and thus blunt the release of proapoptotic cytochrome c and Smac/Diablo from mitochondria. The cellular inhibitor of apoptosis molecules XIAP, c-IAP1, and c-IAP2 also contribute to its protective activity (reviewed in Wright and Duckett, 2005). While some IAPs like XIAP directly block cleavage-mediated activation of pro-caspase-9 and the activity of caspases 3 and 7, others are less potent in this regard (Deveraux et al., 1997; Liston et al., 2003). Recent studies showing that the baculoviral IAP protein (OpIAP) promotes ubiquitination of the IAP antagonist Smac/DIABLO uncovered a novel mechanism whereby cytoprotective IAPs can block apoptosis in a caspase-independent manner (Duckett, 2005; Wilkinson et al., 2004). Of late, the zinc finger protein A20 was shown to suppress cell death by promoting degradation of the TNFR1 complex component RIP1, via its deubiquitinating (DUB) and E3 ligase activities (Wertz et al., 2004). Other NF-κB-regulated candidates include decoy TRAIL receptor 1 (DcR1) (Bernard et al., 2001a) and c-FLIP that interferes with activation of pro-caspases 8 and 10 (Kreuz et al., 2004; Micheau et al., 2001). cFLIP can also work with caspase-8 to enhance NF-κB activation via the B-cell lymphoma 10 (BCL-10) and mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1 (MALT1) that act as E3 ligases for IKKγ/NEMO, along with RIP1 (Zhou et al., 2004; reviewed in Budd et al., 2006). NF-κB-mediated induction of the serine protease inhibitor 2A (Spi2A), that inhibits cathepsin B, was shown to suppress cell killing by TNFα by blocking lysosome-mediated PCD (Liu et al., 2003).

In the antagonistic relationship between the NF-κB and JNK signaling cascades, GADD45β/Myd118 and XIAP were among the first NF-κB targets proposed to block sustained JNK activation (De Smaele et al., 2001; Tang et al., 2001). GADD45β associates with and blocks the catalytic activity of the JNK-activating kinase MKK7/JNKK2 (Kaur et al., 2005; Papa et al., 2004a, b). How XIAP blocks prolonged JNK activation is still not clear, but a recent study suggests that it can inhibit TGF-β1-induced JNK activation and apoptosis by ubiquitinating the kinase TAK1, leading to its degradation (Kaur et al., 2005). It should be noted, however, that homozygous deletion of *gadd45β* or *xiap* had no significant effect on JNK activation in vivo (Amanullah et al., 2003; Sanna et al., 2002), suggesting that compensatory mechanisms may exist or that another NF-κB-dependent inhibitor(s) of proapoptotic JNK signaling remain to be identified. Relevant candidates in this regard are the antioxidant molecules manganese superoxide dismutase (MnSOD), and ferritin heavy chain (FHC) that inhibits JNK by suppressing ROS accumulation through iron sequestration (Bernard et al., 2001b, 2002; Delhalle et al., 2002; Pham et al., 2004; Tanaka et al., 2002; reviewed in Papa et al., 2004b).

Other means have been described to explain the antiapoptotic effects of NF-κB in certain contexts. One of them involves NF-κB-induced destabilization of tumor suppressor p53 as a result of increased expression of Mdm2 (Egan et al., 2004; Tergaonkar et al., 2002). RelA-dependent suppression of caspase-8 and TRAIL receptors DR4 and DR5 was shown to confer survival to TRAIL along with induction of c-IAP1 and c-IAP2 (Chen et al., 2003). The peptidyl prolyl-isomerase Pin1

was reported to enhance nuclear accumulation of RelA/p65 by blocking its association with IκBα and to also lead to p65 stabilization by interfering with its interaction with the ubiquitin ligase SOCS-1 (Ryo et al., 2003). Although direct evidence is still lacking that Pin1 enhances the protective activity of RelA, Pin1 is frequently upregulated in breast cancer compared to normal mammary glands (Currier et al., 2005; Ryo et al., 2003).

5 Mechanisms that Underlie the Pro-Death Activity of NF-κB

Although NF-κB is best known for its ability to antagonize PCD, it should be noted that it can be proapoptotic in certain cells and in response to certain stimuli (reviewed in Kucharczak et al., 2003; and see below). Some NF-κB transcriptional targets that were implicated in this effect include factors that modulate the mitochondrial and death receptor apoptotic pathways including the p53 tumor suppressor, death receptor Fas and its ligand FasL, TNFα, TRAIL receptors DR4, DR5, DR6, TRAIL itself, and the proapoptotic Bcl-2 family members Bcl-xS and Bax.

Work in recent years uncovered an interesting new way whereby the typically antiapoptotic NF-κB subunit RelA can behave as a pro-death factor in response to certain stimuli (reviewed in Perkins and Gilmore, 2006). Cell treatment with atypical activators of NF-κB such as UV-C radiation or the chemotherapeutic drugs daunorubicin and doxorubicin switches RelA from a transcriptional activator into a gene-specific transcriptional repressor of antiapoptotic genes (like *Bcl-x_L*, *XIAP*, and *A20*), but not IκBα by promoting association of RelA with histone deacetylase HDAC1, resulting in cell death (Campbell et al., 2004). This occurs in a RelA phosphorylation-independent manner. Tumor suppressor alternative reading frame (ARF) can also suppress the protective activity of RelA by using a slightly different mechanism, i.e., by directing ATR- and Chk1-dependent phosphorylation of the RelA transactivation domain (Thr 505). This creates a potential docking site for HDAC1 to suppress expression of antiapoptotic genes and sensitize cells to TNF-induced killing (Rocha et al., 2003, 2005). Lately the DNA cross-linking chemotherapeutic drug cisplatin was identified to imitate ARF's activity, by promoting Chk1-dependent phosphorylation of RelA to repress expression of Bcl-x_L (Campbell et al., 2006). It should be noted, however, that not all genotoxic drugs convert RelA into a transcriptional repressor, as etoposide promotes RelA-dependent activation of the antiapoptotic genes *Bcl-x_L* and *XIAP* (Campbell et al., 2006).

Targeting of RelA to the nucleolus was recently suggested as a novel means to antagonize its transcriptional and antiapoptotic activities in colorectal cancer cells treated with aspirin, serum deprivation, or UV-C radiation (Stark and Dunlop, 2005), although others previously reported that aspirin suppresses NF-κB activation by interfering with the activity of the IKK complex (Kopp and Ghosh, 1994; Yamamoto et al., 1999; Yin et al., 1998).

6 NF-κB's Role in PCD has Important Developmental and Physiological Consequences

The phenotypes of mice deficient for individual Rel/NF-κB subunits highlighted the crucial contribution of NF-κB in the control of apoptosis during development and/or homeostasis in the hepatic, epidermal, immune, and nervous systems (reviewed in Kucharczak et al., 2003; Li and Verma, 2002). For example, homozygous inactivation of RelA or of its upstream activating kinase IKKβ, alone or together with IKKα, is embryonic lethal due to massive liver apoptosis (Beg and Baltimore, 1996; Li et al., 1999a, b, 2000; Rudolph et al., 2000; Tanaka et al., 1999). That this phenotype is rescued by concerted deletion of RelA with TNFα, or of IKKβ with TNFR1, indicates that developing hepatocytes undergo apoptosis induced by circulating TNFα (Alcamo et al., 2001; Doi et al., 1999). Although RelA is believed to protect developing hepatocytes from TNF-induced killing by upregulating the expression of antiapoptotic genes, expression of Bcl-2 recently failed to rescue fatal liver apoptosis in RelA-deficient mice (Gugasyan et al., 2006). It therefore appears that the protective activity of RelA against physiological levels of TNF requires activation of other NF-κB targets in developing hepatocytes. The protective role of NF-κB in hepatocytes is also evident in cells treated with transforming growth factor β (TGF-β), which induces cell death by promoting synthesis and stabilization of the NF-κB inhibitor IκBα (Arsura et al., 2003; Cavin et al., 2003). Induction of apoptosis in this context coincides with suppression of the prosurvival NF-κB targets Bcl-x_L and XIAP, as well as alpha-fetoprotein that suppresses TNF-induced cell death by inhibiting TNFR1 signaling (Cavin et al., 2004).

The protective role of NF-κB in the immune system is also well documented. NF-κB orchestrates survival and differentiation during early lymphopoiesis, where RelA suppresses apoptosis of precursor cells in presence of high levels of TNF (Prendes et al., 2003); reviewed in Claudio et al., 2006; Gerondakis and Strasser, 2003; Siebenlist et al., 2005). Later in development, NF-κB activation via the pre-B-cell receptor (pre-BCR) is key for suppressing apoptosis and promoting proliferation and developmental progression. Combined inactivation of c-Rel and RelA impairs maturation to the IgM(lo)IgD(hi) stage and causes premature cell death (Feng et al., 2004; Grossmann et al., 2000; reviewed in Gilmore et al., 2004). NF-κB activation downstream of the BCR is also crucial for survival and proliferation of mature peripheral B cells. Homozygous deletion of c-Rel renders primary B cells exquisitely susceptible to apoptosis following stimulation with mitogens, as does B-lineage-specific inactivation of IKKβ or IKKγ/NEMO (Grumont et al., 1998, 1999; Kontgen et al., 1995; Leitges et al., 2001; Li et al., 2003; Martin et al., 2002; Owyang et al., 2001; Pasparakis et al., 2002b; Petro and Khan, 2001; Petro et al., 2000; Tan et al., 2001; Tumang et al., 1998). The recent analysis of mice deficient for the B-cell adaptor for phosphoinositide 3-kinase (BCAP), that signals through c-Rel, is consistent with this (Yamazaki and Kurosaki, 2003). NF-κB also reduces apoptosis induced by the cytokine BlyS/BAFF that is involved in peripheral B-cell

development. Its protective activity in this context coincides with induction of Bcl-2, Bcl-x_L, and Bfl-1/A1 (Do et al., 2000; Hsu et al., 2002; Schiemann et al., 2001). Combined, these results highlight a crucial role for NF-κB in B-cell survival, maturation, and function.

NF-κB is also prominent in determining the fate of T cells, in which it serves either in an antiapoptotic or a proapoptotic fashion. NF-κB activation following T-cell receptor (TCR) engagement together with CD28 costimulation fosters survival and proliferation of naïve T cells (Khoshnan et al., 2000). Both p50/NF-κB1 and c-Rel were implicated in inducing expression of cell death inhibitors Bcl-x_L, Bcl-2, and Bfl-1/A1 (Verschelde et al., 2003; Zheng et al., 2003). Despite its protective role, it appears that only an appropriate dose of NF-κB activity is tolerated as survival of B and T lymphocytes is compromised in mice deficient for both inhibitory subunits IκBα and IκBε in which NF-κB is highly activated, akin to the phenotype of mice lacking NF-κB activity (Goudeau et al., 2003). There is also evidence suggesting that NF-κB can be proapoptotic in double-positive thymocytes (Hettmann et al., 1999). NF-κB-dependent induction of Fas ligand (FasL) in mature T cells undergoing activation-induced cell death (AICD) has also been reported (Kasibhatla et al., 1999; Lin et al., 1999; Zheng et al., 2001).

Inhibition of apoptosis by NF-κB is also important for the development of most ectodermal appendages, as tissue-specific suppression of NF-κB activity leads to impaired development of hair follicles and exocrine glands due to increased apoptosis (Headon et al., 2001; Pasparakis et al., 2002a; Schmidt-Supprian et al., 2000; Schmidt-Ullrich et al., 2001; Yan et al., 2002). In this regard tumor suppressor cylindromatosis (CYLD), whose loss predisposes patients to tumors of hair follicles, sweat, and scent glands acts as a deubiquitinating enzyme for IKKγ/NEMO and TRAF2, and suppresses NF-κB activation of the TNFR family members CD40, XEDAR, and EDAR (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). It thus seems that the antiapoptotic activity of NF-κB may contribute to cancer development in these tissues.

In the nervous system too, NF-κB can either block or induce apoptosis depending on the cell context and the stimulus. It is neuroprotective in response to injury as illustrated in experimental models of stroke or seizure, where it induces expression of the prosurvival genes *IAP*, *Bcl-2*, *Bcl-x_L*, and *MnSOD* (reviewed in Kucharczak et al., 2003; Mattson and Camandola, 2001). While increased NF-κB activity is observed in neurodegenerative disorders like Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, epilepsy, and stroke, it was postulated that it helps to protect against oxidative stress and mitochondrial dysfunction (reviewed in Mattson and Camandola, 2001). This is supported by the increased susceptibility of p50/NF-κB1-deficient mice to neuronal damage following treatment with a mitochondrial toxin in an experimental model of Huntington's disease (Yu et al., 2000). However, others reported that NF-κB promotes cell death in models of neuronal injury following ischemia/reperfusion and excitotoxic insult in which tumor suppressor p53 was implicated as a harmful downstream effector (Crumrine et al., 1994; Morrison et al., 1996; Xiang et al., 1996). Interestingly, recent work indicates that K^+ loss in cortical neurons subjected to serum withdrawal leads to increased

levels of NF-κB and that apoptosis is associated with upregulation of the pro-death factor Bcl-xS (Tao et al., 2006).

The cell type in which NF-κB is activated appears to significantly influence whether NF-κB is neuroprotective or neurodegenerative. While its activation in neurons is often cytoprotective, NF-κB activation in microglia promotes neuronal cell death (Mattson and Camandola, 2001). In this regard, it was suggested that NF-κB activation in glial cells might induce neuronal apoptosis by promoting production of proinflammatory cytokines, ROS, and excitotoxins (John et al., 2003; Mattson and Meffert, 2006). Consistent with this idea, inactivation of astroglial NF-κB by transgenic expression of a superrepressor IκBα was recently shown to reduce production of proinflammatory cytokines and to dramatically improve recovery after spinal cord injury (Brambilla et al., 2005).

7 NF-κB's Role in PCD Fosters Cancer Development and Progression

Constitutive activation of NF-κB contributes to the pathogenesis of a large number of human cancers (reviewed in Rayet and Gelinas, 1999; Kim et al., 2006). Many tumor cells, including those derived from activated B cell-like diffuse large B-cell lymphoma (ABC-DLBCL), primary mediastinal B-cell lymphomas (PMBL), classical Hodgkin's lymphoma (cHL), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), adult T-cell leukemia (ATL), breast, lung, or head and neck cancer show constitutively high levels of nuclear Rel/NF-κB factors and depend upon them for survival (Alizadeh et al., 2000; Bargou et al., 1997; Davis et al., 2001; Hinz et al., 2001; Kordes et al., 2000; Shipp et al., 2002). Suppression of NF-κB activity using a degradation-resistant form of IκB blocks tumor cell proliferation and sensitizes them to apoptosis (reviewed in Baldwin, 2001; Barkett and Gilmore, 1999; Kucharczak et al., 2003; Sonenshein, 1997). This agrees with the acute oncogenicity of the viral NF-κB oncoprotein v-Rel of reticuloendotheliosis virus strain T that causes fatal leukemia/lymphoma in animal models (reviewed in Fan et al., 2006; Gilmore, 1999). NF-κB activation is also implicated in malignant cell transformation by many viruses, as reviewed previously (Fan et al., 2006; Hiscott et al., 2001; Kucharczak et al., 2003; Santoro et al., 2003). These include Epstein-Barr virus (EBV) implicated in Burkitt's lymphoma, human Herpesvirus 8/Kaposi's sarcoma-associated Herpes virus (HHV8/KHSV) associated with Kaposi's sarcoma, and primary effusion lymphoma (PEL) and human T-cell leukemia virus type-1 (HTLV-1) associated with ATL.

In a majority of human cancers, persistent NF-κB activity results from constitutive activation of the IKK complex, although the mechanisms responsible for IKK activation in these tumors have remained elusive. Using an RNA interference screen Staudt's group recently uncovered that CARD11, that signals though MALT1 and BCL10, is a key factor responsible for the constitutive activation of IKK in ABC-DLBCL (Ngo et al., 2006). In other instances, constitutively high levels of nuclear Rel/NF-κB proteins are due to chromosomal rearrangement,

amplification and/or overexpression of the *rel/nf-κb* genes, or in some cases due to mutations in IκB (reviewed in Fan et al., 2006; Gilmore et al., 2002; Karin et al., 2002; Rayet and Gelinas, 1999). For example, c-*rel* is overexpressed in PMBL, certain follicular large cell lymphoma, and in cHL (Barth et al., 1998, 2003; Feuerhake et al., 2005; Houldsworth et al., 1996; Joos et al., 1996, 2002; Lu et al., 1991; Rao et al., 1998; Savage et al., 2003; Wessendorf et al., 2003). In several of these cases, this was correlated with accumulation of nuclear c-Rel protein (Barth et al., 2003; Savage et al., 2003). There is emerging evidence that NF-κB may also contribute to brain cancer, as constitutive NF-κB activity coincides with expression of a novel TrkA splice variant (trkAIII) in neuroblastoma cell lines (Tacconelli et al., 2004). In addition, reduced expression of the candidate tumor suppressor ING4 is correlated with increased expression of NF-κB target genes that foster survival, growth, and angiogenesis of brain tumors, and ING4 was proposed to regulate NF-κB activity by directly interacting with RelA/p65 (Garkavtsev et al., 2004).

A large number of studies have delineated a cell autonomous role for NF-κB in tumor cell survival, but recent publications provided compelling evidence that activation of NF-κB in the tumor microenvironment plays a vital role in promoting tumor cell growth (Fig. 11.3; reviewed in de Visser and Coussens, 2005). In a

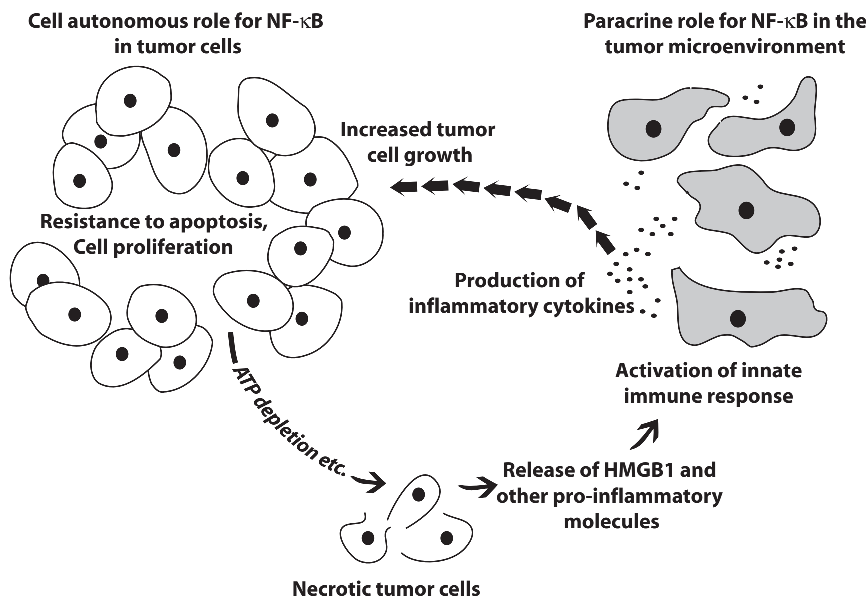

Fig. 11.3 NF-κB plays an essential role in tumor cells and in the tumor microenvironment. Activation of NF-κB in tumor cells acts in a cell autonomous fashion to increased cell resistance to apoptosis, cell proliferation, and metastatic capacity. Rapidly dividing tumor cells that depend on glycolysis can undergo PCD via necrosis under conditions where ATP is depleted. Necrotic cells release potent proinflammatory factors like HMGB1, which trigger activation of the innate immune response in the tumor microenvironment, resulting in NF-κB-dependent production of proinflammatory cytokines that promote tumor cell growth in a paracrine fashion

mouse model of colitis-associated cancer, abrogation of NF-κB activity either in intestinal epithelial cells or in myeloid cells significantly reduced tumor incidence following administration of a carcinogen that promotes colonic tumor formation together with dextran sulfate sodium (DSS) salt to induce inflammation and accelerate tumor growth (Greten et al., 2004). Inactivation of IKKβ in intestinal epithelial cells decreased tumor incidence due to increased apoptosis, coincident with decreased expression of antiapoptotic proteins like Bcl-x_L, but it had no effect on tumor cell proliferation. In contrast, ablation of IKKβ in myeloid cells decreased tumor incidence by inhibiting epithelial cell proliferation due to reduced expression of proinflammatory genes, but had no effect on tumor cell survival. Consequently, it seems that NF-κB activation promotes tumor cell survival, whereas its activation in myeloid cells promotes production of cytokines that accelerate tumor cell growth in a paracrine fashion (Greten et al., 2004). A similar correlation has emerged between NF-κB activation and inflammation-associated tumor growth in a mouse model of chronic hepatitis that evolves into hepatocellular carcinoma (HCC) (Pikarsky et al., 2004). In this system, chronic liver inflammation triggers production of TNFα by endothelial and inflammatory cells that leads to chronic activation of NF-κB in hepatocytes. While inactivation of NF-κB in hepatocytes had no effect on the onset of early neoplastic events, its inactivation at later stages increased hepatocyte apoptosis and blunted progression to carcinoma (Pikarsky et al., 2004).

Further evidence that NF-κB plays a prominent role in inflammation-associated tumor growth and metastasis came to light in studies in which administration of bacterial LPS induced systemic inflammation and production of TNFα by cells in the tumor microenvironment that accelerated the growth and metastasis of colon and breast cancer cell lines (Luo et al., 2004) Inhibition of NF-κB in the tumor cells themselves prompted tumor regression in response to LPS-induced inflammation, where reduced tumor cell proliferation and increased apoptosis resulted from induction of TRAIL receptor DR5 on NF-κB-deficient tumor cells and of its ligand TRAIL on surrounding immune cells. Together these studies highlight a critical role for NF-κB in inflammation-associated tumor promotion, progression, and metastasis.

Exposure to carcinogens is an important contributing factor to the onset of sporadic human cancer. NF-κB plays an important role in this scenario as well, as evidenced in various mouse models of chemically induced cancer. An interesting link between inflammation and chemical carcinogenesis was unveiled in a mouse model of diethylnitrosamine (DEN)-induced HCC, in which IKK-mediated NF-κB activation plays a critical role both in hepatocytes and in hematopoietic-derived Kupffer cells (Maeda et al., 2005). A surprising finding was that hepatocyte-specific deletion of IKKβ noticeably enhanced tumor development, as hepatocyte apoptosis was offset by proliferation of surviving hepatocytes, coincident with increased ROS production, and JNK activation. Administration of antioxidant or compound inactivation of IKKβ in both hepatocytes and hematopoietic-derived Kupffer cells reduced the incidence of HCC in this model. Although the mechanism whereby DEN triggers this inflammatory response is unclear, it was proposed that hepatocytes undergoing

necrosis release factors like HMGB1 that can trigger a strong inflammatory response in the microenvironment, that in turn promotes the growth and tumorigenesis of surviving hepatocytes (Scaffidi et al., 2002). This model is supported by: (1) the observation that supernatant from necrotic hepatocytes can activate NF-κB in primary macrophages (Maeda et al., 2005); (2) an increasing number of studies showing that inflammation and necrosis support tumor growth (Vakkila and Lotze, 2004; reviewed in Lotze and Tracey, 2005; Zeh and Lotze, 2005); and (3) work indicating that HMGB1 released from necrotic cells is an important mediator of inflammation (reviewed in Lotze and Tracey, 2005; Zeh and Lotze, 2005; Fig. 11.3).

In contrast to its well-documented growth-promoting effects in most cell types, NF-κB inhibits cell growth in the epidermis and loss of NF-κB activity promotes epidermal cell proliferation and hyperplasia (Seitz et al., 1998, 2000; van Hogerlinden et al., 1999). Furthermore, suppression of NF-κB activity in epidermal keratinocytes in conjunction with expression of oncogenic Ras promotes invasive neoplasia reminiscent of squamous cell carcinoma (SCC; Dajee et al., 2003). The growth suppressive effects of NF-κB in epidermal homeostasis were recently shown to result from suppression of the G1 cell cycle kinase CDK4 (Zhang et al., 2005). Suppression of NF-κB in the epidermis was accompanied by upregulation of CDK4 in a TNFR1- and JNK-dependent manner and CDK4 was necessary for epidermal cell hyperplasia under conditions in which NF-κB activity was inhibited (Zhang et al., 2005). This highlights an important tumor suppressor function for NF-κB in certain cells.

Interestingly, NF-κB was found to be preferentially activated in epithelial cells of ER-negative breast tumors and particularly in ER-negative and ErbB2-positive tumors (86%; Biswas et al., 2004). Interestingly, in ER-negative and ErbB2-negative breast cancer samples, nuclear NF-κB was predominantly found in the stroma (Biswas et al., 2004).

8 Approaches for Prevention and Therapy

Many dietary and natural agents that show chemopreventive activity can block NF-κB activity (reviewed in Yamamoto and Gaynor, 2001). These include the green tea polyphenol epigallocatechin-3 gallate, resveratrol, and curcumin that can block tumor initiation and progression by suppressing tumor cell proliferation and by inducing apoptosis (Hofmann and Sonenshein, 2003; Bharti et al., 2003); reviewed in Signorelli and Ghidoni, 2005).

Not only is NF-κB important for the inherent resistance of tumor cells to PCD and for promoting tumor cell growth, but it is also a central figure in the resistance of many tumors to anticancer treatment. Compounds that interrupt NF-κB signaling counteract the growth and survival of many tumor cells in which NF-κB is implicated and can potentiate the efficacy of anticancer drugs (Wang et al., 1996; reviewed in Baldwin, 2001; Karin et al., 2002; Yamamoto and Gaynor, 2001). Since several signaling molecules and posttranslational modifications are necessary

to mediate NF-κB activation, different steps in the pathway can be used as potential therapeutic targets. One of them involves suppression of the proteasome-dependent degradation of IκBα. The proteasome inhibitor Velcade/bortezomib is currently used for the treatment of advanced multiple myeloma (also see Chapter 12) and also shows promise in preclinical models of breast, colon, lung, prostate, and pancreatic cancer (reviewed in Kim et al., 2006; Richardson et al., 2004). Since the proteasome is involved in the turnover of many cellular factors, Velcade's effectiveness does not solely derive from inhibition of the NF-κB pathway, as illustrated by recent evidence that it can also affect mitochondrial function and blunt activation of JNK (Landowski et al., 2005; Small et al., 2004). Moreover, its proapoptotic effects for melanoma cells do not seem to coincide with widespread inhibition of NF-κB, suggesting the need to identify more specific inhibitors of NF-κB (Fernandez et al., 2005).

Another valuable approach is to target phosphorylation events critical for NF-κB activation. In this regard, there is a growing inventory of compounds that can suppress IKK activity and promote apoptosis in tumor-derived cells (reviewed in Kim et al., 2006). A few examples include nonsteroidal anti-inflammatory drugs (NSAIDs) like celecoxib, sulfasalazine or aspirin (e.g. Ashikawa et al., 2004; Robe et al., 2004; Subhashini et al., 2005; Takada et al., 2004). Incidentally, prolonged use of NSAIDs has been linked with a decreased incidence of colon cancer (reviewed in Li et al., 2005). Thalidomide and arsenic, respectively show efficacy in combined therapy for relapsed or refractory multiple myeloma, and in the treatment of acute promyelocytic leukemia (reviewed in Kim et al., 2006; Mathas et al., 2003). However, since the activity of these agents is not selective for NF-κB, there is a great deal of interest in identifying small molecule inhibitors specific for IKK subunits. Among them, the β-carboline derivative PS-1145 was shown to specifically kill ABC- DLBCL and PMBL-derived tumor cell lines that rely on NF-κB for growth and survival (Lam et al., 2005). Other specific and potent IKK inhibitors are undergoing preclinical testing. These include the quinazoline analogue SPC839 and the imidazoquinoxaline derivative BMS-345541 (reviewed in Karin et al., 2004). Their safety and effectiveness in combination therapy is currently under investigation (reviewed in Nakanishi and Toi, 2005).

Lately, there has been a significant new development in the quest to identify new molecular targets critical for the pathogenesis of NF-κB-associated cancers. Using an inducible RNA interference library to identify genes important for tumor cell proliferation and apoptosis resistance, Staudt's group uncovered that CARD11/CARMA1 is responsible for the constitutive activation of IKK in ABC-DLBCL-derived tumor cells (Ngo et al., 2006). CARD11/CARMA1 lies downstream of the BCR and TCR and engages MALT1 and BCL10 to promote ubiquitination of IKKγ/NEMO. This new finding opens the possibility to develop strategies to inhibit signaling by CARD11, an approach that may have limited side effects since CARD11 expression is restricted to lymphoid cells. Moreover, the work provides compelling evidence that RNA interference screens might be particularly useful to uncover new therapeutic targets that are crucial for tumor cell survival and proliferation (Ngo et al., 2006).

Although compounds that suppress NF-κB activity offer promising avenues to antagonize tumor development or progression and to enhance the efficacy of existing therapeutic agents, it is important to remember that NF-κB can be proapoptotic in certain cell types and in response to certain stimuli. This suggests that the particular cell context may be important for the therapeutic outcome. For example, the chemotherapeutic drug doxorubicin triggers apoptosis in colon cancer cells by activating NF-κB (Ashikawa et al., 2004), but others found that NF-κB protects HeLa cells from apoptosis induced by this agent (Baldwin, 2001; Nakanishi and Toi, 2005). The tumor suppressor activity of NF-κB in the epidermis is another consideration, as a possible adverse effect of long-term inhibition of NF-κB might be an increased susceptibility to develop certain tumors associated with suppression of NF-κB, such as SCC. Lastly, recent work indicating that certain chemotherapeutic drugs can convert RelA into a transcriptional repressor of antiapoptotic genes suggests that the response of tumor cells to particular chemotherapeutic regimens may differ significantly depending on the tumor type, the status of endogenous tumor suppressors, and the stage of tumor development (Perkins, 2004; Perkins and Gilmore, 2006). Ongoing efforts to clarify the mechanisms that govern the anti- vs pro-death effects of NF-κB in different cell contexts will certainly be very informative to help predict the impact of NF-κB inhibition in different tumor cell contexts and the outcome of therapy.

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Chapter 12
Targeting Proteasomes as Therapy in Multiple Myeloma

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Abstract The Ubiquitin-proteasome pathway (UPP) regulates normal intracellular protein degradation processes essential for cell cycle progression, inflammation, transcription, DNA replication, and apoptosis. Blockade of UPP using proteasome inhibitor Bortezomib (Velcade) is an effective therapy for relapsed/refractory multiple myeloma (MM). Both oligonucleotide microarrays and proteomic studies are delineating the molecular mechanisms mediating Bortezomib-induced cytotoxicity, defining targets of sensitivity vs resistance, allowing for the development of next generation therapies, and providing the rationale for combination therapies.

Keywords proteasomes, apoptosis, drug resistance, myeloma

1 Introduction

The proteasome is a multisubunit complex with catalytic activities mediating proteolysis of ubiquitinated intracellular proteins (Adams, 2004; Goldberg and Rock, 2002) The 26S proteasome complex consist of 19S units flanking a barrel-shaped 20S proteasome core; the 19S units regulate entry only of those proteins marked for degradation into the 20S core chamber (Adams, 2004; Goldberg and Rock, 2002). Proteasomal protein degradation is a multistep process: protein is first earmarked with a chain of ubiquitin molecules; E1 ubiquitin enzyme then activates ubiquitin and links it to the ubiquitin-conjugating enzyme E2 in an ATP-dependent manner; E3 ubiquitin ligase then links the ubiquitin molecule to the protein; a long polypeptide chain of ubiquitin moieties is formed; and finally, proteasomes degrade the protein into small fragments and free ubiquitin for recycling (Goldberg and

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Rock, 2002; Pickart, 2004). Protein degradation is predominantly regulated by caspase-like (CT-L) (beta-5), trypsin-like (beta-1), and caspase-like (beta-2) proteolytic activities residing within the 20S proteasome core. Importantly, the substrates of proteasomes include many cellular proteins that maintain normal cell cycle progression, growth, and survival. Most of the damaged or misfolded, short or long-lived, proteins in the cell are eliminated by UPP; conversely, blockade of protein degradation by proteasome inhibitors (PIs) causes intracellular accumulation of redundant proteins, resulting in induction of heat-shock response and apoptosis (Adams, 2004; Goldberg and Rock, 2002).

Since the proteasome regulates normal cellular functions, its value as a possible therapeutic target was viewed with skepticism due to cytotoxicity to normal cells. However, various studies suggest that PIs are more cytotoxic to proliferating malignant cells than quiescent normal cells, thereby providing an acceptable therapeutic index (Adams, 2004). The mechanism whereby cancer cells are more susceptible to PIs than normal cellular counterparts is unclear. One possibility is that cancer cells have altered cell cycle machinery, leading to an increase in their proliferation rate. These cells therefore accumulate damaged proteins at a much higher rate than do normal cells, which in turn increases dependency on proteasomal degradation. In contrast, quiescent cancer cells may be more susceptible to proteasome inhibition than normal cells. PIs also inhibit prosurvival signaling pathways. For example, nuclear factor-kappa B (NF-κB) is linked to proliferation and drug resistance in cancer cells (Haefner, 2002); conversely, PIs downregulate NF-κB activation, thereby enhancing the cytotoxic effects of chemotherapy. Together, these findings support the notion of targeting proteasomes in novel therapeutics.

Naturally occurring and synthetic inhibitors of the ubiquitin-proteasome pathway (UPP) include peptide aldehydes, peptide boronates, nonpeptide inhibitors, peptide vinyl sulfones, and peptide epoxyketones (Adams, 2004). All of these PIs differentially affect proteasome activities and also show activity against other proteases. For example, peptide aldehydes (MG-132, MG-115, ALLN, or PSI) potently, but reversibly, block the chymotrypsin-like (CT-L) (beta-5) activity of the proteasome; they also inhibit lysosomal cysteine and serine proteases, as well as calpains, thereby limiting their clinical utility. Lactacystin is a natural, irreversible, nonpeptide inhibitor; the clasto-lactacystin beta-lactone, an analog of its active metabolite, is currently in phase I clinical trails. Importantly, studies by Adams et al. led to the development of peptide boronic acid PIs (Adams, 2004). The dipeptidyl boronic acid Bortezomib/PS-341 is a potent and reversible inhibitor of CT-L (beta-5) activity. Moreover, our recent study using radiolabeled active site-directed probe specific for proteasome catalytic subunits showed that Bortezomib targets beta-5 (CT-L activity) and beta-1 (C-L activity), as well as beta-5i and beta-1i catalytic subunits of the immunoproteasome (Berkers et al., 2005). Initial NCI screening showed remarkable antitumor activity of Bortezomib in a panel of 60 tumor cell lines. We have shown that Bortezomib induces MM cell apoptosis, downregulates adhesion molecules, inhibits constitutive and MM cell adhesion-induced cytokine secretion, and blocks angiogenesis in the BM milieu (Chauhan et al., 2005b). It also

inhibits human MM cell growth and prolongs host survival in a severe combined immunodeficient (SCID) mouse model of human MM (Chauhan et al., 2005b). Phase I trials showed safety and acceptable toxicity, as well as early signs of anti-MM activity (Richardson, 2004; Voorhees and Orlowski, 2006) Phase II clinical trails demonstrated durable responses (including complete responses) with associated clinical benefits, providing the basis for the Food and Drug Administration (FDA) approval to treat relapsed refractory MM (Richardson, 2004). A randomized phase III trial showed higher responses as well as prolonged time to progression and survival in patients treated with Bortezomib vs Dexamethasone (Richardson et al., 2005), providing the basis for FDA approval extended to include relapsed MM. Although Bortezomib is a major advance, treatment is associated with toxicity and the development of drug-resistance in most patients. Recent studies have therefore delineated the mechanisms mediating Bortezomib-induced cytotoxicity and drug resistance, in order to design novel therapeutic strategies.

2 Bortezomib-Triggered Signaling Pathways

The proteasome is the primary target of PIs; however, PIs also affect growth/survival and apoptotic molecules. A major mechanism whereby PIs inhibit growth and survival of cancer cells is by blocking prosurvival NF-κB signaling (Adams, 2004). Constitutive activation of NF-κB, associated with growth/proliferation and drug resistance, occurs via these sequential events: IκB-a kinase (IKK) activation; IκB phosphorylation; ubiquitination and degradation of IκB; and nuclear translocation of p50/65 NF-κB. Nuclear localization of NF-κB induces transcription of genes-encoding cytokines (IL-6, TNF-α), survival factors (inhibitors of apoptosis proteins [IAPs], Bcl-x_L), and cell adhesion molecules (intracellular adhesion molecule [ICAM], vascular cell adhesion molecule [VCAM], and E-selectin). NF-κB activation is also associated with growth and survival of MM cells; specifically, adhesion of MM cells to bone marrow stromal cells (BMSCs) triggers NF-κB-mediated transcription and secretion of IL-6 and insulin-like growth factor-I (IGF-I) (Chauhan et al., 2005b), both of which promote survival and conventional drug resistance in MM cells in the BM milieu. Moreover, patient MM cells and BMSCs have upregulated NF-κB activity relative to normal cells; within the tumor cell population, drug-sensitive MM cells have lower NF-κB activity than drug-resistant MM cells. Importantly, treatment of MM cells with Bortezomib inhibits NF-κB activation and related cytokine production, thereby overcoming the survival advantage for MM cells conferred by BMSCs. Our work also shows that NF-κB inhibition alone is unlikely to account for the total anti-MM activity of Bortezomib. Both PS-1145, a specific inhibitor of IκB, and Bortezomib block TNF-α-induced NF-κB activation by inhibiting phosphorylation and degradation of IκB-α; in contrast to Bortezomib, however, PS-1145 only partially inhibits MM cell growth (Hideshima et al., 2002).

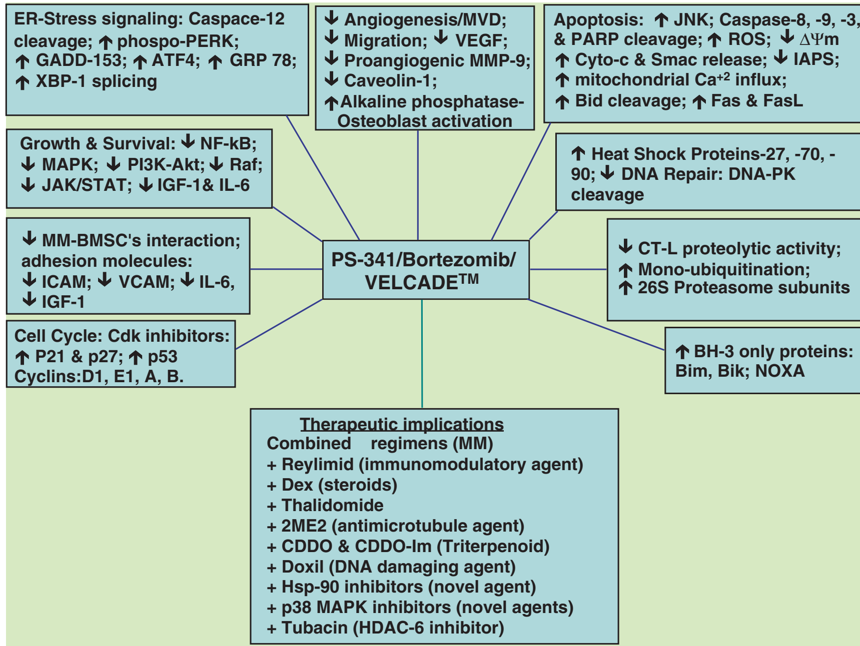

Fig. 12.1 Identification of Bortezomib/Velcade/PS-341-triggered molecular mechanisms mediating growth/survival, apoptosis, and drug resistance in tumor cells, including host-BM microenvironment and angiogenesis ("↑" arrow: induction/upregulation; "↓" arrow: reduction /inhibition/downregulation). Delineation of the Bortezomib signaling profile allow us to combine it with agents that utilize either similar or additional apoptotic pathways to enhance its tumor cytotoxicity, reduce toxicity to normal cells, prevent development of drug-resistance, and improve patient outcome. Shown are the ongoing therapeutic strategies in MM using the combination of Bortezomib with various conventional and novel agents

Recent oligonucleotide microarray and proteomic studies show that Bortezomib affects various signaling pathways (Figure 12.1). For example, Bortezomib-induced apoptosis is associated with: (1) activation of stress response proteins such as heat shock proteins, Hsp-27, Hsp-70, and Hsp-90 (Chauhan et al., 2003; Mitsiades et al., 2002); (2) upregulation of proapoptotic c-Jun-NH2-terminal kinase (JNK) (Chauhan and Anderson, 2003); (3) alteration of mitochondrial membrane potential (MMP) and generation of reactive oxygen species (ROS); (4) induction of the intrinsic cell death pathway via the release of mitochondrial proteins cytochrome-c/Smac into cytosol, resulting in activation of caspase-9 > caspase-3 cascade; (5) activation of extrinsic apoptotic signaling through Bid and caspase-8 cleavage (Mitsiades et al., 2002); (6) upregulation of ubiquitin/proteasome pathway members (Mitsiades et al., 2002); (7) inactivation of DNA-dependent protein kinase (DNA-PK) (Mitsiades et al., 2003), which is essential for the repair of DNA double-strand breaks; and (8) inhibition of MM cell growth factor-triggered MAPK and PI3-kinase/Akt signaling (Hideshima et al., 2003). Our studies using dominant negative

strategies and knockout cell line models have established a direct role for JNK and Bax/Bak during Bortezomib-induced apoptosis (Chauhan et al., 2005b). Stress stimuli that induce mitochondrial outer membrane permeabilization (MOMP) use BH3-only proteins to facilitate Bax/Bak translocation to mitochondria; treatment of various cell types with Bortezomib induces stabilization of the BH3-only proteins Bim and Bik, whereas Bik or Bim and Bik-deficient MEFs are less susceptible to Bortezomib-induced killing. Moreover, Ca^{2+} influx into mitochondria triggers cyto-c and caspase-9-mediated apoptosis; conversely, treatment of MM cells with mitochondrial Ca^{2+} uptake inhibitor abrogates Bortezomib-triggered apoptosis (Landowski et al., 2005). These findings suggest that Bortezomib also affects signaling events upstream of mitochondria.

Recent studies link endoplasmic reticulum (ER)-related stress signaling to Bortezomib-induced death in MM cells (Landowski et al., 2005; Mitsiades et al., 2002). Oligonucleotide microarrays show a predominant induction of gene products associated with endoplasmic reticulum secretory pathways in MM cell lines following short-term exposure to high-dose Bortezomib (Landowski et al., 2005). Bortezomib triggers: expression of proteins associated with ER secretory pathways; activation of ER-resident caspase-12; and dysregulation of Ca^{2+} homeostasis, thereby resulting in cell death. Specifically, Bortezomib activates ER membrane-resident stress kinase PERK, accompanied by steady-state levels of ER protein-folding chaperone GRP-78; as well as proapoptotic ATF-4 and CHOP/GADD153, coupled with a simultaneous decrease in general protein synthesis. Another study in head and neck squamous cell carcinoma cells suggests that Bortezomib enhances efficacy of chemotherapeutic drugs via activation of the proapoptotic ER stress-ROS pathway (Fribley et al., 2004). Both caspase-12 and caspase-4 have been implicated in ER stress-induced apoptosis; however, neither caspase-12 nor caspase-4 are required for ER stress-induced apoptosis (Obeng and Boise, 2005), and it remains unclear how ER stress causes caspase activation. Nonetheless, Bortezomib-induced apoptosis involves activation of ER-related stress pathways, including activation of caspase-12. Together, these findings suggest that inhibition of growth/survival signaling cascades and concurrent activation of apoptotic signaling pathways mediate overall Bortezomib-induced cytotoxicity in MM cells.

Although Bortezomib triggers remarkable antitumor activity in MM cells, intrinsic or acquired drug resistance occurs in most cases. Recent studies have therefore focused on defining mechanisms mediating Bortezomib resistance. Our study shows that treatment with Bortezomib induces apoptosis in SUDHL6 (DHL6), but not SUDHL4 (DHL4), lymphoma cells (Chauhan et al., 2003). Microarray analysis demonstrates high RNA levels for heat shock protein-27 (Hsp27) in DHL4 vs DHL6 cells, correlating with increased Hsp27 protein expression. Blockade of Hsp27 in DHL-4 cells using antisense (AS) strategy restores the apoptotic response to Bortezomib; conversely, overexpression of Hsp27 renders Bortezomib-sensitive DHL6 cells resistant to Bortezomib. These data suggest that Hsp27, at least in part, accounts for Bortezomib resistance. MM cells obtained from patient's refractory to Bortezomib treatment show elevated Hsp-27 levels, further supporting this view. Of note, Hsp-27 negatively regulates the mitochondrial release of cytoc-c and Smac,

thereby preventing activation of intrinsic cell death-signaling cascade. Upregulated expression of IAPs, such as XIAP, may also contribute to Bortezomib resistance (Mitsiades et al., 2002); conversely, inhibition of these prosurvival molecules may sensitize tumor cells to Bortezomib and even overcome Bortezomib resistance.

3 Therapeutic Implications

It is unlikely that one specific mechanism accounts for Bortezomib-induced cytotoxicity or the development of resistance, suggesting that combinations of Bortezomib with other conventional and/or novel agents may enhance its cytotoxicity and overcome drug resistance. For example, combined Bortezomib and irinotecan treatment triggers apoptosis in pancreatic tumor xenografts and enhances chemosensitivity in colorectal cancer xenograft models (Cusack et al., 2001). Preclinical studies in MM demonstrate that combining Bortezomib with conventional agents such as Dex, Doxorubicin, Melphalan, or Mitoxantrone induces additive or synergistic antitumor activity (Mitsiades et al., 2003). Treatment of MM cells with Bortezomib and novel agents Relvimid or triterpenoids CDDO-Imidazolide also induces synergistic anti-MM activity and overcomes Bortezomib resistance by targeting both intrinsic and extrinsic apoptotic signaling, thereby providing the basis for clinical protocols using combination regimens. Importantly, gene profiling studies show that Bortezomib induces Hsp-90 in MM cells; conversely, blockade of Hsp-90 with 17-AAG enhances sensitivity and even overcomes Bortezomib resistance (Mitsiades et al., 2002). Clinical trials already show promise of combined therapy in Bortezomib refractory MM. Our laboratory has recently demonstrated the significance of the alternative aggresome cascade for protein catabolism in MM cells; identified histone deacetylase-6 (HDAC-6) to be essential in the chaperoning of ubiquitinated proteins for aggresomal degradation; and validated the preclinical anti-MM activity of HDAC-6 inhibitor Tubacin (Hideshima et al., 2005). Importantly, dual inhibition of proteasomes and aggresomes with Bortezomib and tubacin, respectively, triggers synergistic cytotoxicity, setting the stage again for clinical translation of this new class of cancer therapeutics. Finally, correlative science studies of samples from patients on Bortezomib treatment protocols show that resistance is associated with upregulation of Hsp-27; already preclinical and clinical studies of p38 MAPK inhibitors to downregulate Hsp-27 and thereby overcome Bortezomib resistance have been completed.

4 Novel Proteasome Inhibitor Npi-0052 and its Clinical Utility *Vis-À-Vis* Bortezomib

Besides the combination therapeutic strategies, our recent study also shows that a novel proteasome inhibitor NPI-0052 can overcome Bortezomib resistance in MM cells. NPI-0052 is a small molecule derived from fermentation of Salinospora, a new marine gram-positive actinomycete (Chauhan et al., 2005a; Feling et al., 2003;

Macherla et al., 2005). NPI-0052 is a nonpeptide PI with structural similarity to Omuralide (Feling et al., 2003; Groll et al., 2006; Macherla et al., 2005), a beta-lactone derived from naturally occurring lactacystin. NPI-0052, in contrast to Omuralide, possess a uniquely methylated C3 ring juncture, chlorinated alkyl group at C2, and cyclohexene ring at C5 (Macherla et al., 2005), which accounts for its higher antitumor activity than omuralide. Initial screening of NPI-0052 against the NCI panel of 60 tumor cell lines showed GI_{50} of <110 nM in all cases. Our data showed that (1) NPI-0052 triggers apoptosis in MM cells sensitive and resistant not only to conventional, but also to Bortezomib therapies; and (2) The IC_{50} of NPI-0052 for MM cells is within the low nanomolar concentration (Chauhan et al., 2005a). Importantly, NPI-0052 similarly triggered apoptosis in purified tumor cells from several MM patients relapsing after various prior therapies including Bortezomib and thalidomide.

The mechanism whereby NPI-0052 overcomes Bortezomib resistance in MM cells is unclear; however, this may be due to its differential mode of action than Bortezomib. For example, NPI-0052 and Bortezomib differentially affect 20S proteasomal activities: (1) NPI-0052 inhibits CT-L and T-L activities at much lower concentrations than Bortezomib, and (2) higher concentrations of NPI-0052 than Bortezomib are required to inhibit C-L activity (Chauhan et al., 2005a). Animal studies using whole blood lysates showed that NPI-0052 blocked CT-L activity, which was recoverable to near basal levels by day 7; whereas inhibition of CT-L activity is significantly restored at 24 h after Bortezomib. NPI-0052 inhibits 50% of T-L activity, which is restored by day 7; whereas Bortezomib enhances T-L activity, which remains upregulated even at day 7. Interestingly, both NPI-0052 and Bortezomib inhibited C-L activity, which recovered only at day 7. The comparative kinetics of proteosomal activities suggest that NPI-0052, in contrast to Bortezomib, triggers a sustained inhibition of CT-L, T-L, and C-L (up to 7 days), which may therefore allow for a less-frequent administration schedule in patients. In this context, previous studies showed that CT-L activity is inhibited in peripheral blood cells of patients within 1 h of Bortezomib administration, and recoverable before the next dose (Adams, 2002; Hamilton et al., 2005).

A recent study showed that simultaneous inhibition of multiple proteasome activities is a prerequisite for significant (i.e., >50%) proteolysis (Kisselev et al., 2006). Another study showed that 50% inhibition of cystic fibrosis transmembrane conductance regulator degradation in reticulocytes extracts required concurrent blockade of CT-L and C-L proteasome activities (Oberdorf et al., 2001). Importantly, our study showed that MM cells exhibit higher constitutive levels of T-L proteasome activity than either CT-L or C-L activities (Crawford et al., 2006). These data, together with the results that NPI-0052, but not Bortezomib, efficiently inhibits CT-L + T-L activities (Chauhan et al., 2005a), suggest that NPI-0052 may block more protein breakdown than Bortezomib in MM cells. Moreover, mechanisms conferring Bortezomib resistance may not be effective against NPI-0052. Importantly, our study suggests that NPI-0052 is a potent inducer of MM cells apoptosis in tumor cells obtained from Bortezomib-refractory MM patients.

Another distinction between NPI-0052 and Bortezomib is their toxicity profile against normal cells. NPI-0052 does not significantly decrease normal lymphocyte

viability at the IC_{50} doses for MM cells, with only modest effects at higher concentrations. By contrast, Bortezomib decreased the survival of lymphocytes at concentrations close to the IC_{50} doses for MM cells. NPI-0052 inhibits CT-L activity at doses which does not trigger apoptosis in MM cells. Previous observations that Bortezomib inhibits 20S proteasome activity in murine WBCs at 1 h postinjection, and that a similar degree of proteasome inhibition was noted in blood from responders vs nonresponders to Bortezomib therapy (Adams, 2002; Richardson, 2004), suggest that inhibition of proteasome activity in blood may not correlate to tumor cell cytotoxicity. Nonetheless, the above data suggest that (1) NPI-0052, in contrast to Bortezomib, is likely to have less toxic effects than Bortezomib on normal cells; and (2) NPI-0052 has a larger therapeutic index, which may allow for dose escalation therapy.

In vivo efficacy of NPI-0052 was shown using a human plasmacytoma xenograft mouse. Model (LeBlanc et al., 2002). Specifically, NPI-0052 inhibited MM tumor growth and prolongs survival of these mice at concentrations which were well tolerated and without significant weight loss or any neurological behavioral changes. Analysis at day 300 showed no recurrence of tumor in 57% of NPI-0052-treated mice.

Examination of signal transduction pathways showed that (1) NPI-0052 is a more potent inhibitor of NF-κB and related cytokine transcription and secretion than Bortezomib; (2) NPI-0052-induced MM cell death is predominantly mediated by caspase-8; and (3) Bortezomib-induced apoptosis requires both caspase-8 and caspase-9 activation. These findings further confirm differential actions of NPI-0052 vs Bortezomib in MM cells. The mechanistic differences between NPI-0052 and Bortezomib, i.e., their effect on proteasome activities and their dependence on specific apoptotic signal transduction pathway, provide a rationale for combination regimens for the treatment of MM. Indeed, the combination of NPI-0052 with Bortezomib induced synergistic anti-MM activity, without significantly affecting the viability of normal lymphocytes. The mechanisms mediating enhanced cytotoxicity of the combination regimen may simply reflect higher levels of proteasome inhibition with the two-drug regimens and/or activation of differential apoptotic signaling pathways. These data provide the framework for clinical trials of combined PIs to improve patient outcome in MM.

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Chapter 13
Histone Deacetylase Inhibitors: Mechanisms and Clinical Significance in Cancer

HDAC Inhibitor-Induced Apoptosis

Sharmila Shankar and Rakesh K. Srivastava*

Abstract Epigenic modifications, mainly DNA methylation and acetylation, are recognized as the main mechanisms contributing to the malignant phenotype. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. While histones represent a primary target for the physiological function of HDACs, the antitumor effect of HDAC inhibitors might also be attributed to transcription-independent mechanisms by modulating the acetylation status of a series of non-histone proteins. HDAC inhibitors may act through the transcriptional reactivation of dormant tumor suppressor genes. They also modulate expression of several other genes related to cell cycle, apoptosis, and angiogenesis. Several HDAC inhibitors are currently in clinical trials both for solid and hematologic malignancies. Thus, HDAC inhibitors, in combination with DNA-demethylating agents, chemopreventive, or classical chemotherapeutic drugs, could be promising candidates for cancer therapy. Here, we review the molecular mechanisms and therapeutic potential of HDAC inhibitors for the treatment of cancer.

Keywords HDAC inhibitors, HAT, SAHA, MS-275, TSA, TRAIL, apoptosis, caspase

1 Introduction

Recent years have seen major advances in elucidating the complexity of chromatin and its role as an epigenetic regulator of gene expression in eukaryotes. Epigenic modifications, mainly DNA methylation and acetylation, are recognized as additional

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mechanisms contributing to the malignant phenotype (Jones, 2002; Plass, 2002). Acetylation and deacetylation of histones play an important role in the regulation of gene expression (Grunstein, 1997). Histone acetylation is a reversible process whereby histone acetyltransferase (HAT) transfers the acetyl moiety from acetyl coenzyme A to the lysine; histone deacetylase (HDAC) removes the acetyl groups, reestablishing the positive charge in the histones. HATs and HDACs have recently been shown to regulate cell proliferation, differentiation, and apoptosis in various hematological and solid malignancies (Kouzarides, 1999). Altered HAT or HDAC activity is associated with cancer by changing the expression pattern of selected genes (Grignani et al., 1998; Lin et al., 1998). Hyperacetylation of histones correlates with gene activation, whereas deacetylation mediates eukaryotic chromatin condensation and gene expression silencing (Johnstone and Licht, 2003; Strahl and Allis, 2000). Recently, new roles of histone acetylation have been uncovered, not only in transcription, but also in DNA replication, repair, and heterochromatin formation (Kurdistani and Grunstein, 2003).

2 Histone Deacetylases

HDACs catalyze the removal of an acetyl group from the ε-amino group of lysine side chains of the core nucleosomal histones (H2A, H2B, H3, and H4), thereby reconstituting the positive charge on the lysine. Recent studies have revealed 12 human HDAC enzymes, HDAC1-11 (Emiliani et al., 1998; Gao et al., 2002; Grozinger et al., 1999; Taunton et al., 1996; Yang et al., 1996) and HDAC-A (Fischle et al., 1999). Based on the structural properties, HDACs can be divided into three classes (Gray and Ekstrom, 2001). Class I members (HDAC 1, 2, 3, 8, and 11) are transcriptional corepressors homologous to yeast RPD3 and have a single deacetylase domain at the N-termini and diversified C-terminal regions (de Ruijter et al., 2003). Class II members (HDAC 4, 5, 6, 7, 9, and 10) have domains similar to yeast HDA1 with a deacetylase domain at a C-terminal position (Verdin et al., 2003). In addition, HDAC 6 contains a second N-terminal deacetylase domain, which can function independently of its C-terminal counterpart. Class III HDACs are distinct from class I and II and are homologous of the yeast silent information regulator 2 (Sir2). All of these HDACs apparently exist in the cell as subunits of multiprotein complexes. Class II HDACs translocate from the cytoplasm to the nucleus in response to external stimuli, whereas class I HDACs are constitutively nuclear and play important roles in dynamic gene regulation (McKinsey and Olson, 2005).

Sir2 enzymes (or sirtuins) are NAD(+)-dependent deacetylases that modulate gene silencing, aging, and energy metabolism. Previous work has implicated several transcription factors as Sir2 targets. Sir2 silences transcription at silent mating loci, telomerese, and ribosomal DNA (rDNA), and this also suppresses recombination in rDNA. Earlier experiments have shown that the overexpression of Sir2 in yeast induced the global deacetylation of histones, indicating that Sir2 was an

HDAC (Braunstein et al., 1993). Later, it was shown that *cobB*, a bacterial homologue of Sir2, had ribosyltransferase activity, leading to experiments showing that Sir2 was also able to transfer adenosine diphosphate-ribose (ADP-ribose) from nicotinamide adeninedinucleotide (NAD) (Frye, 1999). Subsequently, it was confirmed that Sir2 was an NAD-dependent HDAC (Imai et al., 2000). The ADP-ribosylation of an acetylated lysine residue is an intermediate state of the enzymatic reaction catalyzed by Sir2. Only class III enzymes use NAD as a cofactor. Therefore, they are known as NAD-dependent HDACs.

Recently, Sir2 has attracted much attention, because it is related to longevity (Bordone and Guarente, 2005). The overexpression of Sir2 extends the life span of budding yeast, while its knockout shortens the life span by about 50% (Kaeberlein et al., 1999). Sir2 is conserved from bacteria to humans. In the nematodes, the gene most homologous to yeast *Sir2* gene is Sir-2.1. A duplication containing the *Sir-2.1* gene confers a life span that is extended by up to 50% (Tissenbaum and Guarente, 2001). The mammalian homologues consist of seven members, Sirt1–Sirt7. In mammalian cells, Sirt1 downregulates stress-induced p53 and FOXO pathways for apoptosis, thus favoring survival under stress. In the absence of applied stress, Sirt1 silencing induces growth arrest and/or apoptosis in human epithelial cancer cells (Ford et al., 2005). In contrast, normal human epithelial cells and normal human diploid fibroblasts seem to be refractory to Sirt1 silencing. Further studies have revealed that the Sirt1-regulated pathway is independent of p53, Bax, and caspase-2. Alternatively, Sirt1 may suppress apoptosis downstream from these apoptotic factors. FOXO4 (but not FOXO3) is required as proapoptotic mediator. Caspase-3 and caspase-7 act as downstream executioners of Sirt1/FOXO4-regulated apoptosis. These data suggest that Sirt1 as a novel target for selective killing of cancer vs noncancer epithelial cells. Upregulation of Sirt1 may be a double-edged sword that both promotes survival of aging cells and increases cancer risk in mammals.

Histones are part of the core proteins of nucleosomes. The recruitment of HATs and HDACs plays an important role in proliferation, differentiation and apoptosis (Glass and Rosenfeld, 2000; Kouzarides, 1999). Altered HAT or HDAC activity is associated with the development of cancer by changing the expression of several genes (Grignani et al., 1998; Lin et al., 1998). Treatment of malignant cells with HDAC inhibitors regulates only a small number (1–2%) of genes, as examined by DNA microarray studies (Van Lint et al., 1996). HDAC1 interacts directly with other transcription repressors, including all three of the pocket proteins, Rb, p107 and p130, and YY1. HDAC1 causes transcription repression by locally deacetylating histones, leading to a compact nucleosomal structure that prevents transcription factors from accessing DNA to promote transcription. Furthermore, HDAC1 knockout mice were embryonic lethal, possibly due to a proliferative defect upon unrestricted expressions of the cell cycle inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Lagger et al., 2002). Overexpression of HDAC I confers resistance to sodium butyrate-mediated apoptosis in melanoma cells through a p53-mediated pathway (Bandyopadhyay et al., 2004). We and others have shown that inhibition of HDAC activity induces apoptosis in various types of cancer (Fandy et al., 2005; Fang, 2005; Marks et al., 2003; Rosato et al., 2001; Singh et al., 2005).

Stability of HDACs is an important factor in determining the biological activity. HDAC4 is unusually unstable, with a half-life of less than 8 h (Liu et al., 2004). Consistent with the instability of HDAC4 protein, its mRNA was also highly unstable (with a half-life of less than 4 h). The exposure of cells to ultraviolet (UV) irradiation resulted in the degradation of HDAC4. This degradation was not dependent on proteasome or CRM1-mediated export activity but instead was caspase-dependent and was detectable in diverse human cancer lines. Of two potential caspase consensus motifs in HDAC4, both lying within a region containing proline, glutamic acid-, serine-, and threonine-rich (PEST) sequences, Asp-289 as the prime cleavage site was identified by site-directed mutagenesis (Liu et al., 2004). Notably, this residue is not conserved among other class IIa members, HDAC5, HDAC7, and HDAC9. Finally, the induced expression of caspase-cleavable HDAC4 led to markedly increased apoptosis. These results therefore link the regulation of HDAC4 protein stability to caspases, enzymes that are important for controlling cell death and differentiation.

3 Histone Deacetylase Inhibitors

It is well established that hyperacetylation of the N-terminal tails of histones H3 and H4 correlates with gene activation, whereas deacetylation mediates transcriptional repression (Strahl and Allis, 2000). Revived interest in these enzymatic pathways and how they modulate eukaryotic transcription has led to the identification of multiple cofactors whose complex interplay with HDAC affects gene expression. Concurrent with these discoveries, screening of natural product libraries yielded new small molecules that were subsequently identified as potent inhibitors of HDAC. While predominantly identified by using antiproliferative assays, the biological activity of these new HDAC inhibitors also encompasses significant antiprotozoal, antifungal, phytotoxic, and antiviral applications. During the past decade, a number of HDAC inhibitors have been shown to induce growth arrest, differentiation, and/or apoptosis in cancer cells (Boyle et al., 2005; Fandy et al., 2005; Kwon et al., 2002b; Marks et al., 2004; Singh et al., 2005), and inhibit tumor growth in various xenograft models (Bordin et al., 2004; Butler et al., 2000; Park et al., 2004; Sakajiri et al., 2005; Shao et al., 2004; Takimoto et al., 2005; Tang et al., 2004; Zhang et al., 2004c). HDAC inhibitors induce expression of cell cycle regulatory (e.g., p21$^{/WAF1/CIP1}$) and apoptotic proteins (e.g., Bax, PUMA, and Noxa), downregulate survival signaling pathways (e.g., Raf/MAPkinase/ERK), and disrupt cellular redox state (e.g., reactive oxygen species, ROS). Therefore, HDAC inhibitors are considered candidate drugs in cancer therapy (Johnstone, 2002; Marks et al., 2001b; McLaughlin and La Thangue, 2004).

Seven classes of HDAC inhibitors have been characterized and include short-chain fatty acids (e.g., sodium butyrate and phenylbutyrate); hydroxamic acids (e.g., suberoylanilide hydroxamic acid [SAHA], LAQ824, and trichostatin A [TSA]); benzamides (e.g., MS-275, CI994); cyclic tetrapeptide containing a 2-amino-8-oxo-9,

10-epoxy-decanoyl (AOE) moiety (e.g., trapoxin A); cyclic peptides without the AOE moiety (e.g., FK228/depsipeptide, apicidin); and epoxides (e.g., depudecin). These inhibitors induce a dose-dependent inhibition of either class I or class II HDACs, or both. Newly characterized HDAC inhibitors are now available that preferentially inhibit specific HDAC classes, including SK7041 (inhibits class I HDACs) and splitomicin (inhibits class III HDACs). A wide variety of HDAC inhibitors of both natural and synthetic origin has been reported. Except for depsipeptide (FK228), natural HDACs (TSA, depudecin, trapoxins, and apicidins), as well as sodium butyrate, phenylbutyrate, and SAHA, while effective in vivo, are marked by instability and low retention. Subsequently, synthetic analogs isolated from screening libraries (oxamflatin, scriptaid) were discovered as having a common structure with TSA and SAHA: a hydroxamic acid zinc-binding group linked via a spacer (5 or 6 CH2) to a hydrophobic group. Second-generation HDAC inhibitors such as LAQ824 and PDX101 are currently under clinical trials. Synthetic benzamide-containing HDAC inhibitors (e.g., MS-275 and CI-994) are also being evaluated in the clinics.

3.1 Short-Chain Fatty Acid

Butyrate inhibits HDAC activity at micromolar concentrations. It is generated by the fermentation of dietary fibers in the lumen of the large intestine. The aromatic fatty acids phenylbutyrate and phenylacetate, which has been used to treat patients with disorders of urea metabolism, also inhibits HDAC activity and possess anticancer activity (Appelskog et al., 2004; Boivin et al., 2002; Pili et al., 2001; Sowa and Sakai, 2000; Warrell et al., 1998; Zhang et al., 2004a). Valproic acid (VPA), an anticonvulsant, has been shown to have HDAC inhibitory activity at relatively high concentrations (Catalano et al., 2005; De Felice et al., 2005; Facchetti et al., 2004; Sakajiri et al., 2005; Shen et al., 2005; Takai et al., 2004a). VPA also inhibits angiogenesis, but displays no toxicity in endothelial cells (Michaelis et al., 2005). VPA increases extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation in human umbilical vein endothelial cells. Moreover, the combination of VPA with PD98059, a pharmacological inhibitor of the mitogen-activated protein kinase kinase 1/2, synergistically inhibited angiogenesis in vitro and in vivo.

3.2 Hydroxamic Acids

Essential characteristics of hydroxamic acid-based inhibitors are the polar hydroximic group – a six-carbon hydrophobic methylene spacer, a second polar site, and a terminal hydrophobic group. TSA from *Streptomyces hygroscopicus* was initially identified as an antifungal agent (Tsuji et al., 1976). TSA and SAHA act as noncompetitive inhibitor of HDAC by mimicking the lysine substrate as well as chelating a

zinc atom crucial for enzymatic activity (Yoshida et al., 1990b). TSA and SAHA inhibit both class I and II HDACs. Simple analogs of cyclic tetrapeptides that contain suberic acid linkers and hydroxamate, instead of epoxyketone or ketone functional group, inhibit HDAC activity (Hoffmann et al., 2000). The structurally related hybrid polar compounds (HPCs) were shown to induce differentiation in a wide variety of transformed cells (Marks et al., 1996). The first representative was hexamethylene bisacetamide (HMBA) which induced differentiation of transformed cells in millimolar range (Marks and Rifkind, 1988). HMBA regulates genes that control G1-to-S phase transition, leading to G1 arrest and inhibition of DNA synthesis. Among the inducer-mediated changes, suppression of cyclin-dependent kinase cdk4, which may be required for phosphorylation of the retinoblastoma protein pRB and perhaps p107, is critical in the pathway of terminal differentiation. HMBA induces an increase in the level of $p21^{WAF1/CIP1}$ which inhibits cyclin-dependent kinase activity and, in turn, may cause cells to arrest in G1. p107 complexes with transcription factor E2F, which may alter E2F-dependent gene transcription. HMBA has also been shown to induce differentiation of neoplastic cells in patients. Furthermore, a second generation of HPCs have been synthesized which are up to 1,000-fold more potent than HMBA. Second-generation HPCs such as oxamflatin, SAHA, suberic bishydroxamic acid (SBHA), and m-carboxycinnamic acid bishydroxamide (CBHA) inhibited HDAC activity and induced cancer cell differentiation and apoptosis (Richon et al., 1998; Shankar et al., 2005b). Polyaminohydroxamic acids (PAHAs) represent an important new chemical class of HDAC inhibitors and appear to be more specific than SAHA, TSA, and MS-275, because they are selectively directed to chromatin and associated histones by the positively charged polyamine side chain. Several other analogs of hydroxamic acids are being developed (Hoffmann et al., 2000; Qiu et al., 2000).

These HDAC inhibitors inhibits proliferation, causes cell cycle arrest, and induces differentiation and/or apoptosis in numerous models of lymphoma, leukemia, multiple myeloma, and solid tumors (Fandy et al., 2005; Fronsdal and Saatcioglu, 2005; Inoue et al., 2002; Monneret, 2005; Shankar et al., 2005b; Taghiyev et al., 2005; Toth et al., 2004; Tsatsoulis, 2002; Vanhaecke et al., 2004a; Vanhaecke et al., 2004b; Wang et al., 2002; Yamashita et al., 2003). TSA is also effective in xenograft models (Canes et al., 2005; Touma et al., 2005). TSA attenuates the development of allergic airway inflammation by decreasing expression of the Th2 cytokines, IL-4 and IL-5, and IgE, which results from reduced T-cell infiltration, suggesting that HDAC inhibition may attenuate the development of asthma by a T-cell suppressive effect (Choi et al., 2005). Other analogs of TSA such as oxamflatin, scriptaid, and amide derivatives have been reported to have anticancer activity (Jung et al., 1999; Kim et al., 1999c; Monneret, 2005; Su et al., 2000). Scriptaid induces reticulocytosis and human gamma-globin synthesis (Johnson et al., 2005), suggesting its potential as a treatment option for sickle cell disease. The suppressed RARβ expression in head and neck carcinoma (HNSCC) can be reactivated by TSA (Wang et al., 2005). Additionally, TSA alone or in combination with 5-aza-2′-deoxycytidine (5-AzaC) increases lysine-9 (Lys-9) acetylation and Lys-4 methylation of the first exon at the *RAR*β gene, while decreasing the methylation

of Lys-9. Similarly, treatment of gastric carcinoma with 5-aza-C, and/or TSA resulted in reexpressed caspase-1 mRNA (Jee et al., 2005). DNA methylation-mediated repression of eNOS promoter activity was partially reversed by TSA treatment, and combined treatment of TSA and 5-AzaC synergistically induced eNOS expression in nonendothelial cells (Gan et al., 2005). Furthermore, TSA downregulates DNMT3B mRNA and protein expression in human endometrial cancer cells (Xiong et al., 2005). This decrease in DNMT3B mRNA results in a significant reduction in de novo methylation activities, suggesting that TSA may not only modify histone acetylation, but also potentially alter DNA methylation. The above findings suggest that epigenetic events such as DNA methylation and histone deacetylation play important roles in the regulation of cancer-related genes.

3.3 Benzamides

Several benzamides have been found to inhibit HDAC activity in the low micromolar range. A 2′-hydroxy or amino function seems to be essential for the optimum activity (Suzuki et al., 1999). A newly synthesized benzamide derivative with HDAC inhibitory activity, MS-275 is believed to enter the catalytic site and bind the active zinc, inhibits HDAC at micromolar concentrations. MS-275 is the first HDAC inhibitor discovered with oral anticancer activity in several animal models. Pretreatment of human leukemic cells with MS-275 significantly enhances the abrogative capacity of an established nucleoside analogue, fludarabine (Maggio et al., 2004). The study indicates that apart from promoting acetylation of histones and regulation of genes involved in differentiation and apoptosis, MS-275 also induces multiple perturbations in signal transduction, survival and cell cycle regulatory pathways that increase the fludarabine-mediated cell death. CI-994 (*N*-acetyl dinaline), originally synthesized as an anticonvulsant, does not seem to directly inhibit HDAC, but causes accumulation of acetylated histones by an unknown mechanisms. MS-275, acetyldinaline, and CI-994 are in clinical trials for the treatment of several cancers (Monneret, 2005; Ryan et al., 2005).

3.4 Cyclic Tetrapeptides Containing AOE Moiety

Hydrophobic cyclotetrapeptides contain common amino acid (*S*)-2-amino-9,10-epoxy-8-xodecanoic acid (L-Aoe) and have been reported to inhibit HDACs (Brosch et al., 1995; Kijima et al., 1993). The epoxyketone was first thought to be essential for activity, as reduction or nucleophoilic attack resulted in inactivation of compounds (Brosch et al., 1995; Kijima et al., 1993). Trapoxin A, a microbially derived cyclotetrapeptide, is an irreversible inhibitor in the low nanomolar range (Kijima et al., 1993). Trapoxin A irreversibly inhibits histone deacetylation in vivo

and causes mammalian cells to arrest in the cell cycle (Taunton et al., 1996). On the other hand, related HC toxin (host-selective toxin of *Cochliobolus carbonum*) inhibits maize enzyme activity reversibly (Brosch et al., 1995). K-trap (an analogous of trapoxin A) inhibited HDAC1 activity. A number of derivatives, such as 9-acyloxyapicidins and 9-hydroxy, have been prepared and are under investigation. Trapoxin analogs that combine cyclotetrapeptide and hydroxamic acid moieties have been prepared. The inhibitors of quinolone analogs and the hydroxamic acid analogs of apicidin yielded promising results (Meinke et al., 2000; Meinke and Liberator, 2001). Depudecin, a natural epoxide derivative isolated from the fungus *Alternaria brassicicola*, induces hyperacetylation of histones and morphological reversion in v-ras-transformed NIH 3T3 cells (Kwon et al., 1998).

3.5 Cyclic Peptides that do not Contain an AOE Moiety

Cyclic peptides such as depsipeptide (FR901228/FK228) isolated from *Chromobacterium violaceum* inhibits HDAC activity at nanomolar concentrations. Depsipeptide induces differentiation, growth arrest and apoptosis, and inhibits metastasis and angiogenesis (Aron et al., 2003; Doi et al., 2004; Khan et al., 2004; Klisovic et al., 2003a, b, 2005; Kwon et al., 2002a; Mie Lee et al., 2003; Sasakawa et al., 2002, 2003; Sato et al., 2004; Sawa et al., 2004; Vanoosten et al., 2005). Depsipeptide is also very promising antitumor agent against osteosarcoma, inducing apoptosis by the activation of the Fas/FasL system (Imai et al., 2003). A novel fungal metabolite, apicidin (cyclo(*N-O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)), exhibits potent, broad spectrum antiprotozoal activity in vitro against apicomplexan parasites (Darkin-Rattray et al., 1996). Apicidin's antiparasitic activity appears to be due to low nanomolar inhibition of HDAC, which induces hyperacetylation of histones in treated parasites. Since apicidin and apicidin A possess only a ketone functional group and are active in the low nanomolar concentrations, it appears that the presence of the epoxy group is not essential for activity. Apicidin induces differentiation, cell cycle arrest and apoptosis, and inhibits metastasis and angiogenesis in several cancer models (Cheong et al., 2003; Han et al., 2000, 2001; Hong et al., 2003; Kim et al., 2001a, 2004b, c; Kouraklis and Theocharis, 2002; Kwon et al., 2002b). It promotes histone acetylation and gene transcription. Its activity is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells (Khan et al., 2004). Preclinical studies with depsipeptide in chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) have demonstrated that it effectively induces apoptosis at concentrations at which HDAC inhibition occurs. A dose-dependent increase in H3 and H4 histone acetylation was noted in depsipeptide-treated AML1/ETO-positive Kasumi-1 cells and blasts from a patient with t(8;21) AML (Klisovic et al., 2003b). A phase I and pharmacodynamic study of depsipeptide in CLL and AML have yielded promising results (Byrd et al., 2005).

Opening of the disulfide bridge leads to a thiol that may be able to enter the active site and complex the zinc ion. In this regard, garlic constituents and their metabolites such as diallylsulfide and allylmercaptan inhibited HDAC activity. Diallyl disulfide caused increased acetylation of H3 and H4 histones in DS19 mouse erythroleukemic cells and K562 human leukemic cells (Lea et al., 1999), suggesting that differentiation in erythroleukemic cells by diallyl disulfide and allyl mercaptan may be mediated through induction of histone acetylation. Acetylation was also induced in rat hepatoma and human breast cancer cells by diallyl disulfide or its metabolite, allyl mercaptan. Diallyl disulfide increased histone acetylation and $p21^{WAF1/CIP1}$ expression in human colon tumor cell lines (Druesne et al., 2004).

3.6 Epoxides

The naturally occurring epoxide depudecin (a microbial metabolite containing two epoxide groups) irreversibly binds to HDAC and inhibits its activity at micromolar concentration. Depudecin inhibited embryonic angiogenesis, involving the chorio-allantoic membrane of growing chick embryo (Oikawa et al., 1995). It also affected the growth of vascular endothelial cells, a key event in the process of angiogenesis in vivo. Depudecin reverts the rounded phenotype of NIH 3T3 fibroblasts transformed with v-ras and v-src oncogenes to the flattened phenotype of the nontransformed parental cells (Kwon et al., 1998). These data suggest that depudecin could be promising as an antiangiogenic agent and that its antiangiogenic action involves an inhibitory effect on vascular endothelial cell growth.

3.7 Psammaplins

Psammaplins, isolated from a marine sponge *Pseudoceratina purpurea*, inhibited HDAC and DNA methyltransferase activities (Pina et al., 2003). Psammaplin A (PsA) contains an α-oximatoamide functional group, which inhibits the HDAC activity at the catalytic site. The disulfide group is also an essential feature for HDAC inhibition. PsA showed a potent cytotoxicity against several cancer and endothelial cells (Jiang et al., 1995, 2004; Kim et al., 1999a, b; Nicolaou et al., 2001; Park et al., 2003; Pham et al., 2000; Shim et al., 2004). PsA-induced cytotoxicity may correlate with its inhibition on DNA replication (Jiang et al., 2004). Furthermore, PsA was found to inhibit mammalian aminopeptidase N (APN) that plays a key role in tumor cell invasion and angiogenesis (Shim et al., 2004). Interestingly, the antiproliferative effect of PsA was dependent on the cellular amount of APN expression. PsA suppressed the invasion and tube formation of endothelial cells stimulated by basic fibroblast growth factor. Several synthetic analogs of PsA are currently being developed as antiangiogenic and anticancer agents.

4 Mechanism of Actions of HDAC Inhibitors

HDAC inhibitors regulate several biological events including cell cycle, differentiation, and apoptosis in vitro and in vivo (Donadelli et al., 2003; Fandy et al., 2005; Fang, 2005; Fenic et al., 2004; Fronsdal and Saatcioglu, 2005; Henderson and Brancolini, 2003; Hu and Colburn, 2005; Imai et al., 2003; Mai et al., 2005; Marks et al., 2001a; Marks and Jiang, 2005; Nome et al., 2005; Sasakawa et al., 2003; Strait et al., 2005; Takimoto et al., 2005; Yoshida et al., 1990a, 2003). The mechanisms by which these inhibitors induce cell cycle arrest, differentiation, and apoptosis appear to involve multiple genes. In addition to inducing growth arrest and apoptosis, they also inhibit metastasis and angiogenesis (Deroanne et al., 2002; Kim et al., 2001b, 2004c; Sasakawa et al., 2003; Sawa et al., 2002; Williams, 2001; Zgouras et al., 2004). These biological processes are described in this section.

Inhibition of ErbB signaling pathway has been an attractive target for cancer therapy. Several studies have shown that HDAC inhibitors decreased expression of ErbB1 and ErbB2 in DU145 and ErbB2 in SKBr3 cancer cell lines (Chinnaiyan et al., 2005b). HDAC inhibitors also inhibited caveolin-1 and hypoxia-inducible factor 1α (HIF-α), and upregulated gelsolin, p19 (INK4D) and Nur77 expressions in DU145 cells (Chinnaiyan et al., 2005b). Synergistic effects of HDAC inhibitor and ErbB blockade have been shown on cell proliferative, apoptosis, and signaling pathways in cancer cells. Thus, anti-ErbB agents and HDAC inhibitors may offer a promising strategy of dual-targeted therapy. The beneficial effects of these agents may not derive solely from modulation of ErbB expression, but may result from effects on other oncogenic processes including angiogenesis, invasion, and cell cycle kinetics.

The ability of HDAC inhibitors to deactivate Akt through the reorganization of PP1 complexes not only provides a unique mode of Akt regulation, but also represent first example of modulating specific PP1-protein interactions by small-molecule agents. HDAC inhibitors have been reported to lower the apoptotic threshold of several molecularly targeted agents in cancer therapy. This therapeutic strategy is illustrated by the synergistic combination of HDAC inhibitors with other therapeutic agents, Hsp-90 antagonist 17-AGG (George et al., 2005; Rahmani et al., 2005), including the Bcr-Abl kinase inhibitor imatinib (Kim et al., 2004b; Nimmanapalli et al., 2003), the purine analog flutarabine (Maggio et al., 2004), the HER2 antibody trastuzumab (Fuino et al., 2003), the receptor tyrosine kinase FLT-3 inhibitor PKC412 (Bali et al., 2004), the proteosome inhibitor Bortezomib (Yu et al., 2003), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Shankar et al., 2005b; Singh et al., 2005). These chemosensitization effects may be mediated through both histone acetylation-dependent and acetylation-independent effects of HDAC inhibitors, of which the underlying mechanism warrants investigation.

MS-275 upregulates TGFβ signaling pathway via transcriptional activation of the TGFβ type II receptors (TβRII) (Lee et al., 2001), as a result of PCF recruitment to the NF-Y complex on the type II receptor promoter and selective

hyperacetylation of histones associated with the TβRII promoter (Park et al., 2002). Thus, MS-275induces TβRII promoter activity by the recruitment of the PCAF protein to the NF-Y complex, interacting with the inverted CCAAT box in the TβRII promoter. TβRII is often inactivated by mutation or transcriptionally repressed in many cancers, and is therefore a potential candidate for reactivation by HDAC inhibitor treatment.

HDAC inhibitor may also enhance tumor-cell immunogenicity through transcriptional activation of MHC class I and II genes, costimulatory molecules (CD40, CD80, and CD86), intercellular adhesion molecule ICAM1, and type I and II interferons (Johnstone, 2002). These proteins play important roles in host defense mechanisms and cell signaling.

Nonepigenic mechanisms of HDAC inhibitors have recently been described. A number of tumor-associated proteins that mediate cell cycle, growth and/or apoptosis, including Ku70 (Cohen et al., 2004a, b; Subramanian et al., 2005), FOXO1 (Yang et al., 2005), p300 (Bouras et al., 2005), androgen receptor (Fu et al., 2003; Gaughan et al., 2002, 2005), Smad7 (Simonsson et al., 2005), Stat3 (O'Shea et al., 2005; Yuan et al., 2005), p53 (Juan et al., 2000; Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001), Hsp90 (Kovacs et al., 2005), NF-κB/RelA (Greene and Chen, 2004; Quivy and Van Lint, 2004; Yeung et al., 2004), and SRY (Thevenet et al., 2004) have been identified as substrates for various HDACs isoforms. Targeting the acetylation status of these signal mediators might underlie the antiproliferative activities of HDAC inhibitors in cancer cells. Furthermore, various HDACs have been shown to form complexes with cellular proteins including 14-3-3 proteins, α-tubulin, ubiquitin, and PP1(Brush et al., 2004; Canettieri et al., 2003; Grozinger and Schreiber, 2000; Hook et al., 2002; Kawaguchi et al., 2003; Yang and Gregoire, 2005). These protein–protein complexes may be responsible for altering the biological functions. HDACs 1 and 6 formed complexes with PP1 (Brush et al., 2004; Canettieri et al., 2003), of which the combined deacetylase/phosphatase activities underlie the ability of HDAC1 to modulate transcriptional activity of the cAMP-responsive element-binding protein (CREB) and that of HDAC6 to regulate microtubule dynamics. These studies provide new insight into the mechanism by which HDAC inhibitors elicited coordinate changes in cellular protein phosphorylation and acetylation and suggested that changes in these protein modifications at multiple subcellular sites may contribute to HDAC inhibitor's effects to suppress cell growth and transformation.

4.1 Cell Cycle Regulation by HDAC Inhibitors

During the cell-division cycle, chromosomal DNA must initially be precisely duplicated and then correctly segregated to daughter cells. Cell cycle control of transcription seems to be a universal feature of proliferating cells, although relatively little is known about its biological significance and conservation between organisms.

Given the key role of cell cycle integrity in tumor suppression and cancer therapy, a lot of attention has focused on the ability of HDAC inhibitors to alter the levels of cell cycle regulatory proteins. HDAC inhibitors induce growth arrest at both the G1 and G2/M phases of cell cycle and induce differentiation and/or apoptosis of various types of tumor cell lines (Acharya and Figg, 2004; Donadelli et al., 2003; Duan et al., 2005; Fandy et al., 2005; Fang, 2005; Lavelle et al., 2001; Marks and Jiang, 2005; Myzak et al., 2004; Nome et al., 2005; Rocchi et al., 2005; Rosato et al., 2003b; Sakajiri et al., 2005; Sato et al., 2004; Shankar et al., 2005b; Strait et al., 2005). HDAC inhibitors induced both $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ at protein levels, and caused hypophosphorylation of Rb (Fandy et al., 2005; Mitsiades et al., 2005; Nome et al., 2005; Shankar et al., 2005b). Other cell cycle inhibitors that participate in the proliferative arrest elicited by HDAC inhibitors are $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ (Hitomi et al., 2003; Yokota et al., 2004). Moreover, positive regulators of proliferation, such as cyclins D1 and D2, cMyc, or c-Src, are downregulated by HDAC inhibitors (Dehm and Bonham, 2004; Heruth et al., 1993; Lallemand et al., 1996; Souleimani and Asselin, 1993; Takai et al., 2004b). p53 is activated both by inhibitors of HDACs class I/II, as well as by inhibitors of the Sir2 family (Juan et al., 2000; Luo et al., 2000, 2001; Vaziri et al., 2001). Transcription factor Sp1 regulates $p21^{WAF1/CIP1}$ expression in a p53-independent fashion (Han et al., 2001; Sasakawa et al., 2002; Savickiene et al., 2004; Varshochi et al., 2005). Furthermore, $p21^{WAF1/CIP1}$ expression is also transcriptionally regulated by p53 (Parker et al., 1995).

4.2 Apoptotic Induction by HDAC Inhibitors

HDAC inhibitors induce apoptosis in several types of cancers including breast, prostate, lung and thyroid carcinoma, leukemia, and multiple myeloma (Amin et al., 2001; Chen et al., 2005; de Ruijter et al., 2003; Donadelli et al., 2003; Fandy et al., 2005; Fandy and Srivastava, 2006; Kim et al., 2003; Mitsiades et al., 2005; Mori et al., 2004; Papeleu et al., 2005; Rosato et al., 2003a; Sakajiri et al., 2005; Singh et al., 2005; Vigushin and Coombes, 2002; Zhang et al., 2004d). In addition to TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors, the regulation of Bcl-2 family members is also important for inducing sensitivity by HDAC inhibitors. We and others have shown that HDAC inhibitors selectively induce proapoptotic members such as Bax, Bak, Noxa, Bim and Puma and inhibit antiapoptotic Mcl-1, $Bcl-X_L$ and Bcl-2 expression (Fandy et al., 2005; Fandy and Srivastava, 2006; Khan et al., 2004; Mitsiades et al., 2003; Neuzil et al., 2004; Shankar et al., 2005b; Singh et al., 2005; Zhang et al., 2003, 2004b). Bcl-2 family members mainly exert their apoptotic effects by acting at the level of mitochondria and play a crucial role in cancer development (Green and Reed, 1998). HDAC inhibitors cleave poly(ADP-ribose) polymerase (PARP) and caspase-8, caspase-9, caspase-3, caspase-7, and caspase-2. Transfection of Bcl-2 cDNA partially suppressed SAHA-induced cell death. HDAC inhibitors can also induce TRAIL, suggesting the activation of

death receptor pathway without the requirement of exogenous TRAIL. Thus, HDAC inhibitors can induce apoptosis by linking both death receptor and mitochondrial pathways of apoptosis.

Dysregulation in apoptosis has been associated with the development of cancer (Johnstone et al., 2002). Recent studies have shown the involvement of mitochondria in many apoptotic signaling pathways (Kandasamy et al., 2003; Wei et al., 2000). Members of the Bcl-2 family of proteins that regulate apoptotic signaling through mitochondria are key regulators of apoptosis in mammalian development, and their deregulation is associated with disease, particularly cancer (Grimm et al., 1996; Gross et al., 1999). There are three classes of Bcl-2 family members: apoptosis promoters (e.g., Bax and Bak); apoptosis inhibitors (e.g., Bcl-2, Bcl-X_L, and adenoviral E1B 19K); and the BH3-only Bcl-2 family members (e.g., Bid, Puma, Noxa, Bad, and Nbk/Bik) (Gross, 2001). BH-3 only proteins may function as death sensors that mediate activation of the mitochondrial apoptosis pathway in response to oncogenic stress signals or DNA damage. Noxa and PUMA are transcriptionally induced by p53 and mediate apoptosis induced by p53. These proapoptotic activities of certain BH3-only proteins essentially depend on the presence of Bax and Bak. Inactivation of both Bax and Bak was required for tumor growth and was selected for in vivo tumorigenesis (Degenhardt et al., 2002a, b). $Bax^{-/-}$ and $Bak^{-/-}$ double knockout mouse embryo fibroblasts (DKO MEFs) were resistant to death signaling pathway, indicating that they are the required downstream components of mitochondrial signaling pathways (Kandasamy et al., 2003). Bim has been implicated in modulating lymphocyte homeostasis in immune cells. $Bim^{-/-}$ mice succumb to autoimmune kidney disease, accumulation of lymphoid and myeloid cells, and perturbed T-cell development (Bouillet et al., 2002; Bouillet and Strasser, 2002). Therefore, the regulation of Bcl-2 family members by HDAC inhibitor may play important roles on apoptosis by inducing a death activity or by antagonizing a survival activity. Furthermore, HDAC inhibitors can disrupt cellular redox state (e.g., ROS), and damage mitochondria in cells undergoing apoptosis.

Direct inhibitor of apoptosis protein (IAP)-binding protein with low pI/second mitochondrial activator of caspases, HtrA2/Omi and GstPT/eRF3 are mammalian proteins that bind via N-terminal IAP-binding motifs (IBMs) to the baculoviral IAP repeat (BIR) domains of IAPs. These interactions can prevent IAPs from inhibiting caspases, or displace active caspases, thereby promoting cell death (Deveraux and Reed, 1999). IAPs (cIAP-1, cIAP-2, NIAP, Livin/ML-IAP, survivin, and XIAP) protect cells against apoptosis by acting as caspase inhibitors (Deveraux and Reed, 1999). IAPs bind to and directly inhibit caspase-3, caspase-7, and caspase-9 (Deveraux and Reed, 1999; Deveraux et al., 1999). IAP proteins are regulated by interactions with the mitochondrial proteins (e.g., Smac/DIABLO), which may be released into the cytosol upon apoptotic stimulation and through IAP sequestration results in elevated caspase activity (Du et al., 2000; Verhagen et al., 2000). Some IAP proteins are also regulated by proteolysis via the ubiquitin-proteasome pathway and caspase-dependent cleavage of XIAP in cells undergoing apoptosis. The inhibition of XIAP, cIAP1, and cIAP2 expressions by HDAC inhibitors may contribute in sensitization of cells to TRAIL. In this context, we have shown that

TRAIL inhibits the expression of IAPs in breast and prostate cancer cells (Shankar et al., 2005b; Singh et al., 2005). The combination of HDAC inhibitors and TRAIL may further inhibit the expression of some of the IAPs and contribute to the synergistic induction of apoptosis by these agents.

HDAC inhibitors activate the p53 molecule through acetylation of 320 and 373 lysine residues, upregulate PIG3 and NOXA, and induce apoptosis in cancer cells expressing wild and pseudo-wild-type *p53* genes (Terui et al., 2003). SAHA induced polyploidy in human colon cancer cell line HCT116 and human breast cancer cell lines, MCF-7, MDA-MB-231, and MBA-MD-468, but not in normal human embryonic fibroblast SW-38 and normal MEFs (Xu et al., 2005a). The polyploid cells lost the capacity for proliferation and committed to senescence. The induction of polyploidy was enhanced in HCT116 $p21^{WAF1-/-}$ or HCT116 $p^{53-/-}$ cells than in wild-type HCT116. The development of senescence of SAHA-induced polyploidy cells was similar in all colon cell lines (Xu et al., 2005b). The present findings indicate that the HDAC inhibitor could exert antitumor effects by inducing polyploidy, and this effect is more marked in transformed cells with nonfunctioning $p21^{WAF1/CIP1}$ or *p53* genes.

In chronic myelocytic leukemia (CML) the activity of the Bcr-Abl tyrosine kinase is known to activate a number of molecular mechanisms, which inhibit apoptosis (Nimmanapalli et al., 2003; Xu et al., 2005b). SAHA markedly decreases protein expression levels of Bcr-Abl, c-Myc, and HDAC3 in CML, suggesting that SAHA exerts its biological activity by inhibiting survival pathway (Xu et al., 2005b). Differential expression of HDAC has been reported in various cancers. To explore the mechanisms of disease-specific HDAC activity in AML, the expression of HDAC in primary AML blasts and in four control cell types (namely CD34+ progenitors from umbilical cord, quiescent or cycling (postculture) cells, cycling CD34+ progenitors from GCSF-stimulated adult donors, and peripheral blood mononuclear cells) was characterized. Only Sirt1 was consistently overexpressed in AML samples compared with all controls, while HDAC6 was overexpressed relative to adult, but not neonatal cells (Bradbury et al., 2005). HDAC5 and SIRT4 were consistently underexpressed. HDAC inhibitors (valproate, butyrate, TSA, and SAHA) caused hyperacetylation of histones in AML blasts and cell lines (Bradbury et al., 2005). Such treatment also modulated the pattern of HDAC expression, with strong induction of HDAC11 in all myeloid cells tested, and lesser, more selective, induction of HDAC9 and SIRT4. The distinct pattern of HDAC expression in AML and its response to HDAC inhibitors is of relevance to the development of HDAC inhibitor-based therapeutic strategies and may contribute to observed patterns of clinical response and development of drug resistance.

4.3 Antiangiogenic Properties of HDAC Inhibitors

Tumor growth requires the development of new vessels that sprout from preexisting normal vessels in a process known as “angiogenesis” (Folkman, 2002). These new vessels arise from local capillaries, arteries, and veins in response to the release of

soluble growth factors from the tumor mass, enabling these tumors to grow beyond the diffusion-limited size of approximately 2 mm diameter. Tumor growth and metastasis depend upon the development of a neovasculature in and around the tumor (Folkman, 2002, 2003a, b, d; Folkman and Kalluri, 2004; Liotta et al., 1991). Angiogenesis is regulated by the balance between stimulatory (e.g., bFGF, IL-8, MMP-2, MMP-9, TGFβ1, and vascular endothelial growth factor [VEGF]) and inhibitory (e.g., angiostatin, IL-10, and interferon) factors released by the tumor and its environment (Folkman, 2003b, c). For example, overexpression of bFGF (Allen and Maher, 1993; Ravery et al., 1992) and VEGF (Brown et al., 1993a, b; O'Brien et al., 1995) has been found in the tissue, serum, and urine of patients with bladder cancer and has been associated with cancer progression, suggesting a direct involvement of these proteins in angiogenesis.

HDAC inhibitors also modulate angiogenesis in a potentially therapeutic manner. HDAC1 downregulates expression of p53 and the von Hippel–Lindau tumor suppressor gene and stimulates angiogenesis of human endothelial cells. HDAC inhibitors prevent endothelial cell proliferation and angiogenesis by downregulating angiogenesis-related gene expression (Bapna et al., 2004; Caponigro et al., 2005; Chinnaiyan et al., 2005b; Deroanne et al., 2002; He et al., 2005; Kim et al., 2001b, 2004c; Kwon et al., 2002a; Liu et al., 2003; Michaelis et al., 2004, 2005; Mie Lee et al., 2003; Momparler, 2003; Murakami et al., 2004; Nam and Parang, 2003; Pili et al., 2001; Qian et al., 2004; Rossig et al., 2002; Sasakawa et al., 2003; Sawa et al., 2002; Takimoto et al., 2005; Wang et al., 2003; Wiedmann and Caca, 2005; Williams, 2001; Zgouras et al., 2004). Phenyl butyrate, LBH589, LAQ824, and TSA have antiangiogenic activity both in vitro and in vivo (Pili et al., 2001; Qian et al., 2004, 2006; Williams, 2001). Other HDAC inhibitors such as SAHA, FK228, VPA, and apicidin also have antiangiogenic acitivity (Kim et al., 2001b; Kwon et al., 2002a; Michaelis et al., 2004). Angiogenesis inhibition induced by HDAC inhibitors was associated with modulation of angiogenesis-related genes both in cancer cells (e.g., inhibition of HIF-1α and VEGF) and in endothelial cells (inhibition of Tie-2 and survivin), and inhibition of endothelial cell migration and proliferation (Kim et al., 2001b; Kwon et al., 2002a; Michaelis et al., 2004; Williams, 2001). Furthermore, LBH589 inhibited endothelial tube formation and matrigel invasion (Qian et al., 2006). These data suggest that the effects of HDAC inhibitors on angiogenesis can be further enhanced in the presence of TRAIL.

HDAC inhibitors upregulate p53 and von Hippel–Lindau expression (Kim et al., 2001b). The combination of adenoviral vector carrying wild-type *p53* (*Ad-p53*) gene therapy with sodium butyrate resulted in a complete regression of xenografted human gastric tumor (KATO-III) cells in nude mice (Takimoto et al., 2005). Tumors treated with the combination showed higher numbers of TUNEL-positive cells and lower CD34 staining than those treated with a single modality (Takimoto et al., 2005). This was further supported by the finding that the brain-specific angiogenesis inihibitor-1 (BAI-1), an inhibitor of vascularization, was induced by sodium butyrate treatment in cells transfected with Ad-p53 (Takimoto et al., 2005). These data suggest that HDAC inhibitors can be combined with *p53* gene therapy for the treatment of cancer. The HDAC inhibitors have shown the

dual function of targeting both tumor cells and proliferating endothelial cells and to inhibit tumor angiogenesis by gene modulation. Rational clinical testing of these agents either alone or in combination with angiogenesis inhibitors is warranted.

5 Combination of HDAC Inhibitors with Trail/Apo-2L

HDAC inhibitor either alone or in combination with TRAIL can be used in cancer therapy. We and others have shown that several HDAC inhibitors can enhance the apoptosis-inducing potential of TRAIL in TRAIL-sensitive cells and sensitize TRAIL-resistant breast, prostate, and lung cancer cells, and malignant mesothelioma, leukemia, and myeloma cells (Facchetti et al., 2004; Fandy et al., 2005; Goldsmith and Hogarty, 2005; Inoue et al., 2004; Nebbioso et al., 2005; Rosato et al., 2003a; Shetty et al., 2005; Singh et al., 2005; Vanoosten et al., 2005). The sensitization of TRAIL-resistant cells appears to be due to downregulation of the antiapoptotic protein Bcl-2, Bcl-X_L, and Mcl-1, and upregulation of proapoptotic genes *Bax*, *Bak*, *TRAIL*, *Fas*, *FasL*, *DR4*, and *DR5*, and activation of caspases. HDAC inhibitors upregulate proapoptotic genes in cancer cells but not in normal cells (Insinga et al., 2005a, b). Sodium butyrate and TSA enhanced TRAIL-mediated apoptosis to a greater extent than depsipeptide, MS-275, and oxamflatin (Vanoosten et al., 2005). Both sodium butyrate and TSA treatment also increased mRNA and surface expression of TRAIL-R2/DR5 that was dependent on the transcription factor Sp1, thus providing a possible mechanism behind the increased sensitivity to TRAIL. These results show that sensitivity to HDAC inhibitors in cancer cells is a property of the fully transformed phenotype and depends on activation of a specific death pathway. Since HDAC inhibitors sensitize TRAIL-resistant cancer cells to undergo apoptosis by TRAIL, they appear to be promising candidates for combination chemotherapy.

Several studies have demonstrated the engagement of mitochondria during activation of death receptor pathway (Debatin and Krammer, 2004; Sartorius et al., 2001; Shankar et al., 2005b; Suliman et al., 2001). Cross talk between the death-receptor (extrinsic) and mitochondrial (intrinsic) pathways requires caspase-8/caspase-10-dependent cleavage of Bid (Fandy et al., 2005; Shankar et al., 2005b; Singh et al., 2005; Suliman et al., 2001). tBid activates Bax and Bak to release cytochrome c and other mitochondrial proteins (Luo et al., 1998; Wei et al., 2000). Since HDAC inhibitors induced cleavage of Bid, the truncated Bid may trigger activation of mitochondria in the absence of ligand TRAIL. We have shown that the pan-caspase inhibitor z-VAD-fmk completely inhibited TRAIL-induced apoptosis in the presence of HDAC inhibitor (Fandy et al., 2005; Shankar et al., 2005b; Singh et al., 2005). The caspase-8 inhibitor z-IETD and DN-FADD completely inhibited the synergistic interaction between HDAC inhibitor and TRAIL. Furthermore, in the presence of HDAC inhibitors, TRAIL induced caspase-3 and caspase-9 activation and caused cleavage of their substrate poly(ADP-ribose)

polymerase (PARP). Antiapoptotic proteins Bcl-2 and Bcl-X_L inhibit HDAC inhibitors and/or TRAIL-induced apoptosis by blocking cytochrome c release. The phosphorylation deficient mutant of Bcl-2 and Bcl-X_L also blocked HDAC inhibitors and/or TRAIL-induced apoptosis. In cell-intrinsic pathway of apoptosis, mitochondria amplify the apoptotic signals leading to activation of caspase-9 (Kandasamy et al., 2003). Caspase-9 in turn activates downstream caspases and the cleavage of apoptotic substrates that finally kill cells. The synergistic effects of HDAC inhibitors and TRAIL on apoptosis occur through activation of downstream caspase-3, which can be activated by both extrinsic and intrinsic pathways (Fandy et al., 2005; Shankar et al., 2005b; Singh et al., 2005).

The sensitization of cancer cells to HDAC inhibitors appears to be p53 independent. We have recently shown that chemotherapeutic drugs (Singh et al., 2003) or irradiation (Shankar et al., 2004a, b) can sensitize breast and prostate cancer cells by upregulating death receptors DR4 and/or DR5 in cells harboring wild-type (MCF-7) and mutated (MDA-MB-231 and MDA-MB-468) p53. Recent studies have shown that HDAC inhibitors induce apoptosis in leukemia in a p53-independent manner but not in normal hematopoietic progenitors (Insinga et al., 2005b; Nebbioso et al., 2005). Other transcription factors such as NF-κB and SP1 have been shown to regulate the expression of death receptors (Chen et al., 2003; Keane et al., 1999; Nagane et al., 2000; Ravi et al., 2001).

Treatment of nude mice with HDAC inhibitors resulted in acetylation of histone H3 and H4, and downregulation of hypoxia-inducible factor 1-alpha and VEGF expression in tumor cells. Furthermore, control mice demonstrating increased rate of tumor growth had increased numbers of CD31-positive or von Willebrand Factor (vWF)-positive blood vessels, and increased circulating vascular VEGFR2-positive endothelial cells compared to HDAC inhibitor and/or TRAIL-treated mice. Sequential treatments of athymic nude mice with HDAC inhibitors followed by TRAIL cause a synergistic apoptotic response through activation of caspase-3 and caspase-7, which is accompanied by regression of tumor growth, inhibition of angiogenesis, and enhancement of survival of xenografted nude mice. Together with our previous studies showing that cancer chemotherapeutic drugs and irradiation upregulate DR4 and/or DR5 expression, thereby enhancing TRAIL-induced apoptosis in vivo (Chinnaiyan et al., 2000; Shankar et al., 2004b, 2005a; Singh et al., 2003), these studies demonstrate the antitumor interactions of HDAC inhibitors with the TRAIL death-receptor pathway. Similarly, several recent studies including ours have demonstrated the additive or synergistic effects of HDAC inhibitors and TRAIL on apoptosis in vitro (Facchetti et al., 2004; Fandy et al., 2005; Goldsmith and Hogarty, 2005; Inoue et al., 2004; Nebbioso et al., 2005; Neuzil et al., 2004; Rosato et al., 2003a; Shankar et al., 2005b; Shetty et al., 2005; Singh et al., 2005; Zhang et al., 2003). The ability of HDAC inhibitors to sensitize cancer cells to TRAIL suggests that HDAC inhibitors can reduce the minimal effective dose or side effects of TRAIL. Thus, these data provide the framework for clinical evaluation of HDAC inhibitors and TRAIL for the treatment of human cancer.

6 Combination of HDAC Inhibitors with Irradiation

HDAC inhibitors have been shown to radiosensitize prostate, breast, and glioma cell lines (Camphausen et al., 2004; Kim et al., 2004a; Nome et al., 2005). TSA has been shown to radiosensitize human glioblastoma U373MG and U87MG cell lines in a dose- and time-dependent manner (Kim et al., 2004a). VPA enhanced the radiosensitivity of brain tumor SF539 and U251 cell lines in vitro and U251 xenografts in vivo, which correlated with the induction of histone hyperacetylation (Camphausen et al., 2005). Similarly, MS-275 can enhance radiosensitivity of DU145 prostate carcinoma and U251 glioma cells suggesting that this effect may involve an inhibition of DNA repair (Camphausen et al., 2004). The combination of HDAC inhibitors with irradiation may be useful for the treatment of cancer and merit further investigation. Given the limited efficacy of standard treatments for patients with cancer, these data provide support for clinical trials integrating HDAC inhibitor with radiation therapy.

Caspase-2 and caspase-3 cleave HDAC4 in vitro, and caspase-3 is critical for HDAC4 cleavage in vivo during UV-induced apoptosis (Paroni et al., 2004). After UV irradiation, GFP-HDAC4 translocates into the nucleus coincidentally/immediately before the retraction response, but clearly before nuclear fragmentation. Together, these data indicate that caspases could specifically modulate gene repression and apoptosis through the proteolytic processing of HDAC4. Among molecular cell cycle-targeted drugs currently in the pipeline for testing in early-phase clinical trials, HDAC inhibitors may have therapeutic potential as radiosensitizers.

7 Combination of HDAC Inhibitors with Chemotherapeutic Drugs

Chemotherapeutic treatment with combinations of drugs is frontline therapy for many types of cancer. Combining drugs which target different signaling pathways often lessens adverse side effects while increasing the efficacy of treatment and reducing patient morbidity. It has recently been shown that HDAC inhibitors facilitate the cytotoxic effectiveness of the topoisomerase I inhibitor camptothecin in the killing of tumor cells (Bevins and Zimmer, 2005). SAHA has been shown to act as a chemopreventive agent in mammary tumors in the rat (Cohen et al., 1999) and inhibited the growth of established tumors (Butler et al., 2000; Chinnaiyan et al., 2005a; Cohen et al., 1999). SAHA and sodium butyrate interacted synergistically with camptothecin in inducing apoptosis of breast and lung cancer cell lines. Experiments have shown that cells arrested in G2-M by camptothecin were most sensitive to subsequent addition of HDAC inhibitor. In camptothecin-arrested cells, sodium butyrate decreased cyclin B levels, as well as the levels of the antiapoptotic proteins XIAP and survivin. Overall, these findings suggest that reducing the levels

of these critical antiapoptotic factors may increase the efficacy of camptothecin in the clinical setting if given in a sequence that does not prevent or inhibit tumor cell progression through the S phase.

MS-275 also synergistically interacted with fludarabine in inducing apoptosis of human lymphoid and myeloid leukemia cells (Maggio et al., 2004). Prior exposure of Jurkat lymphoblastic leukemia cells to MS-275 increased mitochondrial injury, caspase activation, and apoptosis in response to fludarabine, resulting in highly synergistic antileukemic interactions and loss of clonogenic survival. Simultaneous exposure to MS-275 and fludarabine also led to synergistic effects, but these were not as pronounced as observed with sequential treatment. Similar interactions were noted in the case of (a) other human leukemia cell lines (e.g., U937, CCRF-CEM); (b) other HDAC inhibitors (e.g., sodium butyrate); and (c) other nucleoside analogues (e.g., 1-beta-D-arabinofuranosylcytosine, gemcitabine). Potentiation of fludarabine-induced apoptosis by MS-275 was associated with acetylation of histones H3 and H4, downregulation of the antiapoptotic proteins XIAP and Mcl-1, enhanced cytosolic release of proapoptotic mitochondrial proteins (e.g., cytochrome c, Smac/DIABLO, and AIF), and caspase activation. These events were accompanied by the caspase-dependent downregulation of $p27^{/KIP1}$, cyclins A, E, and D1, and cleavage and diminished phosphorylation of retinoblastoma protein. Prior exposure to MS-275 attenuated fludarabine-mediated activation of MEK1/2, extracellular signal-regulated kinase, and Akt, and enhanced c-Jun NH(2)-terminal kinase phosphorylation; furthermore, inducible expression of constitutively active MEK1/2 or Akt significantly diminished MS-275/fludarabine-induced lethality. Combined exposure of cells to MS-275 and fludarabine was associated with a significant increase in generation of ROS; moreover, both the increase in ROS and apoptosis were largely attenuated by coadministration of the free radical scavenger L-*N*-acetylcysteine. Finally, prior administration of MS-275 markedly potentiated fludarabine-mediated generation of the proapoptotic lipid second messenger ceramide. Taken together, these findings indicate that MS-275 induces multiple perturbations in signal transduction, survival, and cell cycle regulatory pathways that lower the threshold for fludarabine-mediated mitochondrial injury and apoptosis in human leukemia cells.

A synergistic interaction of retinoic acid and CBHA was shown in a mouse model of neuroblastoma. DNA hypomethylating agents have been found to have synergistic effects with HDAC inhibitors. The combination of TSA with azacytidine caused a dramatic potentiation in the activation of silenced genes (Baylin and Bestor, 2002; Baylin et al., 2001; Chen et al., 1997). Depsipeptide and TSA induced apoptosis in human lung cancer cells. HDAC inhibitor-induced apoptosis was greatly enhanced in the presence of the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, suggesting the DNA methylation status plays an important role on the effectiveness of HDAC inhibitors (Zhu et al., 2001). Furthermore, HDAC inhibitors enhanced paclitaxel-induced cell death in ovarian cancer cell lines independent of p53 status (Chobanian et al., 2004). Similarly, commonly used anticancer drugs doxorubicin and decitabine have been reported to have synergistic effects with HDAC inhibitors (Blagosklonny et al., 2000; Gozzini and Santini, 2005).

Thus, the combination of anticancer drugs with other epigenetic therapies provides potentially safer therapeutic options.

8 Chemoprevention by HDAC Inhibitors

In recent years, the use of naturally occurring chemopreventive agents have attracted many investigators because of their nontoxic effects. The preclinical data on selected chemopreventive agents have been very promising. Evidence indicates that a diet high in fresh fruits and vegetables decreases risk of certain cancers because they contain fiber, folate, and vitamins with antioxidant activity (Howe et al., 1992; Janne and Mayer, 2000). Studies have shown that the dietary fiber provides a protective effect against colon cancer (Howe et al., 1992; Trock et al., 1990). It appears that the fermentation of dietary fiber in the lumen of the colon produces the short chain fatty acid *n*-butyrate, which has anticarcinogenic activity on a variety of cellular functions, including differentiation, motility, invasion, adhesion, proliferation, and apoptosis. There is a positive correlationship between high fecal butyrate levels and decrease tumor incidence and tumor growth (Cassidy et al., 1994; Hylla et al., 1998; McIntyre et al., 1993). Butyrate is a physiological regulator of colonic epithelial cell proliferation, differentiation, and survival; and it induces histone hyperacetylation and inhibits methylation (de Haan et al., 1986; Riggs et al., 1977). Butyrate induces expression of $p^{21/WAF1/CIP1}$ through a process involving histone hyperacetylation and recruitment of Sp3 to the proximal p21 promoter (Sowa et al., 1999), and p21 is required for butyrate-mediated growth arrest in colon carcinoma cells (Archer et al., 1998). Although p21 is a p53 target gene, p21 induction by butyrate and other HDAC inhibitors is p53-independent (Xiao et al., 1997). Thus, HDAC inhibitors can induce p21-associated growth arrest in the absence of wild-type p53 function.

Sulforaphane (SFN), a compound found at high levels in broccoli and broccoli sprouts, is a potent inducer of phase 2 detoxification enzymes and inhibits tumorigenesis in animal models. SFN also has a marked effect on cell cycle checkpoint controls and cell survival and/or apoptosis in various cancer cells. SFN dose-dependently increased the activity of a β-catenin-responsive reporter, without altering β-catenin or HDAC protein levels (Myzak et al., 2004). SFN inhibits HDAC activity in colon and prostate cancer cells (Myzak et al., 2005). The inhibition of HDAC was accompanied by an increase in acetylated histones. SFN caused enhanced interaction of acetylated histone H4 with the promoter region of the $p^{21/WAF1/CIP1}$ gene and the bax gene. SFN induced cell cycle arrest and apoptosis through caspase activation. These findings provide new insight into the mechanisms of SFN action in benign prostate hyperplasia, and they suggest a novel approach to chemoprotection and chemotherapy of prostate cancer through the inhibition of HDAC.

In summary, several reports have described butyrate, diallyl disulfide, and SFN as HDAC inhibitors, and many other dietary agents likely will be discovered to attenuate HDAC activity. Dietary HDAC inhibitors, as weak ligands, regulate the

expression of genes involved in cell growth, differentiation, and apoptosis. An important question is the extent to which dietary HDAC inhibitors, and other dietary agents that affect gene expression via chromatin remodeling, modulate the expression of genes so that cells can respond most effectively to external stimuli and toxic insults.

9 Clinical Trials with HDAC Inhibitors

Phase I and II clinical trials indicate that HDAC inhibitors from several different structural classes are very well tolerated and exhibit clinical activity against a variety of human malignancies; however, the molecular basis for their anticancer selectivity remains largely unknown. Furthermore, HDAC inhibitors have also shown preclinical promise when combined with other therapeutic agents, and innovative drug delivery strategies, including liposome encapsulation, may further enhance their clinical development and anticancer potential. An improved understanding of the mechanistic role of specific HDACs in human tumorigenesis, as well as the identification of more specific HDAC inhibitors, will likely accelerate the clinical development and broaden the future scope and utility of HDAC inhibitors for cancer treatment.

Several HDAC inhibitors (SAHA, MS-275, CI-994, and depsipeptide) are currently undergoing clinical trials (Blanchard and Chipoy, 2005; Hess-Stumpp, 2005; Kelly et al., 2005). HDAC inhibitors represent a relatively new group of targeted anticancer compounds, which are showing significant promise as agents with activity against a broad spectrum of neoplasms, at doses that are well tolerated by cancer patients. SAHA is most advanced in development, currently in phase I and II clinical trials for patients with both hematologic and solid tumors (Kelly et al., 2005). Clinical trials on depsipeptide alone have shown low toxicity and evidence of antitumor activity (Sandor et al., 2002). Additionally, the compound has potential for synergism with radiotherapy, chemotherapy, and biologicals. Second-generation HDAC inhibitors, such as LAQ824 and PDX101, are currently under phase I clinical trials. Simultaneously, synthetic benzamide-containing HDAC inhibitors, CI-994 and MS-275, have reached phase I and II clinical trials, respectively.

10 Conclusions

Epigenetic modifications causing gene transcriptional repression have been associated with malignant transformation and are intriguing new targets in the treatment of cancer. In contrast to genetic deletions causing irreversible loss of gene function, epigenetic gene silencing mediated by DNA methylation and histone deacetylation can be reversed via pharmacologic inhibition of DNA methyltransferases and HDACs, respectively. When this occurs, normal patterns of gene expression, cell

differentiation, and apoptosis may be restored and disease response obtained. The HDAC has been considered an attractive target molecule for cancer therapy. The inhibition of HDAC activity by a specific inhibitor induces growth arrest, differentiation, and apoptosis of several cancer cells.

Our studies have shown that HDAC inhibitors upregulate proapoptotic members of Bcl-2 family and death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5), and downregulate antiapoptotic genes of Bcl-2 family; thus it is possible that sensitization of cancer cells to chemotherapy, irradiation, or TRAIL by HDAC inhibitors may occur at various stages of apoptotic pathways. Furthermore, the ability of HDAC inhibitors to inhibit angiogenesis may further affect tumor growth by regulating angiogenesis-related signaling pathways. Preliminary studies in animal models have revealed a relatively high tumor selectivity of HDAC inhibitors, strengthening their promising potential in cancer chemotherapy. Some of these inhibitors are undergoing phase I and phase II clinical trials. Furthermore, the combination of HDAC inhibitors with commonly used anticancer drugs, irradiation, or TRAIL will be useful for cancer therapy. Since the HDAC inhibitors are frequently used in epigenetic studies and are considered to be promising anticancer drugs, these findings will have implications in both laboratory and clinical settings.

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Chapter 14
RNA Interference and Cancer: Endogenous Pathways and Therapeutic Approaches

Derek M. Dykxhoorn, Dipanjan Chowdhury, and Judy Lieberman*

Abstract The endogenous RNA interference (RNAi) pathway regulates cellular differentiation and development using small noncoding hairpin RNAs, called microRNAs. This chapter will review the link between mammalian microRNAs and genes involved in cellular proliferation, differentiation, and apoptosis. Some microRNAs act as oncogenes or tumor suppressor genes, but the target gene networks they regulate are just beginning to be described. Cancer cells have altered patterns of microRNA expression, which can be used to identify the cell of origin and to subtype cancers. RNAi has also been used to identify novel genes involved in cellular transformation using forward genetic screening methods previously only possible in invertebrates. Possible strategies and obstacles to harnessing RNAi for cancer therapy will also be discussed.

Keywords RNA interference, microRNA, cancer, microarray, tumor profile, siRNA, therapy, prognosis

1 Introduction

RNA interference (RNAi) is an endogenous, ubiquitous, and evolutionarily conserved pathway for regulating gene expression. Noncoding stem-loop RNAs, encoded within exons or in intergenic regions, are processed by specialized intracellular RNase III enzymes into small RNAs, called microRNAs or miRNAs.[1–4] The microRNAs are taken up by a multiprotein cytoplasmic complex, called the RNA-induced silencing complex (RISC), which directs the posttranscriptional silencing of a partially complementary mRNA target. Silencing of highly complementary mRNAs can occur through mRNA degradation, but for less complementary targets, gene silencing occurs by inhibiting translation. Most mammalian microRNAs work by the latter

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pathway. The rules for identifying silenced target genes are still poorly defined; therefore, only a handful of mammalian genes have been clearly shown to be regulated by the endogenous RNAi pathway. However, current bioinformatic estimates suggest that the expression of a third or more of all genes may be regulated by microRNAs. In other species, such as plants, worms, and flies, RNAi regulates critical genes involved in cellular differentiation and survival. In fact, the first identified endogenous microRNAs regulated the progression from one larval state to another in *Caenorhabditis elegans* development.

There is increasing evidence of a role for microRNAs in cancer.[5–8] This should not be surprising since malignant transformation results from abnormally regulated cell differentiation and survival – processes regulated by microRNAs in other organisms. Here, we review how microRNAs are processed within mammalian cells and then describe the evidence for microRNA regulation of genes implicated in cancer and apoptosis. Recent studies provide examples of emerging networks that regulate the expression of microRNAs and transcription factors to control terminal differentiation in a variety of cell types, a step that is aberrant in cancer. We will discuss how microRNA expression profiles are altered in cancer and might be used for diagnosis and prognosis. We will also discuss recent examples of RNAi-based screens to identify tumor-promoting and suppressor-coding genes and microRNAs. Lastly, we will discuss the therapeutic prospects for harnessing RNAi to silence oncogenes or other genes involved in cell proliferation and survival or for interfering with microRNAs that play a role in tumorigenesis.

2 microRNA Biogenesis and the Endogenous RNAi Pathway

Most microRNAs are transcribed within coding mRNAs or as independent transcripts by RNA polymerase II as long precursor primary transcripts that are capped and polyadenylated (Fig. 14.1).[4,9–11] microRNA transcripts are highly structured with an elongated hairpin that contains frequent mismatches, bulges, and non-Watson–Crick base-pairings. In some cases, several microRNAs are coordinately expressed as polycistrons from the same primary transcript.[12–16] The microRNA precursors, called pri-miRNAs, have a characteristic fold-back structure that is recognized in the nucleus by an RNase III-type enzyme, Drosha, and its binding partner, variously called DiGeorge syndrome critical region gene 8 (DGCR8) protein in mammals and partner of Drosha (Pasha) in *Drosophila* and *C. elegans*.[15,17–21] Drosha cleaves the pri-miRNA into a ~70 nt fold-back structure, termed the pre-miRNA, which is exported into the cytoplasm by exportin 5.[22–25] The pre-miRNA is then recognized by Dicer and cleaved into a small dsRNA intermediate that contains both the mature microRNA and the accompanying complementary strand.[26–31] The strand whose 5′-end is less tightly bound to its complementary strand is incorporated into the effector RISC or miRISC.[32,33] The complementary strand is rapidly lost when the microRNA is taken up into RISC.[34,35] In some cases, presumably when both ends are comparably paired, microRNAs can be found that correspond

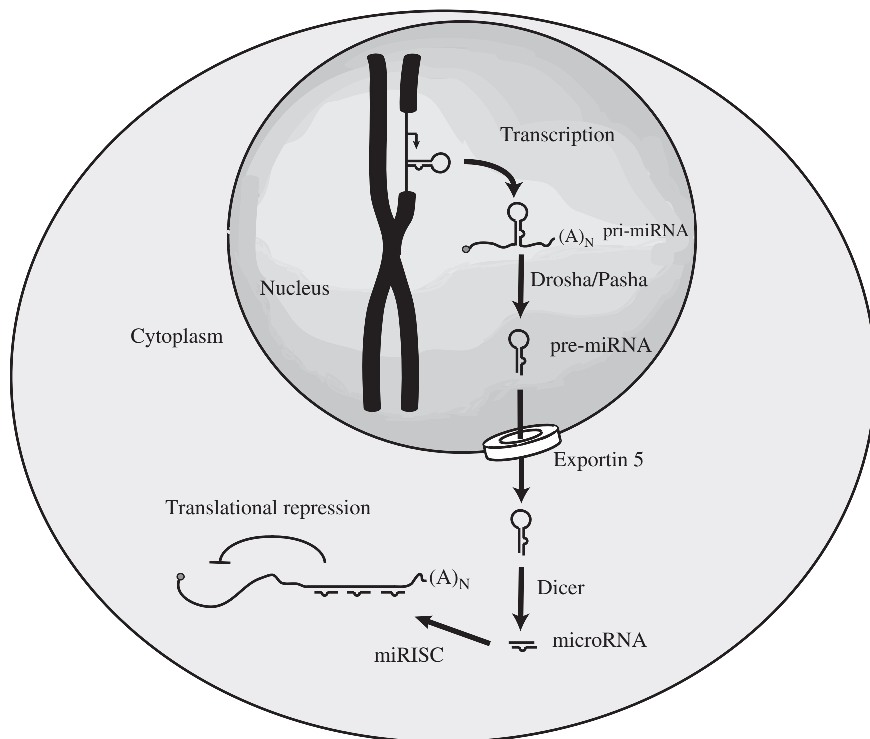


Fig. 14.1 RNA interference pathway. microRNAs that direct the posttranscriptional silencing of gene expression are derived from longer primary transcripts that are expressed from RNA polymerase II promoters.[4,9–11] These primary transcripts, termed pri-miRNAs, can range from several hundred to thousands of nucleotides long with the microRNA sequence encoded in a highly structured RNA hairpin that contains frequent bulges and mismatches.[4] These long hairpins are recognized and cleaved into shorter (~70 nt) hairpin RNAs, pre-miRNAs, in the nucleus by Drosha in conjunction with the double-stranded RNA recognition protein, termed Pasha in *Drosophila* and *Caenorhabditis elegans* and DGCR8 in mammalian cells.[15,17–21] pre-miRNAs are exported from the nucleus into the cytoplasm by Exportin 5 where they are recognized and cleaved into the ~22 nt microRNA by Dicer in conjunction with another dsRNA-binding protein, called Loquacious in *Drosophila* and TRBP in mammals.[22–25,28–30] The miRNA is taken up by the effector complex, miRISC, and the passenger strand is lost, leaving the mature microRNA to guide the recognition of the microRNA-binding sites on the target mRNA, leading to silencing of target gene expression.[34,35] Originally, mammalian microRNAs were thought to mediate target gene silencing by binding to sites on the mRNA that had incomplete complementarity with the microRNA and inducing translational repression, in contrast to small interfering (si)RNAs which have complete (or nearly complete) homology and direct mRNA cleavage.[2,4] However, microRNAs with partial complementarity can facilitate some mRNA degradation, in addition to inducing translational repression[177]

to both strands of the microRNA precursor. The exact composition of the RISC is still unknown. (In fact, this term probably refers to several complexes that may have some core components in common but have additional factors that determine their individual function.) A key component of the RISC is an Argonaute family protein, often Ago 2, which is the RISC endonuclease.[36]

Regulation of gene expression by microRNAs operates through several mechanisms including two that work posttranscriptionally – degradation of the targeted mRNA by cleavage and inhibition of translation.[2,4] The first mechanism has a more potent effect on gene expression, probably because the same RISC-incorporated small RNA can be used repeatedly to guide the degradation of multiple target mRNAs.[37] It is controversial whether the other mechanism (translational inhibition) involves blocking the initiation of translation or a more distal step in translation, and possibly multiple mechanisms may operate in different circumstances. A less well-understood mechanism of gene silencing by noncoding RNAs involves inhibition of transcription by the formation and maintenance of regions of silenced chromatin.[38] In fact, Dicer-deficient cells are impaired in heterochromatin formation.[39] The specificity of posttranscriptional silencing is determined by complementarity of the microRNA to the target mRNA, usually at sites in the 3′ untranslated region (UTR) of the message. The 5′-end of the guide strand is buried into a pocket of the Ago protein, while nucleotides 2–8 are exposed on the surface of the molecule forming a seed sequence that directs target mRNA recognition.[40,41] How strongly base-pairing of the remaining nucleotides of the typical 19–23 nucleotide sequence of the microRNA to the target sequence influences gene silencing is uncertain. Other properties of the target mRNA that might influence gene silencing (such as lack of secondary structure of flanking sequences) are not well understood, making prediction of microRNA gene targets still a challenge.

mRNAs undergoing microRNA-induced translational inhibition appear to be sequestered in distinct cytoplasmic foci.[42–47] These sites, referred to by a variety of names including processing (P)-, cytoplasmic-, GW-, Dcp-, or Lsm-bodies, serve as foci for the accumulation of mRNAs that are destined for degradation.[48–50] In addition to the mRNA, these sites contain essential components of the mRNA degradation pathway, the mRNA decapping enzymes (Dcp1/Dcp2), as well as the 5′-3′ exonuclease Xrn1, Dhh1p, and Pat1p, and in mammalian cells, GW182.[51] The first hint of the interaction of the microRNA machinery with these sites of mRNA turnover was the demonstration that the mammalian Ago proteins implicated in RNAi colocalize with components of mammalian P-bodies.[43,46] Another family of Argonaute proteins, the Piwi family, that have not been found to be associated with microRNAs, do not colocalize.[46] A direct physical interaction between Ago1 and Ago2 with Dcp1 and Dcp2 was also shown by co-immunoprecipitation, even in the absence of RNA or using Ago2 protein, mutated in the Piwi Argonaute Zwille (PAZ) domain required for small RNA binding.[46] However, Ago2 localization to P-bodies is a microRNA/siRNA-dependent process.[45] This was further confirmed by following the fate of reporter mRNAs containing multiple MS2-binding sites that can be visualized with a fluorescently tagged MS2 protein and sites for either an endogenous microRNA or an exogenously introduced siRNA that mimics microRNA function by binding to multiple imperfectly complementary sites on the mRNA.[45] The tagged mRNAs, but not reporter mRNAs that lack the microRNA-binding sites, localize in P-bodies only in the presence of their respective microRNA. A functional link between P-bodies and RNAi-mediated silencing was shown by silencing GW182, which disrupts the formation of P-bodies and

significantly impairs gene silencing by both translational repression and mRNA cleavage.[44,45]

The physical and functional link between the sites of mRNA turnover and microRNA/siRNA-mediated silencing raises questions about the potential role of the RNAi machinery in other translational regulation mechanisms. P-bodies are increasingly thought to be sites for the storage of translationally repressed mRNAs, with mRNAs being able to move between the active and inactive pool as needed. One hypothesis put forward by the Parker and Hannon groups is that microRNAs may mediate their repressive function by selectively transporting and possibly even maintaining their mRNA targets in these sites of translational repression, segregated from the translational machinery. It is possible to envision a variety of potential mechanisms by which the RISC could inhibit translation, including impairing various steps in translation (e.g., blocking the processivity or binding of ribosomes along the mRNA) or "tagging" newly formed proteins for degradation, in addition to sequestering the target mRNA from the translational apparatus. The interaction of RNAi components with other sites of mRNA storage and translational regulation, such as stress granules, which are distinct structures that interact with P-bodies, remains to be clarified.

3 Changes in microRNA Expression in Cancer

Mammalian microRNAs were first predicted using RNA-folding algorithms that identified evolutionarily conserved sequences that form into energetically favorable short hairpins that are structurally similar to microRNAs identified in other organisms.[52,53] These algorithms identified about 200 predicted microRNAs in mammalian genomes. A substantial subset of the predicted microRNAs was then verified by cloning small RNAs from a variety of cells. However, when the requirement for evolutionary conservation was relaxed, additional microRNAs were predicted (and the actual number may well exceed 1,000) and some of these have been cloned.[54] These less conserved microRNAs may regulate specialized functions (such as immune responses) that have evolved recently. The makeup and size of the universe of functional mammalian microRNAs is still uncertain, but will soon be more accurately defined using recently available methods for efficiently cloning small RNAs.

microRNAs are expressed in temporally regulated patterns during cell differentiation with distinct expression patterns in different cell types and tissues.[55–62] The total number of microRNAs in a cell can also vary during differentiation and typically constitutes about 1% of the total cellular RNA. Highly expressed microRNAs can be present at as many as 10^4 copies/cell. Highly efficient cloning has enabled researchers to identify microRNAs in rare cell types that are expressed at fewer than 100 copies/cell.[54,63] The functional significance of these rare microRNAs on gene expression is unclear. Figuring this out will be challenging because the gene targets of most microRNAs are unknown, and current target gene prediction

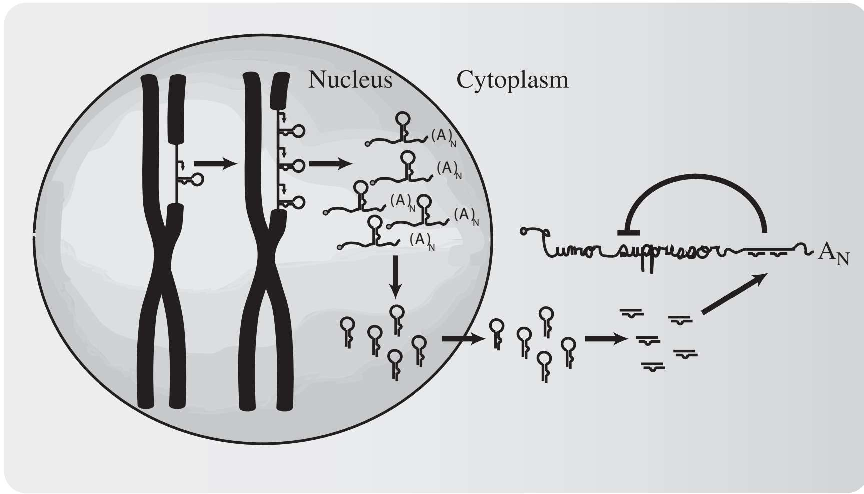
algorithms are poor at identifying them. Moreover, the effect on gene expression of a single microRNA binding to an mRNA may be small, particularly when silencing is via translational inhibition. In fact, when single microRNAs are genetically deleted or inhibited, it is rare to find any significant difference in cellular function or fate.[64] However, binding of multiple microRNAs to different sites in the 3′ UTR of a gene can coordinately have an impact on its expression.[65] This model of cooperative regulation is reminiscent of models of transcriptional regulation by groups of transcription factors binding to promoter sites on the DNA.

microRNAs have been associated with the regulation of a variety of biological processes from fat metabolism and insulin secretion to cell proliferation, apoptosis, and developmental timing.[66–70] Since microRNAs play such an important role in the regulation of invertebrate development and differentiation, it is not surprising that dysregulation of microRNA expression would be associated with oncogenic transformation in mammals. microRNAs might function as either tumor suppressors or oncogenes depending on their target genes and could contribute to cancer either by enhanced or reduced expression in tumor cells (Fig. 14.2). The first hint that microRNAs might be associated with the development of cancer was the identification of two microRNAs, miR15 and miR16, encoded in a small region of chromosome 13 that is frequently deleted in B-cell chronic lymphocytic leukemia (CLL).[71] These two microRNAs were later found to suppress the expression of bcl-2, an antiapoptotic protein that is frequently overexpressed in B-cell lymphomas and other malignancies. Similarly, expression of miR143 and miR145 is significantly decreased in colorectal cancer specimens compared to matched normal tissue.[72] Expression of these microRNAs is also reduced in a variety of colorectal, breast, prostate, lymphoid, and cervical cancers. In addition, miR26a and mir99a, expressed from regions associated with loss of heterozygosity in lung tumors, have reduced expressed in lung tumors and lung cancer cell lines. Bioinformatic analysis of the regions encoding microRNAs found that most microRNA genes (98 of 186 microRNAs examined) are encoded in regions of the genome associated with cancer, including regions associated with loss of heterozygosity, gene amplification, common break point regions, and fragile sites.[73] Importantly, one of these breakpoint region translocations (t(8,17)) associated with aggressive B-cell lymphoma places the MYC oncogene downstream of the miR142s gene promoter leading to MYC overexpression.[74] Although these studies correlate decreased microRNA expression with the development of cancer, they do not generally identify targets of the microRNAs that can explain their role in tumorigenesis.

Other microRNAs are overexpressed in specific malignancies. A conserved noncoding RNA termed BIC was first identified as a site of insertion of avian leukosis retroviruses, and enhanced expression in chicken B-cell lymphomas.[75] Recently, BIC was found to encode for miR155[74] and was found to be upregulated in human diffuse large B-cell lymphomas (DLBCL) with an activated B-cell phenotype. miR155 overexpressing tumors have poorer prognosis than B-cell lymphomas of the germinal center phenotype.[76–78]

Cancers result from the accumulation of multiple spontaneous and/or inherited mutations that lead to dramatic changes in the pattern of gene expression, particularly

miRNA functioning as an oncogene



miRNA functioning as a tumor suppressor

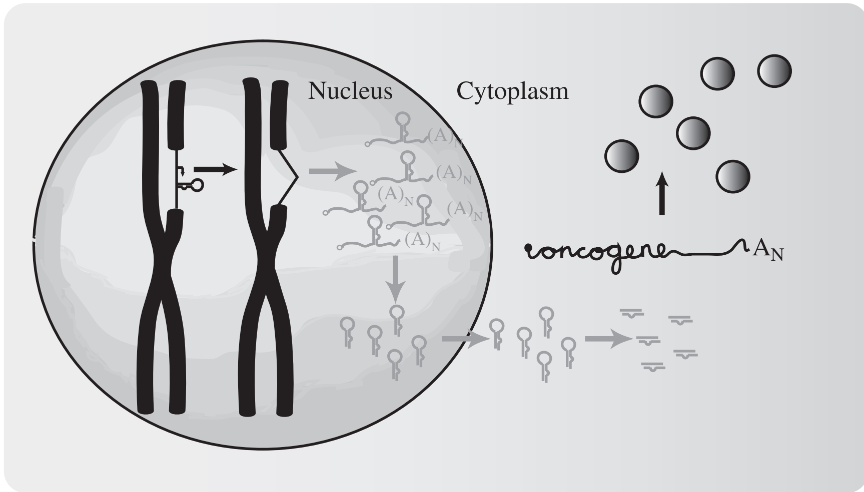


Fig. 14.2 microRNAs can act as either tumor suppressors or oncogenes depending on their targets. (A) *microRNAs as oncogenes*. microRNAs that target a tumor suppressor gene and are overexpressed because of gene amplification (e.g., the miR17–92 polycistron),[86] inappropriate expression of factors that upregulate transcription of the miRNA (e.g., c-Myc upregulation of the miR17–92 cluster)[94] or translocation into a genome locus that alters microRNA expression[73] can lead to cellular transformation, dysregulated proliferation, and tumor formation. (B) *microRNAs as tumor suppressors*. Tumor formation can be induced by the loss or decreased expression of a microRNA whose normal function would be to suppress expression of an oncogene. Inappropriate expression of the oncogene would then lead to cellular transformation. In either case, tumor formation could be a result of increased proliferation, angiogenesis or invasiveness, decreased levels of apoptosis, or alteration of the state of cellular differentiation

in pathways that control cell proliferation and cell cycle regulation, cell signaling, angiogenesis, apoptosis, protein degradation, transcriptional regulation, and the immune response. The assessment of changes in gene expression profiles in specific cancers by mRNA microarrays can be used to some extent to enhance definition of tumor subtypes and improve diagnosis and prognosis. However, a better understanding of the changes that are necessary for oncogenic transformation is seen when the mRNA microarray data are analyzed for changes in groups of related molecules, "molecular modules." This allows for the identification of specific pathways and biological processes that are disrupted in particular cancers. Changes in microRNA expression in different tissues, developmental and differentiation states were initially assessed using a cloning strategy that took advantage of the unique structure and size of Dicer cleavage products to isolate and sequence microRNAs.[13] Cloning microRNAs, however, is not suitable for high-throughput analysis and often does not provide reliable quantitative comparisons of expression. Until recently, low-abundance microRNAs were not readily detected by cloning. To address some of these concerns, microarray technologies used for profiling mRNA levels have been adapted to analyze microRNA expression[79–84] in a variety of normal and cancerous tissues, including CLL[85,86] and solid tumors, including lung, breast, stomach, prostate, colon, and pancreatic cancer.[87,88]

To begin to explore the role of microRNAs in CLL, Calin and colleagues compared the microRNA profile of CLL patient samples with that of normal CD5+B cells.[85] The CLL samples fell into two distinct clusters of microRNA expression. Some microRNAs were upregulated in both groups compared to CD5+B cells (e.g., miR183, miR190, and miR24–1) and some downregulated (e.g., miR213 and miR220). CLL patients can be grouped into two major subtypes according to whether their tumor cells express high levels of the signaling molecule ZAP70 and unmutated immunologlobulin heavy chain (more rapid disease progression) or low or undetectable ZAP70 and mutated immunoglobulin (slower disease progression). Expression of 13 microRNAs differed between the two groups.[89] When patients were classified by the interval between diagnosis and initiation of therapy (another indication of tumor grade), expression of 9 of the 13 microRNAs identified the slower progressing tumors. Eight of the nine differentially expressed microRNAs were overexpressed in the more rapidly progressing tumors. Some of the changes in microRNA expression could be linked to mutations within or near the microRNA sequences.

Microarray analysis also found that microRNA expression differed between normal and cancerous tissue in solid tumors, as well as between solid tumors arising from different organs.[90] microRNA expression by prostate, colon, stomach, and pancreatic adenocarcinomas tend to cluster together. On the other hand, lung and breast cancer samples have distinct patterns of microRNA expression. A few microRNAs (miR21, miR17–5p, and miR191) are overexpressed in a majority of solid tumors. One would expect that these common microRNAs might be involved in dysregulating cellular processes, such as cell proliferation, that are aberrant in all malignancies, while the tissue-specific microRNAs might be involved in oncogenic or differentiation events relevant to specific tissues.[91]

To facilitate microRNA profiling in human cancers, Lu et al. developed a highly effective and specific bead-based solution hybridization procedure.[90] This technique uses oligonucleotide-capture probes linked to polystyrene beads impregnated with a variable combination of fluorescent dyes, a specific combination for each microRNA that is being tested, to analyze rapidly the microRNA composition of large numbers of samples. By binding the oligomer-capture probes to the microRNAs in solution, as opposed to on a solid support (e.g., glass slides), microRNA family members that differ from one another by only a single nucleotide can be distinguished without much cross-reactivity. This method has a robust dynamic range with linear detection over a 100-fold range of microRNA expression. It was used to analyze the microRNA profile from 334 primary tumors representing a variety of tumor types and tissues of origin. Tumor samples showed decreased overall microRNA expression. Tumors of related lineage (i.e., epithelial, endodermal, and hematopoietic) clustered together, and expression patterns differed between tumors and their normal cellular counterparts. Moreover, tumors whose histology was not diagnostic could be assigned based on their microRNA expression profile with much more assurance than would be possible from mRNA profiling.

Early indications suggest that microRNA expression patterns will be more informative than mRNA microarrays in characterizing cancer cells.[90] It is likely that microRNA profiling will soon be used to refine diagnosis and subtype tumors to improve prognostic information and guide the choice of therapies. As the targets of the microRNAs whose expression is altered in various cancers are elucidated, this information will hopefully shed light also on the key events that contribute to the development and progression of cancers. It would not be surprising, for example, if alterations in microRNA genes might underlie poorly understood processes, such as metastasis.

4 microRNAs as Oncogenes or Tumor Suppressor Genes

Recently, a few pathways for microRNA regulation of genes implicated in cellular transformation have begun to be uncovered, but it is clear that this is just the beginning (Table 14.1). let-7, one of the first identified and most well-conserved microRNAs, regulates developmental timing in *C. elegans*. Upregulation of let-7 is necessary for the terminal differentiation of seam cells in adult animals by facilitating their exit from the cell cycle. In worms that lack let-7 expression, seam cells continue to divide and fail to differentiate, similar to cancer cells. In fact, let-7 is downregulated in lung cancer cells and cell growth of a lung cancer cell line is inhibited by overexpression of let-7.[92] These results suggest that let-7 acts as a tumor suppressor. This was demonstrated to be the case when the RAS oncogene was identified as a let-7 target in mammalian cells.[93] In *C. elegans* as well, the RAS homolog, let-60/RAS, is inhibited by the let-7 family members, let-7 and miR84, which bind to multiple target sites in the 3′ UTR of the let-60/RAS mRNA. Overexpression of miR84 in vulval cells

Table 14.1 Validated microRNA targets in mammalian cell proliferation, differentiation, and apoptosis

microRNA	Target gene	Function	Reference
let-7 family	Ras and its homologues	Cell proliferation	93
miR17–5p	E2F1	Transcription and cell proliferation	94
miR20a	E2F1	Transcription and cell proliferation	94
miR181	Hox A11	Hematopoiesis	99
		Skeletal myoblast differentiation	108
miR223	Nuclear factor I-A (NFI-A)	Granulopoiesis	100
miR221	c-kit receptor	Erythropoiesis	101
miR222	c-kit receptor	Erythropoiesis	101
miR130a	Transcription factor MAFB	Platelet physiology	102
miR10a	HoxA1	Megakaryocyte differentiation	102
miR196a	HoxB8	Limb development	106,107
miR1	Histone deacetylase 4 (HDAC 4)	Skeletal myogenesis	112
	Hand2	Cardiac development	111
miR133	Serum response factor (SRF)	Myoblast proliferation	112
miR134	Lim-domain-containing protein kinase 1 (Limk1)	Dendritic spine development	115
miR375	Myotrophin	Insulin secretion	66
miR143	ERK5/BMK1	Adipocyte differentiation	117
miR15a	bcl-2	Antiapoptosis	120
miR16–1	bcl-2	Antiapoptosis	120
miR372	LATS2	Tumor suppressor (germ cells)	135
miR373	LATS2	Tumor suppressor (germ cells)	135

(vulval development being a good model for let-60/RAS function) leads to abnormal vulval development and precocious seam cell terminal differentiation. In addition, miR84 overexpression suppresses the effects of activating mutations of let-60/RAS. Similar to the *C. elegans* let-60/RAS, the human RAS homologues, HRAS, KRAS, and NRAS, contain multiple putative let-7 family member binding sites in their 3′ UTRs. The introduction of a let-7a siRNA that mimics the let-7a microRNA suppresses RAS expression in a liver cancer cell line. Reciprocally, inhibiting let-7a in HeLa cells by transfection of complementary 2′-*O*-methyl antisense oligomers increases RAS expression (Fig. 14.3). Of note, several of the human let-7 family members, let-7a, let-7c, and let-7g, are encoded in chromosomal locations that are commonly deleted in lung cancer samples,[73] and let-7 expression is reduced in lung tumor samples relative to normal adjacent tissue. In fact, the extent of let-7 reduction is an important independent prognostic indicator; patients with the most drastic reductions in let-7 have the poorest prognosis after potentially curative tumor resection. Moreover, reduced let-7 expression is inversely correlated with the level of NRAS protein. These experiments suggest that let-7 family members are tumor suppressors.

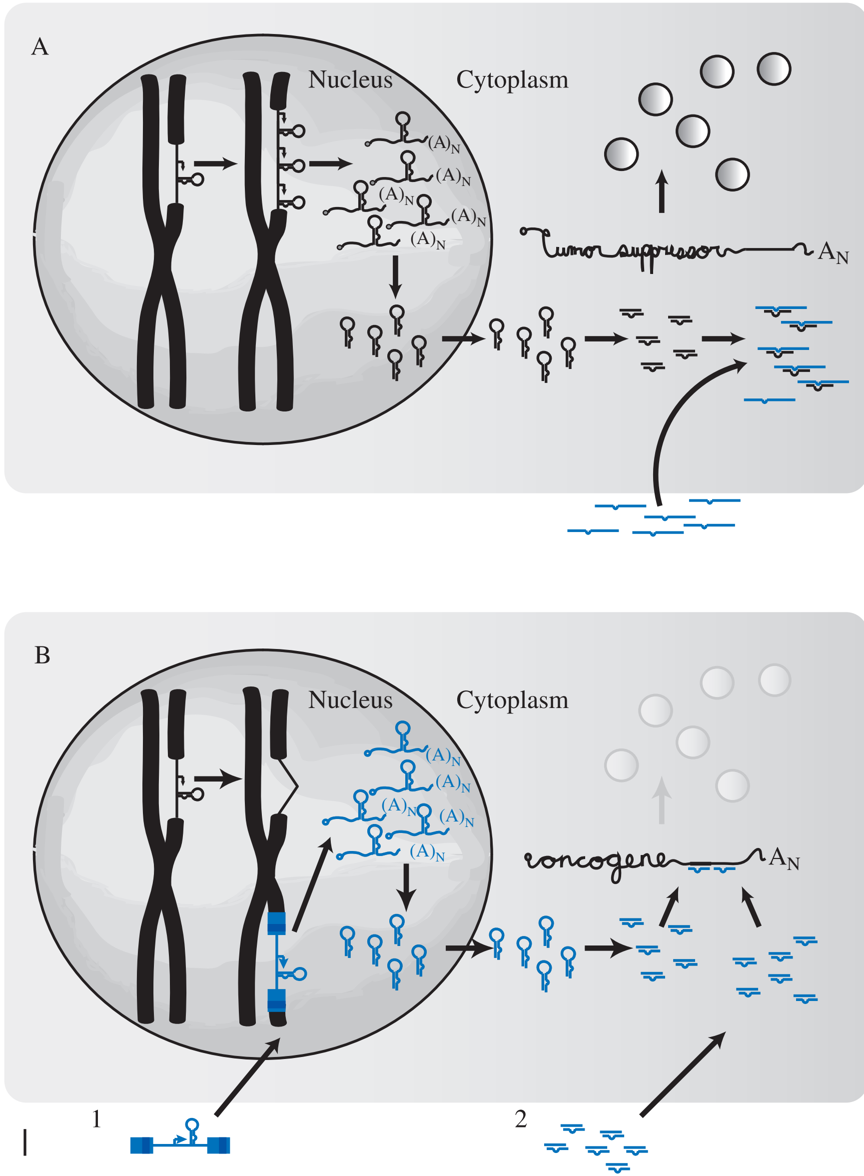


Fig. 14.3 Potential therapeutic approaches to inhibit tumorigenesis associated with altered microRNA expression patterns. (A) Tumor cells resulting from the overexpression of a micro-RNA that functions as an oncogene can be treated with cleavage-resistant single-stranded RNA molecules (e.g., chemically modifying the RNA by replacing the 2′-hydroxy groups on the sugar backbone with 2′-*O*-methyl groups)[178] that are complementary to the mature miRNA. These RNA molecules can effectively bind to the microRNA, preventing the association of the microRNA with its target gene(s) and thereby restore expression of the tumor suppressor gene and inhibit tumor growth. (B) Tumors that result from the loss of expression of a microRNA that acts as a tumor suppressor can be treated by reintroducing the microRNA into the cells. This can be

(continued)

In some instances, multiple microRNAs are encoded as polycistrons from a single common transcript. One of these microRNA clusters, the miR17–92 microRNA polycistron, maps to a region of chromosome 13 (13q31–q32) that is frequently amplified in B-cell lymphomas.[86] microRNA microarray analysis of several B-cell tumor lines that carry known amplifications of this region show increases in five of the six microRNAs in the miR17–92 cluster, compared to normal B cells and leukemia, and lymphoma cell lines lacking amplification of this region. In fact, expression of these microRNAs correlates with the copy number of the amplified region. Expression of the primary miR17–92 transcript is elevated in DLBCL and follicular lymphomas, suggesting that increased expression of this microRNA cluster might contribute to tumor formation. To test this hypothesis, He et al. overexpressed the first five of the microRNAs (miR17–19b), in the context of c-myc overexpression from the immunoglobin heavy chain enhancer (Eμ-myc), a well-established mouse model of B-cell lymphomas. While Eμ-myc transgenic mice typically develop B-cell lymphomas by 4–6 months of age, tumor formation was accelerated in Eμ-myc mice overexpressing miR17–19b, with a mean age of tumor formation of 51 days. Overexpression of each of the microRNAs in the miR17–19b cluster separately failed to enhance the rate of tumor formation. These tumors were particularly aggressive, invading visceral organs outside the lymphoid compartment, including liver, lung, and kidneys. One potential clue to the oncogenic nature of this microRNA cluster was reduced apoptosis in the miR17–19b/Eμ-myc tumors compared to control tumors.

The choice of the Eμ-myc transgenic mouse to test the oncogenic potential of miR17–92 may have been especially apt since O'Donnell et al. (2005) showed that the miR17–92 promoter contains c-Myc E-box binding sites and c-Myc activates the expression of this miR cluster.[94] This study also identified the transcription factor gene E2F1, which regulates progression through G1/S, as a target of two members of the miR17–92 polycistron. c-Myc is known to activate transcription of E2F1, and E2F1 activates c-Myc expression, suggesting a positive feedback loop to enhance cell proliferation. c-Myc induction of miR17–92 then serves to dampen this loop by suppressing c-Myc-induced E2F1 expression. This study illustrates the potential of microRNAs to fine-tune gene expression patterns for important genes that regulate cell cycle progression. It is likely that other genes involved in regulating cell proliferation will also be targeted by this microRNA cluster. These studies provide an example of how dysregulating microRNA expression might disrupt the

Fig. 14.3 (continued) achieved either by (1) introducing a DNA-based microRNA expression construct (e.g., using an oncoretroviral or lentiviral vector) that stably expresses the microRNA or (2) by directly introducing a chemically synthesized duplexed form of the microRNA that can enter the microRNA pathway and direct the silencing of the target oncogene. Alternatively, therapeutic benefit could be achieved by introducing siRNAs that silence expression of the dysregulated oncogene or any gene that will inhibit tumor growth (e.g., genes involved in cell cycle progression or angiogenesis) or make the tumor more sensitive to radiation or chemotherapy

fine balance that regulates cell growth, disrupted during oncogenic transformation. However, these regulatory networks may be more complicated than this story suggests; although the miR17–92 cluster is amplified in some B-cell lymphomas and clearly promotes tumor formation in the Eμ-myc mouse, the same region is associated with loss of heterozygosity in some hepatocellular carcinomas.

5 microRNAs and Differentiation in Mammalian Cells

Cancer cells are sometimes considered to be "frozen" in an undifferentiated or partially differentiated state. Until recently, differentiation research primarily focused on transcriptional regulation by regulatory DNA sequences (promoters, enhancers, and locus control regions) that are proximal to protein-coding sequences, paying little attention to the "noncoding" genomic DNA. The discovery of microRNAs has focused attention on mechanisms of posttranscriptional regulation of differentiation. As a general rule, total microRNA expression is higher in terminally differentiated cells than in less-differentiated cells and is higher in adult tissues than in embryos. Moreover, microRNAs have well-defined and distinct expression patterns in different tissues, particularly in cells of different developmental lineages. These findings suggest that microRNAs might play an important role in regulating terminal differentiation in different lineages. In fact, several recent studies provide compelling examples of regulatory networks (or the beginnings of networks) involving microRNAs, discussed below, that hint at an important role for microRNAs in controlling terminal differentiation, a step that is aberrant in cancer. These emerging regulatory circuits often involve intimate connections between transcription factors and microRNAs with, on the one hand, microRNA gene expression being regulated by transcription factors known to be important in lineage determination and, on the other, microRNAs suppressing the expression of key transcription factors.

The first evidence that microRNAs play a role in the differentiation of mammalian cells came from the conditional deletion of Dicer1. (Loss of Dicer1 is lethal early in development.[95]) Conditional deletion of Dicer1 in embryonic stem cells,[39] T cells,[96] limb mesoderm,[97] and skin[98] showed gross defects in differentiation in all these lineages. The logical inference is that impaired production of microRNAs in the absence of Dicer1 interferes with cellular differentiation.

microRNAs in hematopoiesis Much of the initial work in studying the role of microRNAs in mammalian cell differentiation has been elucidated in hematopoiesis, probably the best-studied system of mammalian cellular differentiation. The first example implicated miR181, whose expression is increased in thymus, lymphoid tissues, and bone marrow, in promoting B-cell differentiation. Ectopic expression of miR181 in mouse hematopoietic precursor cells leads to a dramatic increase in B lineage cells.[99] Another microRNA, miR223, expressed in the bone marrow, is important in granulopoiesis.[100] An elegant network involving miR223 and two competing transcription factors, C/EBPα and NFI-A, appears to control

the differentiation of promyelocytes into granulocytes. During in vitro and in vivo retinoic acid-induced differentiation of leukemic promyleocytes, miR223 expression is upregulated. The miR223 promoter contains overlapping sites for C/EBPα and NFI-A binding. C/EBPα upregulates and NFI-A inhibits miR223 expression. Upon retinoic acid treatment, NFI-A expression declines, while C/EBPα is upregulated. C/EBPα then binds and displaces NFI-A from the miR223 promoter to enhance miR223 expression. This molecular circuit is complete when miR223 binds the 3′ UTR of NFI-A transcripts, blocking further expression of NFI-A. The importance of miR223 in regulating granulocyte differentiation was shown by inducing differentiation of promyelocytes by ectopic expression of miR223 without retinoic acid and by blocking retinoic acid-induced differentiation by inhibiting miR223.

Two clustered microRNAs, miR221 and miR222, abundantly expressed in CD34 + hematopoietic precursor cells, are downregulated upon in vitro differentiation into the erythroid lineage.[101] One likely target of these microRNAs is the kit receptor, required for proliferation and erythroid differentiation in response to kit ligand. Overexpressing either of these microRNAs reduces kit expression, cell proliferation under erythroid-promoting conditions, and engraftment of CD34+ cord blood cells into immunodeficient mice. Because constitutively activated c-kit has been implicated in leukemias and gastrointestinal stromal tumors, inducing expression or transducing cells with these microRNAs (or their siRNA analogues) might have therapeutic benefit.

Another study that looked at in vitro differentiation of CD34+ progenitor cells into megakaryocytes found a group of downregulated microRNAs, one of which might be involved in targeting the transcription factor *MAFB*, upregulated during megakaryopoiesis and involved in activating transcription of the megakaryocyte-specific gene GPIIB.[102] Another downregulated microRNA miR10a is embedded in the HOX gene cluster and potentially targets HoxA1.

HOX gene microRNAs miR10 and miR196 microRNA families are embedded within the four HOX clusters of mammalian homeobox transcription factor genes,[103] which play an important conserved role in determining the identity of cells in the developing embryo. The intricate expression pattern of HOX genes persists in adult tissues, but their roles are modified according to specific cellular needs (reviewed in[104]). The embryonic expression of the HOX-embedded microRNAs closely follows that of their “host” HOX cluster genes.[105] Moreover, the HOX-embedded microRNAs have been shown in a few examples to regulate the expression of HOX genes. miR196a binds to the HOXB8 3′ UTR and inhibits HOXB8 expression by cleaving the HOXB8 transcript.[106] HOXB8 and miR196a also have complementary expression patterns during embryogenesis, supporting the idea that miR196a regulates the expression of HOXB8 during development.[103] miR196a is overexpressed in embryonic mouse hindlimbs compared to forelimbs, while the expression pattern of HOXB8 is the opposite. At least in chickens, miR196a appears to impede the retinoic acid-induced expression of HOXB8 and sonic hedgehog (Shh) in forelimb development to establish anterior–posterior patterning.[107] However, loss of microRNAs in Dicer-deficient hindlimbs does not induce

HOXB8 expression. This result suggests that the primary regulation of HOX gene expression might not be via microRNAs or that multiple microRNAs might be involved in more complicated regulatory networks.[107]

Another illustration of HOX gene regulation by microRNAs is the regulation of HOXA11 in differentiating myoblasts by miR181.[108] miR181 is upregulated in differentiating muscle cells during development or during regeneration in response to injury, but is not expressed in undifferentiated myoblasts or fully differentiated muscle cells. Its target HoxA11, which inhibits myoblast differentiation,[109,110] is reciprocally expressed in myoblasts and (at low levels) in adult muscle cells, but turned off during differentiation. Inhibiting miR181 interferes with myoblast differentiation, but does not completely restore HoxA11 expression, suggesting that multiple microRNAs or other pathways contribute to this process.[108] Moreover, the ectopic expression of miR181 does not induce myoblast differentiation, again suggesting a more complex regulatory network. Nonetheless, the involvement of miR181 in both B-cell and myoblast differentiation suggests that miR181 might be involved in regulating common pathways activated during the terminal differentiation of cells of mesodermal origin.

microRNAs and muscle development The miR1 family and miR133 genes are specifically and highly expressed in adult skeletal and cardiac muscle tissues and to a lesser extent during development of these tissues. Expression of miR1 genes is activated in the heart by the serum response factor (SRF) transcription factor and its cofactor myocardin and in skeletal muscle by the Mef2 and MyoD transcription factors. One of the targets of miR1 is the Hand2 transcription factor that promotes proliferation of cardiac muscle precursor cells. Cardiac embryonic development is activated when Hand2 begins to be expressed. Although Hand2 mRNA persists in adult cardiac tissue, Hand2 protein is downregulated coincident with miR1 expression. Precocious expression of miR1 in the developing heart leads to severe defects in heart formation because of decreased cell division.[111] Therefore, miR1 controls terminal differentiation of myocardiocytes.

Another microRNA (miR133) is clustered with miR1-1, and they are transcribed as a single transcript beginning late in embryonic development.[112] However, miR1 and miR133 have opposing effects on myoblast fate – as it does for the heart, miR1 promotes skeletal myoblast differentiation, whereas miR133 promotes myoblast proliferation and inhibits differentiation.[112] One of the targets of miR1 in skeletal muscle is HDAC4, which globally represses transcription, including transcription of the muscle-specific transcription factor MEF2C.[113] One way that miR133 inhibits differentiation is by suppressing expression of SRF, which activates myoblast differentiation.[114] Recall that SRF activates expression of miR1 (and thus miR133). This negative feedback loop indicates a complicated microRNA-transcription factor-regulated mechanism for controlling muscle cell differentiation. Likely, there will be more to this story.

microRNAs also regulate the function of terminally differentiated cells microRNAs are especially abundant in terminally differentiated cells compared to their precursors, suggesting that they may not only suppress the genes required for proliferation and progenitor cell pluripotency, but may also regulate their effector functions.

Regulating the function of terminally differentiated cells might not be directly related to cellular transformation and cancer. However, a few instructive examples of tissue-specific microRNAs and their role in differentiated cell function will be briefly described. miR375 appears to be exclusively expressed in pancreatic β cells and regulates insulin response to glucose.[66] Increasing miR375 in β cells suppresses glucose-induced insulin secretion, while inhibiting miR375 has the opposite effect. This effect is mediated by the effect of miR375 on Myotrophin (Mtpn), a protein previously not know to play a role in insulin secretion. Silencing Mtpn by siRNA reproduces the miR375-suppressive effect on insulin secretion.

Another interesting example involves the role of miR134, highly expressed in brain, in regulating dendritic spine development of neurons in response to synaptic stimulation.[115] miR134 inhibits translation of Lim-domain-containing protein kinase 1 (Limk1) which regulates dendritic spine formation.[116] miR134 is localized near synapses where Limk1 synthesis takes place. The authors speculate that miR134 might bind to Limk1 mRNA as it is being transported from the cell body to the dendrites and be responsible for suppressing Limk1 translation during transport and before synaptic stimulation. In response to activating stimuli, such as brain-derived neurotrophic factor (BDNF), the inhibitory effect of miR134 on Limk1 translation is reversed.[115] A surprising observation is that even after BDNF stimulation when Limk1 mRNA is being translated, miR134 continues to associate with the Limk1 transcript. How BDNF stimulation might interfere with miR134-mediated silencing of Limk1 translation or bypass it remains a puzzle.[117]

6 microRNAs and Apoptosis

Deregulation of cell death is an important feature of many cancers (reviewed in[118]). Highly conserved caspase-dependent pathways are often inactivated in transformed cells, principally by overexpression of inhibitors of apoptosis, including antiapoptotic bcl-2 family members, survivin, and other IAP family members. The first example of a role for microRNAs inhibiting apoptosis was in *Drosophila*, where expression of the proapoptotic factor hid is repressed by the microRNA bantam.[67] Bantam not only blocks apoptosis, but also directly increases cell proliferation.[67] In flies, miR14, the miR2 gene family and miR278 also act as potent cell death suppressors.[68,119,120]

In mammals the first evidence for a role of microRNAs in regulating apoptosis comes from conditional deletion of Dicer, which in embryonic limbs causes extensive apoptosis.[97] Deletion of Dicer in the T-cell lineage reduces the numbers of mature T cells, which both proliferate more slowly and are more prone to apoptosis in response to stimulation.[96] Expression of antiapoptotic Bcl-2 in B-cell lymphomas is a likely target of miR15a and 16-1, which are deleted in many high-grade B-cell malignancies.[121] Not only is bcl-2 expression tightly correlated with expression of these microRNAs, but transfection of bcl-2+ leukemia cells with an expression plasmid for either or for both of these microRNAs leads to downregulation of bcl-2

and induction of apoptosis. Glioblastoma cells and some other tumors strongly overexpress miR21.[122] Depletion of miR21 in cultured glioblastoma cells activates caspases and leads to increased apoptosis through an unknown mechanism. Interestingly, another study using antisense microRNA inhibitors in cervical adenocarcinoma HeLa cells identified miR21 as an inhibitor of cell growth with no direct effect on apoptosis.[123] The biological effects of any particular microRNA, including miR21, in different cells are likely to vary depending on the cell-specific repertoire of expressed target genes. Although these studies support a role for microRNA regulation of apoptosis, understanding the target genes and pathways in mammalian cells awaits further research.

7 RNA Interference-Based Screens to Identify Novel Tumor Suppressor Genes and Oncogenes

RNAi has provided new opportunities to identify novel genes implicated in a variety of diseases by forward genetic screens. Before the discovery that RNAi worked in mammalian cells, the power of unbiased screens to identify unexpected participants in biologically important pathways was only available in invertebrates. Libraries of retroviruses encoding short hairpin RNAs (shRNAs) or arrays of siRNAs mixed with a transfection reagent, designed to silence a large proportion of human-expressed genes or functionally related subsets of genes (i.e., all kinases and phosphatases, all known ubiquitin ligases), can be used to identify genes involved in cellular transformation, susceptibility to apoptosis, or drug resistance. Similarly, libraries of retroviruses encoding microRNAs can be used to identify microRNAs involved in cancer. Identifying a gene candidate in any screen is only the first step to validating its role in a biological pathway or disease. Some illustrative examples of RNAi-based cancer screens are given below.

Cancer cells are especially sensitive to apoptosis induced by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). To identify genes that might enhance or suppress TRAIL-mediated apoptosis, TRAIL was added to HeLa cells transfected in microtiter plates with a panel of siRNAs targeting 510 genes, including 380 kinases.[124] This screen was able to identify several unknown genes whose silencing either sensitized or desensitized cells to TRAIL-induced apoptosis and to identify several signaling pathways (WNT, MYC) required for maintaining TRAIL sensitivity, since silencing multiple genes in these pathways differentially affected cell survival in response to TRAIL. Another siRNA-based loss-of-function screen surveyed hundreds of kinase and phosphatase genes to identify those that enhance or suppress apoptosis of HeLa cells either on their own or in conjunction with chemotherapeutic drugs.[125] A large proportion of these enzymes (i.e., more than one third of the phosphatases) affected cell survival by at least twofold. The large number of “hits” suggests that more refined screens or biological verification would be needed to winnow through these leads to identify attractive targets for drug development. For example, identifying kinases or phosphatases that are preferentially

needed for survival of different types of cancer cells vs normal cells or are only required for survival in the face of radiation or chemotherapy would be a first step.

Another screening approach uses libraries of DNA or viral vectors to express shRNAs, processed intracellularly into active siRNAs. Most libraries have used retroviral vectors because of the transduction efficiency and stability of gene expression afforded by these vectors. Brummelkamp et al.,[126] examining what effect silencing the expression of 50 deubiquitylating enzymes had on TNFα activation of NF-κB, singled out the cylindromatosis (CYLD) tumor suppressor gene, which is linked to a familial proliferative skin disease. With this lead they were then able to pinpoint a role for CYLD in deubiquitinating TRAF2, which activates IKK and consequently NF-κB. By interfering with NF-κB activation using sodium salicylate, they could enhance apoptosis of CYLD-silenced cells. This result was rapidly translated to show that topical aspirin derivatives could be used to treat this rare disfiguring disorder.

Although this study screened a small set of genes, large-scale plasmid and retroviral shRNA expression libraries targeting a large proportion of the human and mouse genome have been constructed and validated by several groups.[127,128–133] Some of these vectors express the shRNA within a microRNA sequence to enhance its processing and increase the efficiency of silencing.[129] In screens for tumor suppressor genes, cells at the brink of transformation because of expression of combinations of oncogenes are transduced to express shRNAs and then selected for outgrowth of transformed cells. One retroviral-based RNAi screen took advantage of a conditionally transformed cell line that expresses the catalytic subunit of telomerase (hTERT) and a temperature-sensitive allele of SV40 large T antigen (tsLT), which allows cells to proliferate at 32°C (the temperature at which tsLT is functional and can inactivate pRb and p53), but not at 39°C at which growth arrest occurs, to identify novel factors that modulate p53-dependent proliferation arrest.[133] After infection with the library, positive colonies, containing cells able to proliferate at 39°C, were selected and sequenced to identify the gene being silenced. shRNAs targeting six genes were pulled out of the screen, including the p53 gene, as well as five novel genes – RPS6KA6 (ribosomal S6 kinase 4, RSK4), Tip60 (histone acetyltransferase), HDAC4 (histone deacetylase), KIAA0828 (putatitive *S*-adenosyl-L-homocysteine hydrolase, SAH3), and CCT2 (T-complex protein 1, β-subunit). These novel genes were validated by showing that shRNAs targeting each of the genes selected in the screen were able to inhibit growth arrest induced by ionizing irradiation or p19^{ARF} overexpression. In a similar manner, Westbrook et al.[134] used another shRNA expression library to look for potential tumor suppressor genes that inhibit transformation of human mammary epithelial cells (HMECs) expressing hTERT and SV40 large T antigen. Colonies of cells that demonstrated anchorage-independent growth after infection with the shRNA library were isolated. The silenced genes were identified by DNA sequencing and bar code (a sequence identifier specific for each shRNA construct) microarray analysis. This approach identified several previously known tumor suppressor genes, including TGFBR2 and PTEN, as well as a gene that had not been previously shown to have tumor suppressing properties, REST/NRSF (RE1-silencing transcription

factor/neuron-restrictive silencing factor). The role of REST as a tumor suppressor was confirmed by expressing a dominant negative REST gene. The tumor suppressor activity of REST was then found to be mediated by its ability to suppress PI(3)K-dependent signaling. REST is often deleted in colorectal cancer cell lines.

In a similar assay system, a retroviral shRNA-expressing library was used to screen for potential tumor suppressor genes whose silencing could substitute for overexpression of a constitutively active form of RAS (RAS^{V12}) and permit anchorage-independent growth in fibroblasts that overexpressed the catalytic subunit of telomerase (hTERT), SV40 small t antigen, and had silenced p53 and $p16^{INK1A}$[130]. The homeodomain pituitary transcription factor PITX1 was identified and confirmed as a tumor suppressor gene by showing that inhibiting PITX1 expression activates the RAS pathway by activating the promoter of RASAL1, a RAS-GTPase activating protein that connects Ca^{2+} signaling to RAS activity.

These previously described screens could identify tumor suppressor genes whose silencing promotes cellular proliferation or anchorage-independent growth, but they could not be used to identify potential oncogenes, whose silencing would cause growth arrest or cell death. To identify putative oncogenes, Staudt and colleagues[135] used an inducible shRNA retroviral library to identify by microarray analysis shRNAs that were depleted in abundance when transduced and induced DLBCL lines were cultured for 3 weeks. All of the depleted shRNAs silenced NF-κB pathway components, including IKBKB, CARD11, MALT1, and BCL10. Interestingly, these genes were required for the proliferation of only activated-type DLBCL and not germinal center-type DLBCL, suggesting that they might be good selective drug or siRNA targets.

Another type of screen was used to identify microRNAs that act as oncogenes. Using a retroviral library to express many of the known human microRNAs, Voorhoeve and colleagues[136] identified two microRNAs, miR372, and 373, which share the same seed sequence, that cooperate with a constitutively active form of RAS (RAS^{V12}) to transform primary human fibroblasts that express wild-type p53. miR372 and 373 expression was elevated in testicular germ cell tumors, which mostly contain functional p53, but not in normal testes or in samples from breast, colon, lung, and brain tumors. Expression of a putative tumor suppressor gene, large tumor suppressor homolog 2 (LATS2), predicted to contain two potential miR372/373-binding sites, is decreased in cells overexpressing these microRNAs, and its silencing may be contributing to the oncogenic effect of these microRNAs.

8 Harnessing RNA Interference for Cancer Target Validation and Therapeutics

Although we are just beginning to understand the role of microRNAs in cancer, many investigators are already exploring the possibility of exploiting the power of RNAi for cancer therapy and drug target validation in animal models. (A discussion

of this extensive body of work is beyond the scope of this review [see, e.g.,[137,138]]. RNAi has become a standard tool to identify the importance of any particular gene in diverse biological pathways, including those implicated in cellular transformation. siRNAs can be highly (but not completely) specific and can distinguish a single nucleotide polymorphism, as was first demonstrated by targeting the point mutation that constitutively activates a RAS oncogene, leaving wild-type RAS unaffected. RNAi can be used effectively to silence the expression of any gene in any cell in vitro.[139] Transgenic mice expressing shRNAs can also be used to identify the importance of particular genes or microRNAs in cancer formation in vivo.[140]

Cleavage of the target mRNA is likely the most potent RNAi mechanism to harness for therapy, because the same RISC-incorporated small RNA can direct the cleavage of many transcripts and because the transcript is eliminated, not merely repressed. (The relative effectiveness of mechanisms in which chromatin is silenced to inhibit transcription is unknown. This mechanism is too poorly understood to use as the basis for therapy at present.) The RISC-stabilized small RNA is highly stable within the cell – probably with a half-life of 1 week or more. The major determinant of durability of silencing is the rate of cell proliferation, where small RNAs are diluted with each cell division.[141] In terminally differentiated nondividing cells silencing can last for weeks, while in rapidly dividing cell lines silencing peaks 3 days after transduction and is gone by 1 week. For cancer cells, frequent and repetitive dosing will likely be required for siRNA-based drugs. However, less rapidly dividing precancerous lesions or potentially cancer stem cells might be particularly effective targets requiring infrequent treatments. In addition to silencing transcripts for oncogenes, RNAi could be used either to mimic microRNAs identified in promoting differentiation, inducing apoptosis or reducing proliferation or to inhibit cancer-promoting microRNAs. If the studies reviewed here that suggest that microRNAs may be master regulatory switches for terminal differentiation hold up, then transducing cells with siRNAs that mimic such microRNAs may be a highly attractive strategy for cancer therapy.

RNAi-based therapy for cancer could be used to target more than oncogenes. Genes implicated in cell cycle progression[142–146] and angiogenesis[147–150] would be good targets. Particularly, if siRNAs can be targeted preferentially to tumor cells, then any gene required for viability is a potential target, although genes needed only for cell division are particularly attractive since they will cause less toxicity to the majority of nondividing cells. Growth factors or their receptors required for tumor growth are also possible targets.[151–154] Targeting viral oncogenes encoded by EBV,[155–157] HPV,[158,159] and other oncogenic viruses also provides an opportunity for specificity. RNAi-based therapy could also be used in conjunction with chemotherapy or radiation to make cells more susceptible to these agents, particularly by silencing genes involved in drug resistance (i.e., transporters that efflux drugs), DNA repair, or metabolic pathways targeted by these drugs.[160–166] It may also be possible to target cells involved in either supporting the growth of the tumor or eliminating it rather than the tumor itself. For example, tumor infiltrating lymphocytes are

largely incapacitated in their ability to destroy tumor cells; by targeting inhibitory receptors or regulatory cells, these tumor-specific immune cells might be activated to eliminate residual tumor cells.[138]

Two strategies can be used to harness RNAi for therapy – one is gene therapy (transducing cells with viral vectors that encode for shRNA precursors processed intracellularly like endogenous microRNAs); the other is to develop siRNAs as small molecule drugs.[167,168] The latter is more suitable for cancer therapy and closer to clinical application. siRNAs can be chemically modified to enhance their pharmacokinetics and reduce potential off-target effects caused by binding to Toll-like receptors, immune sensors of pathogenic double-stranded RNAs. The main obstacle to using siRNAs is delivering them into the cytoplasm of cells, where they work. Cancer cells can be transfected in vitro, but except for superficial sites, this is not a viable strategy for treating most cancers, particularly micrometastases and macrometastases. Although most cells do not readily internalize siRNAs, mucosal surfaces appear to be especially susceptible to topically applied siRNAs.[168] Initial siRNA phase I and II studies targeting the eye and lung (to treat age-related macular degeneration and respiratory syncytial virus infection, respectively) have not met with any unexpected toxicity. Therefore, malignancies that are located at these sites or spread locally are good initial targets. Attractive examples for initial studies might include HPV-related cervical cancer (targeting the E6 and E7 oncogenes), EBV-related nasopharyngeal cancer, lung squamous cell carcinomas, retinoblastoma, or head and neck cancer.

However, for most cancers a method for effective systemic administration is needed and is the major obstacle to using siRNAs for cancer therapy. Recently, several systemic siRNA delivery strategies have begun to be described in animal models. These involve covalently coupling the passenger strand of the siRNA to a targeting molecule (e.g., cholesterol),[169] incorporating the siRNA into liposomes,[170,171] lipoplexes,[172–174] or nanoparticles,[149,175] or mixing the siRNA with fusion proteins, capable of specific targeting by binding to cell surface receptors.[176] This latter approach was used to target and inhibit the outgrowth of a subcutaneous mouse melanoma cell line by intravenous injection of 1 mg/kg of a cocktail of siRNAs. siRNA delivery was highly specific since adjacent normal tissues did not take up the siRNAs.

As for other cancer therapies, drug resistance caused by mutating the target site sequence is an anticipated problem. This may be more of an obstacle for siRNAs than for other types of drugs, since conservative mutations that do not alter the encoded protein may interfere with gene silencing. However, dealing with drug resistance to siRNAs is a much simpler problem than for other small-molecule drugs, which usually work by targeting a single active site on a protein. Since multiple sequences can be used to target any gene, alternate siRNAs can readily be designed. Combinations of siRNAs that target more than one sequence in a gene or multiple genes at once are likely to work synergistically to enhance tumor suppression and reduce the likelihood of emerging drug resistance.[141]

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Chapter 15
Cancer Stem Cells and Impaired Apoptosis

Zainab Jagani* **and Roya Khosravi-Far**

Abstract For more than 100 years scientists have fervently sought the fundamental origins of tumorigenesis, with the ultimate hope of discovering a cure. Indeed, these efforts have led to a significant understanding that multiple genetic and molecular aberrations, such as increased proliferation and the inhibition of apoptosis, contribute to the canonical characteristics of cancer. Despite these advances in our knowledge, a more thorough understanding, such as the precise cells, which are the targets of neoplastic transformation, especially in solid tumors, is currently lacking. An emerging hypothesis in the field is that cancer arises and is sustained from a rare subpopulation of tumor cells with characteristics that are highly similar to stem cells, such as the ability to self-renew and differentiate. In addition, more recent studies indicate that stem cell self-renewal pathways that are active primarily during embryonic development and adult tissue repair may be aberrantly activated in various cancers. This chapter introduces the cancer stem cell hypothesis; explores evidence for the presence of cancer stem cells, particularly in leukemia; and discusses various classical stem cell self-renewal pathways in relation to cancer. Investigating the role of cancer stem cells in the context of the major characteristics of cancer, especially impaired apoptosis, offers great promise for the design of superior tumor-selective and apoptosis-inducing therapies.

Keywords cancer stem cell, therapy, leukemia, Notch, Hedgehog, Wnt, Bmi1

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1 Introduction

The inability of conventional chemotherapeutic drugs and even various targeted therapies to produce complete remissions demands a more in-depth understanding of the key cellular events underlying tumor formation, maintenance, and progression, and the molecular pathways that dictate such processes. It has become increasingly apparent that the tumor, rather than consisting of a uniform population of rapidly proliferating cells, is actually composed of a heterogeneous population of cells with variable cellular and molecular characteristics (Foulds, 1965; Heppner, 1984). Therefore, one possible explanation for the failure of chemotherapy is that it cannot eliminate this entire mixed composition of tumor cells, thus necessitating multiple treatment approaches. Along these lines, it has been proposed that a rare group of cells with stem cell-like properties lies within the tumor and gives rise to the heterogeneous tumor cell population (Reya et al., 2001). The existence of these cells indicates that while our current anticancer therapeutics may be successful in debulking a tumor, they remain ineffective in targeting the minute, yet crucial, population of tumor cells that ultimately sustains the tumor. While the "cancer stem cell hypothesis" is supported by seminal findings from hematopoietic cancers, especially acute myeloid leukemia (AML) (Warner et al., 2004), its importance and application in other types of cancers are not clearly understood.

1.1 The Cancer Stem Cell Hypothesis

One intriguing and emerging area of cancer research concerns the striking parallels between cancer cells and stem cells. Both of these cell types have the capacity to self-renew and differentiate. Unlike the highly regulated self-renewal and differentiation decisions of normal stem cells, however, it has been proposed that cancer cells undergo uncontrolled self-renewal and abnormal differentiation. Coincidently, the pathways that regulate stem cell self-renewal and differentiation, such as Notch, Hedgehog (Hh), Wnt, and Bmi1 are dysregulated in various cancers (Reya et al., 2001). In addition, key findings revealing the presence of leukemic stem cells and providing evidence for a stem cell origin for AML are in support of the hypothesis that cancers arise from a small population of tumor-initiating cells known as cancer "stem cells" (Bonnet and Dick, 1997; Buick and Pollak, 1984; Jordan and Guzman, 2004; Lapidot et al., 1994; Mackillop et al., 1983; Reya et al., 2001). These cancer stem cells give rise to the clinically observed, phenotypically diverse tumor population consisting of cells displaying varied capacities for abnormal differentiation, uncontrolled proliferation, and a reduced rate of apoptosis. While the precise identity of a cancer stem cell is difficult to pinpoint, it is possible that cancer stem cells can arise either from the malignant transformation of a stem cell, or the abnormal re-activation of self-renewal pathways in a more committed progenitor cell (Al-Hajj et al., 2004; Burkert et al., 2006; Reya et al., 2001).

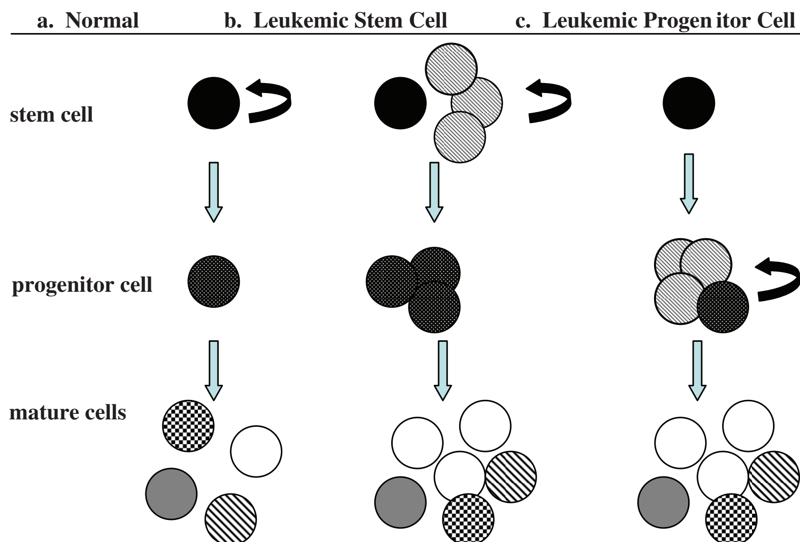

Fig. 15.1 Cancer stem cells and leukemia. (a) A simplified demonstration of normal hematopoietic development in which the self-renewing stem cell is highly regulated leading to normal progenitor and mature cell production. In leukemia however, and according to the cancer stem cell hypothesis; (b) transformation of a stem cell can lead to uncontrolled self-renewal resulting in an abnormal growth and differentiation program; (c) alternatively, transformation of a progenitor cell can abnormally reactivate self-renewal resulting in the abnormal growth and differentiation of hematopoietic cells

1.1.1 Cancer Stem Cells in Leukemia and Other cancers

Since the cellular and developmental biology of the hematopoietic system is well understood, the cancer stem cell hypothesis has been most thoroughly tested in the context of hematopoietic malignancies (Fig. 15.1), such as AML (Dick, 2005). AML is characterized by the uncontrolled growth and accumulation of abnormally differentiated blood cells, or leukemic blasts, which rapidly overwhelm normal blood cell function. Initial studies using various in vitro systems, such as the clonogenic; suspension culture-initiating cells (SC-IC); and long-term culture-initiating cells (LTC-IC) quantitative stem cell assays revealed that only a minor fraction of AML cells are capable of supporting growth in vitro (Warner et al., 2004). These studies were followed by key experiments performed in vivo using the NOD/SCID-leukemia xenotransplantation model. In this model, transplantation of leukemic cells from AML patients into mice can produce leukemic disease resembling human AML (Bonnet and Dick, 1997). It was demonstrated that only a minor percentage (0.1–1%) of AML cells with primitive CD34 + CD38– surface expression was capable of initiating AML in the NOD/SCID mice, thereby providing the first evidence for the presence of cancer stem cells (Bonnet and Dick, 1997; Lapidot et al., 1994). The discovery of leukemic stem cells thus set the groundwork for an

investigation of the existence of cancer stem cells in other types of cancers. While the origin of cancer stem cells has not been conclusively defined, recent studies have also identified a subpopulation of tumor-initiating cells in solid tumors, such as breast (Al-Hajj et al., 2003), melanoma (Grichnik et al., 2006), brain (Singh et al., 2003), prostate (Xin et al., 2005), and ovarian (Bapat et al., 2005) cancers. Together, these studies raise important questions regarding the target cells of our current anticancer therapeutics, and the study of cancer signal transduction pathways in the appropriate cellular context.

1.1.2 Targeting Cancer Stem Cells

In the case of the CML-causing oncogene *BCR-ABL*, accumulating evidence suggests that the target cell for transformation is a hematopoietic stem cell (HSC) rather than a committed progenitor cell (Elrick et al., 2005; Huntly and Gilliland, 2005; Huntly et al., 2004). Unfortunately, research has shown that while the Abl kinase inhibitor, Gleevec, can eradicate the majority of proliferating CML progenitors and differentiated granulocytes, it is unable to target the minute population of CML progenitor stem cells that can sustain the disease (Bhatia et al., 2003; Elrick et al., 2005; Graham et al., 2002). In accordance with the cancer stem cell hypothesis, Gleevec treatment can be used continuously to manage chronic phase CML, but not to eliminate leukemic disease, since the remaining cancer stem cells are still able to sustain the disease. Further research must specifically target this cancer stem cell population.

It remains important to determine whether abnormal survival and antiapoptotic signaling, as has been intensively investigated in primary tumor cells, tumor cell lines, and mouse tumor models, actually plays a significant role in the transformation and maintenance of the tumor-initiating cell, or, more specifically, the cancer stem cell population. One goal of such studies is to determine how to selectively induce apoptosis in leukemic stem cells, but not in normal HSCs. Recent studies have shown that the prosurvival pathways, such as NF-κB and PI3-K, are highly activated in the leukemic stem cell population in AML (Guzman et al., 2001; Xu et al., 2003; Zhao et al., 2004). Interestingly, AML leukemic stem cells preferentially undergo apoptosis, unlike normal HSCs, upon combined treatment with the chemotherapeutic agent idarubicin and the proteasome inhibitor MG-132 (Guzman et al., 2002). Such treatments lead to the inhibition of NF-κB activity, along with other currently unidentified mechanisms, and also activate p53, causing the expression of target genes, such as *GADD45, p21*, and the proapoptotic gene *Bax* (Guzman et al., 2002).

1.2 The Role of Stem Cell Regulation Pathways in Tumorigenesis

As early as 1855, the scientist Rudolph Virchow recognized elements of dysregulated embryonic development in tumors, proposing his embryonal-rest hypothesis. In accordance with these earlier findings, there is now evidence for a molecular link between the pathways that regulate stem cell self-renewal during

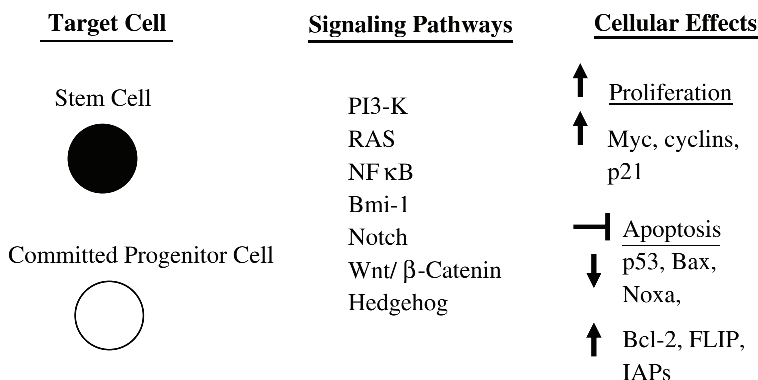

Fig. 15.2 Cancer stem cells and signaling pathways. A summary of the signaling pathways implicated in the survival of cancer stem cells. In general, these signaling pathways could either be aberrantly activated in a stem cell or a committed progenitor cell. Whereas, the outcomes of activating such pathways are numerous, key cellular effects include increase in cellular proliferation, and the inhibition of apoptosis. This figure outlines only a few of the various downstream genes that play important roles in proliferation and apoptosis

development and tumorigenesis (Fig. 15.2) (Burkert et al., 2006; Reya et al., 2001). The major developmental pathways such as Notch, Hh, and Wnt, which intricately control the self-renewal of stem cells during both embryonic development and adult tissue repair and homeostasis, are found to be upregulated in various cancers. These observations have brought forth important questions as to whether these pathways critically contribute to tumor formation and maintenance and whether their inhibition can be utilized in future anticancer therapeutic strategies. Selective inhibition of these developmental pathways in tumor cells may also have the potential to eliminate the elusive population of tumor-initiating cells that share common characteristics with stem cells. Furthermore, determining the direct impact of inappropriate activation of self-renewal pathways on apoptosis in a tumor cell will lead to a better understanding of how to combine therapies that attack upstream self-renewal pathways, with those that unleash downstream apoptotic cascades.

1.2.1 Bmi-1

The *Bmi-1* proto-oncogene was first identified as a target of the Moloney murine leukemia viral insertion in the Eμ-myc lymphoma mouse model (Haupt et al., 1991; van Lohuizen et al., 1991), with further studies suggesting a cooperative role with c-myc in inducing murine lymphogenesis (Haupt et al., 1993). Bmi-1 is a Polycomb-group gene which functions as a transcriptional repressor and plays a role in regulating cellular proliferation and senescence through repression of the INK4A locus (Jacobs et al., 1999). Recently, the *Bmi-1* gene has been shown to play a critical role in the generation of self-renewing adult HSCs, as mice deficient in Bmi-1 show reduced numbers of HSCs (Park et al., 2003). In addition, the *Bmi-1*

gene has not only been implicated in regulating the proliferative activity of normal hematopoietic cells, but also of leukemic stem and progenitor cells, in which lack of Bmi-1 leads to proliferation arrest and characteristics of differentiation and apoptosis (Lessard and Sauvageau, 2003).

1.2.2 Notch Signaling

Notch signaling functions in a diverse set of cellular processes during embryonic and postnatal development, including the maintenance of stem cells, cell fate specification, differentiation, and proliferation (Artavanis-Tsakonas et al., 1999; Kadesch, 2004). Interestingly, research points to a role for constitutively active Notch signaling under certain cellular contexts, such as in tumorigenesis (Callahan and Egan, 2004; Hansson et al., 2004; Radtke and Raj, 2003), yet the precise mechanisms underlying this effect remain to be determined. In mammalian systems, the Notch signaling pathway consists of four receptors (NOTCH1–4) and five ligands, Delta-like 1, 3, 4 (DLL1, DLL3, and DLL4), Jagged 1 and Jagged 2 (JAG1, JAG2) (reviewed in Artavanis-Tsakonas et al., 1999; Hansson et al., 2004; Kadesch, 2004). Notch receptors are synthesized as precursors, with Notch receptor activation occurring in a series of proteolytic cleavages upon interaction with its ligand. While the first cleavage is facilitated by TACE (tumor-necrosis factor α-converting enzyme/metalloproteinase) (Brou et al., 2000), the second is mediated by the γ-secretase activity of presenilins, and results in the release of the intracellular cytoplasmic portion of Notch, which then translocates to the nucleus (De Strooper et al., 1999; Mumm et al., 2000; Saxena et al., 2001). The known targets of Notch activation are the HES (hairy/enhancer of split) and HERP (Hes-related repressor protein) families of transcription factors, which regulate the transcription of various genes through development (Bailey and Posakony, 1995; Davis and Turner, 2001). The set of target genes activated by Notch signaling has not been completely defined, and may vary with cellular context. In transformed cells, transcription of the *erbB2* (Chen et al., 1997) and *cyclin D1* (Ronchini and Capobianco, 2001) genes have been reported to be upregulated in response to activated Notch.

The earliest evidence for the involvement of activated Notch in human cancers arose from the identification of a translocation involving the *Notch1* gene in cases of T-cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). In particular, the t(7;9) chromosomal translocation fuses a truncated Notch consisting mainly of the intracellular domain (NOTCH1-IC) to the TCRβ promoter/enhancer locus. The oncogenic property of NOTCH1-IC was confirmed by a murine bone marrow transplant model wherein reconstitution with hematopoietic progenitors expressing NOTCH1-IC led to the development of T-cell leukemias (Pear et al., 1996). The presence of activated Notch is not limited to leukemias, as its overexpression or gain-of-function mutations, resulting in expression of a truncated active Notch, have also been observed in tumors of epithelial origin such as breast, cervical, and colon carcinomas (Callahan and Egan, 2004; Callahan and Raafat, 2001; Gray et al., 1999; Zagouras et al., 1995). A role for constitutive Notch signaling

in the development of mammary tumors was first found with the discovery that the *Notch4* gene is a common integration site for the mouse mammary tumor virus (MMTV) in about 18% of virus-induced mouse mammary tumors (Gallahan and Callahan, 1997; Gallahan et al., 1987). MMTV interruption of *Notch4* results in the expression of a transcript that encodes the transmembrane and intracellular regions for Notch4, but that lacks the extracellular regulatory domain. Transgenic mouse models expressing the Notch4 intracellular domain develop mammary tumors (Jhappan et al., 1992; Smith et al., 1995), and therefore support a causative role for activated Notch signaling in mammary tumorigenesis. The relevance of Notch activation in human breast cancers has recently been investigated using tissue microarrays of breast tumor samples from various clinical stages. In these studies, elevated expression of Notch-1 and the Notch ligand, Jag1, was associated with poor survival (Reedijk et al., 2005).

Among the primary mechanisms for Notch-induced tumorigenesis, in addition to increased proliferation, is the inhibition of apoptosis. Activated Notch-1 renders T cells resistant to Fas receptor-mediated signaling, as well as to drugs including dexamethasone and etoposide, via upregulation of antiapoptotic molecules such as Bcl-2, FLIP, and IAPs (Sade et al., 2004). Additional mechanisms for Notch-induced survival include inhibition of p53 tumor suppressor expression, and activation of the RAS, PI3-K, and NF-κB pathways (Leong and Karsan, 2006).

While the precise value of Notch signaling inhibition in cancer therapy remains to be determined, preliminary studies have shown the potential for gamma secretase inhibitors (GSI) (Lanz et al., 2004; Wong et al., 2004), which can block Notch proteolytic processing, to induce apoptosis in various tumor cell lines (Curry et al., 2005; Nickoloff et al., 2005). Treatment of chemoresistant melanoma cells with a small molecule, GSI, induced the expression of the proapoptotic BH3 family member, NOXA, and caused apoptotic cell death (Nickoloff et al., 2005). Future studies will determine which downstream survival or antiapoptotic pathways play a role in the context of Notch activation in leukemias, as well as in solid tumors. In addition, the precise role of each of the four Notch receptors in tumorigenesis, and the development of specific inhibitors and/or antibodies against these receptors, will be crucial for an understanding of the overall role of Notch signaling in cancer and for investigating the potential of Notch inhibition in anticancer therapy. Finally, it will also be important to perform these studies at the cancer stem cell level in order to determine the cellular context in which dysregulated Notch signaling can potentially exert its oncogenic effects.

1.2.3 Hedgehog Signaling

The Hh pathway, first discovered in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), is highly conserved across vertebrates, with important functions during embryonic development, as well as in adult tissue homeostasis, such as in postembryonic tissue repair and stem cell regulation (Lum and Beachy, 2004; Taipale and Beachy, 2001; Zhang and Kalderon, 2001). The mammalian Hh pathway includes

three secreted Hh ligands (Sonic, Indian, and Desert), their 12-pass transmembrane receptors Patched1 (PTCH1) and Patched2 (PTCH2), and the 7-pass transmembrane signal transducer Smoothened (SMO). Hh ligands activate the Hh pathway by inducing the activation of SMO, followed by a signal transduction cascade that causes the nuclear translocation of the GLI family of transcription factors (GLI1, 2, 3), and the subsequent induction of a distinct transcriptional regulatory program (Cohen, 2003; Hooper and Scott, 2005; Kalderon, 2005). The targets of Hh pathway activation include various cell cycle, proliferation, and survival-regulating genes such as the cyclins (Kenney and Rowitch, 2000), c-myc (Kenney et al., 2003), and Bcl-2 (Bigelow et al., 2004; Regl et al., 2004), and also Hh pathway genes themselves, such as Ptch1, Gli1, and Hip (Hh-interacting protein), which in turn regulate pathway activation (Chuang and McMahon, 1999; Goodrich et al., 1996; Lee et al., 1997).

Notably, gene mutations within the Hh pathway have been linked with several human diseases. Mutations resulting in unrestrained Hh pathway activity have been found in Gorlin's syndrome, which is characterized by developmental defects in the brain, spinal cord, and skeleton, and a predisposition for skin and brain cancers, such as basal cell carcinomas (BCCs) and medulloblastomas, respectively (Hahn et al., 1999). Subsequent investigations have substantiated aberrant Hh signaling in BCCs and medulloblastomas (Gailani et al., 1996; Xie et al., 1998). Recent studies have revealed that the Hh pathway is also active in more common tumors such as those of the lung, breast, pancreas, stomach, and prostate (Berman et al., 2003; Karhadkar et al., 2004; Kubo et al., 2004; Pasca di Magliano and Hebrok, 2003; Sheng et al., 2004; Thayer et al., 2003; Watkins et al., 2003). Cyclopamine is a plant-derived steroidal alkaloid that inhibits the Hh pathway by antagonizing SMO (Taipale et al., 2000). Various studies have shown the ability of cyclopamine to induce apoptosis in a variety of tumor cell lines, and to inhibit tumor progression in medulloblastoma, pancreatic, and lung mouse tumor models (Berman et al., 2002; Thayer et al., 2003; Watkins et al., 2003).

1.2.4 Wnt/β-catenin Signaling

Similar to the Notch and Hh pathways, the Wnt signal transduction pathway also plays a critical role during development. Among several functions, Wnt signals regulate the self-renewal of hematopoietic, epidermal, and intestinal stem cells. The canonical Wnt pathway involves signaling through the cytoplasmic protein, β-Catenin. The binding of a Wnt ligand to a complex of a Frizzled receptor and the LRP5/6 receptor leads to a series of signaling events resulting in the inhibition of a destruction complex that promotes the proteasomal degradation of β-Catenin. Therefore, Wnt pathway activity causes the accumulation of β-Catenin and its translocation to the nucleus where it binds to the Lef/Tcf family of transcription factors. This binding elicits the transcriptional activation of various target genes involved in the promotion of cellular proliferation and invasion, and the inhibition of apoptosis (reviewed in Fuchs et al., 2005; Reguart et al., 2005; Reya and Clevers, 2005).

Interestingly, the first *Wnt* gene was identified in mouse mammary tumors induced by the integration of the MMTV (Rijsewijk et al., 1987). Since then, there have been numerous studies on the aberrant activation of Wnt signaling in various cancers, including those of the colon, ovary, prostate, pancreas, breast, and lung, along with melanomas, multiple myeloma, and even leukemias (Fuchs et al., 2005; Janssens et al., 2006; Reguart et al., 2005; Reya and Clevers, 2005). While mutations in the Wnt ligands and receptors have not been identified in cancers thus far, mutations have been identified in downstream effectors of the Wnt pathway, especially in colorectal cancers (CRC). Gain-of-function mutations in oncogenic β-Catenin, and loss-of-function mutations in adenomatous polyposis coli (APC) and Axin, the latter of which are components of the destruction complex, can all lead to uncontrolled β-Catenin-mediated Lef/Tcf target gene expression (Fuchs et al., 2005; Janssens et al., 2006). Wnt pathway target genes involved in the inhibition of apoptosis include *MDR1/PGP, COX-2, PPAR-δ*, and Survivin, each of which has been found to be upregulated in CRCs (Fuchs et al., 2005). Considering the activation of the Wnt pathway in various cancers, inhibition of the Wnt pathway may serve as an attractive and promising therapeutic approach. Recent studies have demonstrated the potential for small-molecule antagonists of the TCF/β-Catenin complex to decrease expression of the Wnt target genes, *Myc* and *Cyclin D*, and to inhibit cellular proliferation in colon carcinoma cell lines (Lepourcelet et al., 2004). In another approach, monoclonal antibodies against Wnt-1 and Wnt-2 ligands have shown promise in inducing apoptosis in a variety of tumor cell lines overexpressing Wnt ligands, both in vitro and in vivo (He et al., 2004; You et al., 2004a–c;). Interestingly, the Wnt-2 antibody was shown to downregulate the expression of Survivin and induce apoptosis in various human non-small-cell lung cancer (NSCLC) cells, while failing to induce apoptosis in normal human airway cells that do not express Wnt-2. In contrast, primary NSCLC tissues showed elevated expression of Wnt-2 (You et al., 2004c).

2 Conclusion and Perspectives

Even though the cellular heterogeneity of tumors has long been recognized, the exact reasons for this feature have not always been clearly understood. The genomic instability that is inherent in cancer cells offers one explanation. Interestingly, recent studies, especially in leukemia, have revealed that the abnormal behavior of a malignant stem cell can give rise to the abnormally differentiated and diverse cellular hierarchy observed in tumors. The cancer stem cell hypothesis proposes that the tumor is actually sustained by a minority of cells, the cancer stem cells. The identification of cancer stem cells in leukemia and some solid cancers has yielded great insight into the cellular underpinnings of cancer, and will greatly affect the consideration of which cells to target critically in future anticancer therapeutics. Together, the study of signal transduction pathways that govern the survival of cancer stem cells, the precise role of cancer stem cells in different cancers, and an

analysis of stem cell regulation pathways in cancer offers great promise for the development of more effective treatments in the future.

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