cell death (inhibited by Bcl-2/Bcl-x_L) even in yeast, which lack caspases (*10, 22, 48*).

Conclusions

Although mitochondrial involvement and cyto c release may not be universal aspects of apoptosis, they appear to be frequently involved in vertebrates. In contrast, there is currently no evidence that cyto c plays roles in apoptosis in either nematodes or insects. In the former, caspase activation through interaction with Ced-4 (whose role presumably is similar to that of Apaf-1) proceeds without a requirement for cyto c. A more extensive phyletic survey will help establish whether the involvement of cyto c is a relatively late evolutionary innovation or whether it was secondarily lost from some lineages.

Nevertheless, the role of mitochondria as stress sensors and executioners makes intuitive sense for maintenance of the process of apoptosis in multicellular organisms. That is, cells cannot gain a growth advantage simply by losing mitochondria, because these organelles are also needed for efficient energy metabolism, production of membrane lipids, and cell growth. Defects in the apoptotic pathway downstream of mitochondria (for example, caspases) might also fail to provide a growth advantage, because cells may nevertheless die because of the mitochondrial effects (albeit more slowly), and thus caspase mutations may not promote oncogenesis.

Most intriguing is the structural similarity of Bcl-2 family proteins to channel-forming proteins such as the colicins, which are used as weapons by bacteria to kill competing bacteria. This ancient system involving secreted channel-forming proteins and opposing immunity proteins that bind colicins, preventing channel formation, is highly reminiscent of the scenario in mammalian cells where apoptotic stimuli induce translocation of cytosolic Bax to mitochondrial membranes, thereby killing the cell, unless opposed by Bcl-2. Perhaps a few hundred million years ago, either convergent or divergent evolutionary processes allowed the same fundamental framework for bacterial warfare to be incorporated into the cell death mechanisms used by animal cells, thereby establishing mitochondria as important participants not only in animal cell life but also in active cell death.

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Caspases: Enemies Within

Nancy A. Thornberry and Yuri Lazebnik

REVIEW

Apoptosis, an evolutionarily conserved form of cell suicide, requires specialized machinery. The central component of this machinery is a proteolytic system involving a family of proteases called caspases. These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell. Understanding caspase regulation is intimately linked to the ability to rationally manipulate apoptosis for therapeutic gain.

Apoptosis is a type of cell death that is accomplished by a specialized cellular machinery. That this machinery exists and is highly conserved was predicted from observing a stereotypical morphology of cells dying either under physiological conditions or after mild injury (*1*). These changes reflect complex biochemical events carried out by a family of cysteine proteases called caspases. In this review, we describe properties of caspases, how they kill a cell, how they are regulated, and discuss the potential therapeutic utility of caspase modulation. Because relatively little is known about caspase regulation, it is instructive to first review lessons learned from well-studied proteolytic systems, which provide a framework for understanding the biology of caspases and can serve as guiding principles for ongoing research in this area.

Proteolysis is irreversible, unlike most other posttranslational modifications. This implies that regulation of proteases is limited to control of their activity and availability of substrate since the only known way of "correcting" a cleaved protein is to make it afresh. Considering this feature of proteolysis, it is not surprising

to find these enzymes at the helm of such irreversible processes as development, the cell cycle, and most irreversible of all, cell death.

Most proteases are synthesized as precursors that have little, if any, catalytic activity. The precursor is usually converted to the active enzyme by proteolytic processing. This is mediated either by another protease or by autocatalysis, triggered by the binding of cofactors or removal of inhibitors. Hence, large amounts of a precursor can be accumulated in advance and activated on demand.

Proteases can regulate their own activation. This is achieved through positive and negative feedbacks, one of which is the amplification loop. In this case, the active protease can directly or indirectly activate its own precursor, resulting in an exponential rate of activation and ensuring that the protease can accomplish its goal quickly.

Where there are proteases, there are inhibitors. The inhibitor can establish a threshold that regulates the concentration of active protease in the cell. Such thresholds prevent the consequences of spontaneous activation, an undesirable event if the active enzyme can kill a cell.

Proteolytic reactions can be specific. Protease specificity is often determined by a combination of primary, secondary, or tertiary structures of protein substrates. Proteolysis that governs critical biological processes (for example, cell cycle or cell death) is highly specific, involving a restricted set of substrates.

What Are Caspases?

Caspases were implicated in apoptosis with the discovery that CED-3, the product of a gene required for cell death in the nematode *Caenorhabditis elegans*, is related to mammalian interleukin-1β–converting enzyme (ICE or caspase-1) (*2, 3*). Although caspase-1 has no obvious role in cell death, it has become the first identified member of a large family of proteases whose members have distinct roles in inflammation and apoptosis (Fig. 1A). In apoptosis, caspases function in both cell disassembly (effectors) and in initiating this disassembly in response to proapoptotic signals (initiators).

Caspases share similarities in amino acid sequence, structure, and substrate specificity (*4*). They are all expressed as proenzymes (30 to 50 kD) that contain three domains: an NH_2-terminal domain, a large subunit (~20 kD), and a small subunit (~10 kD) (Fig. 1, B and C). Activation involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer. Crystal structures of two active caspases (caspase-1 and caspase-3) have been determined: in both cases, two heterodimers associate to form a tetramer, with two catalytic sites that appear to function independently (*5–7*). Within each catalytic domain, the large and small subunits are intimately associated, with both contributing residues necessary for substrate binding and catalysis.

Two features of the proenzyme structure are central to the mechanism of activation of these enzymes. First, the NH_2-terminal domain, which is highly variable in sequence and length, is involved in regulation of activation (see below). Second, all domains are derived from the proenzyme by cleavage at caspase consensus sites, implying that these enzymes can be activated either autocatalytically or in a cascade by enzymes with similar specificity.

Caspases are among the most specific of proteases, with an unusual and absolute requirement for cleavage after aspartic acid (*8*). Recognition of at least four amino acids NH_2-terminal to the cleavage site is also a necessary requirement for efficient catalysis. The preferred tetrapeptide recognition motif differs significantly among caspases and explains the diversity of their biological functions (*9*). Their specificity is even more stringent: not all proteins that contain the optimal tetrapeptide sequence are cleaved, implying that tertiary structural elements may influence substrate recognition. Cleavage of proteins by caspases is not only specific, but also highly efficient ($k_{cat}/K_m > 10^6\ M^{-1}\ s^{-1}$). The strict specificity of caspases is consistent with the observation that apoptosis is not accompanied by indiscriminate protein digestion; rather, a select set of proteins is cleaved in a coordinated manner, usually at a single site, resulting in a loss or change in function.

How Do Caspases Kill a Cell?

Apoptotic events include DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into membrane-enclosed vesicles (apoptotic bodies). In vivo, this process culminates with the engulfment of apoptotic bodies by other cells, preventing complications that would result from a release of intracellular contents. These changes occur in a predictable, reproducible sequence and can be completed within 30 to 60 min.

How do caspases contribute to this process? The overall picture is not fully understood, largely because many of the known substrates have been found serendipitously. As a result, of the 40 or so that have been identified, the relationship of their cleavage to cell death is well understood for only a handful (reviewed by *10, 11*). Nonetheless, these few examples suggest that a subset of caspases (effectors) is responsible for the cellular changes that occur during apoptosis and provide insights into the mechanisms that they employ (Fig. 2).

One role of caspases is to inactivate proteins that protect living cells from apoptosis. A clear example is the cleavage of I^{CAD}/DFF45 (*12, 13*), an inhibitor of the nuclease responsible for DNA fragmentation, CAD (caspase-activated deoxyribonuclease). In nonapoptotic cells, CAD is present as an inactive complex with I^{CAD}. During apoptosis, I^{CAD} is inactivated by caspases, leaving CAD free to function as a nuclease. This system is not as simple as it appears: CAD synthesized in the absence of I^{CAD} is not active, implying that the

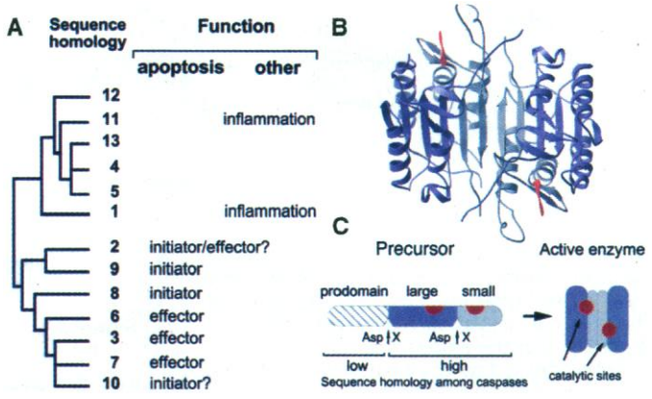

Fig. 1. Proposed caspase functions and structure. (**A**) Caspases have been found in organisms ranging from *C. elegans* to humans. The 13 identified mammalian caspases (named caspase-1 to caspase-13) have distinct roles in apoptosis and inflammation. The family includes two murine homologs (11 and 12) that have no known human counterparts. In apoptosis, caspases are directly responsible for proteolytic cleavages that lead to cell disassembly (effectors) and are also involved in upstream regulatory events (initiators) (more details in Fig. 3). The functions of caspases have been tentatively assigned based on phenotypes of knockout animals, studies of enzyme specificity, and the results of numerous other biochemical studies. (**B**) Shown is the crystal structure of caspase-3 in complex with a tetrapeptide aldehyde inhibitor (red). The active enzyme is composed of a large (~20 kD, lavender) and small (~10 kD, gray) subunit, each of which contributes amino acids to the active site. In the two crystal structures that are available, two heterodimers associate to form a tetramer. (**C**) In common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation. The NH_2-terminal domain, which is highly variable in length (23 to 216 amino acids) and sequence, is involved in regulation of these enzymes.

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CAD-I^{CAD} complex is formed co-translationally, and that I^{CAD} is required for both the activity and inhibition of this nuclease.

Other negative regulators of apoptosis cleaved by caspases are Bcl-2 proteins (*14–16*). It appears that cleavage not only inactivates these proteins, but also produces a fragment that promotes apoptosis. That such positive feedbacks are involved in the control of apoptosis is not surprising, given their importance in the regulation of other proteolytic systems.

Caspases contribute to apoptosis through direct disassembly of cell structures, as illustrated by the destruction of nuclear lamina (*17, 18*), a rigid structure that underlies the nuclear membrane and is involved in chromatin organization. Lamina is formed by head-to-tail polymers of intermediate filament proteins called lamins. During apoptosis, lamins are cleaved at a single site by caspases, causing lamina to collapse and contributing to chromatin condensation.

Caspases also reorganize cell structures indirectly by cleaving several proteins involved in cytoskeleton regulation, including gelsolin (*19*), focal adhesion kinase (FAK) (*20*), and p21-activated kinase 2 (PAK2) (*21*). Cleavage of these proteins results in deregulation of their activity. For example, in the case of gelsolin (a protein that severs actin filaments in a regulated manner), caspase cleavage generates a fragment that is instead constitutively active.

Dissociation of regulatory and effector domains is a hallmark of caspase function. For example, they inactivate or deregulate proteins involved in DNA repair (such as DNA-PK_{cs}), mRNA splicing (such as U1-70K), and DNA replication (such as replication factor C) (*11, 22*). Although the relationship of these cleavages to cell death is not clearly understood, it is likely that the disabling of critical homeostatic and repair functions facilitates cellular disassembly.

A survey of these and other substrates suggests that caspases participate in apoptosis in a manner reminiscent of a well-planned and executed military operation. They cut off contacts with surrounding cells, reorganize the cytoskeleton, shut down DNA replication and repair, interrupt splicing, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis, and disintegrate the cell into apoptotic bodies. As systematic approaches for finding new caspase substrates are developed (*23*), better understanding of the mechanisms used to accomplish these changes will emerge.

Consideration of potential therapeutic strategies for selectively inducing apoptosis in cells (for example, cancer cells) raises several questions about caspase substrates. For example, what is the minimal set of substrates that must be cleaved in order to harmlessly dispose of a cell? Clearly, cleavage of I^{CAD} is likely to result in cell death, but unlikely to induce cell engulfment. On the other hand, perhaps triggering engulfment would be sufficient by essentially burying the cell alive.

Besides these practical issues, the finding that many key proteins have caspase cleavage sites challenges the understanding of life and its evolution. Indeed, how did it happen that cells are made to be so quickly disassembled? How is it that cell components can have two functions: to support cell life, but also to kill? Answering these questions may make the notion that cells die by default seem not quite so radical (*24*).

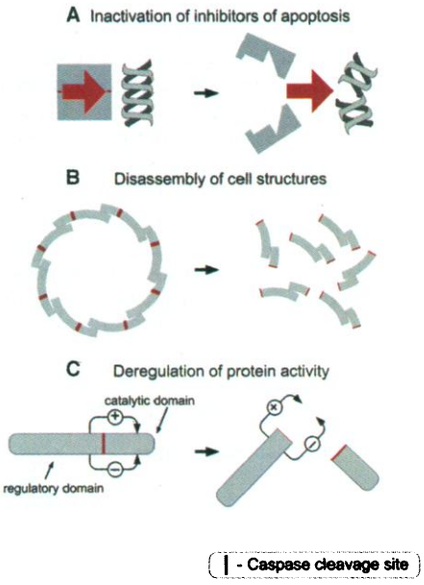

Fig. 2. How caspases disassemble a cell. Caspases kill cells by cleaving a discrete set of proteins, using a variety of strategies. They (**A**) inactivate inhibitors of proteins that promote apoptotic changes (for example, CAD/I^{CAD}), (**B**) destroy cell structures (such as lamina), and (**C**) deregulate proteins by separating regulatory and catalytic domains, resulting in loss or gain of function (for example, gelsolin or DNA-PK_{cs}).

How Are Caspases Regulated?

The observations that caspase precursors are constitutively expressed in living cells (even in neurons that can live for a lifetime) but that apoptosis can be induced quickly indicates that caspase regulation is

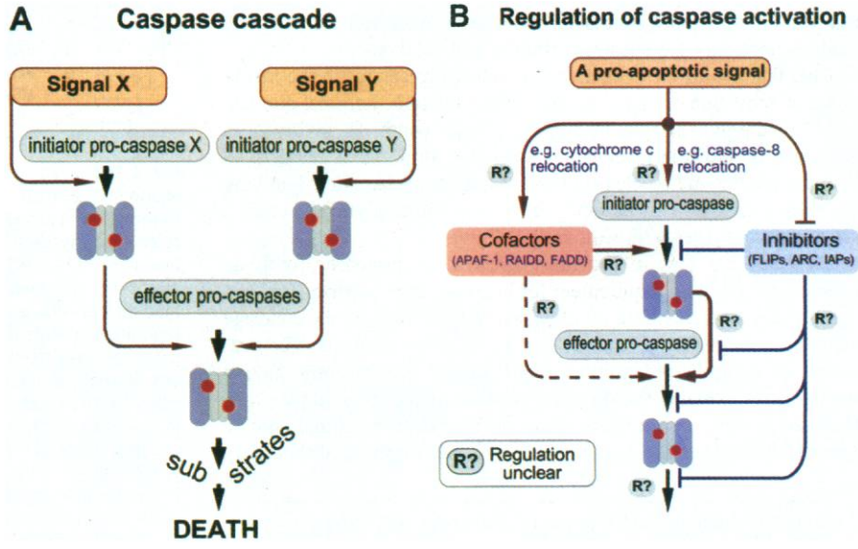

Fig. 3. Caspase cascade in apoptotic cells and a model for caspase regulation. (**A**) Early observations that, during apoptosis, common morphological changes occur in tissues and species led to the suggestion that this process is governed by a conserved biochemical system. It is now clear that these changes are due to the activities of a common set of effector caspases. The observation that distinct death signals result in the same manifestations of apoptosis is explained by the finding that effector caspases are activated by different initiator caspases, each of which is activated by a set of proapoptotic signals. (**B**) Available evidence suggests that caspases are regulated by opposing effects of activators and inhibitors. A signal apparently initiates three pathways involving cofactors, initiator caspases, and inhibitors. Activation of cofactors (for example, cytochrome c relocation from mitochondria to cytoplasm), modification of the caspase (for example, relocation of caspase-8 to a receptor complex), and inactivation of inhibitors (no examples yet) together result in activation of the initiator caspase. The dashed line from cofactors to effector caspases reflects the possibility that effector caspases may be activated by an autocatalytic mechanism. Regulation is likely to be even more complicated; for example, active caspases may be involved in feedback mechanisms.

sophisticated and effective. Complex proteolytic systems often involve a combination of regulatory proteases, cofactors, feedbacks, and thresholds that converge to control the activity of an effector protease, that in turn carries out the function of the whole process (*25*). This intricate regulation accounts for a spectacular feature of these systems: they keep the effector protease inactive but are able to rapidly activate large amounts of it in response to minute quantities of an appropriate inducer. Given the function of caspases as mediators of cell death, the complexity of their regulation is likely to rival that of the coagulation and complement systems.

Activation of effector caspases. A large body of genetic and biochemical evidence supports a cascade model for effector caspase activation (Fig. 3): a proapoptotic signal culminates in activation of an initiator caspase which, in turn, activates effector caspases, resulting in cellular disassembly. Different initiator caspases mediate distinct sets of signals. For example, caspase-8 is associated with apoptosis involving death receptors (*26*). In contrast, caspase-9 is involved in death induced by cytotoxic agents (*27, 28*). This model explains how distinct apoptotic signals induce the same biochemical and morphological changes.

Activation of initiator caspases. The available evidence indicates that activation of initiator caspases requires binding to specific cofactors, a mechanism commonly observed with proteases. This binding is triggered by a proapoptotic signal and mediated through one of at least two distinct structural motifs that reside in both the caspase prodomain and its corresponding cofactor. Activation of procaspase-8 requires association with its cofactor FADD (Fas-associated protein with death domain) through the DED (death effector domain) (*29, 30*), while procaspase-9 activation involves a complex with the cofactor APAF-1 through the CARD (caspase recruitment domain) (*31*). Activation of caspase-9 also requires cytochrome c and deoxyadenosine triphosphate, indicating that caspase activation may require multiple cofactors. How does the binding of cofactors lead to activation? Because a crystal structure of a caspase precursor is unavailable, the proposed models are based on indirect evidence.

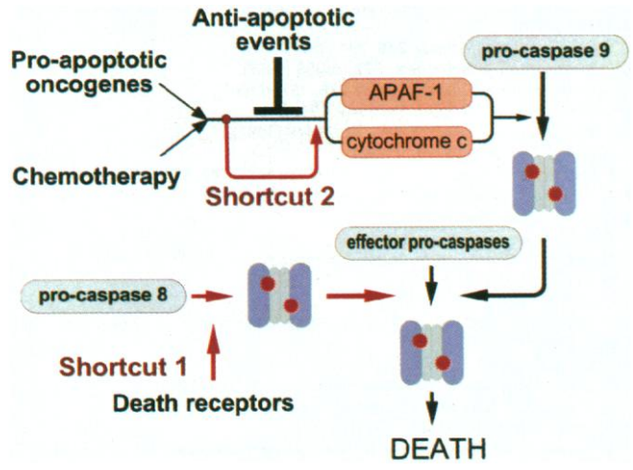

Fig. 4. How can caspases be activated in cancer cells resistant to therapy? Chemotherapeutic drugs induce apoptosis indirectly. They inflict cell damage that is then translated through several poorly understood steps into activation of caspase-9 (*27, 28*). In drug-resistant cells, the apoptosis fails because of defects in signaling pathways (antiapoptotic events, such as p53 mutation or overexpression of bcl-2) that lead to caspase activation. Therapeutic opportunities may lie in bypassing these defects. One possibility (shortcut 1) is to activate the death receptor complexes, resulting in activation of their corresponding initiator caspase (for example, caspase-8). Another potential approach involves bypassing the defective part of the pathway (shortcut 2) to restore the signal triggered by chemotherapeutic drugs or by the oncogenic transformation itself, which can be thought of as a proapoptotic signal that is present only in transformed cells.

The induced proximity or oligomerization model is based on three observations: procaspases have low but detectable activity (*32*), dimerization is required for activation (*33*), and procaspases that are overexpressed in cells and artificially cross-linked become active (*32, 34*). The model argues that caspases are latent in the cell because they exist at low concentrations as monomers. The cofactors serve to bring two or more caspase precursors in close proximity, allowing for intermolecular autoproteolytic activation. If this model is correct, it is indeed an elegant explanation. However, several questions remain unanswered. For example, it is not clear that initiator caspase precursors are indeed monomers in living cells.

The facilitated autocatalysis model postulates that caspase precursors are present in cells in a conformation or a complex that prevents autocatalysis. The cofactors facilitate activation by changing the conformation of the precursors either directly or by removing an inhibitor. Two approaches are likely to be instrumental in understanding the mechanism of activation: obtaining the structure of a caspase precursor and studies of caspase activation using purified components from cells.

Very little is known about the regulation of the interaction between procaspases and their cofactors. One example was provided by the discovery of FADD-like ICE inhibitory proteins (FLIPs) (*35, 36*). These proteins are similar in sequence to procaspase-8, except that they lack essential catalytic residues. These proteins probably compete with procaspase-8 for binding to its cofactor, FADD, thus preventing caspase activation. It appears that procaspase-8 is not the only one to have a decoy, as suggested by the recent discovery of the CARD domain–containing protein, ARC (apoptosis repressor with caspase recruitment domain) (*37*). The factors that determine how cofactors select between procaspases and their decoys are not known.

Compartmentalization of caspases and their cofactors is likely to be another way of regulating caspase activation. This notion is supported by the finding that extracts from some live cells can activate caspases spontaneously, suggesting that all of the components required for caspase activation are present, but sequestered, in living cells (*38*). This observation led to the discovery that cytochrome c is required for caspase-9 activation in vitro, and the subsequent hypothesis that apoptosis can be triggered by inducing mitrochondrial changes that result in the release of this cofactor [reviewed by (*39*)]. Another example is provided by the finding that caspase-8 is activated when recruited to the Fas receptor complex. Determining where caspases and their cofactors are in cells is an area of intense research that will likely contribute to a better understanding of caspase regulation.

Inhibitors as regulators. Given the importance of inhibitors in the regulation of complex proteolytic systems, it is not surprising to find them also involved in control of cell-death proteases. Identification of caspase inhibitors has come out of work on viruses, which attenuate apoptosis to circumvent the normal host response to infection. Three distinct classes of viral inhibitors have been described: CrmA (*40*), p35 (*41, 42*), and a family of IAP (inhibitors of apoptosis) proteins (*43*). Cowpox virus CrmA is a member of the serpin family that is a potent inhibitor (K_i <1 nM) of some active initiator caspases and those involved in inflammation. The baculovirus protein p35 has no known homologs, and its selectivity for caspases is not clearly defined. The IAP proteins are a large family and the only one known to have mammalian members.

The precise caspase targets of the IAPs remain elusive. Potent, selective inhibition of caspase-3 and caspase-7 was observed in vitro with x-linked IAP (*44*), suggesting that IAPs inhibit apoptosis through inhibition of effector caspases. The story is not so simple, however, because they also prevent the activation of these enzymes upon overexpression, suggesting that effector caspase proenzymes or other proteins in the activation complex are the real targets in cells (*45, 46*). Alternatively, if effector caspases amplify the apoptotic signal by activating initiator caspases, IAPs may function as negative regulators of this feedback.

How are IAPs and other caspase inhibitors involved in the regulation of apoptosis? In analogy to other proteolytic systems, inhibitors may establish thresholds that determine the concentration of active effector caspases required to initiate cell disassembly, thus also preventing the consequences of accidental or spontaneous proenzyme activation. Inhibitors may also be used to confine the activity of these enzymes to specific cellular locations.

Can We Target Caspases for Treatment of Disease?

Two opposite types of diseases that involve deregulation of apoptosis are those involving excessive apoptosis, causing damage to normal tissues, and those in which apoptosis is prevented, allowing malignant tissues to grow. Accordingly, the two strategies for therapeutic intervention involve either caspase inhibition or induction of apoptosis through caspase activation.

Caspase inhibition. Excessive apoptosis has been blamed for several serious pathologies for which there are currently limited therapeutic options, including neurodegenerative diseases, ischemia-reperfusion injury, graft-versus-host disease, and autoimmune disorders. Caspases are attractive potential targets for the treatment of these conditions because of the requisite role of these enzymes in apoptosis and the appealing prospect of small-molecule inhibitor therapy. Indeed, there is accumulating evidence that this approach may be successful. For example, preventing apoptosis through p35 expression prevents blindness in *Drosophila* mutants with retinal degeneration, indicating that inhibition of caspases can functionally rescue cells from death (*47*). In addition, peptidyl caspase inhibitors are effective in animal models of stroke, myocardial ischemia-reperfusion injury, liver disease, and traumatic brain injury.

Many significant questions remain to be addressed, particularly regarding the treatment of chronic disorders. What are the best caspase targets for inhibition of apoptosis? To answer this requires a better understanding of the roles of individual caspases in different tissues. Could chronic diseases be safely treated without the risk of autoimmune disease or tumor progression? Tissue-specific delivery of selective inhibitors may be required. It is probably more realistic, at least initially, to consider instead the prospects for treatment of acute disorders, assuming there is an adequate therapeutic window of opportunity.

On a more practical level, can a caspase inhibitor be identified that has appropriate properties for use in vivo? Although two classes of major drugs work by inhibiting proteases, angiotensin converting enzyme inhibitors and human immunodeficiency virus protease inhibitors, there are few other examples. This reflects, in part, the formidable difficulties involved in generating small molecule, nonpeptide inhibitors of proteolytic enzymes that are selective, stable, and penetrate membranes effectively. This notwithstanding, elegant work on cysteine protease inhibition has provided a starting point, leading to the identification of several classes of potent reversible and irreversible caspase inhibitors (*48*). Studies with these compounds will no doubt provide answers to the many questions regarding the therapeutic potential of caspase inhibition.

Cancer treatment. Two main problems of chemotherapy are toxicity to normal cells and failure to kill cancer cells. Both problems stem from the indirect mechanism by which both chemotherapeutic drugs and irradiation kill cells (Fig. 4). They damage both normal and cancer cells, and this damage is then translated through multiple steps into cell death, likely through activation of caspases and apoptosis. When these steps are compromised, the therapy fails.

An alternative strategy is to design a treatment that activates caspases directly. One possibility involves activation of death receptor complexes that are directly linked to initiator caspases (Fig. 4, shortcut 1) [reviewed by (*26*)]. Because death receptors are also expressed in normal cells, the main challenge for this strategy is to activate these receptors selectively in cancer cells.

Another approach may come from the observation that oncoproteins that deregulate the cell cycle can activate caspases and induce apoptosis [reviewed by (*49*)]. Therefore, the oncogenic transformation can be thought of as a proapoptotic signal that is present only in transformed cells. When this signal is uncoupled from caspase activation, transformed cells survive. Understanding how this signal can be recoupled to caspase activation (Fig. 4, shortcut 2) may provide an opportunity to selectively kill transformed cells.

Whether these or other approaches will be beneficial is not clear. Hopefully, in the future, tumor cells will be analyzed for defects in caspase activation, as is currently done with blood clotting proteases, and treatment will be based on this information rather than on a compilation of empirical rules that are the basis for classical chemotherapy.

In summary, substantial progress has been made in understanding the structural and catalytic properties of active caspases and their contribution to apoptosis. The goal for future research is to understand the regulation of these enzymes. This should facilitate efforts to rationally manipulate the apoptotic machinery for therapeutic gain.

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