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Review

Apoptosis signalling: A life or death decision

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Abstract

Apoptosis occurs during the normal development of multicellular organisms and continues throughout adult life. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. More recent evidence has indicated that apoptosis depends upon a tightly regulated cellular program for its successful initiation and execution. Molecular participants in this program are present in different subcellular compartments, including the plasma membrane, cytosol, mitochondria and nucleus. The interplay among these compartments and the exchange of specific signaling molecules are critical for the systematic progression of apoptosis. The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential. Therefore, research continues to focus on the elucidation and analysis of the cell cycle machinery and signaling pathways that control cell cycle arrest and apoptosis. For this reason, the field of apoptosis research has been moving forward at increasingly rapid rate. The aim of this review about general overview of current knowledge on apoptosis signalling considers recent progress in understanding the nature of the suicide process and how it is controlled.

Key words: Apoptosis, Bcl-2, caspase, programmed cell death

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Introduction

Today we know that morphological changes are the result of the activation of an intracellular signal transduction pathway, probably the only function of which is to kill the cell and to organize the disposal of the body. The apoptotic pathway results in characteristic morphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells and macrophages (Hacker, 2000). There are two major mechanisms of cell death-necrosis and apoptosis. Cells that are damaged by external injury undergo necrosis, while cells that are induced to commit programmed suicide because of internal and external stimuli undergo apoptosis. Although understanding of the detailed signalling pathways that trigger apoptosis is incomplete, this process is controlled by a number of complex proteins, which are activated by various triggers and arranged in sequential signalling modules. Apoptosis is an evolutionarily conserved form of cell death that was first described by Kerr and colleagues in 1972 (Kerr *et al.,* 1972). Programmed cell death is internal component of *Caenorhabditis elegans* development. *C. elegans,* was shown to act down-stream of *Ced-3* and *Ced-4* (Hortvitz, 1999). In this organism 1090 somatic cells are generated in the formation of the adult worm, of which 131 of these cells undergo apoptosis or programmed cell death. By contrast, more complex animals can not survive without apoptosis: mutations that inhibit apoptosis in the fruitfly *Drosophila melanogaster,* for example, are lethal early in development, as are mutations in mice that inhibit apoptosis mainly in the developing brain (Raff, 1998). Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Although there are a wide variety of stimuli and conditions, both physiological and pathological, that are trigger apoptosis, not all cells will necessary die in response to the same stimulus. In some cases it's the type of stimuli and /or the degree of stimuli that determines if cells die by apoptosis or necrosis. Since

apoptosis typically does not induce neighbouring cell death, inflammation or tissue scarring, it is well suited for its role in normal cell turnover during embryogenesis and in adult tissue. Depending on the apoptotic stimuli and the affected cell type, this process can last from a few hours to a few days (Zang *et al.,* 2004; Elmore, 2007). Moreover, in some cases, the engulfment of apoptotic cells by phagocytes appears to be necessary for DNA degradation, suggesting that the Nuc-1 nuclease may be expressed by, and fulfill its function within, engulfing cells (Robertson *et al.,* 2000). Apoptosis is a coordinated and often energy dependent process that involves the activation of a group of cysteine proteases called 'caspases' and a complex cascade of events that link the initiating stimuli to the final demise of the cell (Elmore, 2007).

Initiation of apoptosis

Apoptosis is a multi-step, multi-pathway cell-death programme that is inherent in every cell of the body. Many physiological growth-control mechanisms that govern cell proliferation and tissue homeostasis are linked to apoptosis. Apoptosis mechanisms are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. Research indicates that apoptosis occurs through two main pathways. The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily (Ricci and El-Deiry, 2007). The second pathway is the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal (Pei *et al.,* 2003; Wouters and Chiu, 2007). Both pathways converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell (Figure 1).

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. Members of the TNF receptor family share similar cyteine-rich extracellular domains. This death domain plays a critical role in transmitting the death

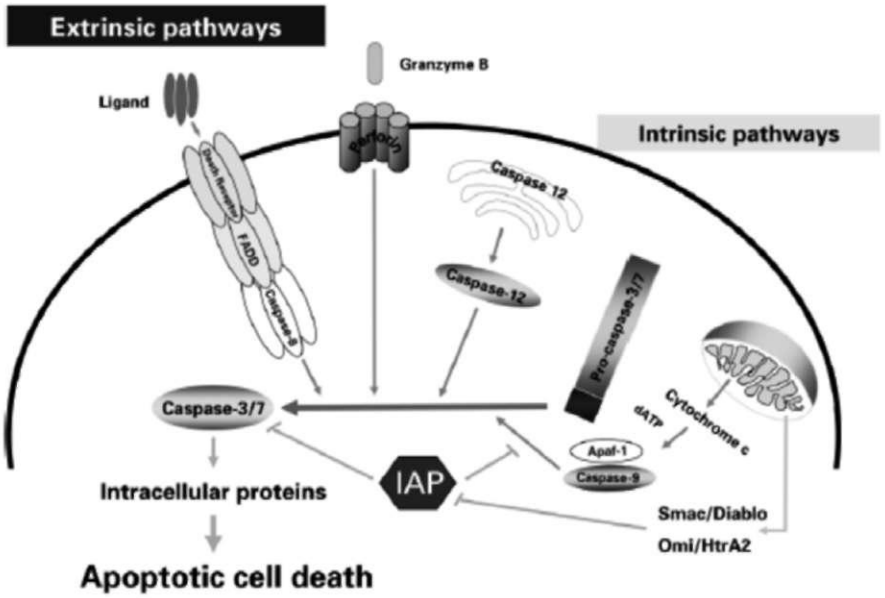

Figure 1. Schematic representation of apoptotic events. The extrinsic pathways involved the delivery of granzyme B through perforin to the cells as well as receptor ligation that triggers caspase-8 activation. Caspase-9 is activated following release of mitochondrial components to form the Apaf complex in the instrinsic pathway. Some intracellular stres can also induce the activation of caspase-12. IAPs gene family can suppress these pathways either blocking the activation of caspase-9 or by directly inhibiting caspase-3 activity (Zang *et al.,* 2004).

signal from the cell surface to the intracellular signaling pathways. To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-a/TNFR-1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Zang *et al.,* 2004; Elmore, 2007).

Chemotherapy, irradiation growth-factor depletion and other stimuli can initiate apoptosis through the mitochondrial (intrinsic) pathway. The intrinsic signaling pathway that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell are mitochondrial-initiated events (Wang, 2001; van Loo *et al.,* 2002; Karbowski and Youle, 2003). Pro-apoptotic Bcl-2 family proteins (for example, Bax, Bid, Bad and Bim) are important mediators of these signals (Hanada *et al.,* 1995). Activation of mitochondria leads to the release of cytochrome c into cytosol, where it binds apoptotic protease factor 1 (APAF-1) to form the apoptosome. At the apoptosome, the initiator caspase-9 is activated. Therefore, in addition to their role in cellular energy metabolism, mitochondria are now recognized as central players in cell death. Crucial for the latter role are not only the Bax/Bak channel, which is open to direct regulation by Bcl-2/Bcl-x_L but also a nonspecific pore in the inner mitochondrial membrane, known as the mitochondrial permeability transition pore (MPTP) (Nicholas *et al.,* 2001; Igney and Krammer, 2002). Apoptosis through mitochondria can be inhibited on different levels by anti-apoptotic proteins, including the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-x_L and inhibitors of apoptosis proteins (IAPs), which are regulated by Smac/DIABLO as shown in Figure 2 (Second mitochondria-derived activator of caspase/direct IAP binding protein) (Igney and Krammer, 2002; van Loo *et al.,* 2002).

There is an additional pathway that involves T-cell mediated cytotoxicity and perforin/granzyme dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A (Pardo *et al.,* 2004). The serine proteases granzyme B and A are the most important component within the granules. Granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of

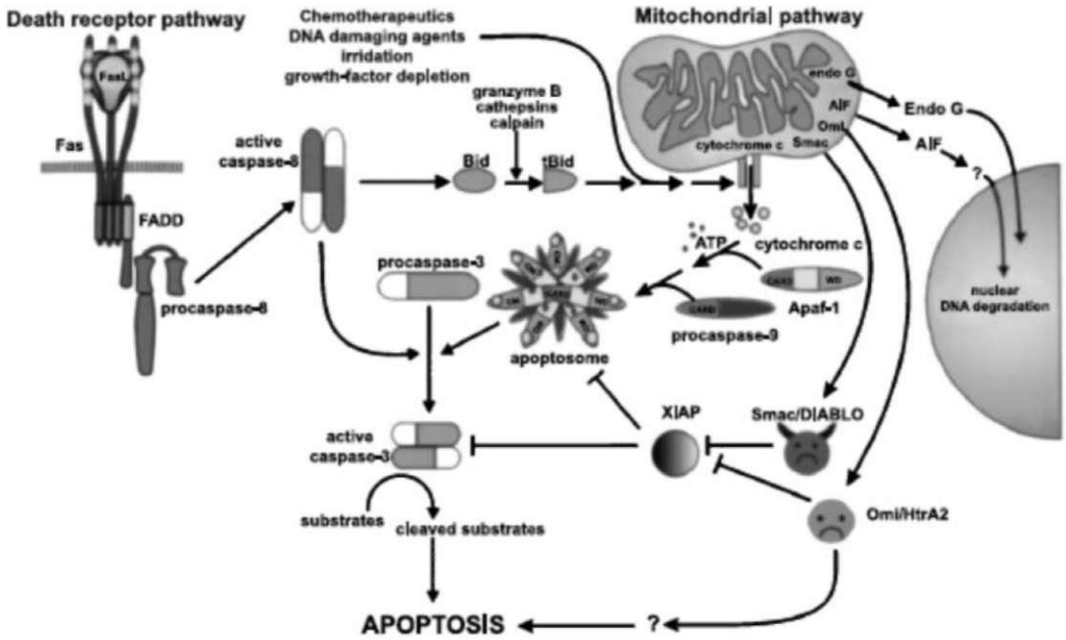

Figure 2. Many death signals converge onto mitochondria and release intermembrane space proteins. A variety of apoptotic stimuli trigger mitochondria, which results in the release of apoptotic proteins including cytochrome c, AIF, endonuclease G, Smac/DIABLO and Omi/HtrA2 (van Loo *et al.,* 2002).

cytochrome c release (Barry and Bleackley, 2002). In addition granzyme B can also activate caspase-3. In this way, the upstream signaling pathways are bypassed and there is direct induction of the execution phase of apoptosis. Reports are suggested that both the mitochondrial pathway and direct activation of caspase-3 are critical for granzyme B induced killing (Goping *et al.,* 2003). Granzyme A is also important in cytotoxic T cell induced apoptosis and activates caspase independent pathways (Elmore, 2007). The extrinsic, intrinsic and granzyme B pathways converse on the same terminal, or execution pathway. Although the triggering of either the death receptor mediated extrinsic pathway of apoptosis or the mitochondrial cytochrome c-mediated intrinsic pathways of apoptosis is dependent upon the death stimulus and/or the cell type involved, there is crosstalk between the two pathways. It has been shown that caspase-8 can cleave Bid, a death-inducing member of the Bcl-2 family. The truncated Bid in turn translocates to the mitochondria and induces cytochrome c release which subsequently results in caspase-9-dependent activation of executioner caspases (Chen and Wang, 2002; Wouters and Chiu, 2007). The fact that cytoplasm of activated cells depleted of Bid by immunoprecipitation was no longer able to induce cytochrome c release indicated that Bid was the only effector molecule downstream of caspase-8 that has such a capability. However, apoptosis triggered through Fas was unaffected in lymphoid cells but was largely reduced in liver cells from mice lacking Bid. These findings suggest that the proteolysis of Bid may not be essential for cell death in all cell types, but that the crosstalk between the two caspase activation pathways may help to amplify weaker apoptotic signals and accelerates cell death execution in certain cell types such as hepatocytes (Zang *et al.,* 2004).

Execution of apoptosis

Once the apoptotic program is activated, it initiates the cell disassembly process, which includes nuclear DNA fragmentation, cytoplasm shrinkage, and exposure of 'eat me' signal(s) on the cell surface to induce phacytosis by neighboring cells (Conradt and Xue, 2005). During apoptosis, the controlled destruction of the cell is coordinated, from within, by the caspase family of cysteine proteases. This meeting focused on two of the major apoptotic pathways: one initiated by the activation of death receptors and the other by stress-inducing stimuli. The discovery of a family of cysteine-aspartate proteases (caspases) and their involvement in signaling and executing apoptosis implicated the critical importance of these enzymes in this form of cell death (MarcFarlane and Williams, 2004). The protein encoded by the Ced-3 gene was found to be very similar to a human protein called interleukin-1 converting enzyme (ICE). ICE induces apoptosis when expressed in cells and crmA, an ICE inhibitor suppresses apoptosis; however, ICE is not a granzyme B substrate (Shi *et al.,* 1996). The discovery and characterization of ICE provided no clues to the role of ICE-like proteases in cell death. In most situations, apoptosis is coordinated by caspases, which dismantle the cell by targeting numerous proteins for limited proteolysis, now widely known as caspases, play key role in apoptosis execution (Kumar, 2007).

To date, at least 14 members of this family have been identified in mammals although not all of them function during apoptosis. The caspase family members can be divided into three subgroups depending on inherent substrate specificity, domain composition or the presumed role in apoptosis: they include initiators in apoptosis (caspase-2, 8, 9 and 10), executioners in apoptosis (caspase-3, 6 and 7) and participants in cytokine activation (caspase-1, 4, 5, 11, 12, 13 and 14). A hierarchical relationship is postulated to exist between the initiators and executioners in that the initiators act upstream of the executioners. The activated executioners cleave key proteins required for the maintenance of homeostasis, leading to the collapse and demise of the cell. All the initiator and executioner caspases have either a direct or indirect role in the processing, propagation and amplification of apoptotic signals that results in the destruction of cellular structures (Zang *et al.,* 2004).

Caspases are synthesized as inactive proenzymes which are activated by cleavage at specific Asp residues to active enzymes containing both large (p20) and small (p10) subunits. In some cases these subunits are separated by a linker region of unknown function but which may be involved in regulation of the activation of the caspase. The process and association model postulates that the p20-p10 heterodimer is derived from the same procaspase molecule (Figure 3). Subsequent removal of the linker and prodomains allows the two heterodimers to form the tetrameric structure (Chang and Yang, 2000). All caspases are cleaved at specific residues, raising the possibility that some caspases sequentially activate others, so

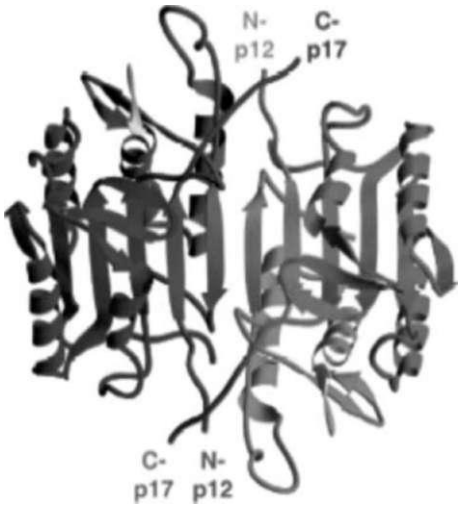

Figure 3. Caspase-3 tetramer structure in complex with Ac-DEV-CHO. The p17 subunits in red and pink, and bound inhibitors in yellow. The C termini of p17 and N termini of p12 are indicated (Chang and Yang, 2000).

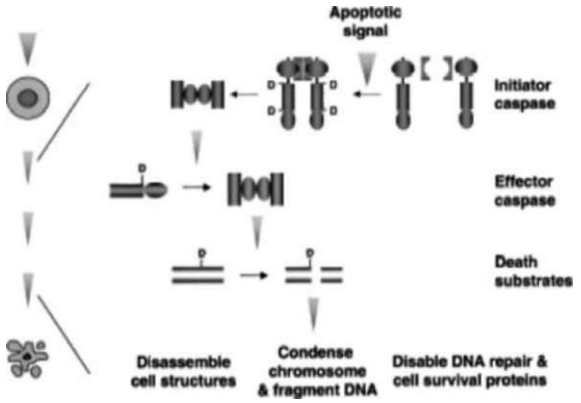

Figure 4. Activation of the caspase cascade. Apoptotic signal trigger oligomerization of death adapter proteins. Active initia¬tor caspases then process and activate effector procaspases. Effector caspases cleave various death substrates to induce apoptosis (Chang and Yang, 2000).

establishing a hierarchy of caspases. A model has been proposed in which caspase-8 has been termed an 'initiator' protease, which activates an 'amplifier' protease such as caspase-1, which in turn activates a 'machinery' protease such as caspase-3 or caspase-7 (Cohen, 1997).

Caspases are among the most specific endopeptidases. The sustrate specificity of human caspases was determined using a systematic approach involving combinatory peptide fluorogenic substrates. Categorizing caspases based on their substrate specificity gives a different result than doing so based on sequence homology among the caspases (Figure 4). This difference is related to the fact that only small numbers of amino acids determine the substrate specificity.

The activation of caspases may be responsible for the neurodegeneration associated with Alzheimer 's disease and several recent studies have suggested that caspases may also play role in promoting pathogenic mechanisms underlying this disease (Rohn and Head, 2009). However, there is now accumulating evidence indicating that cell death can occur in a programmed fashion but in complete absence and independent of caspase activation. Caspase independent cell death pathways are important safeguard mechanisms to protect the organism against unwanted and potential harmful cells when caspase-mediated routes fail but can also be triggered in response to cytotoxic agents or other death stimuli. Endoplasmic reticulum (ER) is an important sensor of cellular stres that can withhold protein synthesis and metabolism to restore cellular homeostasis. If the damage to the ER is too extensive, this can initiate apoptosis via the unfolded protein response or via release of calcium into the cytoplasm (Breckenridge *et al.,* 2003). This leads to activation of caspase-12, possibly via translocation of the Bcl-2 family member Bim to the ER. In addition and independent of caspase-12 activation, ER stres can induce permeabilization of the mitochondrial membrane and thus activate the classic apoptotic pathway as well as other mitochondrial death pathways (Broker *et al.,* 2005).

Regulation of apoptosis

The apoptotic self-destruction machinery is tightly controlled. Various proteins regulate the apoptotic process at different levels. The members of the Bcl-2 family, which regulate apoptosis at the mitochondrial level, are an important class of regulatory proteins. They can be divided into anti-apoptotic and pro-apoptotic proteins according to their function (Hanada *et al.,* 1995; Igney and Krammer, 2002). The

complexity of the apoptotic program began to increase with the discovery of Bcl-2 a gene whose product causes resistance to apoptosis in lymphocytes. Bcl-2 was shown to correct partially the phenotype of a *C. elegans* mutation in Ced-9, a cell survival gene that functions upstream of Ced-4 and Ced-3. This finding suggested an apparent one-for-one correlation between the *C. elegans* and mammalian pro- and antiapoptotic pathways (Nagata, 1997). Although the precise intracellular localization of all proteins contained within this family is unknown, it is clear that Bcl-2 is located in mitochondrial, endoplasmic reticulum and nuclear membrane of different cell types and possesses an anti-apoptotic function. Subsequent analysis demonstrated that Bcl-2 overexpression inhibits cell death (Viera *et al.,* 2002).

Since these early observations, approximately 20 related mammalian polypeptides have been identified. On the basis of functional and structural criteria, these polypeptides can be divided into three groups, the antiapoptotic group I family members and the proapoptotic group II and group III family members. Group I family members, which include Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1/Bfl1, Boo/Diva, Nrf3, and Bcl-B, generally contain four short, conserved BH (Bcl-2 homology) domains, BH1-BH4, and most contain a C-terminal transmembrane domain that targets them to the cytoplasmic surfaces of various intracellular membranes, including the outer mitochondrial membrane and the endoplasmic reticulum. Group II family members, which include Bax, Bak, and Bok/Mtd, lack the N-terminal BH4 domain but contain the other BH domains. Finally, group III family members, which include Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa, PUMA, and Bcl-G, are a more heterogeneous collection of polypeptides that share limited sequence homology only in their 15-amino acid BH3 domains, which is summarized in Figure 5. It has been observed, for example, that cytochrome c release during apoptosis in fibroblasts requires expression of either Bax or Bak, whereas the release of SMAC requires expression of both. The mechanistic basis for this observation remains to be determined (Oliver *et al.,* 2005; Kaufmann, 2007).

The anti-apoptotic members Bcl-2, Bcl-x_L, and Mcl-1 prevent the release of mitochondrial proteins, including cytochrome c, endonuclease G, and AIF, by inhibiting the pore-forming function of BH-3 domain-containing Bcl-2 proteins. Data from human studies suggest that Bcl-2 family proteins might also participate in the regulation of the stress response by interacting with heat shock proteins (Lucianot *et al.,* 2006; Zhang *et al.,* 2005).

Apoptosis can also be regulated by intracellular signal transduction pathways. Several components of the mitogen activated protein kinase (MAPK) pathway extracellular regulated kinase-1/2, c-Jun N-terminal kinase, and p38 pathways are differentially activated depending on the region of brain and timing after injury. Activation of the protein kinase C signaling pathway has also been reported. Pro-survival intracellular signal transduction pathways are also activated after brain

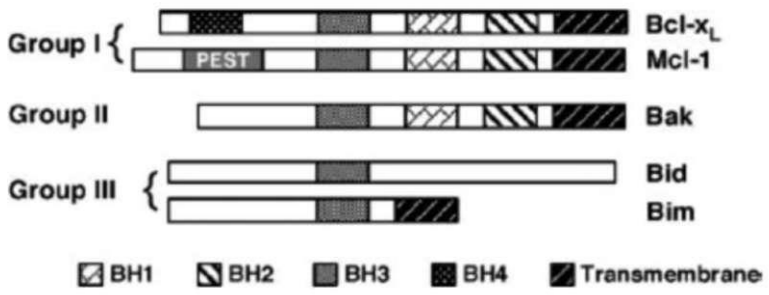

Figure 5. Schematic representation of Bcl-2 family members and related proteins in apoptosis. Group I polypeptides are antiapoptotic and include Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, Boo/Diva, Nrf3, and Bcl-B. Among these, Mcl-1 is unique in lacking a BH4 domain and containing a proline/glutamate/serine/threonine-rich (PEST) sequence, which is often seen in short-lived polypeptides. Group II family members are proapoptotic and include Bax, Bak, and Bok/Mtd. Group III family members, which share limited sequence homology only in their 15-amino acid BH3 domains, include Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa, PUMA, and Bcl-G (Kaufmann, 2007).

injury (Zang *et al.,* 2005). In many tumors, genetic damage apparently fails to induce apoptosis because the constituent cells have inactivated the gene that codes for the p53 protein. This protein, it will be recalled, can lead to activation of the cell's apoptotic machinery when DNA is injured (Duke *et al.,* 1996). The critical role that p53 plays is evident by the large number of tumors that bear a mutation in this gene. Loss of p53 in many cancers leads to genomic instability, impaired cell cycle regulation, and inhibition of apoptosis. After DNA damage, p53 holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered. The mechanism by which p53 promotes apoptosis is still not fully understood (Ghobrial *et al.,* 2005).

Nuclear DNA fragmentation

Apoptosis, or programmed cell death, is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. The nuclear alterations, which are the pre-eminent ultrastructural changes of apoptosis, are often associated with internucleosomal cleavage of DNA, recognized as a "DNA ladder" on conventional agarose gel electrophoresis and long considered as a biochemical hallmark of apoptosis. During apoptosis, nuclear DNA is condensed and degraded into large (50 to 300 kb) and subsequently into small oligonucleosomal fragments of several hundred base pairs. Internucleosomal cleavage of DNA now appears to be a relatively late event in the apoptotic process which in some models may be dissociated from early critical steps (Ghobrial *et al.,* 2005; Elmore, 2007). Biochemical purification by several groups led to the discovery of a caspase regulated DNase complex termed DFF (DNA fragmentation factor) that is composed of a DNase termed CAD (caspase activated DNase: also named DFF40 and CPAN) and its inhibitor ICAD (also named DFF45). In healthy cells, CAD is complexed with ICAD and functionally inactive. In apoptotic cells, ICAD is cleaved by caspase-3 and -7, releasing CAD to degrade nuclear DNA. DFF induces both chromatin condensation and DNA fragmentation *in vitro,* and cells from mice deficient for DFF activity exhibit neither of these classic apoptotic features when induced to die. Another caspase-3-activated factor, named acinus, induces chromatin condensation without affecting DNA fragmentation. The task of DNA fragmentation appears to be separate from other phenotypic aspects of apoptosis. DNA fragmentation is a downstream effect of caspase activation and is dispensable for cell killing. Instead, DNA fragmentation is a way of "hiding the body" and represents one of the multiple parallel execution pathways in apoptosis (Chang and Yang, 2000).

Inhibitor of apoptosis family of proteins

Many natural inhibitors of caspases are known, including several viral and cellular proteins that either act as direct inhibitors of caspases or block the activation of caspases (Kumar, 2007).

IAP gene family is highly conserved in a wide range of organisms ranging from insects to humans. The first IAP was discovered in baculovirus, where it was shown to be involved in suppressing the death of viral-infected host cells. S. Martin discussed the hierarchical nature of the caspase activation cascade that is triggered by cellular stress. Martin's team have shown that Apaf1, caspase-9, caspase-3 and the X-linked inhibitor of apoptosis (XIAP) are the main constituents of the native 'apoptosome', and that cytochrome *c* is not stably associated with the active complex. Martin also presented data obtained from global proteomic analyses of apoptotic cells and discussed the role of specific caspases within this cascade in targeting cellular proteins for degradation. The data suggest that more than 400 proteins are targeted for limited proteolysis during the terminal 'demolition' phase of apoptosis (MarcFarlane and Williams, 2004).

To date, seven mammalian members of the IAP family have been identified; they are XIAP, c-IAP1, c-

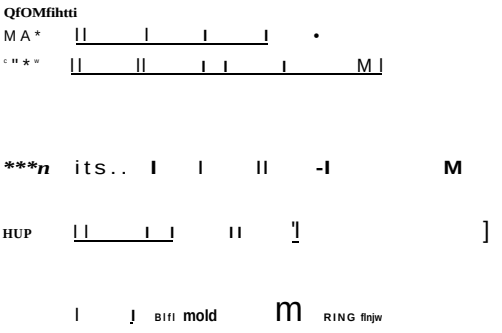

Figure 6. Schematic representation of IAP family members. The proteins were originally identified in baculovirus and are structurally similar (Schulze-Osthoff *et al.,* 1998).

IAP2, NAIP, survivin, livin and Ts-IAP. All of these proteins possess one or more 70- to 80-amino acid baculoviral IAP repeat (BIR) domains, the presence of which is essential for the anti-apoptotic function of the IAPs. Unlike the Bcl- 2 family of proteins, which exert their regulatory effects through the mitochondria, the IAPs directly bind and inhibit caspase-3, 7, and 9 but not other caspases. In addition to the BIR domain, most of the IAPs contain a RING zinc-finger (RZF) near their carboxyl terminus (Figure 6). The balance between IAPs and their negative regulators is postulated to constitute another apoptotic checkpoint, possibly downstream to that comprised by pro- and anti-apoptotic Bcl-2 family proteins (Elmore, 2007; Wauthers and Chiu, 2007). Overall IAPs have a central role in regulating apoptosis. Overexpression of one or more of the IAPs or down regulation of their negative regulator proteins may help suppress apoptosis by allowing unrestricted IAP activity (Zang *et al.,* 2004)

Concluding remarks and future directions

Apoptosis is an essential component of most developmental abnormalities and human diseases and, in many cases, the underlying cause of the resulting pathology. An increased understanding of signalling in apoptosis continues to be a prominent research focus within this field. Apoptosis is a ubiquitous way of cell death in both physiological and pathological conditions. During the past decade there have been major advances in our understanding of the fundamental mechanisms of the apoptotic death program, especially the function of three families of proteins including caspases, Bcl-2 and IAPs. Although significant progress has been made to specifically define and characterize the participation of nuclear changes in apoptotic cell death, this area is still relatively young in so far as a knowledge of essential, compulsory events that must occur in order to elicit nuclear condensation and fragmentation and DNA breakdown is lacking. An understanding of the mechanisms controlling and implementing apoptosis is more than a matter of mere scientific interest. In comparison to the execution phase of the mitochondrial apoptotic pathway, we know a little about how the upstream signaling pathways to the mitochondria are regulated. How are the signals from either developmental cues or damage signals transduced to and integrated in the mitochondria? These questions will be solved in a near future, permitting a general view on the mechanism by which mitochondria regulate apoptosis. From a therapeutic point of view, this knowledge would allow the rational design and use of specific synthetic molecules that mimic Bcl-2-like proteins, IAP-binding factors or the action mode of Smac b. These molecules may not necessarily provoke apoptosis, but they might sensitize cells to apoptotic stimuli, allowing more efficient cancer therapies. Are the BH3- only proteins the major signal transducers? Or are they only part of a more complicated network of proteins? To answer these questions, we will need to develop more sophisticated strategies to identify other players, either through biochemical assays or genetic screens. Only then will we begin to see the big picture of what is happening when cells decide whether to live or die.

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