Apoptosis: Molecular machinery

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The term apoptosis is currently used as a synonym for programmed cell death. It is an essential physiological process required for normal development and maintenance of tissue homeostasis. When dysregulated, apoptosis can contribute to the development of various diseases like cancer, autoimmune or neuro-degenerative diseases. Central components of the apoptotic machinery, which have been conserved during the evolution include Bcl-2, Apaf-1 (apoptotic protease activating factor 1) and caspase family members. Biochemical hallmark of apoptosis includes activation of endonucleases, DNA degradation into oligonucleosomal fragments and activation of a family of cysteine proteases called caspases.

We do not intend to comprehensively review the field, but we have tried to develop a simplified picture of apoptotic mechanism on the basis of recent insights in the area.

APOPTOSIS or programmed cell death (PCD) is a genetically-regulated cellular suicide mechanism that plays a crucial role in the development and defence of homeostasis. The apoptosis process can be divided into at least three functionally distinct phases: initiation, effector and degradation. During the hetrogeneous initiation phase, cells receive death-inducing signals: lack of obligatory survival factors, shortage of metabolite supply, ligation of death-signal transmitting receptors, subnecrotic damage by toxins, heat or irradiation.

During the effector phase, these signals are translated into metabolic reactions and the decision to die is taken. The ultimate fate of the cell is subject to regulatory events. Beyond this stage, during the degradation phase, an increase in the overall entropy, including activation of catabolic enzymes, precludes further regulatory effects. During the late phase, DNA fragmentation and massive protein degradation becomes apparent. Subsequently, fragments are encapsulated into 'apoptotic bodies' that are quietly and unobtrusively consumed by adjacent cells, thereby, leaving little trace of the apoptotic cells' prior existence[1]. Thus, the path to apoptotic cell death is embarked upon when the cell 'senses' or receives a death stimulus[2]. Depending on the type of stimulus and the particular cell type, any one of the many different internal signalling pathways can come into action during the induction phase. However, these pathways converge on one or two control points in the execution or commitment phase of the programme, before the external manifestations of the suicidal or degradation phase become apparent (Figure 1).

Biochemically, apoptotic cells are characterized by reduction in the mitochondrial transmembrane potential, intracellular acidification, production of reactive oxygen species, externalization of phosphatidylserine residues in membrane bilayers, selective proteolysis of a subset of cellular proteins and degradation of DNA into internucleosomal fragments[3–8].

Cells die by apoptosis in the developing embryo during morphogenesis or synaptogenesis and in the adult animal during tissue turnover, immune regulation or at the end of an immune response. Because the physiological role of apoptosis is crucial, aberration of this process can be detrimental. Thus, unscheduled apoptosis of certain brain neurons contributes to disorders such as Alzheimer's and

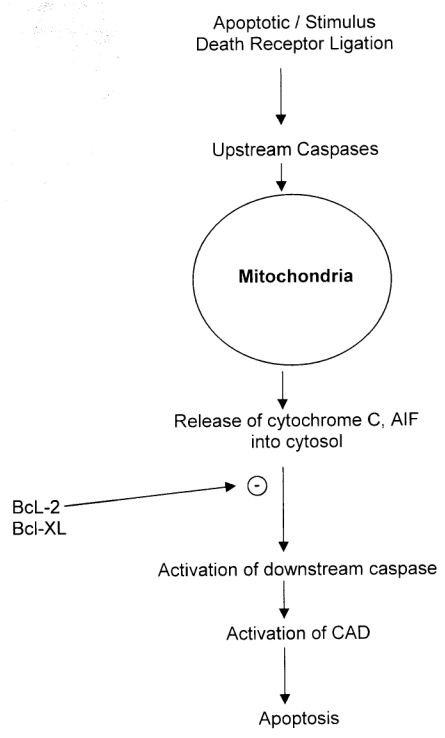

Figure 1. Over simplified view of apoptotic events. See text for description.

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Parkinson's diseases, whereas the failure of dividing cells to initiate apoptosis after sustaining severe DNA damage contributes to cancer[9]. PCD has also been reported in other pathological dysfunctions such as T-cell depletion in HIV infection[10] and mononuclear cell loss in *P. falciparum* and *S. typhimurium* infection[11].

Basic apoptotic machinery

The evolutionary conservation of PCD in animals has meant that genetic studies in worms can illustrate PCD in higher organisms. Genetic analysis in the nematode worm, *C. elegans*, has provided a conceptual framework involving at its heart, three genes. Two of these, cell death defective (*ced*) genes, *Ced3* and *Ced4* are both required for PCD in the worm, whereas another gene, *Ced9*, inhibits the action of *Ced3* and *Ced4* by helping cell survival[12,13]. *Ced3* is a caspase, i.e. a cysteine protease that cleaves certain proteins after specific aspartic acid residues; acts as a zymogen, and is activated through self cleavage[14]. *Ced4* binds to *Ced3* and promotes *Ced3* activation, whereas *Ced9* binds to *Ced4* and prevents it from activating *Ced3* (refs 15–17). Normally, *Ced9* is complexed with *Ced4* and *Ced3*, keeping *Ced3* inactive. Apoptotic stimuli causes *Ced9* dissociation, allowing *Ced3* activation and thereby committing the cell to die by apoptosis. Vertebrates have evolved entire gene families that resemble cell death genes of *C. elegans*. Mammalian caspases are similar to *Ced3* (ref. 14). Apoptosis activating factor 1 (*Apaf-1*) is the only mammalian *Ced4* homologue known so far[18]. The products of the mammalian *Bcl-2* gene family are related to *Ced9*, but include two subgroups of proteins that either inhibit or promote apoptosis[19].

Induction phase

Apoptotic signal can be induced externally, for example, through steroid and cytokine receptors or it may originate inside the cell from the action of a drug, toxin or radiation. Death is rather uncommon consequence of receptor triggering; usually the process is concerned with the transmission of life-enhancing signals such as those that stimulate cell growth and division, Nonetheless, the emerging knowledge of induction of cell death by tumour necrosis factor (TNF) receptors indicates that it happens in the same way as all other receptor-induced effects, i.e. through a series of protein–protein bindings (Figure 2).

The first step involves the binding of specific ligands to the extracellular domains of the receptors. This is followed by sequential binding of cytoplasmic proteins to the intracellular domains of the receptors, eventually leading to the activation of enzymatic function in some of these proteins. In case of death induction, the activated enzymes include caspases, a family of cysteine proteases, whose members occur in cells as latent precursors, becoming activated early in the process of PCD and being central to its development.

Death receptors belong to TNF receptor gene superfamily which is defined by cysteine-rich extracellular domains. The death receptors contain, in addition, a homologous cytoplasmic sequence termed the death 'domain'[20]. Death domains typically enable death receptors to engage the cell's apoptotic machinery, but in some instances they mediate functions that are distinct from or even counteract apoptosis. Some molecules that transmit signals from death receptors contain death domains themselves.

The best characterized death receptors are CD95/Fas/Apo-1 and TNFRI/p55 (refs 21 and 22). Additional death receptors are avian CARI, death receptor 3 (DR3)/Apo3, DR4, TRAMP and DR5/Apo2. The ligands that activate these receptors, with the exception of NGF, are structurally related molecules that belong to the *TNF* gene superfamily[21]. CD95 ligand (CD95L) binds to CD95; TNF and lymphotoxin alpha bind to TNFRI; Apo3 ligand (Apo3L) binds to DR3 and Apo2 ligand (Apo2L/TRAIL) binds to DR4 and DR5.

CD95 signalling

CD95 and CD95L play an important role mainly in three types of physiological apoptosis[22]: (i) peripheral deletion of activated mature T-cells at the end of an immune res-

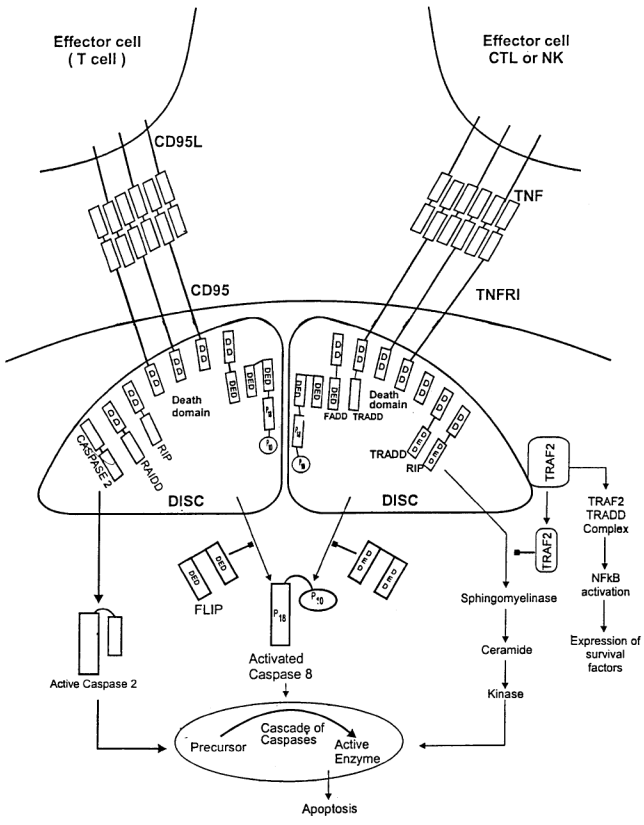

Figure 2. Fas and TNF-mediated apoptotic pathways.

ponse, (ii) killing of targets such as virus-infected cells or cancer cells by cytotoxic T-cells and natural killer cells, and (iii) killing of inflammatory cells at 'immune privileged' sites such as the eye. Evidence for the biological role of CD95 comes from certain mouse strains and from human patients who have defective genes for CD95 and CD95L[22]. Such mutations can lead to accumulation of peripheral lymphoid cells and to a fatal autoimmune syndrome characterized by massive enlargement of lymphnodes. CD95 and CD95L are implicated also in pathological suppression of immune surveillance, namely elimination of tumour-reactive immune cells by certain tumours that constitutively express CD95L[23].

Like other TNF family members, CD95L is a homotrimeric molecule and binds three CD95 molecules[21,22]. Because death domains have a propensity to associate with one another, CD95 ligation leads to clustering of the death domains of the receptors. An adaptor protein called FADD (Fas-associated death domain) then binds through its own death domain to the clustered receptor death domains.

Once incorporated into the ligand bound death receptor complex (the death inducing signalling complex or DISC) FADD relies on a distinct protein–protein interaction module in its amino terminus, the death effector domain (DED) to engage the upstream caspases and induce apoptosis. Specifically, FADD recruits pro-caspase-8 and/or procaspase-10 to the DISC via their respective DEDs. Delivery of these DED-bearing caspase proenzymes to the DISC leads to their proteolytic activation[24]. The death effector domain is a specific example of a more global homophilic interaction domain termed CARD (caspase recruitment domain), which is found in several caspases with large prodomains, including caspases 2, 8, 9 and 10 (ref. 24). Caspase 8, then activates downstream effector caspases such as caspase 9, the mammalian functional homologue of Ced3; committing the cell to apoptosis.

Studies with *FADD* gene knockout mice[25] and transgenic mice expressing a dominant negative mutant of FADD (FADD-DN) in T-cells[26,27] establish that FADD is essential for apoptosis induction by CD95. Surprisingly, these mice display reduced proliferation of mature T-cells in response to antigenic stimulation. Moreover FADD deletion causes embryonic lethality[25,26]. These results are consistent with FADD having other critical signalling functions besides coupling CD95 to caspase 8.

A family of viral proteins called VFLIPs (Fas-linked inhibitory protein) and a related cellular protein called cFLIP[28] contain a DED that is similar to the corresponding segment in FADD and caspase 8. The role of FLIP is controversial, as FLIP over-expression either inhibits or activates apoptosis[28,29].

Signalling by TNFRI

TNF is produced mainly by activated macrophages and T-cells in response to infection. By engaging TNFRI, TNF activates the transcription factors NF-κB and AP-1, leading to induction of pro-inflammatory and immunomodulatory genes[30]. In some cell types, TNF also induces apoptosis through TNFRI. Unlike CD95L, however, TNF rarely triggers apoptosis unless protein synthesis is blocked, which suggests the pre-existence of cellular factors that can suppress the apoptotic stimulus generated by TNF. Expression of these suppressive proteins probably is controlled through NF-κB and JNK/AP-1 as inhibition of either pathway sensitizes cells to apoptotic induction by TNF[31,32].

TNF trimerizes TNFRI upon binding[21], thereby inducing association of the receptor death domains. Subsequently, an adapter termed TRADD (TNFR-associated death domain) binds through its own death domain to the clustered receptor death domains[33]. TRADD functions as a platform adapter that recruits several signalling molecules to the activated receptor: TNFR-associated factor 2 (TRAF2)[34] and receptor interacting protein (RIP)[35] which stimulate pathways leading to the activation of NF-κB and of JNK/AP-1, whereas FADD mediates activation of apoptosis[36,37].

TRAF2 and RIP activate the NF-κB-inducing kinase (NIK), which in turn activates the inhibitor of κB kinase (I-κB kinase complex; IKK)[38–40]. IKK phosphorylates I-κB, leading to I-κB degradation and allowing NF-κB to move to the nucleus to activate transcription. The pathway from TRAF2 and RIP to JNK involves a cascade that includes the mitogen-activated protein (MAP) kinases MEKK1, JNKK (JNK kinase) and JNK[41,42].

TRAF2-deficient cells are totally lacking in JNK activation in response to TNF, demonstrating a critical role for TRAF2 in this response. The picture emerging from RIP-deficient cells is the inverse: NF-κB activation in response to TNF is absent, whereas JNK activation is intact[43]. Hence, RIP is required for coupling TNFRI to NF-κB, but it may not be crucial for coupling TNFRI to JNK.

Both TRAF2 and RIP knockout mice have pathologies that cannot be ascribed to defects in TNF signalling, which suggests that each of these proteins has additional functions. TRAF2 also binds to cIAP1 and cIAP2 (cellular inhibitor of apoptosis 1 and 2)[44], which belong to a family of mammalian and viral proteins with anti-apoptotic activity.

FADD couples with TNFRI–TRADD complex for activation of caspase-8, thereby initiating apoptosis[36]. Cells from FADD knockout mice are resistant to TNF-induced apoptosis, demonstrating an obligatory role of FADD in this response[25]. Besides FADD, TNFRI can engage an adapter called RAIDD, which binds through a death domain of RIP and through a CARD motif to a similar sequence in the death effector caspase 2, thereby inducing apoptosis.

Signalling through DR3, DR4 and DR5

DR3 shows close sequence similarity to TNFRI. Upon expression, DR3 triggers responses that resemble those of

TNFRI, namely NF-κB activation and apoptosis. Similar to CD95 ligand, another TNF family member Apo2L/TRAIL, which binds to DR4 and DR5 triggers rapid apoptosis in many tumour cell lines[45].

Signal transduction pathways to apoptosis

Some physiologically important apoptotic stimuli (e.g. TNF-α and Fas ligand) do not require RNA or protein synthesis but directly link their receptors to ICE-protease. Others like calcium, glucocorticoid or radiation, withdrawal of growth factors and oxidative stress require macromolecular synthesis to activate core apoptotic programme directly. A number of signalling events regulate apoptosis in diverse model systems. Two members of MAP/ERK family are stress activated protein kinase (Jun-N-terminal kinase, JNK) and p38 kinase, both of which are activated in stress. The candidate substrates for these kinases are c-JUN, ATF2 and ELK-1; phosphorylated c-JUN may be required for activation of downstream events in the apoptotic programme[46]. A common feature of stress-induced apoptosis is the rapid generation of ceramide, a lipid mediator liberated by the hydrolysis of sphingomyelin. Ceramide directly activates protein phosphatase A2 and JNK which has a direct role in apoptosis. Enzymatic degradation of membrane phospholipids liberates second messengers that activate serine/threonine kinases. The ability of phorbol esters to potentiate or inhibit apoptosis supports a role for PKC isoforms in cell death. Analysis of individual isoforms has implicated PKCβ and PKCδ. Proteolytic activation of PKCδ appears to involve ICE-like protease[47] and one candidate substrate is Bcl-2. Likely target in the nucleus includes AP-1, a transcription factor. Over-expression of R-Ras can activate JNK or p38 kinase to regulate entry into apoptotic pathway[48]. Cyclin-dependent kinase (CDC2/Cyclin B) activation is required for some forms of apoptosis. Another cell cycle protein, CDC25, a tyrosine phosphatase has been implicated in regulating entry into apoptosis and it co-operates with Ha-Ras in potentiating induction of apoptosis.

Effector phase

In the effector phase, apoptosis initiating events are translated into a regular pattern of metabolic reactions and the 'decision to die' is taken. Thus, the ultimate fate of the cell during the effector phase is subject to regulatory events. It is commonly accepted that Bcl-2, a mammalian Ced-9 homologue and its homologues regulate the effector phase of apoptosis.

Role of p53 in apoptotic machinery

The p53 is a nuclear DNA binding phosphoprotein that normally exists as a homotetramer. It is transcriptionally activated as a specific set of genes and can exert repression. Triggers for p55-dependent apoptosis include DNA damage, cytokines deprivation, hypoxia, shock or abnormal oncogene activation[49]. Activation of p53 by phosphorylation can lead to transcription of its target gene by interaction with p53 response motives. The N-terminus consists of two transcription activating sub-domains extending between residues 1 and 63 and a region spanning residues 60–97, containing several copies of the sequence PXXP, plays an important role in induction of apoptosis by p53 (ref. 50). Among several proposed downstream events following p53 activation, one mechanism requires transcriptional activation perhaps of bax, a member of Bcl-2 family. The p53 can directly interact with proteins like TFII H protein complex. The p53 inhibits helicase activity of this complex by binding to XBP and XPD subunit[51]. TFII H protein complex participates in basal transcription, nucleotide excision repair and cell cycle. In a state of stress, the level and state of p53 is altered, which leads to transcriptional activation of different target genes. The kinetics and spectrum of downstream targets vary from one cell type to another. The p53 can cause a cell arrest at G1 by activating cyclin-dependent kinase inhibitor, p21 and can regulate dependence on cytokine survival factor. Indeed, a plethora of functions have now been ascribed to p53. The p21 activated pRb, a 105 kDa phosphoprotein can induce growth arrest by associating with E2F, a transcription factor complex.

Caspases

The cloning of Ced3 helped to observe that it encoded a protease homologous to the mammalian ICE, a novel cysteine protease with aspartate specificity required for proteolytic conversion of pro-interleukin 1β into its mature form[52,53]. Together with the concurrent finding that apoptosis could be induced in mammalian cells by transient expression of ICE or Ced3 (ref. 54), these studies provided the first inkling that a conserved family of proteases might be a critical component of the cell death machinery. At the present time, at least 10 additional mammalian homologues of Ced3 have been published, and the trivial names of these cysteine proteases with aspartate specificity (caspases) have been replaced with a uniform nomenclature: caspases 1 through 11 (refs 55 and 56) (Figure 3).

In addition to their sequence similarity to Ced3, the members of the caspase family of proteases have several unifying characteristics. As their name indicates, caspases are cysteine proteases, each containing a conserved QACXG pentapeptide surrounding the active site cysteine residue. Several other active site residues that participate directly in catalysis are strictly conserved across the family.

Caspases are constitutively and ubiquitously expressed as catalytically inactive proenzymes composed of variable length amino-terminal prodomain, a large subunit and a

small subunit. Caspase activation requires proteolytic processing of the proenzyme at specific aspartate residues separating these three domains, thereby resulting in the removal of the prodomain and the formation of a heterodimer containing one large and one small subunit. Crystallographic analysis has demonstrated that the active caspase is a tetramer composed of two such heterodimers[57,58]. Although absent from the active enzyme, the amino terminal prodomain plays a critical role in caspase activation by mediating the interaction of caspases with the activating apparatus. True to their family name, these proteases are 'aspases'. They cleave substrates (including pro caspases) carboxy terminal to an aspartate residue (P1site). This distinctive substrate specificity, coupled with their requisite proteolytic activation at Asp sites, immediately suggested that caspases might be responsible for their own activation (autoproteolysis) and/or activation of other caspases.

Despite their uniform requirement for an Asp residues at the substrate P1 site, individual caspases differ in their substrate specificities. These differences are dictated by the amino acids immediately amino terminal to the substrate P1 site, especially the P4 site (four amino acids amino terminal to the cleavage site).

The observation that some pro-caspases (3, 9 and 11) are recruited directly to membrane receptors via their prodomains provided important experimental evidence for the proteolytic cascade theory of caspase signalling. These receptor interacting caspases are at the very top of this cascade. From a functional standpoint, caspases can be loosely divided into upstream 'instigators', which incite the proteolytic cascade and downstream 'terminators', which kill the cell by cleaving key intracellular death targets.

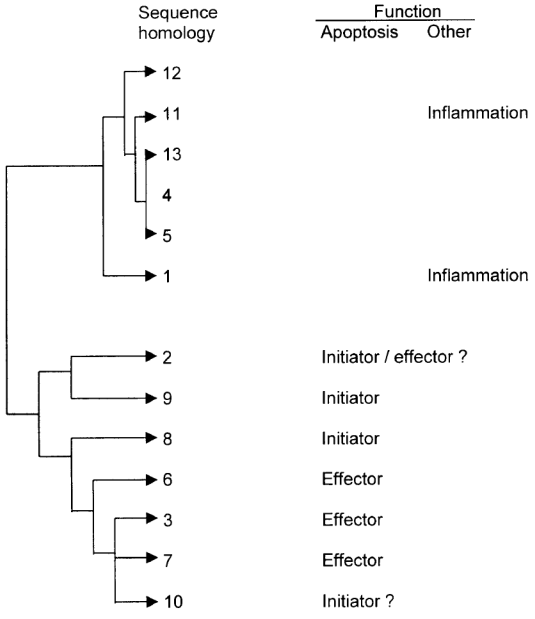

Figure 3. Caspase classification and function.

The instigators include the long prodomain, DED-containing caspases 8 and 10, and the CARD containing caspase-9. Of note is the fact that substrate cleavage preferences of these instigators correspond closely to the sites of proteolytic activation in several effector caspases, suggesting that these upstream proteases may directly activate their downstream counterparts, as has been demonstrated *in vitro*. In contrast, the terminators include the short prodomain caspases 3 and 7, whose predilection for proteolyzing targets at DXXD motifs matches the cleavage sites in vast majority of known apoptotic substrates[59–62].

Following their meticulously guarded activation, the terminator caspases initiate their deadly assault on the cell by selectively cleaving a number of 'death substrates'[63]. In addition to modulating the activity of key regulatory proteins such as kinases, caspases proteolyze structural proteins like gelsolin, microfilament protein Gas2 and nuclear lamins.

Caspases play an essential role in the internucleosomal DNA laddering that typifies apoptotic cell death by activating a latent, cytosolic endonuclease, CAD (caspase-activating deoxyribonuclease). CAD is normally sequestered in the cytoplasm via its binding to an inhibitor (ICAD/DFF45), which suppresses its endonuclease activity and conceals its nuclear localization signal. During the induction of apoptosis, ICAD/DFF45 is cleaved and inactivated by a caspase 3-like protease, thereby resulting in the activation of CAD, its nuclear translocation and subsequent oligonucleosomal DNA fragmentation. Overexpression of wild type or caspase cleavage-resistant ICAD/DFF45 protects cells from apoptotic DNA fragmentation, but does not prevent phosphatidyl serine externalization, mitochondrial injury, or cleavage of other caspase targets, again indicating that ICAD/DFF45 is but one of several downstream caspase targets[64,65]. Nevertheless, this elegant simple strategy directly couples caspase activation to DNA degradation.

Bcl-2 and Bcl-XL, two anti-apoptotic Bcl-2 family members, are also cleaved by caspases, at least during some types of apoptotic cell deaths[66,67]. Caspase cleavage generates a carboxy terminal fragment with radically different properties from their full-length counterparts. They promote, rather than antagonize, cell death.

Thus, it is evident that caspase-mediated proteolysis of specific intracellular targets is both necessary and sufficient to produce many of the characteristic features of apoptosis. However, it is yet to be understood how these seemingly chaotic, multiple proteolytic events, collectively produce a very ordered series of cellular changes culminating in death. Moreover, it is unclear whether caspase proteolysis is responsible for all aspects of apoptotic cell death or whether caspase-independent apoptotic phenomena exist. Recent studies suggest that apoptosis triggered by some stimuli (e.g. over-expression of pro-apoptotic Bcl-2 family members) involves components

(membrane blebbing/permeability alterations, loss of mitochondrial membrane potential and generation of reactive oxygen species) that are refractory to caspase inhibition[68,69]. In these settings, caspase inhibitors do not prevent cell death; death proceeds in a delayed/atypical fashion. These issues are not merely of academic interest, but have a direct bearing on the potential efficacy of therapeutic strategies designed to eliminate cell death by inhibiting caspases.

Caspase activation: Jump-starting the proteolytic engine

Under normal conditions, caspases are present in the cytosol as inert proenzymes that pose no danger to the cell. Two potentially interacting, cascade-initiating pathways converge on the activation of the downstream effector caspases (terminators such as caspase 3), that act to kill the cell by cleaving death substrates. The first of these pathways is initiated in response to apoptotic stimuli such as DNA damaging agents that trigger the release of mitochondrial cytochrome C into the cytoplasm. Once in the cytoplasm, cytochrome C interacts with other factors to form a caspase 3 activating complex.

In the second pathway, caspase proenzymes are recruited to ligand-bound death receptors via homophilic interactions with adapter proteins, thereby leading to the proteolytic activation of these most upstream caspases and initiation of the cascade. These signalling routes may cross communicate, prior to their convergence. For instance, ligand binding to death receptors has been shown to stimulate mitochondrial cytochrome C release in most (but not all) studies[70–73]. Mitochondrial cytochrome C release induced by death receptor ligation may be executed by the upstream caspases themselves[71], whereas its efflux triggered by other apoptotic stimuli is likely to be a caspase-independent event[74–77].

Both of these caspase-activating pathways have multiple barriers to prevent inappropriate cell death. Endogenous inhibitors block the initiation of caspase cascade and/or interrupt its forward progression (Figure 4).

The initially perplexing discovery that cytochrome C, a key component of the mitochondrial electron transport chain, is necessary for the induction of apoptosis in a cell free system, led several investigators to examine its role in apoptosis. Consistent with its pro-apoptotic role *in vitro*, cytochrome C is rapidly released from the mitochondrial intermembrane space into the cytoplasm during the induction of apoptosis by diverse stimuli. Importantly, the release of cytochrome C into the cytoplasm (triggered by stimuli other than death receptor ligands) precedes caspase 3 activation and DNA fragmentation, and is in fact required for these activities. Bcl-2 and its anti-apoptotic relative Bcl-XL are located predominantly in the outer mitochondrial membrane. Their proximity to cytochrome C suggested that they might regulate the latter's release during apoptosis. Indeed, over expression of Bcl-2/Bcl-XL or addition of recombinant Bcl-2 to Xenopus egg extracts containing mitochondria, prevented exodus of cytochrome C from the mitochondria that was triggered normally by apoptotic stimuli such as DNA damaging agents and staurosporine; caspase inhibitors had no effect on this process[74–77]. In these systems, Bcl-2 acts upstream of cytochrome C release within the mitochondria. In contrast, the pro-apoptotic Bcl2 family member Bax, stimulates mitochondrial cytochrome C release[78]. The molecular mechanisms by which Bax stimulates and Bcl-2/Bcl-XL inhibits mitochondrial egress of cytochrome C is unclear, but may be related to their ability to form membrane pores with distinct ion conducting properties; Bcl-2 also inhibits the ability of Bax to form lipid channels[79–81]. Moreover, Bcl-XL can bind to cytochrome C and may thereby act to sequester it in the mitochondria[74]. Furthermore, Bcl-XL inhibits the osmotic swelling of mitochondria and subsequent outer membrane disruption induced by apoptotic stimuli that may directly lead to the cytosolic efflux of cytochrome C[71].

To further complicate matters, recent evidence indicates that Bcl-2 and Bcl-XL may also act downstream of cytochrome C to prevent caspase activation under certain circumstances. For instance, over expression of Bcl-XL or Bcl-2 inhibits apoptosis induced by direct micro injection of cytochrome C into cells[82,83]. Regardless of the precise molecular mechanisms, the antiapoptotic actions of Bcl-2/Bcl-XL reflect their combined ability to prevent cytochrome C release in some situations and to inhibit cytochrome C-induced caspase activation in others, a true testament of its versatility.

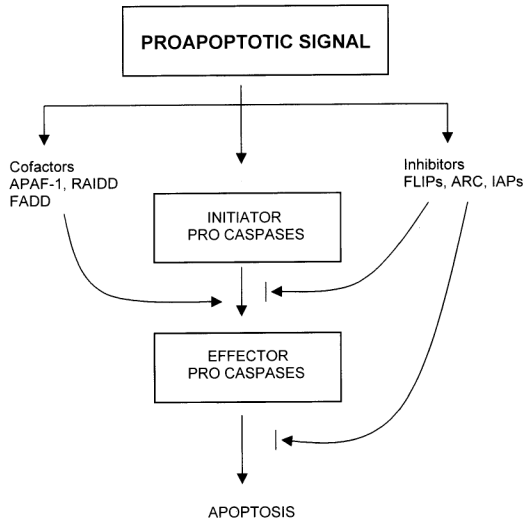

Figure 4. Regulation of caspase activation. Available evidence suggests that apoptotic signal initiates three pathways involving cofactors, initiator caspases and inhibitors. Activation of cofactors (e.g. cytochrome relocation from mitochondria to cytoplasm), modification of caspase 8 to a receptor complex and inactivation of inhibitors, together result in activation of initiator caspase. Active caspases may also be involved in feedback mechanisms.

Role of mitochondria

During the apoptotic process, the mitochondrial transmembrane potential ($\Delta\psi_m$) is frequently disrupted before caspases and endonucleases are activated. This transmembrane potential ($\Delta\psi_m$) collapse is prevented by agents inhibiting the mitochondrial mega channel (also called permeability transition pore or multiple conductance channel), a composite ion channel that traverses the inner and outer mitochondrial membranes at sites of contact between them. The mega channel complex comprises the adenine nucleotide translocator (inner membrane), the voltage-dependent anion channel (outer membrane), cyclophilin D (matrix) and copurifies with several apoptosis-regulating proteins, including Bag-1 and Bax, but not Bcl-2 or Bcl-XL[84]. The mega channel participates in the regulation of matrix Ca^{2+}, pH, ($\Delta\psi$m) and volume functioning as a Ca^{2+}, voltage-, pH-, and redox-gated channel with several levels of conductance and little, if any, ion-selectivity. The mitochondrial mega channel might constitute a point of integration of multiple pro-apoptotic pathways, because several pro-apoptotic second messengers, including Ca^{2+} ions, ROS, p53 induced changes in cellular redox potentials, ceramide-derived ganglioside GD3, recombinant caspases and Bax facilitate its opening. At its high, irreversible level of conductance, the mega channel causes disruption of the ($\Delta\psi$m) (Figure 5).

The reduction in transmembrane potential (depolarization) is a relatively early Bcl-2 inhibitable event in apoptosis[8,85]. However, in several different apoptotic systems (e.g. cell death induced by DNA damaging agents and staurosporine), cytochrome C release preceded mitochondrial membrane potential changes by many hours; in Xenopus egg extracts, cytochrome C release occurred during apoptosis, even though mitochondrial membrane depolarization was never observed[71–74]. Moreover, during apoptosis induced by DNA damage or protein kinase inhibition, disruption of the mitochondrial membrane potential, but not cytochrome C release is prevented by caspase inhibitors[74,75,77,86].

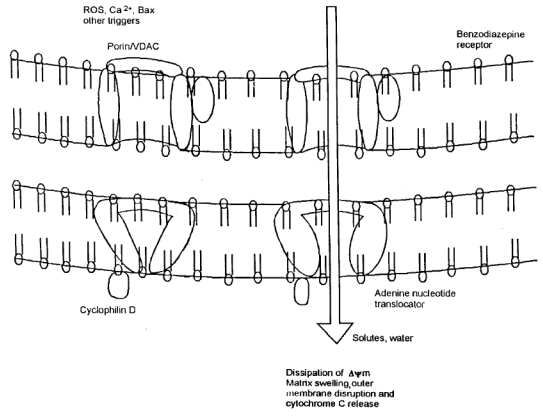

Figure 5. Mitochondrial permeability transition model showing components of the permeability transition pore. In the open state, water and solutes enter the matrix causing membrane swelling, outer membrane disruption and cytochrome C release.

These studies suggest that the loss of the mitochondrial membrane potential, is a consequence, not an antecedent, of caspase activation. Caspase 1 can induce membrane depolarization in isolated mitochondria, a process associated with the release of a pro-apoptotic, yet to be identified caspase–apoptosis inducing factor (AIF) that may play an accessory role in apoptosis[86]. Nevertheless, the caspase-mediated disruption of the mitochondrial membrane potential might serve to amplify the death signal by facilitating the mitochondrial release of cytochrome C during death receptor-mediated apoptosis and the later stages of PCD induced by DNA damaging agents[87].

More recent studies suggest that many apoptotic (and necrotic) stimuli induce progressive mitochondrial swelling that ultimately ruptures the outer mitochondrial membrane, thereby allowing cytochrome C to leak out of the mitochondria into the cytosol[71]. Importantly, these newly recognized apoptotic events in the mitochondria (swelling and subsequent membrane rupture) are prevented by Bcl-XL and precede mitochondrial membrane depolarization; hence they may represent the primary trigger for cytochrome C release during cell death.

Apoptosome

Upon its release into cytoplasm during the initiation of apoptosis, cytochrome C forms a caspase 3-activating complex, the apoptosome. The activating components of this complex have been meticulously teased out in several elegant biochemical studies[18,88,89]. These investigators observed that addition of dATP to a cell free extract prepared from non-apoptotic cells led to caspase 3 activation and DNA fragmentation, when exogenous nuclei were added. This caspase 3-activation complex could be completely reconstituted by four components; dATP, cytochrome C, the mammalian Ced4 homologue Apaf-1, and caspase 9 (Figure 6). Omission or immuno-depletion of any of these factors prevents caspase 3 activation. Apaf-1, the mammalian Ced4 homologue has three functional domains: an amino terminal CARD with homology to Ced-3 and several caspases; a middle Ced4-like domain that includes a conserved nucleotide binding module (P-loop); and a carboxy terminal region with many WD

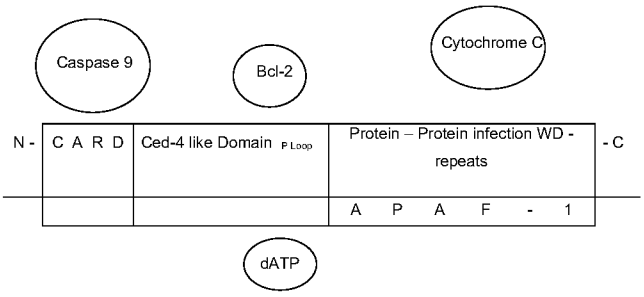

Figure 6. Model of *Apaf-1* regulation by the *Bcl-2* family. Bcl-2/Bcl-XL may bind *Apaf-1* and prevent it from activating pro-caspase 9.

repeats (a presumptive protein interaction motif). Apaf-1 binds to caspase 9 via its respective amino terminal CARD domains, an interaction that requires dATP and cytochrome C. Binding of dATP and cytochrome C to Apaf-1 is likely to alter the latter's conformation and render its CARD domain more readily available to caspase 9. dATP (or higher concentrations of ATP) may interact with the nucleotide binding domain in Apaf-1, that is conserved in Ced4; mutations of this region in Ced4 impair its ability to activate Ced3 and induce apoptosis[15]. Once bound to caspase 9, Apaf-1 triggers proteolytic self-activation of caspase 9; caspase 9 subsequently proteolyzes and activates caspase 3 (Figure 7).

The apoptosome, then is a caspase 3-activating apparatus, whose assembly is dependent on the antecedent release of cytochrome C from mitochondria. Recently, Bcl-XL has been shown to bind specifically to the Ced4-like domain of Apaf-1. This anti-apoptotic Bcl-2 family member is displaced from the apoptosome by its pro-apoptotic relatives Bax and Bak[90]. Although, the functional significance of this binding (and its precise intracellular locale) is yet to be determined, one could certainly envision an 'evolutionary correct' scenario, whereby Bcl-XL would interfere with the ability of Apaf-1 to activate pro-caspase 9, perhaps this is the basis for the inhibition of caspase activation downstream of cytochrome C release by Bcl-2/Bcl-Xl.

Bcl-2 family of apoptotic regulators

Bcl-2, the cardinal member of the family, was identified originally as the protooncogene involved in the translocation in human follicular lymphoma[14,18]. It turned out to be an unusual oncogene whose expression did not provide a proliferative advantage to the cell, but instead enhanced its capacity to survive under suboptimal conditions. A number of Bcl-2-related proteins have now been identified. While some are anti apoptotic-like Bcl-2, others appear to promote apoptosis. Among the death inhibitors are Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, adenovirus E1B19K, Epstein Barr virus (EBV) BHRF1 and Ced9 (*C. elegans*). Death promoters include Bax, Bak, Bcl-Xs, Bok, Bad, Bik, Bid, Bim, Hrk and Blk[91].

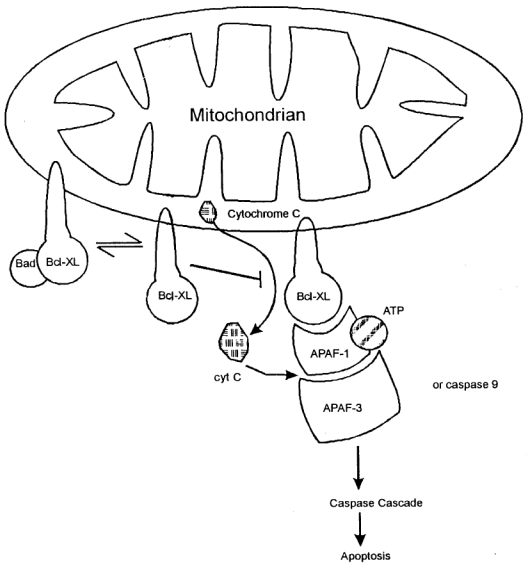

Figure 7. Apoptosome: Association of Bcl-XL and *Apaf-1* block pro-caspase 9 activation. A death signal may provoke interaction of pro-apoptotic Bcl-2 member with Bcl-XL, preventing it from neutralizing *Apaf-1*. *Apaf-1* can bind to pro-caspase 9 in presence of released cytochrome C and ATP and promote its activation.

Bcl-2-related proteins share homology in one to four regions designated the Bcl-2 homology (BH) domains BH1, BH2, BH3 and BH4. While all four homology regions are present in some of the proteins, including Bcl-2 and Bcl-XL, many others lack one or more of these domains. Some pro-apoptotic family members, such as Bad, Bid and Bim, contain only the BH3 region. Most proteins in the Bcl-2 super family also harbour a C-terminal signal which anchors sequences that target them, predominantly to the outer mitochondrial membrane, endoplasmic reticular membrane and the outer nuclear envelope. Another distinguishing feature of this family is the ability of its members to interact with one another to form heterodimers, and in some cases, homodimers. The BH1 and BH2 domains are found in all death antagonists of the Bcl-2 family, but only in one class of death agonists. Residues in the BH1 and BH2 domains are essential in the survival function of the death suppressors such as Bcl-2 and Bcl-XL and for interactions of these proteins with death agonists such as Bax and Bak. In the case of death agonists such as Bak and Bax, which possess intact BH1 and BH2 domains, it is the BH3 region that is important in apoptotic activity (Figure 8).

The crystal structure of Bcl-XL revealed a similarity to pore-forming domains of bacterial toxins such as colicins A1 and E1 and diphtheria toxin. Subsequent studies confirmed that, like bacterial toxins, Bcl-2, Bcl-XL and Bax can insert into synthetic lipid vesicles and planar lipid bilayers and form ion-conducting channels. Domains BH1 and BH2 flank the two hydrophobic core helices $\alpha 5$ and $\alpha 6$, which are predicted to form the channel. It is reasonable to speculate that residue and charge variability in this region between proteins such as Bcl-2 and Bax could translate into differences in ion selectivity and conductance. The ion channels formed by Bcl-2, Bcl-XL and Bax differ slightly in their selectivity at neutral pH, Bcl-2 and Bcl-XL being more selective to cations. All these proteins form channels that display multiple conductance states, which might be a reflection of whether the membrane-inserted proteins are in a monomeric or oligomeric state.

Many instances of the suppression of apoptotic symptoms by Bcl-2 and Bcl-XL point to ability of these proteins to form channels. Mitochondrial permeability transition, alterations in the subcellular distribution of Ca^{2+} and H^{+}, and the subsequent collapse of the mitochondrial

membrane potential almost universally herald the onset of apoptosis[85–92] and over expression of Bcl-2 or Bcl-XL can, for the most part, prevent these events from occurring[75,76]. Apoptosis is preceded by the release of cytochrome C into the cytosol, and Bax insertion into mitochondrial membranes can induce this release[78]. Bcl-XL prevents cytochrome C release possibly by preventing or delaying mitochondrial swelling and outer membrane rupture[71].

Recently, liposomes that contain the mitochondrial porin channel (also called the voltage-dependent anion channel or VDAC) were created[92] to show that the recombinant pro-apoptotic proteins, Bax and Bak, accelerate the opening of VDAC, whereas the anti-apoptotic protein Bcl-XL closes VDAC by binding to it directly. Bax and Bak allow cytochrome C to pass through VDAC out of the liposomes, but passage is prevented by Bcl-XL, pointing to the fact that Bcl-2 family of proteins bind to the VDAC in order to regulate mitochondrial membrane potential and release cytochrome C during apoptosis. VDAC is a part of polyprotein channel called the permeability transition (PT) pore, the other members of which are adenine nucleotide translocator (ANT) and cyclophilin-D. Bax and Bak-dependent changes in mitochondrial membrane permeability that lead to a loss of membrane potential $\Delta\psi m$ and cytochrome C release are mediated by this permeability transition pore[84,93,94].

Genetic control of apoptosis

Despite the emergence of good evidence for the induction and inhibition of apoptosis by specific genes, it is clear that apoptosis independent of genes and of new protein synthesis can be induced by a wide variety of epigenetic factors which are not cell lineage-specific. During the development of nematode *C. elegans*, 131 of the 1090 cells undergo apoptosis. A total of 14 genes control this process. Two genes, *ced3* and *ced4* appear to induce apoptosis and *ced9* inhibits apoptosis in cells destined to live. At least seven genes are involved in dispersal and degradation of dead cells. NUC-1 is required for the degradation of DNA. Nematodes appear to share with mammals, at least part of a common pathway to apoptosis. Thus, over-expression of *ced9* or bcl-2 inhibits apoptosis and over-expression of *ced3* or ICE induces PCD showing sequence and structural homology to mammalian proto-oncogene *bcl-2* (ref. 14). A gene called *reaper* (*rpr*) controls apoptotic death in *Drosophila*. The *rpr* gene product is a small peptide that shows some similarity with mammalian death domains CD95 and TNF R1. Experimental data indicate that the cell death gene is located within an 85 kb interval of the H99 deletion of genome region 75Cl-2 of chromosome 3 and encodes 9 single polyadenylatid transcripts of approximately 1300 nucleotides. RPR mediates its action by activating ICE/*ced3*-like protease in a same fashion as FADD and CD95 in mammals.

The gene *bcl-2* (B cell lymphoma 2) was first identified on human chromosome 18 as the site of reciprocal translocation in follicular B cell lymphoma and encodes a membrane associated protein Bcl-2. Bcl-2 synergises with another oncogene *c-myc*, in mammalian tumour progression, Bcl-2 suppresses *c-myc*-induced apoptosis. There is a general correlation between expression of Bcl-2 (or Bcl-XL) or decreased expression of related protein, Bax (a cell death promotion) and uncontrolled tumour growth[95].

The *c-myc* protooncogene encodes a short-lived sequence-specific DNA-binding nuclear phospho-protein (c-myc), the expression of which is deregulated in virtually all tumours. The c-myc protein appears to be a transcription factor and possesses an N terminal domain with transcriptional activity and C terminal DNA binding/dimerization basic helix–loop–helix leucine zipper domain. The target genes have not been clearly defined, but transactivation of target gene occurs after heterodimerization with another protein partner, Max. Genes such as *c-myc* exhibit a dual role, the pathway depending on associated growth factors and kinase, for example, in the absence of 1L-2, apoptosis is induced but in the presence of 1L-2 lymphocyte proliferation is induced[96].

The tumour suppressor phosphoprotein, p53, has a molecular weight of 53,000 daltons and is involved in mediating the cellular response to DNA damage. The tumour suppressor gene, *p53*, mediates growth arrest through its role as transcription activator, it induces the expression of 21 kDa protein, Waf-1 (wild type p53-activated fragment) or Cip-1 (CDK-interacting protein) which inhibits cyclin–kinase complexes and transactivates genes such as *GADD45* (a growth arrest and DNA damage responsive gene). The

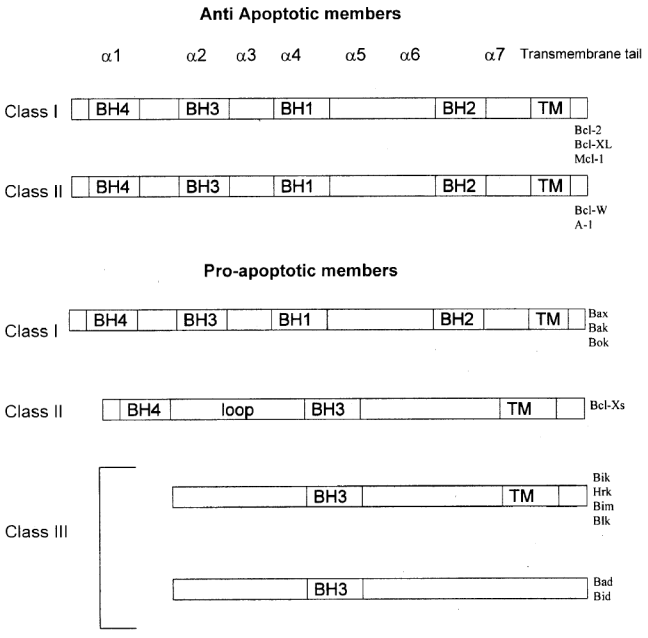

Figure 8. Classification of Bcl-2 family on domain organization. The general architecture is shown. Bcl-2 homology (BH) domains and transmembrane (TM) domains are labelled. A few proteins, such as A-1, Bax and Bak exhibit weak homology to Bcl-2 in the BH4 region. α-helices 5 and 6 are predicted to participate in channel formation.

p53 can control apoptosis by transcriptionally dependent and independent mechanisms. Bax activation and/or Bcl-2 repression by p53 shifts the balance towards cell death; p53-independent negative regulatory element (NRE) has been described in *bcl-2* gene suggesting independent regulation. On the other hand, p53 strongly transactivates bax promotor and thus induces marked elevation in bax protein levels[51].

Protooncogene, *c-fos* has a role in protecting and positively regulating survival, but *c-fos* acts in a tissue-specific manner and exerts stimulus-specific control to selectively protect or suppress apoptosis. Optimal, but not over-expressed levels of *c-fos*, may be required for cell survival. Anti *c-fos* partially suppressed the NF-κB complex[97].

NF-κB controls transcription of *c-myc*, genes for p53 and TNF-α. Promotors of *fas-L* and *fas-R* genes also have NF-κB binding sites. The mechanism of NF-κB action is based on the release of Rel A-p50 from cytoplasmic inhibitor, IκBα. The maintenance of lymphocyte homeostasis by apoptosis is a negative mechanism and transcription factor NK-κB has an important role in protecting death mediated by TNF. Cells not able to activate nuclear factors are more susceptible. Fas itself may activate NF-κB or it may be activated by signal transduction protein R1P that is activated to fNFR type 1 by TRADD protein[98].

Gene expression can be regulated by a number of genetic elements located in the 5′ upstream region. The promotor region of Fas/Apol encompasses 2000 bp sequence divided into promotors, enhancer and silencer region. Fas/Apo1, designated as CD95, is a molecule on the cell surface. *Fas* gene expression has been found in thymus, spleen, ovary and heart, on activated T and B cells, etc. Its expression is upregulated by γ1FN. *Fas* gene has been mapped to the long arm of chromosome10 in human and on syntenic segment of chromosome 19 in mouse. The gene comprises 9 exons and spans T26 kb of DNA and introns varying in length from 152 bp upstream of exon encoding transmembrane domain. Apoptotic signal transduction in the human fas molecule is mediated by a segment at its carboxyl terminal that has homology with human TNF receptor. A computer search for potential transcription factor-binding site revealed no consensus TATA or CAAT box, but a GC-rich sequence at positions – 53 to – 84. A consensus transcription factor k site region identified in the promotor region includes sites for AP-1 at – 455 to – 449, CP2 protein at – 38 to – 32, GF-1 at – 554 to – 549, NF-Y at – 471 to – 467, c myb at – 803 to – 798 and EBP 20 at – 907 to – 900.

CD95 binds to CD95 L which belongs to TNF-α-related type II transduction molecules. Recently, Webei *et al.* identified 2 NFAT sites – 180 and – 35 regions on CD95L promoters. Sequences from – 220 to – 36 are sites for CD95L promoters and DNAse 1 footprint analysis of – 220 CD95L promoter has revealed a novel transcription factor-binding site at – 120 region and at least 2 factors. Constitutively expressed SP.1 and PMA inducible DNA-binding nuclear factors bind to it. Although NFAT does not bind directly at – 120 region, it contributes to transactivation at this region. High induction of regulatory – 120 DNA sequence needs signals from both PMA and ionomycin pathway. NFAT activates – 120 regulatory elements via a protein–protein interaction. NFAT can cooperate with AP-1 (Fas/Jun) to bind and activate week NFAT sites. AP-1 is PMA-inducible transcription factor. CD95L promotors may contain yet unidentified regulatory elements for a fine control[100,101].

Ceramide has emerged as a novel lipid mediator in cell proliferation/apoptosis and to exert its effect, it transduces intracellular signals by activating protein kinase or protein phosphatase and by reducing expression of *c-myc* and nucleus translocation of NF-κB. The protooncogene *C-jun* encodes another transcription factor, i.e. a component of AP-1. C-jun is a member of bzip transcription factor family and has a basic DNA-binding domain and a leucine zipper dimerization motif of C-jun homodimers or heterodimers with c-Fos to bind DNA conserved sequence TCA, found in promotor region of several genes. C-jun expression can be induced by TNF-α, NGF and 1L-1. C-jun expression is induced during cell differentiation and by apoptotis inducing agents like radiation, UV light and cytstatic drugs, or by growth factor deprivation, suggesting that C-jun/AP1 is an important factor for cell growth differentiation and apoptosis. Ceramide induces C-jun and activates AP1 through JNK because both TNF-α and UV light increase ceramide levels via JNK. By using curcumin, (1,7-bis[4-hydroxy-3-methoxy-phenyl]-1,6-heptadiene-3,5-dione) and antisense C-jun oligo as specific inhibitor of C-jun/AP-1, it was shown that C-jun was inhibited and DNA fragmentation was also inhibited, so ceramide induces apoptosis signals via AP-1[46].

Role of baculoviral antiapoptotic gene *p35*

Apoptosis has a central role in normal neuronal development and caspase inhibitors have been demonstrated to be effective in inhibiting neuronal cell death in several apoptotic paradigms. The effects of general caspase inhibition in neurons have been evaluated by creating transgenic mice having normal expression of the baculoviral caspase inhibitor proteins p35. The p35 expression was found to confer functional caspase inhibitory activity and prevent seizure associated neuro-degeneration associated with excitotoxic glutamate analogue kainic acid, *in vitro* and *in vivo*[102]. Expression of p35 has been shown to prevent blindness in *Drosophila* mutants that undergo rational degeneration[103–105]. These models may be useful for exploring the role for caspase inhibition in effective therapeutic treatment for some diseases.

Future directions

The field of apoptosis research appears to be advancing by an amplifying cascade of discoveries. Nevertheless,

there are many gaps in our understanding of the molecular details.

The fundamental questions about the Bcl-2 family remain unresolved. The circuitry conveying upstream death survival signals is hazy, as are critical issues regarding commitment. Is Bcl–XL–Apaf-1 complex present in healthy cells or formed only after a death signal? How does this association restrict Apaf-1 activity? Are pro-caspases part of ternary complexes or do they associate only with liberated Apaf-1? Structural analysis on Ban-like proteins may enlighten us as to why they share so many features with the pro-survival group, yet have opposite function. Clarifying how the Bcl-2 family governs apoptosis might provide the ability to adjust the apoptotic threshold in clinical settings. Degenerative diseases and acute ischemic episodes would clearly benefit from pharmacologic agents that retard further apoptosis. For cancer, delineating the apoptotic defects in specific tumour types may engender therapies that re-establish the normal death programme.

There are many gaps in our understanding of caspase activation and the multifaceted cellular defences against inappropriate activation of these lethal proteases. Moreover, we have identified only a limited number of functionally-relevant downstream caspase targets, whose proteolysis ultimately kills the cell; the number and identity of such additional targets has yet to be determined. Proteolytic cleavage of each one of these caspase substrates presumably deregulates signalling pathways that contributes to one or more components of apoptosis studies on caspases, and suggests that caspases may indeed prove to be important therapeutic targets, particularly with the advent of potent, cell permeable, nonpeptide inhibitors and improvements in gene therapy. Nevertheless, it remains to be seen whether prolonged caspase inhibition will continue to preserve the functional integrity of salvaged cells and whether such inhibition does not itself result in adverse consequences.

At present, it appears plausible that the death and life decision is closely linked to the status of the mitochondrial membranes. The dissipation of the $\Delta\psi m$ resulting from irreversible mega channel spanning is certainly incompatible with the vital role of mitochondria bioenergetics, redox and ion homeostasis. Clearly the release and/or inactivation of cytochrome C would cause disruption in electron transport, resulting in the generation of ROS and a drop in ATP production, both of which can potentially contribute to cell death in the absence of caspases.

The distinction between both choices, viz. death/life decision and the apoptosis/necrosis decision is somehow complicated by the fact that both the mitochondrial events (which dictate the death/life decisions) and the activation of downstream caspases (which are required for the apoptotic death modality) are tied together by the guardian knot of the apoptosome.

Researchers have made substantial progress in delineating signalling pathways that couple CD95 and TNFRI to downstream cellular effectors. The same basic principles probably also apply to signalling by the more recently discovered DR3, DR4 and DR5. Indeed the signalling elements used by DR3 and TNFRI are similar; however, the pathway from DR4 and DR5 to caspases appears distinct and its molecular components have yet to be identified in nontransfected cells. The biological roles of newly identified death receptors and ligands need to be studied and we need to know whether defects in these ligands and receptors contribute to disease.

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