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Review

From sentencing to execution – the processes of apoptosis

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Abstract

Objectives Cell proliferation and apoptosis play a major role in maintaining homeostasis and as such any disruption within these processes can lead to disease states. Apoptosis occurs in three non-distinct phases – induction, effector and degradation – and can be executed through both the extrinsic and intrinsic pathways in addition to recognised sub-pathways such as the p53 and lysosomal pathways. This review article highlights these pathways, incorporating an overview of the molecular regulators of apoptosis.

Key findings These regulators include the prominent apoptotic players 'the caspases' in addition to the main regulators of the Bcl-2 family. Increased understanding of the physiological processes of apoptosis at the molecular level not only offers an insight in disease pathogenesis but, in addition, allows for the development of diagnostic, prognostic and therapeutic tools.

Summary While apoptosis remains the key player in cellular death, other processes cannot be dismissed. Many other proteins, in addition to caspases, within apoptotic pathways have been identified. Research continues into establishing the precise aspects of their molecular mechanisms of action and inter-relationships. Inappropriate apoptosis due to dysregulation of cell death pathways provides a plethora of molecular checkpoints that can be targeted and modulated as part of therapeutic intervention. Increased research into these areas will prove useful for the design of novel chemotherapeutic drugs, an area that is particularly important due to increased risk of chemoresistance.

Keywords apoptosis; Bcl-2 proteins; caspase; inhibitor of apoptotic protein; mitochondria

Introduction

Apoptosis

Apoptosis was first described as physiologically controlled cell death in 1972.[1] In principal, cell proliferation and apoptosis play a major role in maintaining homeostasis. An important process, it plays a critical role in developmental biology, immunology, normal growth, ageing and degenerative processes. Primarily, cell suicide prevents replication of damaged deoxyribonucleic acid (DNA),[2] with removal of self-reactive lymphocytes, tumour cells and cells infected with viruses.[3] The other form of cell death, necrosis, also termed 'cell-murder' where cells are randomly killed, occurs following vital cellular damage. Necrotic cells swell and lyse, effusing their cytoplasmic and nuclear contents uncontrollably into the intercellular milieu provoking inflammation, scarring and damage to neighbouring tissues. Whether a cell undergoes either apoptosis or necrosis is dependent upon various factors, in particular the balance between necrotic and apoptotic signals.

In 2005, a new type of cell death was reported.[4] Necroptosis is a regulated caspase-independent cell death mechanism that can be induced in multiple cell types and is characterised by morphological features resembling necrosis. Indeed, other forms of cell death, such as paraptosis and autophagy, have been described.[5] Paraptosis, another form of programmed cell death, ultimately culminates in necrosis. Characteristics of paraptotic cells include swelling and vacuolisation, with enlarged endoplasmic reticulum and mitochondria.[5] Unlike apoptotic cells, fragmentation does not occur. Autophagy involves cellular self digestion and in many cases, through the removal, or repair, of damaged organelles, can protect against cell death.[6] Its main feature includes the sequestration of cytoplasmic material in vacuoles for bulk degradation by lysosomal enzymes.[7]

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Apoptosis and disease

Inappropriate apoptosis due to dysregulation of the cell death pathways is implicated in various disease states, including neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, stroke, ischaemic injury following myocardial infarction, acquired immunodeficiency syndrome (AIDS), autoimmune disorders, sustained viral infections and tumour development in various forms of cancer.[8–13] Detailing the precise mechanistic aspects of apoptosis in each of these disease states is beyond the scope of this paper; however, we have outlined how excessive and diminished apoptosis contribute to pathogenesis in cancer and neurodegeneration, respectively.

Apoptosis and cancer

Insufficient apoptosis is associated with the pathogenesis of cancer.[9] Effective and efficient programmed cell death contributes to the destruction of cells with damaged DNA. Therefore, prolonged cell viability due to the absence or reduction of apoptosis may permit the accumulation of transforming mutations, thus contributing to cancer formation and progression.[9] Mutations in the tumour suppressor gene *p53* and its regulators occur in 55–70% of human carcinomas.[14] Ordinarily, p53 induces cell cycle arrest and apoptosis in response to DNA damage. Loss of functional p53 correlates with tumour aggressiveness and those with a defect in one of their two copies of this gene develop cancers at a high rate.[15]

Apoptosis and neurodegeneration

Precise regulation of apoptosis is crucial for the normal development and maintenance of the central nervous system (CNS). Members of the apoptotic regulatory family, the caspases, in particular caspase-9 alongside caspase-3, are an essential requirement for programmed cell death during brain development.[16] However, post-development, excessive apoptosis results in loss of function in addition to loss of cells. Gradual loss of specific types of neurons from various parts of the CNS characterises the pathological progression of a variety of neurodegenerative disorders (review[13]) including spinal muscular atrophy, Alzheimer's disease, Huntington's disease and Parkinson's disease.

Inactivating mutations of neuronal apoptosis inhibitory protein is believed to contribute to spinal muscular atrophy.[17] This inhibitory protein is a homologue of the baculoviral inhibitor of apoptotic protein (IAP) genes.

Cathepsin D, an aspartic protease implicated in apoptosis, is elevated in 90% of pyramidal neurons in lamina V and 70% in lamina III in brains of patients with Alzheimer's disease.[18] Mutations in the presenilin 2 gene have been associated with familial Alzheimer's disease.[19] Furthermore, formation of amyloid β plaques in the Alzheimer brain alters the apoptotic threshold of neurons.[20] Active caspases are detected in these neurons. These caspases can cleave amyloid β, generating a pro-apoptotic protein.[21] Furthermore, amyloid β can induce apoptosis in cultured neurons.[22]

Huntingtin, required for neuronal survival, is mutated in Huntington's disease, where the altered form stimulates neuronal apoptosis[23] in addition to inducing mitochondrial abnormalities, the consequences of which contribute to apoptotic progression. Similar to amyloid β in Alzheimer's disease, Huntingtin is also cleaved by caspases and caspase inhibition results in reduced toxicity of the mutant Huntingtin.[24]

Parkinson's disease is characterised by degeneration of nigrostriatal dopaminergic neurons, which are believed to die by both apoptosis and necrosis in response to oxidative damage.[25,26] Treatments for Parkinson's disease ultimately involve the inhibition of apoptosis (review[13]).

Modulation of apoptosis for therapeutic use

Apoptosis occurs as the outcome of various signalling pathways within the cell. It is known that proteases are involved in these pathways, particularly in the cleavage of zymogens necessary for the activation, or maturation of apoptotic enzymes. Unregulated apoptosis, either at increased or decreased rates, can be one of the factors contributing towards, or leading to, a disruption in the balance and control of both intra- and intercellular proteolytic activity.

Research into apoptotic mechanisms and, in particular, the role of proteases has led to investigations into novel therapies for degenerative, neoplastic and autoimmune disorders. Indeed, several current cancer therapies act by inducing cell suicide. A large number of chemotherapeutic drugs, hormone therapies and radiotherapy treatments have been shown to induce apoptosis.[27–29] The efficacy of these treatments depends on successful cellular damage in addition to the cell's ability to respond by inducing the apoptotic machinery. Mutations in this machinery (e.g. loss of function or absence of some apoptotic regulators such as p53) may lead to chemoresistance. Furthering understanding in these areas proves useful for the design of novel chemotherapeutic drugs to target apoptotic suppressors or activate apoptotic effectors.[30]

While many drug therapies tend to focus on inducing apoptosis, prevention of cell death is useful in mitigating against ischaemia associated with stroke and heart attacks in addition to many neurodegenerative disorders. Recent developments in the use of small interfering RNA (siRNA), increases the potential of gene therapy by targeting various apoptotic regulatory and tumour suppressor genes.

In summary, it is important to have an understanding of the apoptotic process and the role proteases play in its execution. This knowledge can be applied to apoptosis-related disease states. Therefore, controlled reversal and initiation of apoptotic processes, particularly through proteolytic targeting, has major clinical and therapeutic implications. Understanding the physiological processes of apoptosis at a molecular level not only offers an insight in disease pathogenesis but also allows for the development of diagnostic, prognostic and therapeutic tools.

Phases of apoptosis

Apoptosis occurs in three non-distinct phases: induction, effector and degradation. Induction involves the initiation of procaspase cascades. This initiation results from DNA damage, physiological stress, inflammation and oncogenic

stimulation, leading to excessive production of pro-apoptotic factors. The first two stages demonstrate a commitment to cell death. The effector stage, also termed the 'execution stage',[8] is characterised by the disruption of the mitochondrial membranes, releasing cytochrome *c*, mitochondrial proteins and an array of proteases into the cytosol. These proteins serve as signalling molecules while the loss of cytochrome *c* has implications for the depletion of energy from the cell. Dramatic stereotypical morphological changes in cell structure mark the outcome of the effector phase.[8,31] The final outcome of the apoptotic process culminates in the cleavage and fragmentation of internucleosomal DNA.[8]

Apoptotic pathways

Molecular pathways control apoptosis through a complex network of cellular substrates. The two main well-established pathways involved are the intrinsic and extrinsic pathways, both of which overlap and interact, allowing components from one pathway to affect those of the other (Figure 1). Intrinsic pathways are initiated within the cell itself and the extrinsic pathway by the extracellular binding of cell signalling molecules to transmembrane death receptors, including those belonging to the tumour necrosis factor (TNF) family. Smaller routes can be detected within each of these pathways.

Intrinsically, the mitochondrial pathway is mediated by the caspases (e.g. activation of caspase-9, followed by caspase-3 and caspase-7 or Bcl-2 proteins),[33] while the extrinsic pathway involves recruitment of caspase-8 and subsequent downstream caspase-3 activation. Both pathways involve the release of cytochrome *c* from the mitochondria into the cytosol.

Other sub-pathways are distinguished within and between the extrinsic and intrinsic pathways – the p53 pathway and the recently characterised lysosomal pathway, which utilises lysosomal proteases. Activation of any particular pathway depends upon both the cell type and the triggering stimulus.

Extrinsic pathway

Induction of the extrinsic pathway (Figure 2), also known as the death receptor pathway, occurs following binding of death receptors (Fas, also known as Apo-1 or CD95), TNF receptor-1 (TNFR-1), interferon and TNF-related apoptosis-inducing ligand (TRAIL) in the cell plasma membrane. Death receptors are transmembrane proteins with cysteine-rich extracellular domains that rely on signalling proteins possessing a distinct set of modular protein motifs capable of homotypic interaction, including death domains (DD) and death effector domains (DED).[34] Following ligation of death receptors, such as Fas, multiple receptors aggregate on the target cell surface, leading to recruitment of an adaptor protein known as Fas-associated death domain protein (FADD). FADD, bearing both DD and DED motifs, binds to the DD of Fas and recruits the initiator protein procaspase-8 via its DED domain, thus forming the death-inducing signal complex (DISC). Furthermore, autocatalytic procaspase-8 activation occurs due to its high local concentration, subsequently leading to the activation of the downstream effector caspase, caspase-3. Consequently, activated caspase-8 cleaves the Bid protein to tBid, which acts on the mitochondrial membrane facilitating release of cytochrome *c*. Fas receptors can activate either mitochondria-dependent or -independent signalling paths in response to ligand binding.

An alternative set of death receptors respond to the different death ligand, TRAIL. However, DISC formation, caspase activation and Bid cleavage downstream of TRAIL receptor ligation is similar to that observed in the Fas pathway.

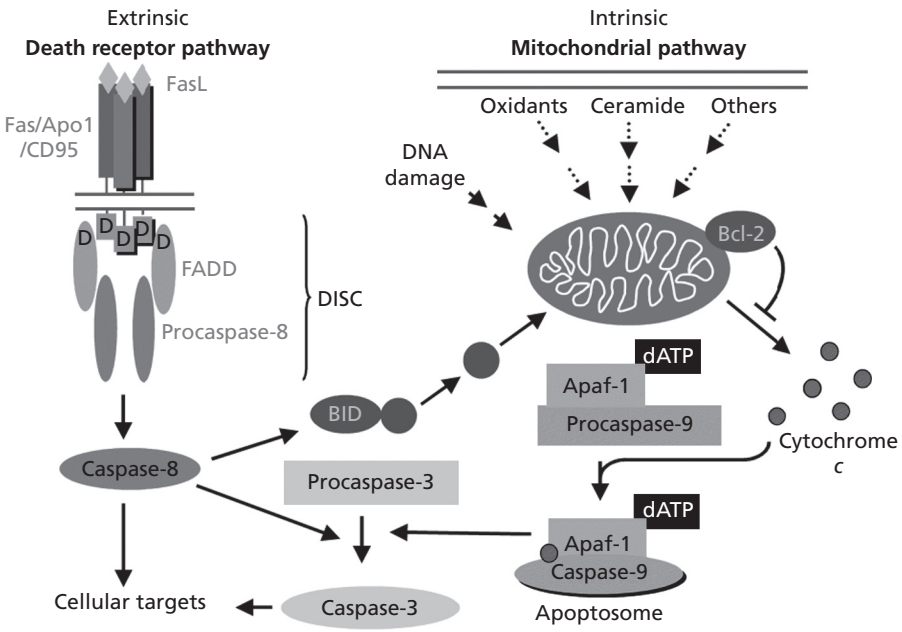

Figure 1 Major apoptotic pathways in mammalian cells. Adapted from Hengartner 2000[32]

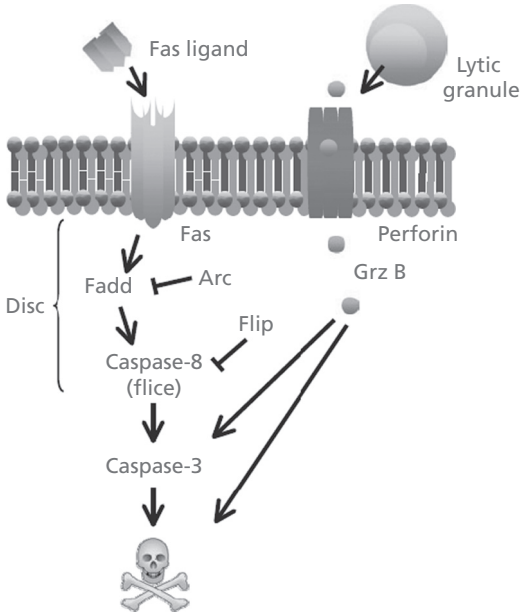

Figure 2 Schematic example of the extrinsic pathway represented by Fas-Fas ligand-dependent signalling. Also shown is caspase-dependent and -independent apoptosis signalling that occurs in CTLs and NK cells via perforin/granzyme B-dependent pathway (taken from Fadeel and Orrenius[34])

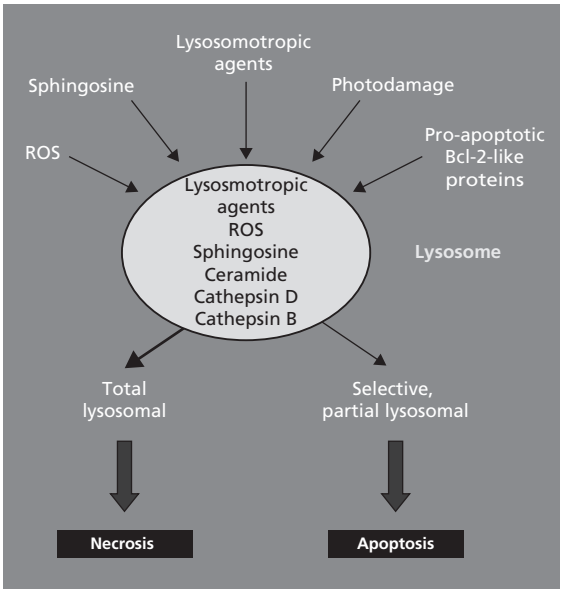

Figure 3 Schematic representation of the mechanisms of lysosomal permeabilisation. Adapted from Guicciardi *et al*[37]

Lysosomal pathway

Recently, it has become clear that, in addition to caspase-mediated proteolysis, other proteases such as cathepsins are involved in the regulation of apoptosis.[35] Ordinarily, cathepsins are localised within lysosomes; however, in response to certain apoptotic stimuli lysosomal permeabilisation occurs and they are released into the cytoplasm to participate in the execution of apoptosis.[36,37] The lysosomal pathway can be activated by death receptors, lipid mediators and photodamage. The pivotal factor in determining the type of cell death mediated by lysosomal enzymes is the magnitude of lysosomal permeabilisation and, consequently, the quantity of proteolytic enzymes released into the cytosol.[38] Partial, selective permeabilisation triggers apoptosis, whereas complete lysosomal breakdown accompanied by release of high concentrations of lysosomal proteases results in unregulated necrosis (Figure 3).[39,40]

Under both conditions the protease concentration is sufficient to overcome the normal regulatory barrier formed by endogenous cytosolic inhibitors, such as cystatins. Following translocation into the cytosol, lysosomal proteases contribute to the execution of the apoptotic programme either by direct cleavage of key cellular substrates or by acting in concert with the caspases.[37]

Damage to the lysosomal membrane can occur following ligation of TNFR-1.[41] Lysosomal destabilisation can also be a feature of oxidative-stress-induced cell damage[42] and involves the generation of reactive oxygen species (ROS). Interestingly, lysosomal enzymes act on mitochondria, increasing their generation of ROS, thus creating a feedback loop leading to increased lysosomal permeabilisation.[43] One lysosomal protease, cathepsin D, is suggested to act upstream of mitochondrial events, activating pro-apoptotic or inactivating anti-apoptotic factors, which ultimately result in the release of cytochrome *c*.[44]

In some respects the lysosomal pathway can be seen as a bridge linking the extrinsic and intrinsic pathways. TNF-α, a component of the extrinsic pathway, is associated with specific lysosomal permeabilisation and the subsequent release of cathepsin B. Cathepsin B consequently initiates the mitochondrial intrinsic pathway.[41]

p53 Pathway

p53 is a DNA-binding transcription factor that initiates programmed cell cycle arrest, cellular senescence or apoptosis.[14] The p53 pathway responds to intra- or extracellular stresses that disrupt DNA replication and cell division. Following DNA damage, the transmitted stress signal leads to a response through post-translational modification and consequential activation of the p53 protein. As p53 levels rise, transcription of downstream target genes occurs. In addition to the transcription-dependent induction of apoptosis, p53 also induces apoptosis through the mitochondrial pathway.[45] It was recently demonstrated that caspase-dependent cleavage of p53 results in the generation of four fragments, two of which lack a nuclear localisation signal and consequently localise to mitochondria. These mitochondrial fragments induce mitochondrial membrane depolarisation in the absence of transcriptional activity[46] in addition to forming complexes with Bcl-X_L and Bcl-2, thus triggering cytochrome *c* release and subsequent caspase activation.

Abnormalities of p53-dependent apoptosis contribute to tumour development, tumour progression and chemotherapeutic drug resistance,[47–50] and p53 mutations are suggested as being of prognostic significance in some cancers.[51]

Intrinsic pathway

The intrinsic pathway is also termed 'the mitochondrial pathway' due to the pivotal role played by the mitochondria (Figure 1). Initiated by cellular stress and chemicals, including anti-cancer drugs, followed by activation of caspase-9, it is characterised by loss of mitochondrial membrane potential with subsequent release of apoptogenic factors, including caspase activators such as the electron transport chain component cytochrome *c*, second mitochondria-derived activator of caspases (Smac/Diablo) and apoptosis-inducing factor (AIF).

Cytochrome *c* initiates the intrinsic pathway in conjunction with the 140 kDa cytosolic protein apoptosis protease-activating factor-1 (Apaf-1). Upon release and binding with Apaf-1, an oligomeric complex is formed with procaspase-9. The CARD (caspase recruitment) domain of Apaf-1 binds to a similar domain on procaspase-9, whereas the central CED-4 domain is involved in Apaf-1 oligomerisation. This complex, termed the apoptosome, can only be formed in the presence of ATP or dATP. The assembly of the apoptosome complex represents the initiating step for the activation of the caspase cascade resulting in cleavage of specific cellular substrate proteins, promoting morphological changes and final cell destruction.

Mitochondrial physiology – cellular poison cupboard

Mitochondria, the cell's energy-producing organelles, play a central role in mediating apoptosis and its associated pathways through providing a link between effector and initiator caspases in several apoptotic pathways. The description of mitochondria being a 'cellular poison cupboard'[52] highlights their destructive potential. They exert their pro-apoptotic action through at least three mechanisms: (1) disruption in ATP production; (2) alteration of Ca^{2+} homeostasis; and (3) release of apoptogenic proteins.[53] The mitochondrial intermembrane space contains several proteins that are liberated through the outer membrane following apoptotic stimulation. During early apoptosis mitochondrial morphology remains intact; however, several biochemical changes take place. Regulation of these changes is largely due to members of the Bcl-2 and Bax family.

Many cell death pathways converge at the mitochondria, whereupon the release of cytochrome *c* and other pro-apoptogenic components is induced by the opening of pores in the inner membrane of mitochondria, a condition known as permeability transition (PT). The primary pore involved in this phenomenon is a multi-protein complex spanning the inner and outer membranes of the mitochondria known as the permeability transition pore (PTP) complex. Opening of transmembrane pores, including the PTPs, permits the equilibrium of ions and respiratory substrates between the cytosol and mitochondrial matrix leading to a decrease in the mitochondrial membrane potential and arrest of ATP synthesis. Failure to produce ATP can also occur following damage to mitochondria. In this instance, the absence of ATP prevents formation of the apoptosome and subsequent caspase activation, leading cells to die by necrosis.[54]

Mitochondrial transmembrane potential

Non-apoptotic cells can be characterised by the presence of a high mitochondrial transmembrane potential ($\Delta\Psi_m^{high}$), which becomes compromised during the apoptotic process.[55] Loss of membrane integrity instigates events resulting in the uncoupling of oxidative phosphorylation, generation of superoxide free radicals and relocation of mitochondrial matrix-associated Ca^{2+} into the cytosol. Additionally, following alteration of transmembrane potential, leakage of key apoptotic mitochondrial proteins occurs. These proteins include cytochrome *c*, HtrA2/Omi, SMAC/Diablo and AIF.[56] Decrease in membrane potential can be used as a characteristic apoptotic marker.

Conversely, however, it has been observed that cytochrome *c* release can occur before, and may not be affected by, mitochondrial transmembrane potential disruption and that subsequent caspase activation, in particular that of caspase-9, leads to loss of mitochondrial membrane potential.[16,57–60] Therefore, inhibition of caspases and Bcl-X_L can prevent loss of transmembrane potential.[61] The change in mitochondrial membrane potential may, after all, not be a universal step in the apoptotic process[58] and its role continues to be debated.

Mitochondrial permeability transition pore

Ordinarily transmembrane potential alongside mitochondrial function is protected by Bcl-2 proteins through maintenance of the mitochondrial PTP.[62] Research indicates that over-expression of Bcl-X_L or Bcl-2 confers protection upon mitochondria, discouraging induction of the PTP opening and subsequent release of cytochrome *c* and AIF.[57,62–64] Opening of the PTP can be triggered by multiple stimuli including oxidative stress, high Ca^{2+} or low ATP concentrations, and leads to $\Delta\Psi_m$ loss as ions equilibrate across this membrane and swelling of the matrix as water enters. This swelling may be sufficient to break the outer mitochondrial membrane leading to permeabilisation.

Structurally, the PTP is composed of several proteins and functions as a calcium-, voltage-, pH- and redox-gated channel with various levels of conductance and poor ion selectivity and permits the release of solutes with molecular masses of up to 1500 Da.[65–68] Its constituents include both inner membrane proteins, such as the adenine nucleotide translocator and outer membrane proteins, such as Porin, which operate in concert, creating a channel through which molecules can pass. When the PTP opens volume dysregulation within the mitochondria occurs due to the hyperosmolarity of the matrix, causing the matrix space to expand. The inner membrane has folded cristae, so possesses a larger surface area than the outer membrane. Therefore, this matrix volume expansion can eventually cause outer membrane rupture, releasing caspase-activating proteins located within the intermembrane space into the cytosol.

The ability of caspases to induce PTP opening, which in turn can induce caspase activation (by release of cytochrome *c* and AIF), creates opportunities for a feed-forward amplification loop.

The channel model

Other studies indicate that the permeability transition pore is not responsible for the release of all apoptotic factors. The

channel model represents the formation of large channels or pores in the outer mitochondrial membrane by members of the Bcl-2 family proteins. Several of these proteins form functional channels that show multiconductance levels and are voltage and pH dependent. Indeed, Bax-induced release of cytochrome *c* is potentiated by Mg^{2+} ions and occurs independently of the PTP.[68] Recent research by Muñoz-Pinedo *et al.*[69] shows caspase-independent, simultaneous release of the pro-apoptotic factors cytochrome *c*, Smac and Omi through a mitochondrial pore.

Cytochrome c

Cytochrome *c* functions as an electron transport carrier in oxidative phosphorylation within the mitochondrial electron transport chain. Here, it interacts with redox partners of complex III and IV.[58,70,71] It is a water-soluble protein with a net-positive charge and is synthesised as a precursor molecule, apocytochrome *c*. Its import to the mitochondrial intermembrane space occurs via the translocase of the outer membrane (TOM) complex located in the outer mitochondrial membrane without any further proteolytic processing.[72,73]

Translocation of cytochrome *c* into the cytosol is complete within 15 min and occurs early in the apoptotic process, preceding the characteristic morphological changes.[58,74] It can proceed independently of Asp-Glu-Val-Asp-specific caspase activity and reduced mitochondrial transmembrane potential.[74] Cytochrome *c* release has been demonstrated to be regulated by the pro-apoptotic Bcl family protein Bax both *in vivo*[75] and *in vitro*.[76]

Upon its release into the cytosol cytochrome *c* binds to Apaf-1, a mammalian homologue of Ced-4, resulting in the activation of caspase-9 and caspase-3 followed by the apoptotic destruction of nuclei.[77] Additional activation targets for cytochrome *c* include initiator caspases-2, -8 and -10; and the effector caspases-3, -6 and -7;[78] however, this activation does not occur in the absence of caspase-9, thus highlighting the indispensable nature of this protease within the cytochrome *c*-dependent caspase cascade.

Not all cytochrome *c* may be released from the mitochondria during apoptosis. It is suggested that an excess of cytochrome *c* not involved in the electron transport chain is translocated, as research has demonstrated that intracellular levels of ATP are maintained for several hours after apoptotic induction with staurosporine.[58] A depletion of the intracellular ATP pool during late apoptosis may be due to an abundance of early ATP-consuming reactions in conjunction with loss of mitochondrial function through later processes.[58]

Molecular regulators of apoptosis

Caspases are the most prominent group of proteases involved in the regulation and execution of apoptosis. However, initiation and progression of apoptosis is tightly regulated by a variety of other factors, including roles played by the Bcl-2 family of proteins, mitochondrial proteins and several cytosolic inhibitors including the IAP family.

Caspases

Caspase activation is a key event in triggering the morphological and biochemical changes associated with cell death. There are at least three distinct pathways for caspase activation; recruitment activation, trans-activation and auto-activation. Recruitment activation can occur following ligation of death domain receptors such as Fas and TNFR-1. In this instance caspase-8 is activated. Equally, oligomerisation of procaspase-9 mediated by APAF-1 and cytochrome *c* fits into this category with recruitment mediated by homophilic CARD-domain interactions.[79] Following recruitment of caspase proenzymes to the oligomerisation site, their low level of endogenous catalytic activity is adequate to initiate full catalytic activation through proteolysis of the Asp-X site at the junction between the large and small subunits. Trans-activation occurs when an upstream caspase cleaves and activates a downstream member, again through proteolysis of the Asp-X site in the linker segment between subunits. Autocatalytic activation is useful for amplification purposes within the cell, thus improving efficacy.

Two main classes of caspases exist: initiator and effector. Initiator caspases include capsase-2, -8, -9 and -10, with the effector caspases incorporating caspase-3, -6 and -7. Caspase-8 and -10 initiate the extrinsic pathway and caspase-9 is the apical caspase in the intrinsic pathway. Activation of these initiator caspases leads to the proteolysis and activation of the effector caspases, which are involved in the cleavage of specific cellular proteins causing the appearance of characteristic morphological cellular changes.

Caspases have a tripartite structure consisting of an N-terminal prodomain of variable length, and a large and small subunit, the p20 and p10 subunits, respectively. The large subunit possesses the conserved active site pentapeptide Gln-Ala-Cys-X-Gly (X = Arg, Gln, Gly), which contains the nucleophilic Cys.[80] The smaller subunit contains residues that bind the Asp carboxylate side chain and others that determine substrate specificity.[80] Initiator caspases contain large domains, while those belonging to effector or executioner caspases are much smaller. Processing into the mature heterotetramer product $(p20p10)_2$ requires cleavage after aspartic acid residues located in the interdomain linkers of the protein.

Caspase-2 contains a large prodomain and can be activated by caspase-3 in the mitochondria-initiated pathway.[78] It induces release of pro-apoptotic mitochondrial proteins[81,82] and is required for mitochondrial permeabilisation in stress-induced apoptosis.

Caspase cascade

A catalytic cascade resembling that of complement or clotting cascade has been suggested for caspase activation.[83] This cascade is central to the progression of apoptosis. Pro-apoptotic stimuli at the origin of the cascade initiation will determine the route taken and caspases activated within each cell. Activation through death receptors induces apoptosis via caspase-8, which induces either directly or indirectly caspase-3, -6 and -7.[84] In comparison, cytotoxic agents activate the caspase cascade through caspase-9, a 45-kDa protein.[16,85,86] An amplification cycle may be set up after activation with some caspase family members further activating the caspase that originally initiated their own activation.[84] Substrates for activated caspases include protein kinases, DNase, retinoblastoma proteins, cytoskeletal proteins, auto-antigens and other

caspases.[87] Cleavage of these major cellular substrates accounts for the characteristic features of apoptosis.

The intrinsic pathway previously mentioned is a prime example of the caspase cascade. As previously described, it is mediated by activation of caspase-9, followed by caspase-3 and caspase-7. The initial cleavage of caspase-9 is thought to occur autocatalytically and is facilitated by the cytosolic protein Apaf-1 via its ATPase domain.[3] This newly formed complex associates with the zymogen caspase-9, forming a multiprotein complex, thus activating procaspase-9.[88] Activated caspase-9 can then proceed with the activation of procaspase-3, an effector caspase, and the execution of apoptosis is carried out.

Caspase-3

Caspase-3 is the most active effector caspase within both the intrinsic and extrinsic pathways, where it is processed and activated by caspase-9 and caspase-8, respectively. A key executioner of apoptosis, it is known also as apopain, stem cell antigen-1 (SCA-1), Yama (Hindu God of death) and cysteine protease 32 (CPP32).[89–91]

The human caspase-3 gene is mapped to chromosome 4q35 and is highly distributed, with particularly high expression in lymphocytic cell lines.[92] This implicates it as an important mediator within the immune system. As a precursor, its inactive form is a unit of 32 kDa.[93] The active enzyme is a heterodimer composed of two p17 subunits and two p11 units at 17 kDa and 11 kDa, respectively. The precursor form is proteolytically cleaved at Asp-175-Ser-176 to generate the mature p11 subunit and a 20 kDa subunit. This 20 kDa subunit is subsequently cleaved at Asp-28-Ser29 to generate the p17 subunit[8,89,93] (Figure 4).

Activation of caspase-3 is dependent upon the presence of cytochrome *c*, dATP and Apaf-1. Additionally, proteolytic maturation of procaspase-3 is performed by caspase-8 and -9 and also by granzyme B, a serine protease found in lytic granules of cytotoxic lymphocytes.[94] Proteasomal inhibitors induce caspase-3 activation and apoptosis in HL60 human leukaemic cells primarily in the G_1 phase of the cell cycle.[95] In particular, a rapid increase in activated caspase-3 has been detected upon treatment of cells with proteasome inhibitor Z-LLL-CHO.[87]

Cleavage of specific substrates by caspase-3 has the effect of either activating effector molecules or triggering characteristic structural changes observed in apoptotic cells.[58] Substrates undergoing proteolysis by caspase-3 each contain a common Asp-Xaa-Xaa-Asp (DXXD) motif[8] highlighting an absolute requirement for Asp in the P_1 position, with a marked preference for Asp in the P_4 position. Several main substrate targets of caspase-3 have been identified and include the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), [89,96] the 70 kDa protein component of the U1 small nuclear ribonucleoprotein[97] and the catalytic subunit of DNA-dependent protein kinase ($DNA\text{-}PK_{cs}$). [93] Furthermore, there are other cytoskeletal targets, including the structural proteins fodrin, actin and gelsolin.[79]

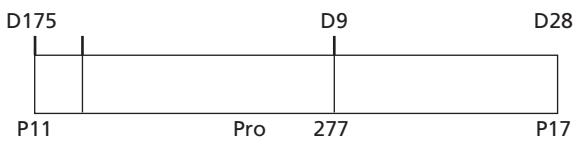

Figure 4 Proenzyme organisation of caspase-3 showing the subunits and corresponding cleavage sites at Asp-28 and Asp-175

Inhibitors of caspases

Due to the potentially irreversible caspase cascade triggered by upstream initiator caspases, it is vital that intracellular caspase activation be tightly regulated. Regulation of caspase activation and activity occurs at several different levels: (1) regulation of zymogen gene transcription and posttranslational modifications; (2) blocking of proximity-induced activation of certain procaspases by anti-apoptotic members of the Bcl-2 family and other cellular polypeptides; (3) binding of IAPs to caspases, causing direct inhibition.

Inhibition of caspases is a general strategy adopted by viruses in their attempt to elude a cellular response to the infectious insult.[98] Cytokine response modifier A (CrmA), a 38 kDa member of the serpin family, is a potent inhibitor of some active initiator caspases and those involved in inflammation.[99] This selectivity suggests that cowpox virus facilitates infection through inhibition of both the host inflammatory response and inhibition of apoptosis, two defence mechanisms utilised by the host to eradicate any potential virus infection. CrmA is able to prevent apoptosis induced by TNF, Fas ligand, serum withdrawal and nerve growth factor withdrawal.[100] Caspase activation and activity are carefully regulated on several levels by an endogenous family of cellular proteins called the inhibitor of apoptosis proteins (IAP). These proteins potently inhibit the enzymatic activity of active caspases and can permanently remove caspases through the ubiquitination-mediated proteasome pathway.[11,101,102]

Further activators of apoptosis

In addition to caspases there are several cytosolic and mitochondrial proteins that contribute to the regulation of apoptosis. This section highlights those responsible for activation of apoptosis. These include Smac, also known as Diablo, apoptosis inducing factor and the B-cell/lymphoma-2 (Bcl-2) family of proteins. Omi/HtrA2 also contributes significantly to apoptosis.

Smac/Diablo

Smac, also known as Diablo, is a mitochondrial protein released in conjunction with cytochrome *c*, which promotes caspase activation and apoptosis. It functions by binding to the BIR3 and BIR2 domains of XIAP (member of the IAP family), thus disrupting its interaction with caspase-9 and the effector caspases (3 and 7).[56,103–105] This direct IAP binding removes their inhibitory activity, thereby lowering the threshold for entry into apoptosis. However, some researchers suggest that IAP binding is a secondary effect, as expression of an NH_2-terminally truncated Smac mutant lacking the entire IAP-interacting domain potentiates apoptosis to the same extent as functional Smac.[106]

Structural studies indicate that Smac is a homodimer[107] and that each dimer binds to the BIR domains of inhibitor

proteins via the Smac N-terminal residues.[103,108,109] Mutations in the hydrophobic interface that disrupt dimer formation significantly compromise Smac function.[104,106]

The 239-amino acid precursor form of Smac is translated within the cytosol. It possesses a 55-amino acid N-terminal mitochondrial targeting sequence, which, following recognition, translocates through the mitochondrial membranes via the translocase in the outer mitochondrial membrane (TOM) complex. Its transfer continues through the inner mitochondrial space to the translocase in the inner mitochondrial membrane (TIM 23) complex.[109] Here, the stop-transfer sequence is cleaved by the inner membrane peptidase (IMP) complex. This IMP complex is responsible for the proteolytic processing of mitochondrial protein presequences, allowing the release of the potent, mature forms to the intermembrane space where it safely remains until apoptotic signalling allows its liberation. Encapsulation with the mitochondria of mature Smac prevents any inappropriate IAP binding. Relocation following apoptotic stimulation occurs during mitochondrial membrane depolarisation, thus having positive implications for the activation of the effector caspases.

Apoptosis inducing factor

AIF is a mitochondrial bound protein found as a peripheral component of the inner mitochondrial membrane.[74] Formed as a precursor protein with a presequence of 101 amino acid residues, its processing occurs following import into the mitochondria.[110] Translocation of AIF into the cytosol follows caspase activation, downstream of pro-apoptotic signalling, mitochondrial outer membrane permeabilisation and cascade reactions. Caspase activation has been suggested as the limiting step for AIF detachment from the inner mitochondrial membrane and subsequent release into the cytosol.[74]

Recombinant AIF causes chromatin condensation in isolated nuclei and large-scale fragmentation of DNA. Overexpression of Bcl-2, which controls the opening of mitochondrial PTP, prevents the release of AIF from the mitochondrion but demonstrates no cytoprotective effect once AIF is present in the cytosol.[110]

Some evidence exists of an AIF-dependent and caspase-independent apoptotic pathway crucial for cell death following growth factor deprivation and early mammalian development.[111]

Apoptogenic protein

Apoptogenic protein (Apop) is a recently reported novel protein with pro-apoptotic functionality.[112] Overexpression of Apop induces apoptotic cell death of cultured smooth muscle cells whereas inhibition prevents cell death following apoptotic stimuli, observations that imply that tight control of Apop is essential for cell viability.

Apop-1, a soluble protein without transmembrane domains, is located in the matrix or in the intermembrane space of mitochondria. It interacts with cyclophilin-D, a component of the PT, and demonstrates no effect on Bax/Bak dependent channels. Mouse Apop-1 induces apoptosis by facilitating the release of cytochrome *c* into the cytosolic space followed by activation of caspase-9 and -3. This apoptosis is not blocked by Bcl-2 or Bcl-X_L, inhibitors of the Bax/Bak-dependent channels, whereas it is completely blocked by ciclosporin, an inhibitor of the PTP.[112]

Bcl-2 family proteins

Bcl-2 family proteins are pivotal regulators of intracellular apoptotic cascade activity. The Bcl-2 gene was first discovered in 1985 in human B-cell lymphomas.[113] Since that date, over two dozen Bcl-2 family members have been discovered. In addition to their role as apoptotic inhibitors, some family members promote programmed cell death, primarily through their channel forming properties. These members include Bax, Bak, Bcl-X_S, Bad, Bid, Bik, Bim, Hrk and Bok.[61] Channels formed are usually pH sensitive and voltage gated. An important characteristic of the Bcl-2 family is their frequent ability to form multimers, including hetero- and homo-dimers, suggesting neutralising competition between the proteins.[114]

The Bcl-2 family is characterised by the presence of Bcl-2 homology (BH) domains. Generally, family members can have up to four BH domains, which correspond to α-helical segments. Pro-apoptotic members do not possess BH4 and are classified into two subgroups: the Bax subfamily (Bax, Bak and Bok), containing BH1, BH2 and BH3, and those possessing only the BH3 domain (Bid, Bad and Bim). Anti-apoptotic proteins, such as Bcl-2 and Bcl-X_L, have all four BH domains.[54]

Human carcinomas frequently display altered expression of Bcl-2 family proteins. Therefore, therapeutic outcome and efficacy of many chemotherapeutic drugs can be heavily influenced by the relative levels and activation state of members of the Bcl-2 family.[62]

Bax, a 21 kDa protein, is the main proapoptotic Bcl-family protein. Both its presence or that of Bak are required for most mitochondrial-dependent cell death processes.[74] Bax, in its monomeric form, safely resides within the cytosol of most normal healthy tissues showing no channel-forming or mitochondrial-targeting activity.[115,116] In apoptotic cells or those over-expressing the protein, Bax targets the outer mitochondrial membrane where, following oligomerisation of between six and eight monomers, anion-selective pore-like channels form, thus permitting the translocation of mitochondrial apoptotic factors such as cytochrome *c*. These anion selective channels are formed optimally under physiologically neutral pH conditions.[117]

In addition to their presence at the outer mitochondrial membrane, Bax and Bak can localise to the endoplasmic reticulum (ER) and nuclear envelope. In response to ER stress, conformational changes and oligomerisation occurs to Bax and Bak at both mitochondrial and ER locations. Within the ER, Bax leads to progressive depletion of Ca^{2+}, and induces caspase-12 cleavage.[118]

Bax, like the anti-apoptotic proteins Bcl-X_L and Bcl-2, possesses a COOH terminal tail of ~20 hydrophobic amino acids. It is proposed that this tail functions as an anchor point within the organelle membranes. Furthermore, deletion of the tail results in loss of cytosolic translocation and inhibition of death-promoting activity, thereby suggesting that post-translation modifications take place in Bax following apoptotic stimuli, to allow the previously inaccessible or blocked hydrophobic tail to insert into membranes.[119]

Overexpression of Bax leads to the induction of cytochrome *c* release from the mitochondria.[68,75,119–122] Equally, the addition of recombinant oligmerised Bax to isolated mitochondria produces a similar release of cytochrome *c*.[68,75,76,116,123] These observations highlight a role for Bax as a necessary component for the release of cytochrome *c* during apoptosis.

During apoptotic signalling, transcription of key genes leads to cell death. DNA damage in some cells induces p53, which in turn activates the Bax promoter resulting in elevated Bax levels.[124–127] Bax is regulated within the cell by Bcl-X_L[119,128] and, as such, its release occurs following sequestering of Bcl-X_L within the cytosol by another member of the Bcl proapoptotic family, Bad.[129]

While Bax is well documented, less is known singularly about Bak. The proapoptotic Bak gene is found on chromosome 6 and codes for a 211-amino-acid protein with a molecular weight of 23 kDa.[130] There is evidence that Bak can form heterogeneous dimers with Bcl-2 or Bcl-X_L to inhibit their anti-apoptotic functions.[131] Studies have shown that Bak deficiency leads to an arrest in cytochrome *c* release, which can be restored following insertion of recombinant Bak into purified mitochondria.[132] Furthermore, this deficiency closely correlates with the occurrence and development of tumours.[133] Conversely, Bak overexpression mediates p53-independent apoptosis-inducing effects on human gastric cancer cells.[134]

Bid is a widely expressed, key member of the BH3 domain-only subgroup of the Bcl-2 family. It is implicated in both the intrinsic and extrinsic apoptotic pathways, where in relation to the latter, it is a substrate protein for the initiator caspase-8. The active form of the 22 kDa protein is its truncated 15 kDa derivative. Truncated Bid (tBid), generated by the proteolytic activity of granzyme B, lysosomal extract and the aforementioned caspase-8, translocates from the cytosol to the mitochondria where it induces a conformational change of Bax, permitting its insertion and oligomerisation at the outer mitochondrial membrane.[135] This process leads to the release of cytochrome *c*[94] and other mitochondrial factors such as Smac/Diablo. Furthermore, in addition to the activation of Bax/Bak, tBid inserts into the outer mitochondrial membrane, thus facilitating the additional release of the mitochondrial factors. This property highlights how BH3 proteins are paramount to the regulation of apoptosis. Several studies indicate that Bid is subject to other post-translational modifications and can be pro-apoptotic without caspase cleavage.[136–139] Other diverse factors that affect the function of Bid include its avid binding to mitochondrial lipids.[136] One lipid in particular, cardiolipin, has been reported to modulate caspase-tBid. The exact role that cardiolipin and other lysolipids play in relation to the direct function of tBid continues to be debated.[136]

Due to Bid playing a principal role in the overall cascade of apoptotic molecular events, it is potentially a molecular therapeutic target in pathologies relating to unregulated cell death.[140] The properties of this target are based on the identification of a deep hydrophobic crevice adjacent to the BH3 region. Binding of a small organic molecule BI-6C9 into this crevice induces the loss of pro-apoptotic activity of Bid *in vitro*.[140] These findings further highlight the importance of the BH3 peptide region of Bid as a critical effector of apoptosis. To this end, BH3 mimetics, small molecule antagonists of anti-apoptotic BCL-2 members, are being developed, some of which are currently undergoing clinical trial investigations (Review[141]).

Bad selectively heterodimerises with anti-apoptotic proteins Bcl-X_L and Bcl-2, but not with other family members.[129] Heterodimerisation of Bad with Bcl-X_L functions to displace the pro-apoptotic protein Bax. Bad contains domains BH1 and BH2, which are important for binding to Bcl-2. Regulation of Bad is dependent upon phosphorylation, which takes place on serine residues in response to select death signals and functions to inactivate the molecule.[114] Phosphorylated Bad cannot bind Bcl-2 or Bcl-X_L and remains sequestered in the cytosol.

Bad plays an important 'gatekeeper' role in relation to cell viability in the presence of growth factors, through regulation of the mitochondrial threshold for apoptosis. When Bad is phosphorylated in the presence of growth factors, it associates with 14-3-3 proteins facilitating the release of sequestered Bcl-X_L and subsequent inhibition of Bax. Upon growth factor deprivation, Bad is dephosphorylated and dissociates from 14-3-3 to bind and sequester Bcl-X_L or Bcl-2 on the mitochondria or within the cytosol.[142] This action permits the release of Bax, resulting in cytochrome *c* release and apoptosis. In-vitro experiments demonstrate that elevated levels of Bax exacerbate the effects of growth factor deprivation in cells.[123,143,144]

Inhibitors of apoptosis

There are several families of apoptotic inhibitors that exert their effect on various intracellular locations and pathways. These include the regulatory Bcl-2 family proteins, IAPs and the baculoviral cell survival factor 35.

Bcl-2 family proteins

Bcl-2 family proteins are intracellular membrane-associated proteins involved in the regulation of apoptosis induced by various stimuli.[61,87] The family members play a role in the regulation of the ion flow across mitochondrial membranes.[58] In addition to Bcl-2, those promoting cell survival include Bcl-X_L, A1, Mcl-1 and Bcl-W.[61,145–147] The ratio of these death antagonists to agonists determines the apoptotic response (Figure 5).[143,148]

Expression of Bcl-2, the prototypic member of the Bcl-2 family, prevents or delays apoptosis induced by a variety of stimuli in numerous cell types.[149] Bcl-2, in addition to Bcl-X_L has a predominantly outer mitochondrial membrane localisation and functions as cation-selective channel-forming protein within low pH conditions.[117] Their overexpression promotes cell survival through the suppression of apoptotic pathways. In addition to its mitochondrial position, other localisations include the nuclear envelope and endoplasmic reticulum.[146,150] Bcl-2 dimerises with other members of the family and the outcome for cell survival depends on the ratio of pro- to anti-apoptotic Bcl-2-like proteins.

The primary function of Bcl-2 (26 kDa) is to interfere with caspase activation. Speculation lies as to the specific regulatory role of Bcl-2 in relation to the mechanisms of caspase-3 inhibition. It may inhibit a protease that cleaves pro-caspase-3 or potentially interact directly or indirectly with

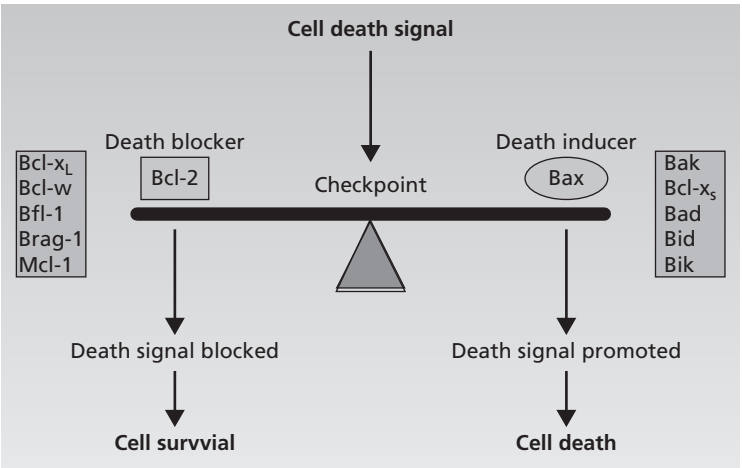

Figure 5 Bcl-2 family proteins are key regulators of apoptosis. Bcl-2 family proteins are able to form homo- and hetero-dimers and the balance achieved will determine the apoptotic fate of the cell (image from www.life.uiuc.edu/.../ figures/bcl2_system.jpg)

pro-caspase-3, rendering it a poorer substrate for its activating protease. Alternatively, Bcl-2 may act further upstream to inhibit signal transduction events that lead to caspase-3 activation.[151] Bcl-2 expression regulates caspase-3 activation normally observed following Fas death stimulus.[151]

Another anti-apoptotic role highlighted for Bcl-2 is the blocking of cytochrome *c* release from the mitochondria through inhibition of the mitochondrial permeability transition pore.[63,152] Prevention of cytochrome *c* release will affect the assembly of the apoptosome and subsequent caspase-3 activation. Furthermore, Bcl-2 is an important regulator of the key Bcl-2 pro-apoptotic protein Bax and can inhibit its ability to auto-activate, which in turn increases mitochondrial membrane permeability.[153] Due to its pivotal role in suppressing apoptosis, the Bcl-2 protein is considered to be an important multi-drug resistance molecule.

The 3D structure of Bcl-X_L, a 27 kDa homologue of Bcl-2, indicates the protein to be structurally related to certain bacterial pore-forming proteins.[154] Increased caspase-3 activity alongside substantial cell death has been observed following inhibition of Bcl-X_L indicating that this protein protects cells from a range of apoptotic stimulants.[61] Bcl-X_L binds to Apaf-1, which alongside caspase-9 forms a ternary complex. Both Bcl-X_L and caspase-9 bind to distinct Apaf-1 domains.[155] Bcl-X_L does not block pro-caspase-9 during the cytochrome *c*/Apaf activation pathway, suggesting its position lies upstream of, or at the level of, cytochrome *c* release.[120,156–158] Moreover, its ability to prevent Bax-induced release of cytochrome *c* from mitochondria suggests this protein cannot confer protection once cytochrome *c* has been released into the cytosol.[76]

The anti-apoptotic effects of Bcl-X_L are antagonised by pro-apoptotic members of the Bcl-2 family, including Bax, Bak and Bik, that are capable of forming heterodimers with Bcl-X_L, thus sequestering its activity.

Inhibitors of apoptotic proteins

The family of IAPs were first identified from baculoviruses (large DNA viruses), their characteristics allowing suppression of host cell death upon viral infection,[159,160] thus permitting survival and propagation of the virus. Two forms of baculoviruses exist – budded and occluded. Both are represented by enveloped nucleocapsids. In the occluded version these are embedded in large protein crystals, an arrangement which serves to protect the virus whilst in the external environment. Both types function to permit the spreading of the virus, the budded version enhancing spread within the host and the occluded version allowing spread between hosts.

IAPs suppress apoptosis through preventing procaspase activation and inhibiting enzymatic activity of mature caspases. Their overexpression has been shown to curb apoptosis following stimulation from various sources including tumour necrosis factor, Fas, menadione, staurosporine, etoposide, taxol and growth factor withdrawal.[11,161–164]

Deregulation of IAPs has been reported in cancers, with particular overexpression in cell lines derived from a variety of leukaemias, lymphomas and other cancers.[165] Furthermore, cIAP-1 overexpression is associated with greater resistance to DNA-damaging chemotherapeutic drugs in many cancer cells.[165]

In humans, eight IAP relatives have been identified: NAIP (neuronal apoptosis-inhibitory protein), c-IAP1, c-IAP2, XIAP (x-linked IAP), ML-IAP (melanoma IAP), ILP2 (IAP-like protein-2), survivin and Bruce/Apollo.[11,166] XIAP, cIAP1 and cIAP2 are potent caspase inhibitors.[120,167] The genes expressing these proteins, in addition to those of survivin, are located at the chromosomal locations Xq25, 11q22–q23, 11q22–23 and 17q25, respectively.[17,162,168,169]

IAP proteins are metalloproteins, and they are characterised by one or more copies of zinc-binding signature motifs called BIRs (baculovirus IAP repeat) – novel domains of ~70 amino acids (Figure 6).[160] The possession of at least one of these functional motifs is a compulsory component for anti-apoptotic activity.[56] BIR motifs have a conserved $CysX_2Cys$ motif near its centre followed by a $HisX_6Cys$ motif near its carboxyl end (X being any amino acid).[170] BIR motifs inhibit apoptotic action through direct caspase

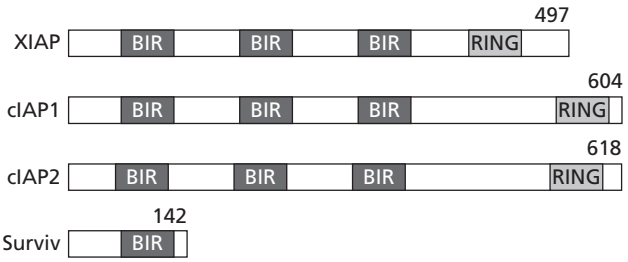

Figure 6 Location of BIR domains within mammalian BIR-containing proteins. Amino-acid length is shown to the upper right of each protein (adapted from Deveraux and Reid 1999[11])

binding.[11,120] Interaction with these motifs by IAP binding proteins such as Smac/Diablo and Omi/HtrA2 disrupts caspase/IAP binding permitting caspase release and subsequent execution of apoptosis. XIAP, c-IAP1 and c-IAP2 contain three BIR domains each and the different BIR domains have distinct functions. In XIAP, the third BIR domain (BIR3) potently inhibits the activity of processed caspase-9, whereas the linker region between BIR1 and BIR2 specifically targets caspase-3 and -7.[101,171]

In addition to BIRs, many IAPs may contain another zinc-binding motif called a RING domain at the carboxyl terminus.[170] First described in 1991, its motif is C_3HC_4.[172] RING functions include involvement in the building of multi-protein complexes via a 'scaffolding arrangement'.[173] This is facilitated via binding of two zinc atoms, thus forming a highly stable platform upon which complexes can be assembled.[174] Further RING functions include involvement in the ubiquitination process of proteins, in particular the permanent removal of active caspases through the proteasomal pathway.[166]

IAPs can be antagonised by pro-apoptotic proteins that bind directly to them via their N-termini. In particular, Smac/Diablo and Omi/HtrA2 bind to XIAP, thus preventing binding of processed caspase-9 and caspase-3, respectively, which in turn will promote cell death.[175] Since Smac and Omi are released from the mitochondria during the early phase of apoptosis, it is hypothesised that they could constitute a positive feedback loop to amplify caspase activation by preventing BIR3 from inhibiting caspase-9.[176]

IAPs are crucial to regulation of the intrinsic pathway; however, they display no binding to, or inhibition of, caspase-8, thus providing some protection from Fas/caspase-8-induced apoptosis. However, this extrinsic pathway can be influenced at the caspase-3 junction, with full activation of the pro-enzyme prevented through binding to the partially processed enzyme.[3]

The principal IAPs within the intrinsic pathway, XIAP, c-IAP1 and c-IAP2, can block cytochrome *c*-induced activation of caspase-9 through binding of its zymogen. This in turn prevents formation of the apoptosome and subsequent activation of pro-caspase-3, -6 and -7.[3] Only active forms of caspases -3 and -7 bind to these particular IAPs.[177] While several IAPs bind to some extent to caspase-9, it is primarily inhibited by XIAP. In relation to the processing of caspase-9, inhibition by IAPs functions by: (1) direct inhibition of auto-activation of pro-caspase-9 induced by Apaf-1, cytochrome *c* and dATP via the anti-apoptotic protein Bcl-X_L and (2) by blocking the cleavage of pro-caspase-9 by active caspase-3.[3]

XIAP, the first IAP protein shown to inhibit caspases directly,[11] inhibits the intrinsic pathway upstream of caspases -3, -6 and -7. In addition it can bind and inhibit processed caspase-3, -7 and -9.[3,120,161,162,178] Caspase-3 and -7 are inhibited to a lesser extent by cIAP1, c-IAP2 and NAIP.[102] Caspase-3 is ubiquitinated in the presence of XIAP, suggesting that XIAP acts as an ubiquitin-protein ligase, promoting the degradation of caspase-3 via the proteasomal pathway, thus enhancing any anti-apoptotic effects.[177]

The IAP survivin blocks apoptosis by inhibiting caspases, particularly active caspase-3 and -7.[179,180] Targeted disruption of survivin in several transformed cell models enhances apoptosis.[181] Survivin is a ~16.5 kDa cytoplasmic protein whose gene locus is 17q25. In contrast to other IAP proteins, it possesses a single BIR and no RING finger.[163,169,170] The single BIR domain shares an amino-acid sequence similar to the BIR2 region of XIAP.[163,182]

Following expression during embryonic and fetal development, where its role may include tissue differentiation, the gene becomes quiescent in most adult tissue medulla.[183] In fact, survivin mRNA is only found occasionally in adult human and mouse tissue.[163] However, survivin becomes aberrantly expressed in almost all cancers, including lung, colon, breast, pancreas and prostate, in addition to haematopoietic malignancies, and furthermore, correlates with poor prognosis.[163] As such, survivin shows increasing notoriety as a potential tumour marker.[11]

Decreased survivin expression has been observed using antisense strategies, leading to apoptosis and sensitisation to anticancer drugs within some tumour cell lines.[169]

p35

p35 is a baculoviral cell survival factor which directly inhibits activation of the caspases in a stoichiometric fashion.[8,184,185] First identified by Friesen in 1987, this protein shows broad inhibitory activity against most of the caspase family enzymes.[11,99,186,187] The structure of p35 has been described as being similar to a teapot with a flexible, solvent-exposed loop containing a caspase cleavage site at the apex.[174,188] Cleavage of this loop by caspase occurs following an aspartate residue, yielding cleavage products of 10 and 25 kDa, which remain associated with the caspase. Below the reactive-site loop is an α-helix and an eight-stranded β-sheet, whose disruption or interaction can eliminate anti-apoptotic function, thus transferring p35 from the role of stoichiometric caspase inhibitor to that of caspase substrate.[174,188]

p35 potently inhibits most caspases both *in vivo* and *in vitro*,[160,189] although it should be noted that p35 would not normally be present in mammals as it is expressed naturally by baculoviruses. Nevertheless, it presents itself as a useful molecule for the pharmaceutical industry for potential therapeutic use.

Summary

Cells can activate or respond to multiple death pathways according to environmental status and stimulus received.

While apoptosis remains the key player in cellular death, other forms cannot be dismissed. Within the processes of apoptosis, caspases have a pivotal role, although many other proteins within apoptotic pathways have been identified. Research continues into establishing the precise aspects of their molecular mechanisms of action and inter-relationships. It is important to close any gaps in our knowledge and understanding of these issues, as cellular death pathways are instrumental in their contribution and association in both health and disease. As such, inappropriate apoptosis due to dysregulation of cell death pathways provides a plethora of molecular checkpoints that can be targeted and modulated as part of therapeutic intervention. In particular, increased research into these areas will prove useful for the design of novel chemotherapeutic drugs, an area that is particularly important due to increased risk of chemoresistance.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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