REVIEW

Swords of Cell Death: Caspase Activation and Regulation

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Abstract

    Apoptosis represents a crucial process in modulating organ development in the embryo, in organ homeostasis in the adult, and in fostering appropriate immunological function. Caspases represent two central class of molecules that are either involved with the stimulation of the apoptotic cascade (initiator caspases), or the various sequential biological pathways required for its execution (effector caspases). With an eye towards therapeutic opportunities, this review discusses in detail the lineage of initiator and effector caspases, how they are each activated, their substrates, their regulation, and maps out how they interact throughout the process from initiation of the first apoptotic signal to the final consequential breakdown of cellular integrity.

Keywords: Apoptosis - caspases - Fas - DNA damage

Apoptosis

    During the last few years, an extensive research of apoptosis becomes increasingly publicized (Elmore, 2007; Lockshin and Zakeri, 2007; Fulda, 2008; 2009; 2010; Li, 2008; Fulda and Debatin, 2008; Taylor et al., 2008; Vazquez et al., 2008; Youle and Strasser, 2008; Chen and Pervaiz, 2009; Deng et al., 2009; Engel and Henshall, 2009; Lu and El-Deiry, 2009; Circu and Aw; 2010; da Fonseca et al., 2010; Fulda and Pervaiz, 2010; Galluzzi et al., 2010; Lamkanfi and Kanneganti, 2010; Speidel, 2010). This wealth of literature is elaborated for investigating the mechanisms by which different proteins and enzymes are altered and consequently lead to deregulation of apoptosis that is implicated in different diseases (Zhivotovsky and Orrenius, 2010) such as cancers (Chen and Lai, 2009; Buggins and Pepper, 2010; Pennarun et al., 2010), immunologic disorders (Elkon, 2006; Muñoz et al., 2010), neurodegenerative diseases (Yin and Dong, 2003), respiratory diseases; COPD (Makriset et al., 2009), pulmonary hypertension (Jurasz et al., 2010), acute respiratory Distress Syndrome (ARDS) (Galani et al., 2010) and many others. Apoptosis is the 20th century scientific revolution that began in 1962 with John Kerr, the Australian scientist. The full story of apoptosis is shown elsewhere (Sluyser, 2005). The word 'apoptosis' (a-po-toe-sis) is used in Greek to describe the 'dropping off' or 'falling off' of petals from flowers, or leaves from trees. Apoptosis or programmed cell death (PCD) is an ordered and fundamental biological process designed safely to dispose of surplus, aged or damaged cells in

order to facilitate maintenance of a viable immune system, embryogenesis, tissue homeostasis and the inflammatory response (Wyllie et al., 1982; Raff, 1992). Apoptosis is distinct from necrosis (Galluzzi et al., 2007; 2008), the latter results in the loss of cell membrane integrity and the release of the dying cell's contents in an uncontrolled, and possibly harmful, manner. In contrast, apoptotic cells shrink in volume, their surface undergoes blebbing, there is deoxyribonucleic acid (DNA) fragmentation, chromatin condensation, formation of apoptotic bodies, and loss of nucleoli. The apoptotic cell is phagocytosed intact or as discrete fragments bound by an intact membrane, thus disposing of these cells without release of their contents. While cell necrosis promotes inflammation, recent evidence suggests that the inflammatory response is provoked by the release of a full spectrum of molecules collectively called alarmins whose specific identity still awaits precise definition (Oppenheim and Yang, 2005), apoptosis is therefore more associated with its resolution. Apoptosis is crucial in the development and maintenance of multicellular organisms. Perturbation of the complex apoptotic mechanisms employed by the cell has been implicated in disease aetiology (Galluzzi et al., 2007; 2008). For example, carcinogenesis is associated with loss of apoptosis induction in mutated cells whereas increased apoptosis is suspected in the pathogenesis of neurodegeneration and immunodeficiency. Apoptosis is an active form of cell death in which the cellular machinery, once initiated, executes a safe, controlled self-destruction. Much work demonstrating clear evidence of the active nature of apoptosis initially utilised the nematode

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Caenorhabditis elegans (*C.elegans*) (Ellis et al., 1991). The apoptotic process requires the activation of specific genes and synthesis of new proteins.

The cellular response to a damaging agent is a sequence of events that lead to cell cycle arrest and repair or alternatively to apoptosis. Cells can be infringed with several genotoxic and non-genotoxic insults that result in a rapid increase of p53. In fact, every type of DNA damage (i.e. radiation, reaction with oxidative free radicals, drugs, virus infection, etc.) is reported to the p53 protein and its pathway. In addition damage to components involved in the proper handling of the genetic material (such as the mitotic spindle), hypoxia, oncogenic activation, ribonucleotide depletion or exposure to nitric oxide, also increase the cellular levels of this protein. Hence, the p53 pathway involves hundreds of genes and their products that respond to a wide variety of stress signals and p53 is arguably the most intensively studied protein today. It plays a key role in monitoring the genetic fidelity of cells, for which it has been appointed as the "guardian of the genome". Its response to DNA-damage or checkpoint failure gives rise to a series of anti-proliferative responses. The importance of p53 in the detection of genetic defects in the early pre and post implantation of mammalian embryos has been demonstrated, as it induces cells with teratogenic damage to die via apoptosis and the defective embryos to abort. Thus, one of the most important functions of p53 is its ability to induce apoptosis, while disruption of this route can promote tumour progression and chemotherapy resistance. The transcriptional program that p53 switches on is remarkably flexible, as it varies with the nature of the activating stimuli, the cell type and the duration of the activation signal. This flexibility may allow cells to mount alternative responses to p53 activation, such as cell cycle arrest or apoptosis.

Upon diverse forms of cellular stress the steady state levels and transcriptional activity of p53 are rapidly (within two hours), and considerably increased. The stabilization and activation of p53 are a result of hindered inhibition by its negative regulators, e.g. Mdmx and Mdm2, while other activators such as HIPK2 and DYRK2 could enhance the p53 response. Once p53 is increased and activated, it gains the ability to bind to p53-responsive DNA sequence elements in the genome. Transcription of more than 150 genes is positive or negatively regulated by p53. There appear to be some p53-regulated genes that are transcribed in response to many different types of stress signals and in all tissues responding to the stress (e.g. MDM-2), while others are either stress- or tissue specific (e.g. PTEN, TSC-2).The functions of the p53 response genes fall into several categories. A set of genes and their products are clearly involved in cell cycle arrest and cellular senescence (e.g. GADD-45). In cells beyond repair, a second set of p53- regulated genes involved in apoptosis are implicated. Both the extrinsic and the intrinsic pathways of apoptosis are stimulated. In the extrinsic pathway p53 regulates Fas production (a secreted protein), as well as DR5/killer, the trail receptor and a membrane protein. The intrinsic pathway is populated with many p53-regulated genes of which the proteins Bax, Noxa and Puma may work in different cell types (reviewed in Campo, 2009). It has

now confirmed the existence of a direct p53-mediated mitochondrial death (Wolff et al., 2008).

Cell death mechanisms operate in both uni- and multicellular organisms, although not all components of the cell death machinery are expressed in all biological species. Death with apoptotic morphology has been observed in a range of organisms from plants to mammals; however, neither plants nor yeast express caspases. A similar expression of apoptotic proteins was described in nematodes, flies, rodents and humans. While the molecular mechanisms of apoptosis and necrosis are now relatively well established, the precise mechanisms of other cell death modalities require additional studies. Moreover, it is still unclear whether autophagy represents a distinct form of cell death, or functions as a survival mechanism that might cause cell death under conditions of prolonged nutrient starvation. The identification of genes and gene products that regulate different modes of cell death and their cross-talk, together with an increased knowledge about their mechanisms of action, have since laid a foundation for novel therapeutic strategies which target cell death pathways. The identification of various "decision points" that regulate the switch between cell life and death has provided a multitude of molecular targets which offer a range of therapeutic options. It is becoming increasingly clear that individual components of the cell death machinery do not operate in isolation, and that the activation of one cell death pathway alone might not be sufficient to kill all damaged cells. Supporting this assumption is accumulating evidence showing that the induction of multiple cell death pathways might be more effective for eradication of damaged cells. Thus, further characterization of signaling events critical for regulation of various cell death modalities and their cross-talk is essential not only for understanding the pathogenesis of cell death related diseases, but also for the development of new therapeutic strategies for their treatment (Zhivotovsky and Orrenius, 2010).

Genetic Regulation of Apoptosis

In the nematode C. elegans, mutants have been identified in which the 131 cells normally fated to die during development survive. Genetic analysis has revealed that two genes, ced-3 and ced-4, are required for the deaths of all but one of these cells and a third gene, ced-9 prevents cell death in the remaining cells (the term, ced, stands for cell death defective (Steller, 1995). Through molecular cloning, at least one mammalian homologue for each gene has been found, each with a role in regulating apoptosis in mammalian cells. Ced-3 is structurally similar to the interleukin 1β converting enzyme (ICE) which has been identified as belonging to a family of aspartate-specific cysteine proteases, collectively known as caspases. Ced-9 is homologous to the human Bcl-2 gene, which is implicated in the suppression of multiple apoptotic pathways in mammalian cells. Ced-4 is homologous to apoptosis protease activating factor-1 (Apaf-1), a polypeptide which can directly promote activation of caspase-3. Other genes have been associated with apoptosis because their expression is increased in cells undergoing the process, for

example, RP-2 and RP-8 (Owens et al., 1991); testosterone-repressed prostate message-2 (TRPM-2), also known as clusterin; and tissue transglutaminase (Fesus et al., 1991). Disruption of apoptotic pathways can allow cells to escape death and increase the possibility of acquiring a mutation in a proto-oncogene and consequently developing into a cancer-causing cell. Indeed, the much-studied p53 gene is responsible for apoptosis induction in cells that have acquired genetic damage. Mutations of p53 have been found in > 50% of human cancers (e.g. colon carcinoma) and are associated with resistance to treatment (Donehower et al., 1992). The gene c-myc is a proto-oncogene that encodes a sequence-specific DNA-binding protein that acts as a transcription factor and induces apoptosis in the presence of p53. Apoptosis pathways include the extrinsic receptor-mediated pathway that is initiated by ligands binding to extracellular death receptors and the intrinsic mitochondria-mediated pathway that is also directly triggered by signals that originate within cells, such as DNA damage. P53 influences both pathways of apoptosis significantly. Through its transcriptional nuclear activities it can induce several apoptosis-promoting factors (including, but not restricted to, Bax, Bid, Apaf1, the death receptors CD95/Fas/Apo-1 and Killer/DR5), whereas the expression of anti-apoptotic effectors (including Bcl-2, Bcl-xL and the IAP survivin) is repressed. By transcription-independent activities, cytoplasmic or mitochondrial p53 promotes the direct activation of Bax and Bak, neutralises the anti-apoptotic function of Bcl-2 and Bcl-xL, and increases the concentration of death receptors at the cell surface (Green and Kroemer, 2009; Vaseva and Moll, 2009). Understanding the intracellular molecular mechanisms involved in the control of apoptosis may enable us to manipulate them for therapeutic intervention.

Caspase Family

Definition and classification

Caspases belong to a conserved family of proteases that use a cysteine residue as a catalytic nucleophile to cleave their substrates specifically after aspartic acid residues. In metazoans, caspases play an essential role in apoptosis and inflammation. The first member of the family, caspase-1, was discovered in humans in 1992 as an important regulator of inflammation and originally called ICE (Cerretti et al., 1992). The role of caspases in apoptosis emerged several months later from a genetic analysis of the nematode *C. elegans*. The team of Robert Horvitz generated *C. elegans* mutants in order to identify genes involved in developmental cell death. In 1993, they cloned the first cell death gene, ced3, and showed that it encodes a cysteine protease (ced3) related to the mammalian ICE (Yuan et al., 1993). Since then, a number of caspases have been cloned from mammals and other species. Fourteen caspases have now been identified in human, ten in mouse, four in chicken, four in zebrafish, seven in Drosophila melanogaster and four in C. elegans (Lamkanfi et al., 2002). Based on their function, structure and substrate specificity, human caspases are classified into three groups. Group-I or inflammatory caspases (caspase-1, -4, -5, -12) are characterized by the presence of a caspase

recruitment domain (CARD) at the N-terminus and they preferentially cleave their substrate after sequence XEHD where X is a hydrophobic residue (Thornberry et al., 1997). Recently, caspase-12 has been shown to regulate negatively the inflammatory response by inhibiting the activity of caspase-1 (Saleh et al., 2004; 2006). Group-II, or effector caspases, (caspase-3, -6 and -7) recognize the tetrapeptide DEXD and are responsible for the cleavage of cellular components during apoptosis. Group-III, or initiator caspases (caspases-2, -8, -9, -10) are characterized by the presence of protein-interacting domains in their N-terminus (either CARD or death effector domains (DED), and they specifically cleave the sequence (I/L) EXD present in effector caspases and other substrates. Despite the separation of caspases into inflammatory and apoptotic classes, some apoptotic caspases have been found to take part in other cellular processes, including cell cycle progression and cell survival.

Cysteine aspartate specific proteases (Caspases)

One common pathway, by which many apoptotic mechanisms may function in a coordinated manner, relies on the activation of the caspase family of proteases. This group of cysteine protease enzymes is structurally related, sharing a specific ability to cleave peptides bound after aspartic acid residues. Caspases appear to be essential components in the apoptotic machinery. They operate by cleaving cytoskeletal and nuclear proteins critical for maintenance of cell structure, and enzymes involved in metabolism and repair (Gorman et al., 1998). At present, 14 mammalian caspases have been identified (Deveraux et al., 1999). The caspases are synthesised as inactive pro-enzymes (zymogens) and, upon appropriate apoptotic triggering, they are converted into mature enzymes by the formation of heterodimers that contain the active catalytic unit. During apoptosis, caspases undergo an hierarchical cascade of activation from the initiator to the effector caspases. Active caspases have a highly conserved, dimeric structure resulting from the association of two identical catalytic subunits. Each catalytic subunit contains one active site and is composed of one large and one small active domain. Upon maturation, pro-caspases are proteolytically processed at the cleavage site located between the large and the small subunits. The enzymes of the caspase family consist of three structural/functional domains, the amino-terminal, plus one large 20 kilo Dalton (kDa) and one small (10kDa) subunit. During caspase activation, the small and large subunits associate, forming a heterodimer with both domains contributing important residues for substrate binding and catalytic activities (Nicholson and Thornberry, 1997; Gupta, 2000). Caspase prodomains range in length from 23 amino acids for caspases-6 and -7, to 219 amino acids for caspase-10. Caspases with large prodomains are thought to be involved in the initiation of the apoptosis response and known as initiator caspases. Caspases with short prodomains are apparently activated by the initiator caspases and known as effector caspases.

Activation of the initiator caspases

Activation of caspases is an important step in the signal
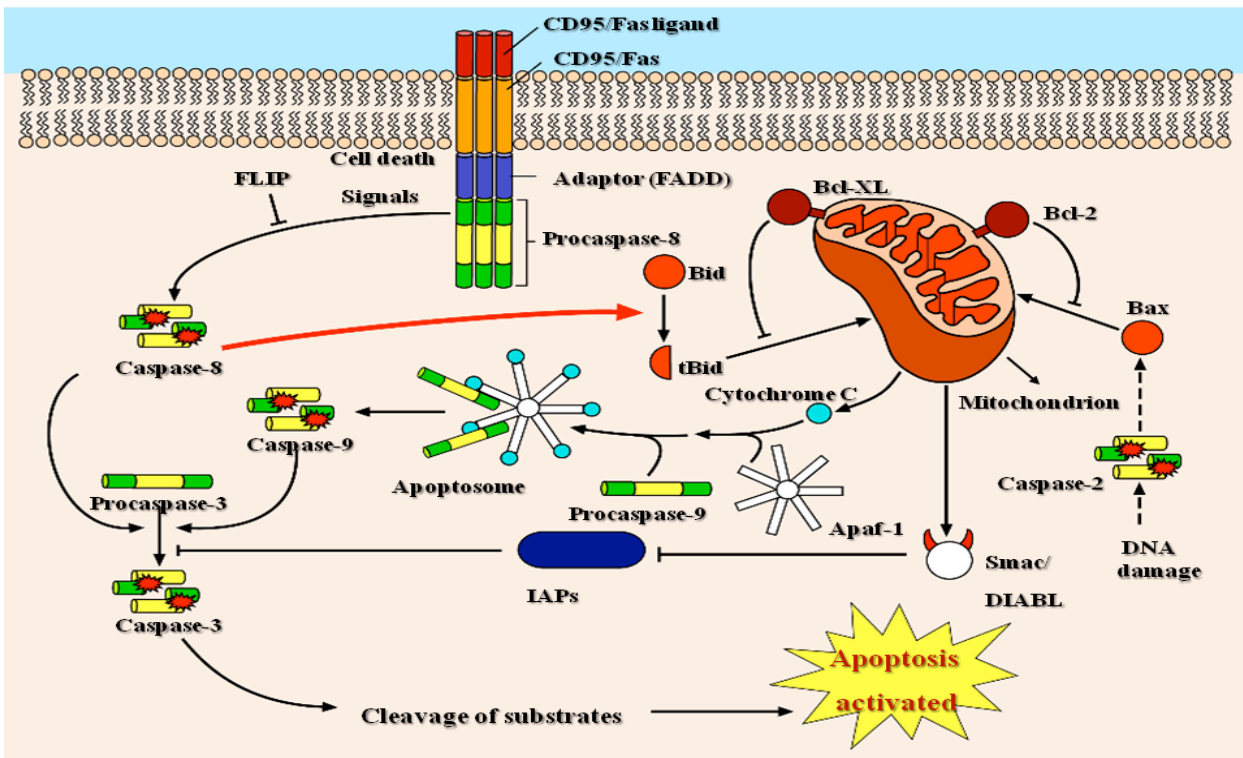
Figure 1. Pathways of Apoptosis Signal Transduction. The apoptotic signal is initiated by direct ligand-mediated trimerization of DR family members like Fas. This leads to the formation of the DISC complex and activation of procaspase-8 by induced proximity. Active caspase-8 can directly cleave procaspase-3, thereby starting the executioner phase of apoptosis. Alternatively, cleavage of the pro-apoptotic Bcl-2 family member Bid leads to amplification of the apoptotic signal by the mitochondrial pathway. This pathway is also used in apoptosis induced by cytotoxic stress. It leads to the release of pro-apoptotic factors like cytochrome-c and Supramolecular activation cluster (Smac) from the mitochondrial inter-membrane compartment to the cytosol. Cytochrome-c release leads to formation of the apoptosome, which activates caspase-3

transduction of apoptosis (Figure 1). Ligation of the death receptor Fas (CD95/Apo-1) can result in the recruitment of a caspase to the cytoplasmic death-inducing signalling complex (DISC). Two groups have independently shown that caspase-8 can be recruited to the DISC through binding to the adaptor molecule FADD, which associates with the cytoplasmic death domain (DD) of Fas (Boldin et al., 1996; Muzio et al., 1996). This observation suggests that recruitment of a caspase to a death complex may be sufficient for its activation. Indeed, caspase-8 is activated by self-processing once it is recruited to the activated Fas receptor (Medema et al., 1997). Caspase-10 was also shown to be activated upon Fas and tumor necrosis factor –receptor (TNFR) ligation (Vincenz and Dixit, 1997). Another adaptor molecule, RAIDD, was subsequently cloned and shown to associate with caspase-2 and RIP, a protein that interacts with TNFR through the adaptor protein TRADD (Duan and Dixit, 1997). Thus, caspase-2, -8 and -10 can be recruited through adaptors to death complexes at the cytoplasmic end of the death receptors. The common feature between these caspases is that they all contain long amino-terminal prodomains, regions that are cleaved off following the proteolytic activation of the caspase molecule. Both caspase-8 and -10 contain two DED, similar to those found in adaptor molecules, such as Fas associated protein with a death domain (FADD), and which mediate the interaction between the caspase and the adaptor (Boldin et al., 1996; Muzio et al., 1996; Duan and Dixit, 1997). FADD also contains DD, which

mediates its recruitment to the death domain (DD) found in the carboxyl cytoplasmic region of the death receptors. Similarly, the caspase-2 prodomain contains a region of homology with the adaptor RIP-associated ICH-1/CED-3-homologous protein with a death domain (RAIDD), while RAIDD can, in turn, interact with TRADD, another death effector domain (DED)- containing protein that is recruited to the TNFR and can interact with the Fas death complex through FADD (Duan and Dixit, 1997). Upon receiving a death signal, activation of the first caspase can be seen as the initiating event in a cascade that commits a cell to apoptosis. Because caspases require processing that occurs after certain aspartic residues, it was predicted that they may have intrinsic pro-enzyme activity that may mediate their autocatalysis (Nicholson and Thornberry, 1997). A number of reports have shown that some upstream caspases, such as caspase-2 and -8, can be autoactivated by homodimerization/ oligomerization (Vakifahmetoglu-Norberg and Zhivotovsky, 2010). In the case of caspase-2, the amino-terminal prodomain region was shown to be essential for dimerization, autoprocessing and activation of the procaspase molecule (Yang et al., 1998). However, a growing body of evidence has led to the re-evaluation of this activation process, and a refined version of the proximity-induced model has been proposed (Chen et al., 2002; Chang et al., 2003). In this model, known as the proximity-induced dimerization, the dimerization provides essential active site rearrangements and this process is considered a prerequisite for the activation of the initiator

caspases. Proteolysis of the inter-subunit linkers is also required for the activation of the enzyme but it occurs after dimerization of the zymogen (Chen et al., 2002). Whereas dimerization is sufficient to trigger self-processing of the initiator caspases, their auto-cleavage alters their substrate specificity and favours processing of the effector caspases (Chen et al., 2002; Chang et al., 2003). In other words, in the proximity induced dimerization model, processing of an initiator caspase is not sufficient to induce its activation unless it is already in a dimeric configuration, and thus, these pre-requisites are required to drive forward the cascade of caspases.

Activation of the effector caspases

In contrast to the initiator caspases that are monomeric as zymogens, the effector caspases are held in the cytosol as inactive dimers. Following apoptotic stimulation, effector pro-caspases are converted catalytically active enzymes by cleavage in the linker region between their large and small active subunits. In most instances, this activating event is catalyzed by active initiator caspases. Analysis of the crystal structure of caspase-7 demonstrated that upon cleavage, the region of the active site of the enzyme undergoes conformational changes that render it accessible to its substrates (Chai et al., 2001). The active site is composed of four surface loops, L1 to L4, all from the same monomer and is stabilized by the L2' loop from the adjacent monomer. Caspase-3 is the major effector caspase, which cleaves a plethora of cellular substrates during apoptosis (Porter and Janicke, 1999). Caspase-7 is highly similar to caspase-3 and they demonstrate similar substrate specificity (Fuentes-Prior and Salvesen, 2004). Mice genetically deficient in either caspase-3 or caspase-7 are viable, whereas those that lack both these caspases have defects in heart development and die immediately after birth (Lakhani et al., 2006). Mouse Embryonic Fibroblast (MEFs) lacking both enzymes are resistant to intrinsic and extrinsic apoptotic stimuli, suggesting that caspase-3 and caspase-7 carry-out redundant, but essential functions during the execution of cell death. In contrast, caspase-6 presents different substrate specificity and does not seem to be required for apoptosis (Loegering et al., 2006).

The downstream (effector) caspases, such as caspase-3 and -7, show poor autoprocessing ability in mammalian cells, suggesting that they require activation by upstream (initiator) caspases. Mitochondria sense apoptotic signals. The initiator caspases (usually caspase-8) cleave Bid. A C-terminal fragment of cleaved Bid goes to the mitochondria, which leads to loss of mitochondrial transmembrane potential (m) and release of cytochrome c into the cytosol (Liu et al., 1997; Li et al., 1998; Steemans et al., 1998). Bid requires Bax, a pro-apoptotic Bcl-2 family member, for its action on mitochondria. Release of cytochrome-c into the cytosol was found to be an early event in apoptosis and could be inhibited by the presence of B cell leukaemia/lymphoma 2 (Bcl-2). Bcl-2 is a 26kD protein, located on the outer mitochondrial membrane, whose over-expression had previously been found to block apoptosis induced by a wide range of stimuli. Together with the activation adapters, Apoptosis protease activating factor-1 (apaf-1) and dATP deoxy-Adenosine Triphosphate

Table 1. Target Proteins of Caspases

Cytoskeletal proteins
Actin, β-catenin, fodrin, gelsolin, gas2, keratins

Nuclear proteins:
Lamins, Rb protein, Sp1, IκB-α, DNA-PK, PARP, Mdm2, U1-70 kDa subunit of small nuclear ribonucleoprotein, topoisomerases I and II, histone H1, hnRP C1 and C2, differentiation specific element binding protein, dentatorubral-pallidoluysian atrophy gene protein, sterol regulatory element binding protein.

Regulatory proteins:
Procaspases, focal adhesion kinases, protein kinase Cδ, presenilin 1 and 2, rabaptin-5, MEKK1, PITSLRE protein kinase, Huntington, D4-GDP dissociation inhibitor, phospholipase A2, inhibitor of caspase activated Dnase, Bcl-2, Bcl-xL, p28 Bap31

(ATP), cytochrome c released from mitochondria activates procaspase-9. Cytochrome-c in the presence of dATP or ATP induces a conformational change of the adapter protein Apaf-1 such that it not only binds pro-caspase-9, but also dimerizes, which allows the juxtaposition of two pro-caspase-9 molecules resulting in autoactivation and release of mature caspase-9 (Hu et al., 1998). Caspase-9 then activates effector caspases such as caspase-3 and caspase-7 (Slee et al., 1999). However, Bratton's group found that caspase-9 only processes effector caspases when bound to Apaf-1 (Bratton et al., 2000). They suggest that WDR domains of Apaf-1 recruit caspases-3 and -7 once the Apaf-1 CARD domain has bound caspase-9. Then caspase-9 processes these effector caspases, initiating their release from the complex (Bratton et al., 2000). Caspase-3 then processes caspases-6 and -2 and, in addition, feeds back to process additional caspase-9. Caspase-6 may subsequently activate caspases-8 and -10, as well as caspase-3. However, in some cell types, caspase-8 can directly activate the effector caspases without requiring release of mitochondrial cytochrome-c (Scaffidi et al., 1998). These effector caspases act on a multitude of substrates, affecting the integrity of the cell skeleton, the nuclear lamin structure, DNA, while repair systems become inactivated.

Substrates of caspases

Caspases facilitate apoptosis through cleavage of specific substrates. More than 70 substrates for caspases have been identified so far. These substrates include proteins involved in cell structure, signalling, cell cycle control, and DNA repair (amongst others). Substrate cleavage by caspases does not always lead to protein degradation because a number of substrates, particularly those involved in signaling, become constitutively activated on proteolytic separation from regulatory domains. A current list of known caspase substrates is provided in Table 1. It is important to note that a large number of caspase substrates are thought to be cleaved by caspase 3. Whereas this is certainly true for some substrates, other caspases with similar specificities may play these roles in vivo. Caspase 3-deficient MCF-7 breast carcinoma cells, for example, cleaves more than just spectrin during apoptosis; in an analysis of the putative caspase 3 substrates, spectrin, poly (ADP)-ribosylating

protein (PARP), Rintoblastma (Rb), DNA-dependent protein kinase (DNA-PK), gelsolin, and inhibitor caspase-activated deoxyribonuclease (ICAD)/ DNA fragmentation factor (DFF) were all identified (Jänicke et akl., 1998; Saikumar et al., 1999).

Regulation of caspase activation

Because caspase activation is a vital step in the initiation of an apoptotic cascade, it is predictably a very tightly regulated process. Mammalian cells seem to have developed several ways to prevent inappropriate activation of caspases (Figure 1). Bcl-2-like proteins are the main players in preventing cell death mediated by most stimuli; however, apoptosis mediated by death receptors, in most circumstances, bypasses the Bcl-2 control mechanisms (Sharad, 1999). For example, overexpression of Bcl-2 or Bcl-xL prevents staurosporine-induced cell death of Jurkat T cells and the processing of caspase-3 and caspase-7 (Chinnaiyan et al., 1996). Apoptosis is an important cellular defence against viral infection. Some viral proteins, such as cowpox virus cytokine response modifier cytokine response modifier A (CrmA) and baculovirus p35, and IAP proteins inhibit apoptosis by directly interacting with caspases (Nicholson and Thornberry, 1997). CrmA is a potent inhibitor of caspase-8, a component of the Fas (CD95) and TNFR death pathways, and which CrmA is able to inhibit with high affinity (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998). Baculovirus p35 is a broader inhibitor of caspases and its overexpression has been shown to inhibit apoptosis in a number of systems (Bump et al., 1995; Xue et al., 1995). In mammalian systems, IAP family proteins have been shown to bind and potently inhibit activated caspases. Among the caspases inhibited by human IAP family members XIAP, cIAP1, and cIAP2 are the effector caspases-3 and -7, as well as the initiator caspase-9 (Deveraux et al., 1997; Roy et al., 1997). Several groups have characterized a cellular protein that shares significant homology with the DED-containing caspases (caspase-8 and -10). This protein, variously called FLICE-inhibitory protein (FLIP), Casper, Flame, CASH, I-FLICE, MRIT and Usurpin (Goltsev et al., 1997; Han et al., 1997; Hu et al., 1997; Srinivasula et al., 1997; Rasper et al., 1998), functions in a manner similar to v-FLIP by inhibiting the recruitment of caspase-8 and -10 to activated death receptors.

Other useful molecules that modulate the activation of caspases are potentially excellent therapeutic targets to control the inappropriate apoptosis characteristic of many diseases. The three most extensively used peptides include YVAD, based on the caspase-1 cleavage site in proIL-1β, DEVD, designed from the caspase-3 cleavage site in PARP, and VAD, a broad inhibitor of caspases (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998). These synthetic peptides are commonly coupled with a C-terminal aldehyde (e.g. Ac-YVAD-CHO or Ac DEVD-CHO) or a ketone (e.g. Ac-DEVD-cmk or zVAD-fmk, where -cmk and –fmk denote chloromethyl and fluoromethyl ketones, respectively), which act as effective competitive, reversible and irreversible caspase inhibitors, respectively (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998). zVAD-fmk, the cell permeable inhibitor that acts on a number of

caspases, has been extensively used to inhibit apoptosis in cultured cells, tissues and whole animals (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998).

Regulation of caspase-8 activity

Caspase-8 was identified in 1996 as a component of the DISC. It is homologous to FADD and the ICE family and was originally named FADD-like interleukin-1 beta-converting enzyme (FLICE) and MACH (Nagata and Golstein, 1995; Wallach et al., 1997. In type I cells (e.g., T cells), procaspase-8 is expressed as two functional isoforms, caspase-8/a and 8/b (55 and 53 kD, respectively) both encoded by the same gene. Pro-caspase-8 is composed of a large prodomain containing a tandem of two DED domains and two active subunits, one of 18 kDa and one of 10 kDa. Caspase-8 is the first caspase to be activated after death receptor (DR) stimulation. As mentioned above, caspase-8 activation is thought to follow the proximity induced-dimerization model. After Fas receptor engagement, cytosolic monomers of pro-caspase-8 are recruited via their DED to the DISC located at the plasma membrane (Scaffidi et al., 1997). This clustering results in the dimerization of the zymogens which then acquire a limited capacity of enzymatic activity (Boatright et alk., 2003). In this context, dimerization favours an enzymatically competent conformation and promotes autoprocessing of the dimer (63). It is more likely that caspase-8 autoprocesses by intramolecular cleavage. In this scenario, formation of the dimeric zymogen is a prerequisite event for the autoprocessing of caspase-8. Upon dimerization, procaspase-8 is cleaved firstly between its two active subunits and secondly in the region linking the prodomain and the large active subunit finally to form the dimeric active enzyme. These two sequential cleavage events are required for the full activity of caspase-8. Additionally, the processed form of caspase-8 shows higher activity compared to the zymogen (called zymogenicity) . Therefore, dimerization of caspase-8 zymogens represents the first activating event that confers sufficient activity for autoprocessing and for further activation. Procaspase-8 is predominantly localized in the cytosol but was also recently shown loosely to associate with the mitochondrial outer membrane (Blanchard et al., 2000; Stegh et al., 2000; 2001). After Fas activation, mitochondria-associated procaspase-8 is cleaved to form an active dimer that moves to the cytosol and specifically targets its substrates (e.g., Bid) (Blanchard et al., 2000; Stegh et al., 2000; 2001). Since the signal is initiated at the cell surface, it is conceivable that a small amount of caspase-8, which is activated at the DISC, moves to the mitochondria and cleaves mitochondria-associated procaspase-8.

In type II cells (e.g., hepatocytes and B cells), the activation of caspase-8 is delayed and requires a mitochondrial step regulated by Bcl-2 and Bcl-xL (Scaffidi et al., 1997). More significantly, it was found that Bcl-xL can block the activity of caspase-8 at the mitochondrial membrane by indirectly sequestering the enzyme at the mitochondrial outer membrane (Stegh et al., 2000; 2001). These data suggest that in type II cells, caspase-8 might be activated at the mitochondrial membrane. Interestingly,

in addition to its crucial role in DR-mediated apoptosis, recent findings suggest that caspase-8 also participates in growth and development mechanisms, not only in lymphocytes, but in other tissues as well. The inactive homologue of caspase-8, c- FLIP, plays an important role in this process. In fact, c-FLIP is able to dimerize and activate caspase-8, which in turn cleaves c-FLIP and results in the recruitment of adaptor proteins that promote the activation of nuclear factor-kappa B (NF-κB) and lead to cell proliferation (Krueger et al., 2001).

Caspase-mediated DNA cleavage

Apoptosis has long been known to be associated with cleavage of DNA in characteristic nucleosomal length fragments. The DNAse responsible for this fragmentation has been identified and termed CAD (caspase-activated DNAse) (Enari et al., 1998). Caspase-activated DNAse normally remains bound to a cytoplasmic inhibitor (ICAD/ DFF45), which is degraded by caspase-3 in apoptotic cells (Enari et al., 1998 ; Luo et al., 1998). The free CAD released from ICAD/ DFF45 degrades chromosomal DNA. Cells lacking caspase-3 can still undergo apoptosis; however, they fail to show characteristic DNA fragmentation (Jänicke et al., 1998; Woo et al., 1998), suggesting that caspase-3 has a non-redundant function in activating CAD, but DNA fragmentation is not necessary *for* apoptosis to occur. Bcl-2 and Bcl-xL proteins are also cleaved during apoptosis and the fragments generated by the cleavage appear to enhance apoptotic changes in dying cells (Cheng et alk., 1997; Clem et al., 1998).

Caspase-mediated inactivation or deregulation of proteins involved in DNA repair

The enzyme poly (ADP-ribose) polymerase (PARP), was the first protein identified as a substrate for caspases. PARP is a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation. It has been suggested it suppress the activity of an apoptotic endonuclease by polyADP-ribosylation. Caspases, in particular caspase-3 and -7, cleave the 116-kD form of PARP from DNA-binding domain at the DEVD site to generate a 85- and a 24-kD fragment. Cleaved PARP is characteristic event of apoptosis in many mammalian cells. It is possible that PARP cleavage accelerates DNA fragmentation by harmfully affecting the DNA repair machinery, but it is unlikely that PARP cleavage is a primary event that commits cells to die or is a component of the main death pathway, since PARP-deficient mice seem to develop normally and are resistant to DNA-damaging agents. DNA-dependent protein kinase (DNA-PK) is another DNA repair enzyme, which is cleaved by a caspase in different cell types exposed to a variety of agents that cause apoptosis.

Caspase-mediated breakdown of cell structure

During the execution phase of apoptosis, a number of plasma-membrane changes occur, resulting in the recognition and subsequent phagocytosis of the apoptotic cell either by professional phagocytes or by a neighbouring cell. Cleavage of important cytoskeletal proteins (e.g., gelsolin) during apoptosis may induce cell shrinkage and membrane blebbing, and alter cell survival signalling systems. For example, in the case of gelsolin (a protein that binds to and breaks (severs) actin filaments in a regulated manner), caspase cleavage generates a fragment that is constitutively active (Kothakota et al., 1997). Also, during apoptosis, a caspase removes 35 C-terminal amino acids from Gas2 (a structural protein involved in actin microfilament reorganisation). It is likely that cleavages such as these promote the cytoplasmic changes seen in cells undergoing apoptosis, including disruption of the actin cytoskeleton, cell rounding, and detachment from the substratum (Earnshaw et al., 1999). Condensation of the nuclear chromatin is also observed during apoptosis, and this may be due to the action of caspases on the nuclear lamina. Lamins are intra-nuclear proteins that maintain the shape of the nucleus and mediate interactions between chromatin and the nuclear membrane. Degradation of lamins by caspase-6 results in the chromatin condensation and nuclear fragmentation commonly observed in apoptotic cells.

Conclusions

Apoptosis represents a crucial process in embryonic development, in maintenance of organ function in the adult, and it is central to normal immunological function. Understanding exactly how caspases initiate, and then propagate the apoptotic process is therefore vital for generation of new therapeutics. Over the last few years intense investigation of the function and activation mechanisms of caspases in apoptosis and in non-apoptotic processes as a novel role, such as cell cycle regulation and DNA repair as of caspase-2 (Shi et al., 2009), have been reported. The exact roles, substrates, activation mechanisms and regulations need further elucidation. Additional studies are required to extend the promising applications of caspases in disease treatment. Caspase-7 inhibition seems especially warranted in neurodegenerative disorders such as Alzheimer's disease and Huntington's disease, where increased caspase-7 expression correlates with excessive neuronal cell death. Another potentially promising application is the prevention of lymphocyte cell death in sepsis. Extensive leukocyte apoptosis is commonly observed in sepsis patients and was suggested to contribute to immune suppression and lethality. Synthetic caspase inhibitors and overexpression of the anti-apoptotic protein Bcl-2 have already shown promising results in experimental sepsis models (Hotchkiss and Nicholson, 2006).

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