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Review

Apoptotic and necrotic cell death induced by death domain receptors

G. Denecker†, D. Vercammen, W. Declercq and P. Vandenabeele*

Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Ghent, K. L. Ledeganckstraat 35, B-9000 Ghent (Belgium), Fax +32 09 2645348,
e-mail: peter.vandenabeele@dmb.rug.ac.be

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Abstract. Apoptosis and necrosis are two distinct forms of cell death. Caspases are indispensable as initiators and effectors of apoptotic cell death and are involved in many of the morphological and biochemical features of apoptosis. Major changes in mitochondrial membrane integrity and release of proapoptotic factors, such as cytochrome c from the mitochondrial intermembrane space, play an important sensor and amplifying role during apoptotic cell death. In vitro studies of cell death in cell lines have revealed that inhibition of the classical caspase-dependent apoptotic pathway leads in several cases to necrotic cell death. Thus, the same cell death stimulus can result either in apoptotic or necrotic cell death, depending on the availability of activated caspase. Therefore, death domain receptors may initiate an active caspase-independent necrotic signaling pathway. In this review, we describe what is known about the apoptotic and necrotic cell death pathways. Principal elements of necrosis include mitochondrial oxidative phosphorylation, reactive oxygen production, and non-caspase proteolytic cascades depending on serine proteases, calpains, or cathepsins.

Key words. Apoptosis; necrosis; death receptor; caspase; mitochondria.

Introduction

Billions of cells are created during embryonic development. Most of these cells are programmed to die before the end of the perinatal period, while over the human life span, during cell renewal, 99.9% of cells die by a physiological suicide process [1, 2]. The term ‘programmed cell death’ was initially introduced by R. A. Lockshin and C. M. Williams [3] to designate cell death that occurred in predictable places and at predictable times during development, emphasizing that cells are somehow programmed to die during the developmental plan of the organism. Subsequently, the morphological features of cell death during development or tissue homeostasis were called ‘apoptosis’ [4]. This process is characterized by cell shrinkage and extensive nuclear fragmentation, while organelles and plasma membrane retain their integrity for a prolonged period. This contrasts with necrosis, the most prominent features of which are cytoplasmic swelling, rapid plasma membrane rupture, and organelle breakdown, involving remarkably few nuclear changes [4, 5]. The basic evidence that a genetic program governs physiological cell death was obtained by developmental studies with the nematode *Caenorhabditis elegans* [6, 7] and the subsequent finding that the genes involved had mammalian homologues [8–10]. The term ‘programmed cell death’ nowadays refers to any kind of cell death mediated by an intracellular death program, irrespective of the trigger. The morphological outcome can be apoptosis, necrosis, or a mixed phenotype [11].

* Corresponding author.
† Present address: Microbial Pathogenesis Unit, Catholic University of Leuven, Avenue Hippocrate 74, B-1200 Brussels, Belgium.

Apoptotic cell death

During the early stages of apoptosis, the plasma membrane of the dying cell characteristically remains intact, avoiding proinflammatory spilling of the cellular content into the surrounding tissue. The apoptotic process has long been considered immunologically silent. Several mechanisms might account for this, such as the production of the immunosuppressive cytokines interleukin (IL)-10 and transforming growth factor-β (TGF-β) [12, 13] as well as rapid engulfment of apoptotic cells in a phosphatidylserine-dependent manner [14]. However, peptides from phagocytosed apoptotic bodies can be presented by dendritic cells to elicit an immune response [15, 16]. Additionally, under some circumstances, the proinflammatory cytokines IL-1α, IL-1β, and IL-18 are processed and released during massive apoptosis [17–19], inducing an inflammatory response. Release of modified intracellular proteins might also contribute to inflammatory processes related to apoptosis. Endothelial monocyte-activating polypeptide II (EMAP-II), a proteolytic fragment of the tRNA multisynthetase complex, is detectable during renal ischemia reperfusion [20–23]. To avoid inflammation, cells signal their apoptotic state at an early stage to their environment, where they are recognized and engulfed by phagocytes. Surface exposure of phosphatidylserine, whose receptor has recently been identified [14], is one of these signals [24–28]. To facilitate engulfment, apoptotic cells reduce their volume by pumping out ions (K^+, Cl^-, organic osmolytes) and contracting the reorganized cytoskeleton [29, 30]. Moreover, the plasma membrane of the cell begins to bleb, a process that will, in the absence of phagocytic cells, eventually lead to formation of apoptotic bodies containing condensed or morphologically normal organelles. Overall shrinkage is usually accompanied by nuclear condensation. The chromatin condenses (pyknosis) and the nucleus is fragmented (karyorhexis). The DNA is degraded into fragments of $\geq$ 50 kb, followed by more advanced internucleosomal DNA degradation into ~180-bp fragments [31–34]. We have reported that exposure of phosphatidylserine is an early event in apoptotic cell death, occurring before a decrease in the mitochondrial transmembrane potential ($\Delta\Psi_m$) and release of cytochrome c, two apoptotic parameters that will be discussed below [35].

Role and activation of caspases in apoptotic cell death

Caspases are a family of cysteine aspartate-specific proteases, to date comprising 14 members [36–38]. They are synthesized as zymogens that become activated either via proximity-induced autoproteolysis by interacting with adaptor proteins, or by cleavage via upstream proteases in an intracellular cascade [39, 40]. The primary structure of procaspases comprises three domains: an N-terminal prodomain of variable length, a domain becoming the large subunit (17–21 kDa), and a domain becoming the small subunit (10–14 kDa). Caspases with a large prodomain are involved in the initiation of the apoptotic cascade (initiator caspases) or in inflammation (inflammatory caspases). Caspases with a short prodomain are implicated in execution of the apoptotic response (effector or executioner caspases). Two protein interaction motifs have been found in the prodomain: the death effector domain (DED) in procaspase-8 and -10, and the caspase activation and recruitment domain (CARD) in procaspase-1, -2, -4, -5, -9, -11, -12, and -13.

Two main pathways of procaspase activation during apoptosis have been proposed: first, *extrinsic* activation of initiator procaspases, triggered by formation of a receptosome complex; second, *intrinsic* activation of initiator procaspases, initiated by formation of an apoptosome complex. Extrinsic activation of initiator procaspases is initiated by death receptors (DRs) upon binding of their cognate ligands [41–44]. Death receptors are characterized by the presence of a C-terminal intracellular death domain (DD), a protein-protein interaction motif found in several receptors, such as tumor necrosis factor (TNF)-RI, Fas, DR3, DR4, DR5, DR6, and CAR1 [45]. This DD serves as a recruitment domain for adaptor molecules also containing a DD, such as Fas-associated protein with DD (FADD), TNF-RI-associated death domain (TRADD), receptor-interacting protein (RIP), and RIP-associated Ich-1/CED3 homologous protein with DD (RAIDD) [45, 46]. Some of these adaptor molecules include other protein-protein interaction domains, such as DED, found in FADD, and CARD, present in RAIDD and RIP2. Initiator procaspases also contain a DED (procaspase-8) or a CARD (procaspase-2) motif. Thus, recruitment of initiator procaspase-8 and -2 by (an) adaptor(s), such as FADD, TRADD-FADD or TRADD-RIP-RAIDD, to activated receptors triggers procaspase autoproteolysis by proximity-induced activation [47]. This complex is called the death-inducing signaling complex (DISC) [48].

Two types of cell death induced by Fas ligation have recently been described [49, 50] (fig. 1). In type I cell death, a large amount of active caspase-8 is generated within seconds at the DISC, leading to an efficient and direct activation of downstream effector procaspases (-3, -6 and -7) (fig. 1). In type II cells, DISC formation is strongly reduced, resulting in lowered and delayed procaspase-8 and -3 activation. In this cell type, propagation and amplification of the apoptotic signal require mitochondrial factors. A molecular link connecting DISC activation and mitochondria is caspase-8-mediated cleavage of Bid, a proapoptotic member of the Bcl-2 family [48, 49]. The C-terminal part of Bid (tBid) translocates to the mitochondria, where it induces release of cytochrome c [51–53]. The latter acts as a cofactor in dATP/ATP-

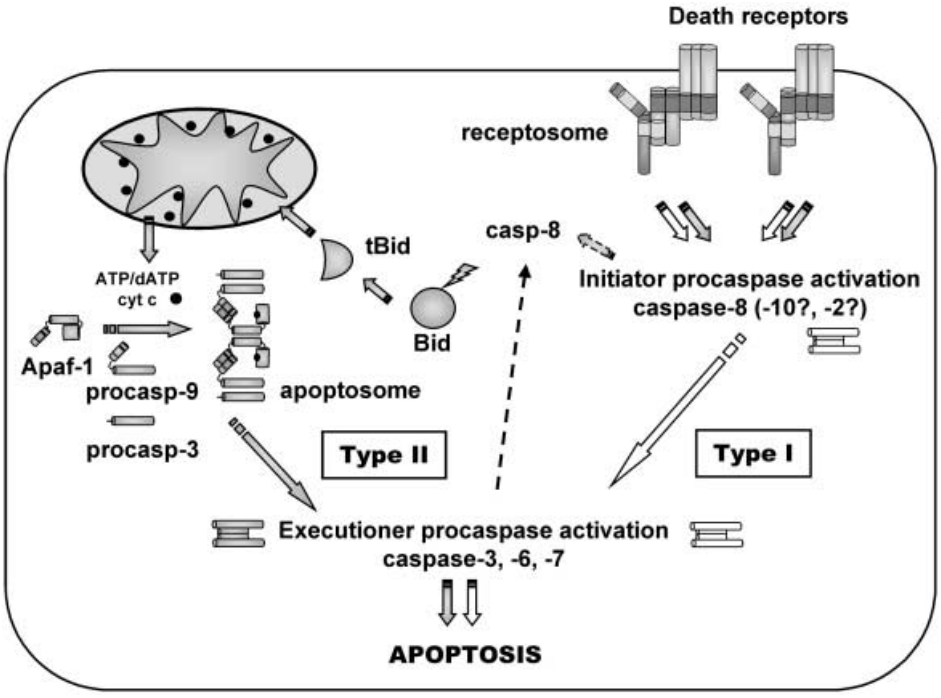

Figure 1. Extrinsic and intrinsic apoptotic signaling pathways [49, 50]. In type I apoptotic cell death (open arrows), a large amount of active caspase-8 is generated at the receptosome complex (efficient DISC formation), which leads to direct activation of downstream procaspases. In type II apoptotic cell death (gray arrows), DISC formation is strongly reduced, resulting in minor procaspase-8 activation. In the latter case, propagation and amplification of the apoptotic signal by mitochondrial factors, such as cytochrome c, are required for activation of downstream procaspases. Cytochrome c and ATP/dATP bind Apaf-1 and induce a conformational change, allowing oligomerization and recruitment of procaspase-9 (formation of apoptosome). Proximity-induced activation of procaspase-9 activates downstream executioner procaspases. Active executioner caspases may further activate procaspase-8 in an autoamplification loop. Executioner caspases proteolyse substrates implicated in the morphological and biochemical features of apoptosis.

dependent apoptotic protease activating factor-1 (Apaf-1)-induced activation of procaspase-9 [54], which in turn activates downstream effector procaspases (fig. 1). The existence of both cell death pathways is supported by the fact that, although overexpression of Bcl-2 or Bcl-X_L may inhibit both the decrease in $\Delta\Psi_m$ and release of cytochrome c in type I and II cells, only in type II cells are procaspase-3 activation and apoptosis inhibited [50].

Intrinsic activation of procaspases, triggered by environmental insults, senescence, and developmental programs, involves release of cytochrome c from the mitochrondrial intermembrane space to the cytosol. Cytochrome c together with dATP/ATP binds to Apaf-1, which initiates the formation of the apoptosome complex [55]. Subsequently, Apaf-1 undergoes a conformational change that allows binding of its N-terminal CARD-like domain to procaspase-9 by CARD-CARD interaction [53, 56, 57] (fig. 1). Self-association of Apaf-1 through its C-terminal WD40 repeats results in procaspase-9 activation [58, 59]. Mature caspase-9 proteolytically activates the executioner procaspase-3, which is also recruited to the apoptosome complex [54, 56]. Caspase-3 then activates procaspase-2, -6, -8, and -10, resulting in a feedback amplification loop [60, 61]. Although the exact mechanism for induction of cytochrome c release during intrinsically induced activation of procaspases has not yet been elucidated, the pro-apoptotic Bcl-2 family members Bax, Bak, Bad, Bik, and Noxa are implicated [62–66]. Another mitochondrial protein that is released during apoptosis is second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low pI (DIABLO), a dimer that binds and neutralizes the caspase-inhibitory action of the IAPs [67–69]. This results in more efficient apoptosome-dependent procaspase-3 activation and type II apoptosis [70]. It also directly promotes the enzymatic activity of caspase-3 in vitro [69]. A strong antiapoptotic modulator of the apoptosome complex is Aven, which prevents homodimerization of Apaf-1 [71].

Recently, several caspase-activated factors have been found to play a role in chromatin condensation and nuclear fragmentation. Acinus is the precursor of a chromatin condensation factor that needs to be cleaved by caspase-3 and an unknown protease to be fully activated. Once activated, acinus induces chromatin condensation without inducing DNA fragmentation, as it does not possess intrinsic DNase activity [33]. Another chromatin condensation factor, which can cause 180-bp internucleosomal

DNA fragmentation, is caspase-activated DNase (CAD/DFF40) [31, 72]. In non-apoptotic cells, CAD is complexed by its cytosolic inhibitor ICAD/DFF45. Cleavage of ICAD by caspase-3 results in release, activation, and nuclear translocation of CAD. In the execution phase of apoptotic cell death, cells reduce their volume through a loss of K^+, Cl^- and organic osmolytes, which prevents them from lytic swelling. A Cl^- efflux-dependent process controls CAD activity and ICAD cleavage is not sufficient to induce CAD activity [73]. Interestingly, phagocytes also contain a CAD-independent backup system for cleaving chromosomal DNA of apoptotically engulfed cells [74]. This system might be attributed to a lysosomal acid DNase, most likely DNase II. Another caspase-independent peripheral chromatin condensation factor is apoptosis-inducing factor (AIF), a flavoprotein normally confined to the mitochondrial intermembrane space [32]. During apoptosis, AIF is translocated to the nucleus, where it participates in chromatin condensation by generating fragments of ≥ 50 kb in the periphery of the nucleus.

The role of mitochondria in apoptotic cell death

During apoptosis, mitochondria undergo major changes in membrane integrity, involving both inner and outer membranes. This process eventually leads to loss of inner $\Delta\Psi_m$ and release of proapoptotic intermembrane proteins in the cytosol, such as cytochrome c. Whether the mitochondrial permeability transition (MPT) pore plays a role in these processes by permeabilizing the mitochondria or by participating in the specific release of cytochrome c is currently unknown. The Bcl-2 family of proteins is definitely implicated in controlling the integrity of the mitochondria.

Proapoptotic and antiapoptotic Bcl-2 family members

Bcl-2, the mammalian homologue of *C. elegans* CED-9, was originally discovered as a proto-oncogene found at the breakpoint of t(14;18) chromosomal translocations of human B cell lymphomas [75]. So far, 18 members of the Bcl-2 family have been identified, including both proapoptotic and antiapoptotic members [76–78]. A characteristic of Bcl-2 family members is their ability to form homodimers and heterodimers. This led to the hypothesis that the balance between proapoptotic and antiapoptotic members is decisive in the relative sensitivity or resistance of cells to a wide variety of apoptotic stimuli [79]. Most antiapoptotic Bcl-2 family members also contain a C-terminal hydrophobic anchor sequence, which is indispensable for targeting to intracellular membranes [80]. In this way, the proapoptotic and antiapoptotic Bcl-2 members are localized in different subcellular compartments in the absence of a death signal. The antiapoptotic ones are integral membrane proteins found in mitochondria, in the endoplasmic reticulum, or in the nuclear membrane. Bcl-2 and Bcl-X_L have been shown to act as 'guardians' of the mitochondria by inhibiting release of cytochrome c and subsequent procaspase activation [49, 81, 82]. In this way they modulate extrinsic and intrinsic apoptotic cell death. The proapoptotic ones are mainly localized in the cytosol or associated with the cytoskeleton [83–86]. After a death signal, the proapoptotic members are targeted to membranes, especially the mitochondrial outer membrane, to fulfill their proapoptotic function. Possible activation mechanisms of the Bcl-2 family members are dephosphorylation (e.g., BAD), cleavage by caspases (e.g., Bid, Bcl-2, and Bcl-X_L), induction of conformational changes (e.g., Bax), dimerization (e.g., Bax) and subcellular redistribution (e.g., Bid, Bax, Bak, Bim/Bod, and Noxa) [51, 52, 66, 84–94]. Recently, Aven was identified as a protein that binds Bcl-X_L, and is required for the antiapoptotic activity of Bcl-X_L. Another level at which Aven interferes with the activation of caspases is its ability to impair self-association of Apaf-1 [71].

The MPT pore

MPT represents a sudden increase in permeability of the mitochondrial inner membrane to solutes with a molecular mass below approximately 1.5 kDa [95, 96]. This phenomenon is caused by opening of the MPT pore, a regulated multiprotein complex [95, 97, 98]. Although the precise architecture of the MPT pore complex is unknown, several proteins participate in MPT pore formation and/or regulation [95, 99]. They include proteins from the matrix (cyclophilin D), the inner membrane (adenine nucleotide translocator), the intermembrane space (creatine kinase), the outer membrane (peripheral benzodiazepin receptor; voltage-dependent anion channel), and the cytosol (hexokinase). These proteins are thought to cooperate at the contact sites between the inner and outer mitochondrial membranes, and to form large Ca^{2+}-, voltage-, pH-, and redox-gated high-conductance channels, allowing diffusion of molecules of < 1.5 kDa. The MPT pore may also interact with proteins of additional multiprotein complexes, viz. TIM and TOM, transporter complexes of the inner/outer membrane [100], and with Bcl-2 family members. In the fully open state, the pore diameter is approximately 3 nm [101], and the open-closed transitions are well regulated. High matrix Ca^{2+}, inorganic phosphate, reactive oxygen species (ROS), oxidant chemicals, high matrix pH, and low $\Delta\Psi_m$ will promote the open state of the MPT pore; Mg^{2+}, ADP, low matrix pH, and high $\Delta\Psi_m$ will favor its closure.

Since many of these regulatory mechanisms are affected in a variety of models of cell death, MPT has been suggested as an early event during the apoptotic process

[102]. Prolonged opening of the MPT pore in the inner membrane during apoptosis will result in diffusion of ions between matrix and cytosol, which will dissipate the H^+ gradient across the inner membrane, leading to uncoupling of oxidative phosphorylation. Due to hyperosmolarity of the matrix, the MPT pore opening leads to volume dysregulation of the mitochondria, which causes the matrix space to expand. Because the inner membrane is folded in pleiomorphic cristae, it has a larger surface area than the outer membrane; hence, the matrix volume expansion results in mitochondrial swelling. The outer mitochondrial membrane disrupts, releasing intermembrane ions, metabolic intermediates, and proapoptotic proteins, such as cytochrome c, to the cytosol. Pharmacological inhibitors of the MPT pore, such as cyclosporin A, targeting cyclophilin D, or bonkrekic acid, targeting the adenine nucleotide translocator, further support an involvement of the MPT pore opening in injury from oxidative stress, anoxia, ischemia reperfusion, and a variety of toxic chemicals [102, 103].

Cytochrome c

Suppression of oxidative phosphorylation in mitochondria during irradiation-induced cell death was reported some time ago [104, 105]. This process has been suggested to be due to controlled release of cytochrome c from the mitochondria to the cytosol [106, 107]. However, the observed loss of cytochrome c could not be correlated with a significant decrease in ATP production [107]. In fact, both release of cytochrome c and maintenance of a certain level of intracellular ATP are necessary to execute the apoptotic cell death program [108, 109]. The exact mechanism by which cytochrome c is released from the mitochondria in response to different apoptotic stimuli remains enigmatic. Moreover, whether all mitochondria or only a subpopulation in apoptotic cells are involved in releasing cytochrome c, and whether affected mitochondria release all cytochrome c at once or only a limited amount requires further investigation [110, 111]. In this respect, two main theories have been advanced as to where the effectors may be interrelated: rupture of the outer mitochondrial membrane or formation of specific channels [78, 82, 112–118].

In addition to cytochrome c, several proteins normally confined to the mitochondrial intermembrane space are released to the cytosol, including certain procaspases, AIF, adenylate kinase 2, and the recently identified Smac/DIABLO [32, 67–70, 119–121]. Furthermore, a systematic mass spectrometric approach has identified over 50 different mitochondria-associated proteins released after opening of the MPT pore [122], but the relevance to the apoptotic process of most of these proteins is unclear.

Necrotic cell death

Necrosis has classically been described as a disordered mode of cell death, occurring in cases of severe and acute injuries (such as abrupt anoxia and sudden shortage of nutrients) or extreme physicochemical injuries (such as heat, detergents, strong bases, and irradiation). However, recent studies have re-evaluated necrotic cell death and shown that it also occurs during normal cell physiology and development [123, 124], confirming the pioneering work of J. U. Schweichel and H. J. Merker [11]. Notably, in several pathological conditions (e.g., brain ischemia) or liver damage induced by cytokines and toxins, cell death can occur both by necrosis and apoptosis [125–129].

Morphologically, necrosis is characterized by cellular swelling. This causes the plasma membrane to burst, which results in release of the cytoplasmic contents into the surrounding tissue, causing an inflammatory response. The generation of an appropriate immune response during necrotic tissue damage may be critical to enforce an antitumor or antiviral response. Organelles also swell and burst, whereas the nucleus remains intact, although nuclear swelling, mottled chromatin condensation, late DNA degradation, and pyknosis have also been reported [11, 124, 130]. Large-fragment DNA degradation during necrosis relies on serine proteases rather than caspases to induce endonuclease activity [131, 132]. Furthermore, the caspase-independent peripheral chromatin condensation factor AIF might also be involved, as it relocalizes to the nucleus during necrosis [133].

The existence of a necrotic-like cell death pathway regulated by a particular *intrinsic* death program distinct from that of apoptosis has also been advanced [123]. The identification of the molecular mechanism involved in necrotic cell death has been hampered, since only recently have several in vitro models of caspase-independent cell death with necrotic-like morphology become available [30, 123, 133–140].

Molecular signaling during death receptor-induced necrosis

While apoptosis and necrosis have clearly distinguished morphological and biochemical features, they can be initiated by the same trigger. A number of reports argue that the same initial stimulus might cause either apoptotic or necrotic cell death, depending on the availability of active caspases (see below). However, in addition to the absence of caspase activation, DD receptors may initiate an active necrotic signaling pathway (fig. 2). This is supported by the observation that TNF signals to necrotic cell death in L929sAhFas cells, whereas anti-Fas treatment leads to apoptosis in the same cells [130]. Thus TNF-RI initiates a necrotic signaling pathway that ignores, for un-

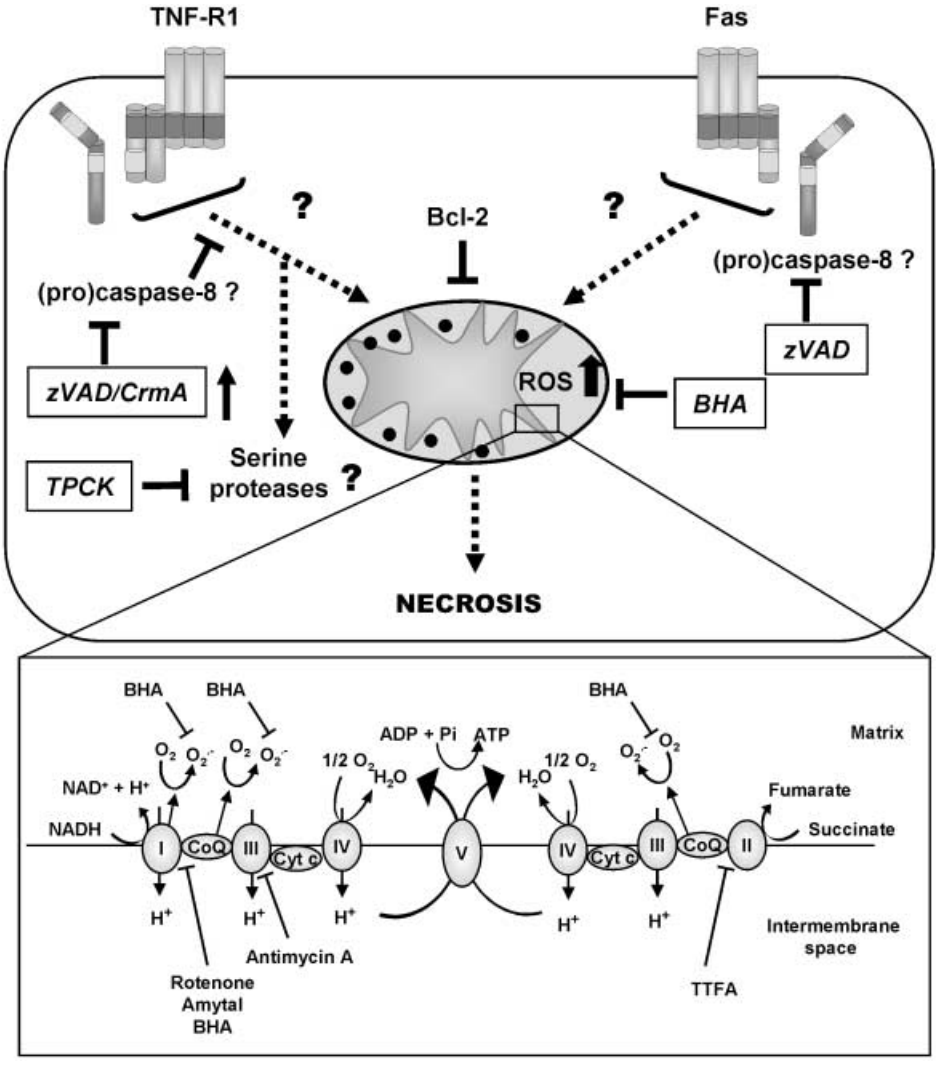

Figure 2. Death domain receptor-induced necrotic signaling pathways in L929sAhFas cells [140, 143]. During TNF-RI-mediated necrosis, procaspases are not activated. Moreover, pretreatment with the broad-spectrum caspase inhibitor zVAD-fmk or overexpression of CrmA, a viral inhibitor of caspase-1 and -8, sensitizes L929sAhFas cells to TNF-induced necrosis. Furthermore, zVAD-fmk converts an initial apoptotic stimulus (anti-Fas) to a necrotic one. TNF-induced necrosis depends on the functionality of oxidative phosphorylation, ROS formation, and serine proteases, as inhibition of oxidative phosphorylation by butylated hydroxyanisole (BHA), amytal, thenoyltrifluoroacetone (TTFA), rotenone, antimycin A, ROS (BHA), and *N*-tosyl-L-phenylalanine chloromethylketone (TPCK) protects cells against death receptor-induced necrosis [130, 147, 149–153]. Overexpression of Bcl-2 can delay TNF-induced necrosis. A possible candidate for further downstream signaling from the death receptors to necrosis is the adaptor FADD [146].

known molecular reasons, the availability of a potent apoptotic machinery. The signal required for necrotic cell death starts from the TNF-RI DD, since mere trimerization of the latter is sufficient to initiate the necrotic pathway [141, 142]. Furthermore, although pretreatment of L929sAhFas cells with a broad-spectrum caspase inhibitor ([benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone, zVAD-fmk]), inhibits anti-Fas-induced apoptosis, cells nevertheless die necrotically [140]. Thus necrotic cell death can also be initiated from the Fas receptor. Importantly, the presence of zVAD-fmk or overexpression of CrmA in L929sAhFas cells induces a 1000-fold sensitization of TNF-induced necrosis [143]. These results suggest that caspases might even be implicated in an antinecrotic or prosurvival pathway [144]. Most probably, the initiator caspase-8 is involved: (i) zVAD-fmk, which inhibits caspase-1, -8, and -9, and CrmA, which inhibits caspase-1 and -8 [145], sensitize TNF-induced necrosis [143]; (ii) acetyl-Tyr-Val-Ala-Asp(OMe)-chloromethylketone (Ac-YVAD-cmk), a caspase-1-specific inhibitor, has almost no sensitizing effect on TNF-induced necrosis [143]; (iii) caspase-8 deficiency favors necrotic cell death [146]. As inhibition of caspases leads to enhanced ROS formation [140, 143, 147], the low levels of active caspases, below the detection limit of enzymatic fluorogenic peptide substrate assays and

Western blotting, might be implicated in controlling TNF-induced ROS formation. In this context, addition of butylated hydroxyanisole (BHA), a lipophilic oxygen radical scavenger, blocks TNF-induced necrosis [148]. Necrosis induced by combined addition of zVAD-fmk and anti-Fas is also blocked by pretreatment with BHA [140]. BHA does not prevent apoptotic cell death induced by treatment with anti-Fas alone. TNF-induced ROS production in L929sA cells has been suggested to result from enhanced electron flow through the electron transport chain complex I [147, 149]. That TNF-induced necrosis depends on the functionality of oxidative phosphorylation is well established. Complex I and, to a lesser extent, complex II inhibitors delay TNF-mediated cell death [150–152]. Futhermore, BHA is able to block complex I activity [153]. Finally, proteases other than caspases might be involved in death receptor-induced signaling to necrosis: N-tosyl-L-phenylalanine-chloromethylketone, a serine protease inhibitor, inhibits necrotic cell death induced either by TNF or by a combination of zVAD-fmk and anti-Fas [140; Denecker et al., unpublished data].

The absence of caspase activity in several necrotic conditions does not rule out the implication of other proteolytic cascades during necrotic cell death. In this respect, involvement of cytosolic calpains in cell death has been reported [154]. Moreover, calpains might proteolytically inactivate caspases [155], promoting caspase-independent cell death [156]. Serine protease inhibitors in some models of TNF-induced cell death delay the necrotic process [130]. Lysosomal proteases such as cathepsin D have also been reported to play a pivotal role in anti-Fas and interferon (IFN)-γ induced cell death [157]. Clearly, a complex interplay between cytosolic, including caspases, and lysosomal proteases defines the outcome of caspase-independent cell death with a necrotic phenotype. Information about the alternative proteolytic cascades besides caspases in the context of cell death is beginning to emerge [123].

Decision points between apoptosis and necrosis

That both cell death pathways are interrelated is also clear from the fact that cellular conditions, including short or prolonged opening of the MPT pore, oxidative stress, cellular ATP content, and high or low Bcl-2 levels, influence the decision between apoptosis and necrosis [4, 108, 133, 158–159]. Strikingly, all these conditions share the absence of caspase activity. There is increasing evidence that caspase inhibition does not always prevent the process of cell death. zVAD-fmk efficiently blocks procaspase activation and oligonucleosomal DNA fragmentation, but cannot inhibit nuclear condensation, cell shrinkage, membrane blebbing, and loss of cell membrane integrity induced by Bax or Bak [160, 161]. Instead, cell death induced in the absence of active caspases is accompanied by cytoplasmic vacuolization, suggesting that the cells die from ‘necrotic-like’ autophagic degeneration, as described originally [11]. Similarly, zVAD-fmk inhibits DNA fragmentation and chromatin condensation induced by etoposide, staurosporine, actinomycin D, and dexamethasone, but not cell death itself [162–164]. Additionally in this case, dying cells show a necrotic-like morphology, such as cytoplasmic vacuolization and lack of pronounced chromatin condensation. Caspase inhibitors also do not block cell death induced by perforin and granzyme B, GD3 ganglioside, class I major histocompatability complex antibodies, intracellular acidification, retinoid 6-[3-1(adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid, anti-CD2, irradiation, nitric oxide (NO), and A23187 [30, 134–139, 165–168]. Although the morphology of dying cells is not always documented in the literature, inhibition of the classical apoptotic pathway at different levels clearly reveals in many cases a necrotic-like cell death, which is essentially caspase independent. Thus the same cell death stimulus can result in apoptotic or necrotic cell death, depending on whether or not procaspases are activated.

FADD

A possible candidate, common to both death receptors and initiating further downstream signaling to necrosis is the adaptor FADD. In this respect, enforced oligomerization of FADD in Jurkat cells, pretreated with zVAD-fmk or deficient for procaspase-8, has been demonstrated to result in necrotic cell death, whereas the same FADD construct induced apoptosis in wild-type Jurkat cells [146]. In addition, overexpression of a dominant-negative FADD molecule (consisting only of the DD and thus blocking DED-dependent caspase-8 recruitment) in TNF-resistant U937 or NIH3T3 cells sensitized these cells to TNF-induced necrotic cell death [169]. The same sensitization was obtained by pretreating such cells with zVAD-fmk. These results demonstrate that, in the absence of caspase activation, FADD may signal to necrosis through its DD, whereas in the presence of active caspases, FADD signals to apoptosis through its DED. We demonstrated that the DD domain of TNF-RI is sufficient for necrotic signaling and that the bifurcation between apoptotic and necrotic signaling, at least during TNF-induced necrosis of L929sA cells, might be situated at the level of FADD. FADD-DED would propagate apoptosis, while FADD-DD would initiate necrotic signaling [142].

Direct inhibition of caspase activity

Besides the proteolytic inactivation of caspases, as mentioned above, several mechanisms lead to inhibition of caspase activity. Caspase activity is optimal under re-

ducing conditions, since their catalytic site contains a nucleophilic cysteine prone to oxidation, thiol alkylation, or thiol nitrosylation. This implies that changes in the redox state in dying cells renders caspases inactive. In the same cellular context, low ROS levels induce apoptosis, whereas high ROS levels are accompanied by necrosis [170–173]. Anti-Fas-induced apoptosis is impaired by pretreatment with hydrogen peroxide [172]. Depending on the degree of initial oxidative stress, procaspases are activated; cells consequently die by apoptosis, or remain inactive followed by necrosis [172]. Accordingly, addition of menadione (a ROS generator) during anti-Fas-induced apoptosis abolishes caspase activity, as measured by cleavage of acetyl-Asp(OMe)-Glu(OMe)-Val-Asp (OMe)-aminomethylcoumarin (Ac-DEVD-amc) [174]. Co-incubation with catalase reduces the levels of menadione-induced ROS and restores caspase activity. Menadione itself induces necrosis in the absence of procaspase-3 and -8 activation, despite cytochrome c release. Furthermore, oxidation of dithiocarbamates to dithiocarbamate disulfides has been shown to inhibit apoptosis by covalently binding to the catalytic cysteine of caspases [175, 176]. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), a bifunctional alkylating/carbamoylating agent, has also been shown to inhibit etoposide-induced apoptosis by blocking caspase activity via its carbamoylating activity [177].

NO may also inhibit apoptosis by interacting with caspases: (i) NO synthase activity can lead to caspase inhibition; (ii) caspases can be S-nitrosylated on their catalytic cysteine residue both in vitro and in vivo; (iii) this modification inhibits caspase activity in a reversible way [178–185]. Moreover, endogenous S-nitrosylation of procaspase-3 was demonstrated in unstimulated lymphocyte cell lines [186]. Upon Fas stimulation, procaspase-3 is denitrosylated and proteolytically activated. Thus procaspase S-nitrosylation may protect cells from unwanted apoptosis by maintaining the zymogen in an inactive state. NO-dependent ATP depletion is an additional mechanism by which NO can affect the execution of apoptosis, resulting in delayed cytochrome c release [187, 188]. Importantly, NO-induced ATP depletion and subsequent inhibition of procaspase activation results in inhibition of the apoptotic cell death program and is accompanied by a switch to necrotic cell death [187, 188].

Oxidative stress

Oxidative stress is implicated both in apoptosis and necrosis. In general, when high ROS levels accumulate in the cell, direct and irreversible damage of cellular components (such as proteins, lipids, and DNA) leads to necrosis. Moderate ROS levels, on the other hand, function as second messengers and regulating molecules, hence ensuring apoptotic cell death. ROS may influence the decision point between apoptosis and necrosis at different levels. As already mentioned above, ROS can directly modulate caspase activity. Moreover, the MPT pore opening is regulated by oxidative stress and will further induce ROS production by uncoupling the respiratory chain and disrupting thiol homeostasis [112, 189]. When the onset of MPT is rapid and profound, high amounts of ROS are produced and necrotic cell death occurs; when MPT is slower and the cell is able to preserve its normal redox state, only moderate levels of ROS are produced and the cell dies apoptotically. However, low ROS levels may also regulate the induction of necrotic cell death. In L929sA cells, relatively low levels of mitochondrial ROS are detectable and sufficient for caspase-independent TNF-induced necrosis [143, 144, 148]. Despite low ROS levels induced by TNF, necrotic cell death follows in less than 2 h once these ROS are formed; high ROS levels generated by treatment with H_2O_2, menadione or t-butyl hydroperoxide do not result in immediate cell death [147]. Hence, the specific subcellular localization of TNF-induced ROS, presumably the mitochondrial inner membrane, might explain the high efficacy in inducing cell death [149]. Furthermore, TNF-induced ROS generation is caused by an enhanced electron flow through complex I of the electron transport chain, which is correlated with the use of glutamine as a respiratory substrate [147]. Mitochondrial ROS fail to induce rapid cytochrome c release and do not lead to procaspase activation, which might explain the necrotic phenotype [147].

Cellular ATP content

Apoptotic cell death in neuronal cells requires ATP, and ATP depletion results in necrotic cell death [189]. Depletion of intracellular ATP inhibits apoptotic cell death induced by anti-Fas, staurosporine, etoposide, calcium ionophore, and dexamethasone, eventually leading to necrosis [188, 190–192]. An important ATP-dependent process during apoptosis is activation of procaspase-9: cytochrome c, together with dATP/ATP and Apaf-1 have been shown to mediate activation of procaspase-9 [53]. ATP depletion/reconstitution experiments revealed that ATP generation, either by glycolysis or by mitochondrial oxidative phosphorylation, is required for nuclear condensation, DNA fragmentation, and exposure of phosphatidylserine at the cell surface [190]. Furthermore, an early, transient burst of poly(ADP-ribosyl)ation of nuclear proteins by poly(ADP-ribose) polymerase (PARP), using NAD as a substrate, occurs during apoptosis in various cell lines [193, 194]. Inactivation of PARP through cleavage by caspases may prevent depletion of NAD and ATP, which are thought to be required for late stages of apoptosis and thus ensure completion of the apoptotic program. Overactivation of PARP after cellular insults, without caspase-dependent inactivation, would

lead to necrotic cell death due to PARP-mediated depletion of cellular NAD and ATP [195]. The ATP dependence of apoptosis, but not of necrosis, might explain the observed appearance of both apoptotic and necrotic cells in pathological conditions, such as the center of solid tumors [196], ischemia [126], or liver damage by cytokines or toxins [127–129]. In areas where blood flow is limited, ATP is rapidly exhausted due to insufficient oxygenation and reduced glycolysis due to the absence of glucose. Thus in vivo ATP availability appears to be one of the factors determining cell death fate.

Bcl-2 family balance

Using two different glioblastoma cell lines, expressing low or high levels of Bcl-X_L (Bcl-X_L^{low} or Bcl-X_L^{high} cells), overexpression of Bax has been shown to result in apoptotic or necrotic cell death, depending on the balance between proapoptotic and antiapoptotic Bcl-2 family members [197]. When overexpressed in Bcl-X_L^{low} cells, Bax-induced mitochondrial damage results in procaspase activation, chromatin condensation, and DNA fragmentation, characteristic for apoptotic cell death [198]. In contrast, in Bcl-X_L^{high} cells, Bax-induced mitochondrial damage does not lead to procaspase activation, so that apoptosis is inhibited. However, due to irreversible mitochondrial damage, necrosis occurs, characterized by the presence of large cytoplasmic vacuoles, dilatation of the endoplasmic reticulum and relatively intact chromatin. As Bcl-X_L does not prevent Bax-induced mitochondrial damage, high levels of Bcl-X_L probably block apoptosis by inhibiting procaspase activation downstream of the mitochondria. This possibly occurs by inhibiting Apaf-1-induced procaspase-9 activation, since Bcl-X_L has been proposed to interact with Apaf-1 and to keep it in a closed conformation [199]. A similar balance between apoptosis and necrosis caused by different Bcl-2 expression levels was also observed [200]. A sustained intracellular calcium rise, induced either by treatment with oxidized low-density lipoproteins or with calcium ionophore, activated both apoptotic and necrotic pathways [201]. In Bcl-2^{low} cells, this calcium rise leads to apoptosis, whereas the same stimulus induces necrosis in Bcl-2^{high} cells [200]. The calcium chelator EGTA may inhibit both apoptosis and necrosis. This suggests that Bcl-2 acts downstream of the calcium rise and inhibits only the apoptotic pathway (probably by inhibiting cytochrome c release [81]), resulting in the appearance of a necrotic pathway. Thus, the relative expression level of Bcl-2 or Bcl-X_L determines whether a certain cell death stimulus results in apoptosis or in necrosis. When irreversible damage has occurred and when high expression levels of antiapoptotic Bcl-2 family members have blocked the apoptotic cell death pathway (presumably by inhibiting caspase activity), cells will enter the necrotic cell death pathway.

Conclusions

In this overview we have presented experimental evidence for, in analogy to the apoptotic cell death program, a necrotic cell death program. In a developmental context, during removal of interdigital cells in the mouse embryo, a fraction of the cells apparently died from necrosis rather than from apoptosis [123, 124]. Remarkably, when apoptosis is blocked by adding a caspase inhibitor or by using Apaf-$1^{-/-}$ mice, interdigital cell death occurs by a process resembling necrosis. Such results are in agreement with earlier studies on programmed cell death in embryogenesis, where necrotic cell death has often been observed [11, 123, 124]. In pathologic conditions, such as ischemia reperfusion or liver damage induced by cytokines and toxins, necrotic and apoptotic cell death occur simultaneously [125–129].

There is no doubt that caspases, Bcl-2 family members, and mitochondrial proapoptotic factors play an important role during the initiation and execution of the apoptotic cell death program. As described above, a number of reports argue that the same initial stimulus might cause either apoptotic or necrotic cell death, depending on the availability of active caspases. If the generation of active caspases is blocked by low ATP levels [108, 158, 188, 190–192], by Bcl-2 overexpression [197], or by caspase inhibitors [123, 140, 146, 159, 169], a given stimulus will eventually lead to necrotic cell death. This means that intracellular balances of ATP, Bcl-2 or endogenous caspase inhibitors might control the apoptotic or necrotic cell death outcome. In addition, there might also be an active necrotic signaling pathway, as exemplified for DD receptor signaling [140, 142, 143, 146]. However, many questions remain open regarding the precise molecular events leading to necrosis and the organelles implicated. Some principal elements of a necrotic signaling pathway that have been discussed in this review are the mitochondrial oxidative phosphorylation pathway and reactive oxygen production [147]. Protease systems other than caspases are also implicated; however, the precise relationship between cytosolic and lysosomal proteases and their executioner role in necrosis remains unclear. At the moment, the necrotic cell death process can merely be discussed in negative terms with the well-studied apoptotic pathways as a reference. Further research on defined models will be required to elucidate the necrotic cell death pathway as clearly distinct or interrelated with the apoptotic cell death pathway.

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