Beyond apoptosis: nonapoptotic cell death in physiology and disease

Claudio A. Hetz, Vicente Torres, and Andrew F.G. Quest

Abstract: Apoptosis is a morphologically defined form of programmed cell death (PCD) that is mediated by the activation of members of the caspase family. Analysis of death-receptor signaling in lymphocytes has revealed that caspase-dependent signaling pathways are also linked to cell death by nonapoptotic mechanisms, indicating that apoptosis is not the only form of PCD. Under physiological and pathological conditions, cells demonstrate a high degree of flexibility in cell-death responses, as is reflected in the existence of a variety of mechanisms, including necrosis-like PCD, autophagy (or type II PCD), and accidental necrosis. In this review, we discuss recent data suggesting that canonical apoptotic pathways, including death-receptor signaling, control caspase-dependent and -independent cell-death pathways.

Key words: apoptosis, necrosis, nonapoptotic programmed cell death, death receptors, ceramides.

Résumé : L'apoptose est une forme de mort cellulaire programmée (MCP) définie morphologiquement qui est contrôlée par l’activité des membres de la famille des caspases. L'analyse de la signalisation issue des récepteurs de mort chez les lymphocytes a révélé que les voies signalétiques dépendantes des caspases étaient aussi liées à la mort cellulaire causée par d'autres formes de mort cellulaire programmée (MCP). Sous des conditions physiologiques ou pathologiques, les cellules font preuve d'un haut degré de flexibilité dans leurs réponses menant à la mort, comme le reflète l’existence d'une variété de mécanismes incluant la MCP apparentée à la nécrose, l’autophagie (ou MCP de type II) et la nécrose accidentelle. Dans cette revue, nous discutons des plus récents résultats suggérant que les sentiers apoptotiques fondamentaux, incluant la signalisation par les récepteurs de mort, contrôlent les sentiers de mort dépendants et indépendants des capsases.

Mots clés : apoptose, nécrose, mort cellulaire programmée non-apoptotique, récepteurs de mort, céramides.

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Apoptosis, nonapoptotic programmed cell death, and accidental necrosis

In general, programmed cell death (PCD) is defined as an active process that depends on the execution of a defined sequence of signaling events that lead to cell demise. Because the precise nature of molecular events associated with various PCD pathways is not well understood, morphological and biochemical criteria employed to define distinctive PCD programs and their molecular pathways will be discussed in this review.


C.A. Hetz.1 Instituto de Ciencias Biomédicas, Universidad de Chile, Santiago, Chile.
V. Torres and A.F.G. Quest.2 FONDAP Center for Molecular Studies of the Cell (CEMC), Universidad de Chile, Av. Independencia 1027, Santiago, Chile.

1Present address: Dana-Farber Cancer Institute, Department of Cancer Immunology and AIDS, Boston, MA 02115, USA.
2Corresponding author (e-mail: aquest@med.uochile.cl).

Apoptosis is a particular morphological manifestation of PCD (Kerr et al. 1972; Lockshin and Williams 1965), and constitutes a highly conserved pathway that, in its basic features, appears to operate in all metazoans. During embryonic development, apoptosis is essential for successful organogenesis and participates in the control of cellular populations (Vaux and Korsmeyer 1999). Apoptosis also operates in adult organisms to maintain normal cellular homeostasis, which is particularly important with respect to the development of disease in humans.

Early studies performed by Kerr et al. (1972) defined the central morphological features of cells undergoing apoptosis during development, such as cytoplasmic condensation, nuclear pyknosis, stage 2 chromatino condensation, membrane blebbing, and generation of apoptotic bodies that are normally eliminated by phagocytic cells in the surrounding tissue. In the past 15 years, additional markers of apoptosis have been found, including DNA fragmentation, phosphatidylserine exposure at the cell surface, and the characterization of key molecular players, such as cysteine proteases of the caspase family (Degterev et al. 2003), many adaptor proteins, and members of the Bcl-2 family of proteins (Danial and Korsmeyer 2004). In essence, pathways
leading to apoptosis via either the extrinsic (death-receptor mediated) or intrinsic (i.e., triggered by endogenous mechanisms related to cellular stress) pathway (Hengartner 2000; Razik and Cidlowski 2002) are becoming increasingly appreciated as highly orchestrated macromolecular assembly processes (Leyton and Quest 2002, 2004). Understanding such processes better holds the promise of promoting or preventing cell-death-related events and, hence, treating a variety of human diseases (Li et al. 2004; Walensky et al. 2004).

Although apoptosis, a caspase-mediated process, is the prevalent form of PCD employed to control cell viability during development and to maintain homeostasis, recent data indicate that alternative PCD pathways exist that may be particularly relevant under pathological conditions. Indeed, a variation of the classic form of apoptosis has been described; it occurs in the absence of caspase activation, is associated with less compact chromatin condensation (stage 1 chromatin condensation), and has been defined as apoptosis-like PCD. The best-characterized regulator of apoptosis-like PCD is apoptosis-inducing factor, a mitochondrial flavoprotein with oxidoreductase activity that translocates to the nucleus in response to defined cell-death stimuli (Cande et al. 2002). Apoptosis-inducing factor, by itself, triggers partial nuclear condensation and large-scale caspase-independent DNA fragmentation (Susin et al. 1999).

Necrosis, also referred to as accidental cell death, is characterized by rapid swelling of the dying cell, rupture of the plasma membrane (as characterized by electron microscopy), and release of the cytoplasmic content to the cell environment. Despite the profound effects of necrotic-like cell death in pathological conditions, such as stroke, ischemia, and several neurodegenerative diseases (Stefani and Dobson 2003), the molecular mechanisms underlying necrotic cell death are poorly understood. Necrosis has traditionally been defined as an unregulated (accidental) cell-death process that often occurs under conditions of cellular injury (Barros et al. 2001a) and is related to the loss of ion homeostasis (i.e., increase in intracellular sodium concentration) and drastic decreases in ATP levels (Majno and Joris 1995). However, in recent years, an increasing number of reports indicate that cell death with necrotic features occurs under normal physiological conditions and during development (Jaattela and Tschopp 2003; Yuan et al. 2003). Also, evidence (Hetz et al. 2002b; Leyton and Quest 2004) indicates that traditional apoptotic pathways may lead to necrotic cell death under certain conditions, and that specific mechanisms regulate this transition, as will be discussed. In this context, it is important to mention that such forms of regulated necrotic cell death have been defined as necrosis-like PCD, thereby emphasizing the active nature of the process and distinguishing it from the fast swelling and lysis normally observed in accidental necrosis, which is a passive process. Such distinction is essential because both forms of cell death are phenotypically alike, but are distinct from apoptosis, and are probably caused by different mechanisms (Jaattela and Tschopp 2003). In particular, at late stages of both accidental necrosis and necrosis-like PCD, nuclear morphology is similar and is characterized by the absence of apoptotic features. However, the rapid increase in cell volume observed before cell lysis can be used as a morphological manifestation of accidental necrosis.

Cell death is often also associated with the presence of numerous cytoplasmic autophagic vacuoles of lysosomal origin. Lysosomes have been referred to as suicide bags, because they contain several unspecific digestive enzymes that, upon release into the cytosol, cause autolysis and cell death (Bursch 2001). Autophagy, the process by which cells recycle cytoplasm and dispose of defective organelles (Shintani and Klionsky 2004), also defined as type II PCD, plays a central role in the maintenance of cellular homeostasis, and participates in the turnover of intracellular organelles and in the regulation of proteins with a long half-life. Lysosome-mediated cell death has been linked to the apoptotic pathway through alterations in mitochondrial function (Boya et al. 2003a, 2003b). Cell death by autophagy may also lead to a necrotic phenotype. However, identification of this form of PCD is difficult, because death by autophagy does not display easily identifiable characteristics, such as chromatin condensation. The main criteria defining a cell-death process, such as autophagy, is the appearance of double-membrane-containing vacuoles in the cytosol, and fusion of autophagosomes with the lysosomes (Bursch 2001). In addition, several genes relevant to autophagy, such as beclin 1 (an autophagic gene), are upregulated and used as indicators of this process. More details concerning autophagy and the molecular processes involved can be found in recent reviews (Edinger and Thompson 2003; Shintani and Klionsky 2004). General interest in understanding the mechanisms leading to autophagy has increased considerably in recent years, because different anticancer drugs are believed to elicit their cytotoxic effects by inducing autophagy (Gozuacik and Kimchi 2004). Here, however, it is important to bear in mind that under conditions of nutrient deprivation, autophagy is thought to operate, at least initially, as a survival rather than a suicide pathway (Klionsky and Emr 2000; Shintani and Klionsky 2004).

In summary, the following criteria can be employed to differentiate accidental necrosis from PCD-necrosis and autophagy. Accidental necrosis is characterized by rapid swelling and a loss of plasma membrane integrity, frequently in connection with dramatic irreversible drops in ATP levels. In contrast, PCD-like necrosis can be a relatively slow process, observed as the consequence of chemical insult or downstream from death receptors, that also leads to membrane rupture but is actively regulated at the molecular level. Finally, autophagy is also a relatively slow process associated with the appearance of double-membrane vacuoles in the cytoplasm, and extensive membrane-fusion events that are controlled by the expression of autophagy-specific genes. In the latter case, neither volume changes nor release of cytosolic contents is observed, and the process can be associated with both cell survival and cell death.

As can be seen from this brief introduction, many excellent reviews exist that summarize molecular events associated with apoptosis or other forms of cell death. Here, we will focus on the available literature that suggests that necrotic-like cell death may not always be accidental, and that this form of cell death may contribute to the regulation of cellular homeostasis under certain conditions.
Caspase-independent cell death and death receptors

Two central pathways involved in the extrinsic stimulation of apoptosis have now been linked to the activation of caspase-independent (nonapoptotic) cell death. When caspases are inhibited, activation of the death receptors Fas (APO-1/CD95) and tumor necrosis factor (TNF) receptor (TNFR)-1 can induce cell death with necrotic-like features in certain cell types (Holler et al. 2000; Vercammen et al. 1998a, 1998b). In general, activation of Fas and TNFR-1 involves the binding of their ligands (FasL or TNF-α, respectively) and the stabilization of the trimeric form of the receptor, thereby allowing the assembly of the death-inducing signaling complex (Peter and Krammer 2003). For example, activated Fas recruits the adapter protein Fas-associated death-domain (DD) - containing protein (FADD). The amino-terminal DD of FADD interacts with a homologous DD within the prodomain of caspase-8 and (or) caspase-10, providing a platform for their activation. Activated caspase-8, in turn, activates downstream effectors, such as the Bcl-2 family member Bid (Li et al. 1998) and caspase-3 (Nagata and Golstein 1995; Suda et al. 1993), ultimately leading to cell death. In addition, stimulation of Fas leads to receptor-interacting protein (RIP) 1 kinase recruitment, and activation of the transcription factor NF-κB via interaction with TNFR-associated factors. Similar protein complexes have been described for TNFR-1, which first recruits TNFR-associated death-domain protein, leading to RIP and caspase-8 activation (Peter and Krammer 2003).

Activation of the TNF-1-pathway may, depending on the cell type, lead to either apoptosis or necrosis-like PCD (Laster et al. 1988). The mechanisms by which TNF triggers a necrosis-like phenotype are poorly understood. Despite many structural similarities between Fas and TNFR-1, FasL was initially thought to trigger only apoptotic cell death. However, as will be discussed, inhibition of Fas-induced apoptosis in L929 fibrosarcoma cells by a general caspase inhibitor (zVAD-fmk) leads to cell death with necrosis-like characteristics that is mediated by oxygen radical production (Vercammen et al. 1998a, 1998b). Also, primary activated T cells can be efficiently killed by FasL, TNF-α, and TNF-related apoptosis-inducing ligand in the absence of active caspases (Holler et al. 2000). These results suggest that Fas, like TNF-1, can trigger apoptotic or necrosis-like PCD.

In an early study, Vercammen et al. (1998a) showed that murine L929 fibrosarcoma cells treated with TNF-α die rapidly by necrosis, owing to excessive formation of reactive oxygen intermediates. In addition, overexpression of CrmA, a serpin-like caspase inhibitor of viral origin, renders L929 cells 1000 times more sensitive to TNF-α-mediated cell death. The presence of zVAD-fmk resulted in a rapid increase of TNF-α-mediated production of oxygen radicals that was associated with cell death. In addition, the same group found that in L929 fibrosarcoma cells overexpressing human Fas receptor, zVAD-fmk treatment augmented cell sensitivity to Fas-mediated cell death with a necrosis-like phenotype (Vercammen et al. 1998b). Interestingly, unlike TNF-α, Fas-activation did not initiate NF-κB-dependent processes. These results demonstrated, for the first time, the existence of 2 different pathways originating from the Fas receptor: a first leads rapidly to apoptosis, and, when this apoptotic pathway is blocked by caspase inhibitors, a second pathway, involving oxygen radical production, redirects the cells to undergo necrosis-like PCD.

Additional evidence is available to support the idea that alternative signaling pathways triggered by Fas can lead to necrosis-like PCD. A caspase 8-deficient subline of human Jurkat cells can be killed by the enforced oligomerization of FADD under artificial conditions. Interestingly, the cell death observed is not accompanied by caspase activation, and is associated with some morphological changes typical of necrosis (Kawahara et al. 1998). In addition, Matsumura and colleagues (2000) showed that the DD of FADD is responsible for the FADD-mediated necrosis-like PCD pathway. This process was accompanied by a loss of mitochondrial transmembrane potential, but not by the release of cytochrome c from mitochondria. Thus, within the same cell, pathways leading to apoptosis and necrosis-like cell death are initiated by death receptors, but are differentially activated, depending on the nature of the stimuli. The results suggest that caspase-dependent pathways leading to apoptosis are temporally faster and, when such rapid events are blocked, alternative pathways appear to result in necrosis-like cell death.

Interestingly, in L929 cells, both the TNF-α and Fasl-L-induced necrosis observed in the presence of the caspase inhibitor zVAD-fmk are prevented by the serine protease inhibitor N-tosyl-L-phenylalanine chloromethylketone and the oxygen radical scavenger butylated hydroxyanisole, whereas Fas-induced apoptosis is not affected, indicating that production of oxygen radicals is mediating necrosis-like PCD in this experimental system (Denecker et al. 2001). These data indicate that cell-death mechanisms triggered by TNFR and Fas are not identical, and that necrosis-like PCD may involve particular regulators not related to the canonical apoptotic pathway. Consistent with this idea, a recent study implicated different FADD domains in the mediation of apoptosis or necrosis-like PCD (Vanden Berge et al. 2004).

Holler et al. (2000) reported that Fas triggering in caspase-8-deficient Jurkat T lymphoma cells promotes cell death associated with mitochondrial damage in the absence of apoptotic markers. The authors showed that induction of caspase-independent cell death was inhibited in primary T cells deficient in either FADD or RIP. Similar observations were made in models of TNF-α and TNF-related apoptosis-inducing ligand toxicity (Holler et al. 2000). More important, RIP requires its own kinase activity for death signaling and is independent of its known role in NF-κB activation, suggesting that RIP may regulate, by phosphorylation, unknown factors involved in the activation of necrotic-like PCD.

The complexity of caspase-independent cell-death regulation has increased in recent years, with the identification of other molecular components associated with this pathway. For example, inhibition of the chaperone Hsp90 with geldanamycin blocked FasL-mediated necrosis-like PCD (Lewis et al. 2000). As a possible mechanism, Hsp90 has been found to regulate the turnover of RIP through the proteasome pathway (Lewis et al. 2000). In addition, L929 cells undergoing necrosis-like cell death in response to TNF-α were shifted to the apoptotic program in the presence of caspase 8 inhibitor. © 2005 NRC Canada
of geldanamycin (Vanden Berghe et al. 2003). This shift was blocked by CrmA but not Bcl-2 overexpression, suggesting that the necrosis-to-apoptosis transition occurred through activation of procaspase-8. A careful examination of the levels of different components of the TNFR-associated proteins revealed that inhibition of Hsp90 alters the general protein composition of the TNFR-1 complex, probably favoring caspase-8-dependent apoptosis (Vanden Berghe et al. 2003). For example, geldanamycin treatment led to a proteasome-dependent decrease in the levels of RIP, the inhibitor of kappa B kinase-alpha, and, to a lesser extent, adaptors like the NF-kB-essential modulator and TNFR-associated factor 2 (Vanden Berghe et al. 2003). The authors proposed that the availability of particular proteins, such as RIP, FADD, and caspase-8, may determine whether TNFR-1 activation leads to apoptosis or to necrosis-like PCD. Similar results were described by the same group when caspase-independent cell death was initiated in L929 cells by the artificial dimerization of FADD. Geldanamycin reverted the induction of necrosis-like PCD to apoptosis in this model of FADD-mediated cell death (Vanden Berghe et al. 2004).

Finally, using a genetic approach, in mouse embryonic fibroblasts (MEFs), RIP, FADD, and TNFR-associated factor 2 (TRAF2) were shown to be critical components in the sequence of events leading to TNF-α-induced necrosis-like PCD upon caspase inhibition (Lin et al. 2004). Inhibitors of NF-κB facilitated TNFR-induced necrosis-like cell death, but other signaling events triggered by TNF-α (like JNK, p38, and ERK activation) were not required for this process. Cell death in this context depends on the generation of reactive oxygen species, which was not observed in RIP(-/-), TRAF2(-/-), or FADD(-/-) MEFs (Lin et al. 2004). These data reinforce the notion that caspase-independent cell death is an active regulated process, and that formation of signaling complexes at the cell surface is crucial in mediating necrosis-like PCD via death receptors.

**Caspase-dependent initiation of apoptosis and necrosis by the Fas receptor**

Our group also studied the signaling events involved in FasL cytotoxicity in B and T lymphoma cells (Hetz et al. 2002b). As a first model for our studies, A20 B lymphoma cells were used. Several known events linked to the onset of apoptosis in A20 cells, including caspase-3 activation, cell shrinkage, and DNA fragmentation, were all activated within the first 3 h of Fas triggering. In these cells, the viability was only partially restored in the presence of a caspase-3 inhibitor, whereas the general caspase inhibitor zVAD-fmk completely protected cells against FasL-induced death. Flow cytometric analysis of cell-death markers was used to distinguish the 2 cell populations. In one, cell death was caspase-3-dependent and paralleled by DNA fragmentation, cell shrinkage, and nuclear fragmentation. In the other, cell death was caspase-3-independent and occurred without any indication of DNA fragmentation or nuclear condensation. In addition, these cells increased in volume. Based on these criteria, the 2 propidium-iodide-positive populations of dead cells were defined as apoptotic and necrotic cells, respectively, (see cell and model in Fig. 1 and Hetz et al. 2002b). A key difference here, with respect to previous reports, is that inhibition of caspases after Fas triggering was not necessary to induce the alternative form of cell death. Interestingly, effects similar to those observed in A20 cells were also detected in Jurkat T cells, whereas for Raji B lymphoma cells lacking phosphatidylserine externalization and delayed ceramide production (Tepper et al. 2000), only Fas-induced apoptosis was observed.

Interestingly, when caspase activity was analyzed in situ in the A20 B lymphoma cells, caspase-3 activation was shown to occur only in the shrunken-cell population (apoptosis), whereas caspase-8 activation was detectable in both the apoptotic and necrotic populations (Hetz et al. 2002b). These unexpected results identified, for the first time, that Fas ligation leads simultaneously to apoptosis and necrosis-like cell death, in which activation of initiator caspases, such as caspase-8, represents a key initial step triggered by FasL (Fig. 1).

As discussed above, initiation of dual Fas-signaling events that lead to both apoptosis and necrosis-like PCD have been described in several experimental systems when caspase activation is blocked (Holler et al. 2000; Vercammen et al. 1998a). Such results suggest that necrosis is favored either when pathways normally leading to apoptosis are blocked or when an alternative caspase-independent pathway is triggered. Our results expanded on these observations by showing that apoptosis and necrosis-like cell death require caspase activation to a different extent and can be triggered in the absence of caspase inhibitors. Caspase inhibitors were employed to help distinguish between the 2 types of cell death. Low concentrations of zVAD-fmk (less than 1 μmol/L) selectively reduced Fas-induced apoptosis without modulating necrosis, whereas at higher concentrations, both modes of cell death were affected (Hetz et al. 2002b). Thus, elevated levels of initiator caspase-8 activity appear to favor FasL-induced apoptosis and, as a prerequisite, caspase-3 activation. Under conditions in which stimulation via Fas leads only to low levels of caspase-8 activity (i.e., presence of zVAD-fmk, low levels of death receptor or ligand), apoptosis may not be initiated and signaling events leading to necrosis become the predominant cell death pathway. Based on these findings, as a more general rule, it is possible that subthreshold stimuli may be employed to ensure cell death by mechanisms other than apoptosis.

**Role of ceramide, a lipid second messenger, in Fas-mediated necrosis**

In addition to caspase activation, treatment with either FasL or TNF-α leads to the formation of ceramide (Garcia-Ruiz et al. 1997; Gudz et al. 1997). Sphingomyelin hydrolysis, by either neutral (nSMase) or acidic sphingomyelinases (aSMase), is generally implicated in ceramide production. However, it has also been suggested that de novo biosynthesis of ceramides plays a role in the induction of apoptosis (Hannun and Obeid 2002; Ogretmen and Hannun 2004). In some experimental systems, apoptosis is accompanied by caspase-dependent delayed (by several hours) ceramide production, owing to the hydrolysis of sphingomyelin associated with a loss of lipid asymmetry in the plasma membrane. As a result, phosphatidylserine is externalized and sphingomyelin is internalized, acting as a substrate for cytosolic SMases, which
release ceramides. Interestingly, Raji B cells that are deficient in lipid scrambling do not produce ceramides upon Fas activation; however, these cells still die by apoptosis upon Fas triggering (Tepper et al. 2000).

Several reports have suggested that ceramide production participates in the apoptotic cell death of lymphoid cells, mainly by correlating the simultaneous appearance of apoptotic markers with ceramide production (Garcia-Ruiz et al. 1997; Gudz et al. 1997). However, experiments in which genetic manipulation was employed to analyze the contribution of different SMases in Fas-induced apoptosis failed to implicate any of these enzymes (Brenner et al. 1997; Cock et al. 1998; Tepper et al. 2001), arguing against a role for ceramide in promoting apoptosis in such situations. Furthermore, experiments in HEK293 and HeLa cells established that ceramide production is an initiator caspase-dependent proximal event in Fas signaling that occurs independent of commitment to the effector phase of apoptosis (Grullich et al. 2000).

As mentioned, and in contrast to the situation described for A20 and Jurkat cells, Fas triggering in Raji B cells neither leads to late ceramide production (Tepper et al. 2000) nor results in Fas-induced necrosis-like PCD (Hetz et al. 2002b). These observations indicate that lipid scrambling and ceramide production may represent the crucial molecular events linking the Fas receptor to necrosis. In our studies, lipid scrambling was detectable in essentially all cells committed to death (apoptotic and necrotic-like cells) after Fas triggering, and was blocked with low concentrations of zVAD-fmk but not with caspase-3 inhibitors, as described elsewhere (Tepper et al. 2000). In agreement with such observations, caspase-independent (Holler et al. 2000) or necrotic (Krysko et al. 2004) cell death is also associated with phosphatidylserine externalization. Taken together, these observations link Fas-induced lipid scrambling, late ceramide production, and cell death by necrosis. Furthermore, they suggest that caspase-3-dependent apoptosis and other forms of cell death may not be clearly distinguished when phosphatidylserine externalization is used as a criterion.

Ceramide production is specifically linked to the induction of necrosis, because cell-permeable C6- or C2-ceramide induced cell death without caspase-3 activation, DNA fragmentation, cell shrinkage, or chromatin condensation (Hetz et al. 2002b). Instead, cells increased in size and were filled with vacuolar structures, resembling FasL-induced cell death by necrosis-like PCD. Likewise, increases in endogenous ceramide levels after treatment with bacterial SMase also
promoted cell death with a necrotic phenotype. In addition, the ceramide effect was shown to be dominant, in the sense that the earlier ceramide was present, the higher the observed percentage of necrotic cell death. These data lead us to speculate that after Fas triggering, ceramide production downstream of caspase-8 may be temporally delayed, in comparison to caspase-3 activation and apoptosis induction, so necrosis is only triggered in those cells not already committed to apoptosis.

**Ceramide signaling and cell death: initiation of necrosis or autophagy?**

Nonapoptotic cell death has been shown to be modulated by the genetic manipulation of survival signaling pathways. For example, when caspases are inhibited, cytotoxic agents, such as staurosporine and dexamethasone, induce necrosis-like cell death, which can be prevented by the overexpression of Bcl-2 (Amarante-Mendes et al. 1998). Also, there are several examples in which deletion of crucial apoptosis-regulating genes in mice and Caenorhabditis elegans leads to PCD by other mechanisms, most often necrosis-like PCD (Yuan et al. 2003). For instance, Apaf-1-null embryonic stem cells undergo nonapoptotic cell death after treatment with various cytotoxic stimuli that can be inhibited by Bcl-2 overexpression (Haraguchi et al. 2000).

Other groups have also found that ceramide induces caspase-independent cell death. Synthetic ceramides have been shown to induce a necrosis-like morphology in neuroblastoma cells (Ramos et al. 2003). Also, ceramides kill normal lymphocytes and cell lines in the absence of caspase activation, triggering a nonapoptotic morphology in dying cells (Mengubas et al. 1999). In addition, ceramide-induced cell death in leukemia cell lines was accompanied by minimal activation of caspase-3, and was not inhibited by zVAD-fmk (Belaud-Rotureau et al. 1999). We confirmed a number of these observations in B and T lymphoma cells (Hetz et al. 2002b). In addition, caspase-3-deficient cells have been shown to be sensitive to ceramide cytotoxicity (Belaud-Rotureau et al. 1999). Remarkably, cell viability was restored when cells were treated with the protease inhibitor leupeptin. Mochizuki et al. (2002) reported that ceramide induced cell death in human glioma cells with a necrosis-like phenotype that was efficiently inhibited by the activation of the AKT – protein kinase B pathway. This unexpected result reinforces the emerging concept that signaling pathways controlling apoptosis and necrosis-like PCD overlap. Taken together, these observations suggest that nonapoptotic cell death triggered by ceramide is an active and regulated process.

Two recent reports have yielded new insights into the mechanisms underlying ceramide-induced cell death. Using different cell lines, ceramide was shown to stimulate autophagy by 2 nonexclusive mechanisms (Scarlati et al. 2004). In human colon cancer HT-29 cells, C2-ceramide, a cell-permeable ceramide analog, stimulates autophagy by increasing the intracellular pool of long-chain ceramides. Ceramides stimulated the expression of the autophagic gene beclin 1 (Scarlati et al. 2004), linking, for the first time, known molecular mediators of autophagy (Liang et al. 1999) to lipid second messenger signaling. Ceramide also mediates the tamoxifen-dependent accumulation of autophagic vacuoles observed in human breast cancer MCF-7 cells. Ceramide-dependent expression of beclin 1 in tamoxifen-treated cells was impaired in the presence of myrioc, an inhibitor of the serine palmitoyltransferase, the rate-limiting enzyme of de novo synthesis of ceramide. These data suggest a novel function for ceramide in controlling a major lysosomal pathway, and provide a molecular link between autophagy and cell responses to stress (Scarlati et al. 2004).

Another report linked ceramide toxicity to autophagy in malignant glioma cells. Ceramide toxicity was accompanied by several specific features characteristic of autophagy, such as the presence of numerous autophagic vacuoles in the cytoplasm, development of the acidic vesicular organelles, autophagosome membrane association of microtubule-associated protein light chain 3 (LC3), and a marked increase in expression of 2 forms of LC3 protein (LC3-I and LC3-II). They also demonstrated that ceramide activates the expression of death-inducing mitochondrial protein BNI3 (Daido et al. 2004). BNI3 is a new member of the Bcl-2 protein family; it contains the Bcl-2 homology domain-3 (Hengartner 2000) that has been shown to promote necrosis-like PCD when overexpressed (Vande Velde et al. 2000). In this particular study, the authors showed that BNI3 triggered autophagy per se via ceramide release (Daido et al. 2004).

In summary, taking ceramide as an example, signaling in nonapoptotic PCD may be linked to common signaling components of the classic apoptotic pathway. In doing so, a novel concept in cell-death regulation is emerging, in which precisely coordinated sequences of molecular events, with at least some elements in common, determine whether cells die by apoptosis or by another form of PCD. These differences are likely to have significant biological consequences at the physiological and pathological levels, and to offer interesting possibilities for therapeutic intervention once the governing principles are understood.

**Nonapoptotic PCD in physiology, pathology, and disease**

In contrast to apoptosis, cell death by a necrosis-like mechanism is typically associated with inflammation. This difference in the physiological response is related to the activation or maturation of phagocytic cells, like macrophages and dendritic cells (Fadok et al. 2000; McDonald et al. 1999; Sauter et al. 2000). Sauter et al. (2000) showed that immature dendritic cells phagocytose a variety of apoptotic and necrotic cells. However, only exposure to necrotic cells provided the signals required for dendritic cell maturation, resulting in the upregulation of maturation-specific markers, costimulatory molecules, and the capacity to induce antigen-specific CD4+ and CD8+ T-cells. Thus, dendritic cells are able to distinguish between the 2 types of dead cells and respond in a distinct manner. The interaction between apoptotic and phagocytic cells induces an anti-inflammatory response (Fadok et al. 2000), whereas necrosis appears to be critical for initiation of an immune response. Because phosphatidylserine externalization is critical for the recognition of dead cells (probably apoptotic and necrotic cells), the data discussed reinforce the notion that lipid scrambling and phosphatidylserine externalization do not provide the molecular basis to distinguish between cells dying by apoptosis and those...
dying by necrosis, and, as a consequence, to elicit different responses of the immune system.

Simultaneous or delayed activation of pathways leading to apoptosis or necrotic-like PCD in the same cells is likely to occur fairly frequently under pathological conditions (Ankarcrona 1998; Barros et al. 2001a; Dypbukt et al. 1994; Hetz et al. 2002a; Jonas et al. 1994). One possibility is that catastrophic or accidental events rapidly deplete cells of ATP and, in doing so, preclude organized cell demise by ATP-requiring mechanisms, such as apoptosis. Thus, necrosis occurs as a default pathway when apoptosis is blocked, as has been suggested in models of accidental necrosis (Leist et al. 1997). Alternatively, the cell may actively undergo death by activating pathways leading to necrosis. This could be achieved by directly manipulating ATP levels and (or) other mechanisms, including the production of reactive oxygen species and the liberation of ceramides.

An interesting parallel can be drawn with models of necrosis-like PCD in C. elegans. Mutations in a sodium channel of the degenerin gene family of C. elegans that increase channel activity cause neuronal degeneration through necrotic events, including intracellular vacuole formation and swelling. In mammals, the activity of sodium channels has also been linked to cell death by accidental necrosis mediated by oxidative stress, a phenomena generally associated with several pathological conditions (Barros et al. 2001a, 2001b). More recently, Simon et al. (2004) demonstrated that hydroxyl radicals increase the open probability of a roughly 20-pS calcium-activated, nonselective cation channel in liver cells, and reduce calcium dependence. Thus, the presence of reactive oxygen species effectively modulates the activity of these channels and, in doing so, may contribute to the regulation of necrosis.

Interestingly, loss-of-function mutations in calreticulin, a major Ca\(^{2+}\)-binding chaperone in the endoplasmic reticulum (ER), or double mutations in both the ryanodine and inositol-1,4,5-trisphosphate receptors that block Ca\(^{2+}\)-release channels, suppress death in C. elegans by necrosis (Xu et al. 2001). Thus, an increase in cytoplasmic Ca\(^{2+}\) concentration, elicited by release from the ER, may play a major role in initiating necrosis-like cell death events triggered by abnormal functioning of plasma membrane Na\(^{+}\) channels. Conditions that activate accidental necrosis may be incompatible with the execution of apoptotic or necrosis-like PCD pathways (such as fast swelling and lysis). The ability of high levels of Ca\(^{2+}\) to induce swelling and disruption of mitochondria, causing permeability transition and loss-of-energy metabolism, is likely a key event leading to necrosis. Interestingly, ceramide treatment has also been shown to increase cytosolic and mitochondrial calcium levels (Belaud-Rotureau et al. 1999; Pinton et al. 2001). Furthermore, ceramides have been shown to directly modulate mitochondria function, for instance, by inhibiting the mitochondrial respiratory complex III (Garcia-Ruiz et al. 1997; Gudz et al. 1997; Quillet-Mary et al. 1997). In this context, it is possible that the induction of nonapoptotic cell death by ceramide may result from a decrease in ATP levels due to mitochondrial dysfunction and calcium release from the ER.

Alterations in ER homeostasis (denominated ER stress) have been linked to specific apoptotic pathways associated with alterations in calcium homeostasis and accumulation of misfolded proteins in this organelle (Breckenridge et al. 2003). Under pathological conditions that affect the nervous system, ER stress plays a central role in controlling cell death observed during stroke, ischemia, and neurodegeneration (Rao and Bredesen 2004). For example, in models of Alzheimer’s disease (Nakagawa et al. 2000), prion disease (Castilla et al. 2004; Hetz et al. 2003), and Huntington’s disease (Nishihito et al. 2002), ER stress has been linked to neuronal dysfunction. Hence, under conditions of extensive ER stress, cells may preferentially undergo necrosis-like cell death. It remains to be investigated whether the common mediators of ER stress that trigger apoptosis also participate in the induction of nonapoptotic PCD.

Recent reports (Kegel et al. 2000; Petersen et al. 2001; Stefanis et al. 2001) have shown that autophagic–necrotic cell death is also involved in neurological diseases, highlighting the importance of understanding the mechanisms associated with nonapoptotic cell death (Bahr and Bendiske 2002). Moreover, the relevance of such understanding is underscored by the fact that anticancer therapies are now known to use nonapoptotic mechanisms to promote cell death and tumorogenesis (Edinger and Thompson 2003; Liang et al. 1999; Paglin et al. 2001). This is relevant for the design of novel therapeutic strategies, because cancer cells are often resistant to apoptotic stimuli. In fact, animals with one of the alleles for beclin 1 knocked out suffer from a high incidence of spontaneous tumors, suggesting that beclin 1 is a tumor-suppressor gene (Yue et al. 2003). Examples that emphasize such potential include a possible ceramide-based therapy to treat gliomas (Daido et al. 2004), and the use of anticancer drugs, such as tamoxifen and arsenic trioxide, to induce tumor cell death by autophagy (Bursch 2001; Kanazawa et al. 2003). This may explain the fact that, in many forms of cancer, increased resistance to apoptosis is a crucial factor in cancer progression, and mutations in genes involved in the regulation of apoptosis are genetically linked to some forms of cancer. Thus, drugs that trigger nonapoptotic cell death are likely to have therapeutic benefits. Along these lines, a recent report indicates that, in knockout cells for crucial proapoptotic genes (such as Bax and Bak), stimulation of cell death by classic proapoptotic stimuli promotes cell death by autophagy as a default pathway (Shimizu et al. 2004).

**Conclusions**

Nonapoptotic cell death plays an important role in the control of cell viability under normal physiological conditions, and when cell death is triggered by injury or disease. Clearly, a more refined understanding of the molecular elements that determine whether cell death occurs by accidental necrosis, necrotic-like PCD, autophagy, or apoptosis is required. The need for such insight becomes all the more apparent when considering the fact that these different forms of cell death share some characteristic features at the morphological level. Multiprotein complex formation is crucial to signaling per se and to apoptosis in particular (Leyton and Quest 2002, 2004). Recent advances indicate that peptidomimetics, which specifically target protein–protein interactions relevant to either the extrinsic or intrinsic apoptotic pathway, represent potentially valuable tools for selectively triggering...
tumor-specific cell death (Li et al. 2004; Walensky et al. 2004). With this in mind, it is easy to appreciate how a better understanding of nonapoptotic cell-death mechanisms may translate into the development of more efficient therapeutic strategies for human disorders, such as cancer, autoimmune, and neurological diseases.

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