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Caspase-independent cell death

Marcel Leist and Marja Jäättelä

1. Introduction

Caspase-mediated apoptosis is the death program of choice in many developmental and physiologic settings (Kerr *et al.*, 1972; Strasser *et al.*, 2000; Kaufmann and Hengartner, 2001). It would, however, be very dangerous for the organism to depend on a single protease family for clearance of unwanted and potentially dangerous cells. Indeed, the exclusive role of caspases in the execution of programmed cell death (PCD) has been challenged recently (Borner and Monney, 1999; Kitanaka and Kuchino, 1999; Johnson, 2000; Wang, 2000; Kaufmann and Hengartner, 2001; Leist and Jäättelä, 2001[**Q1**]). Since the first reports on caspase-independent PCD in the late 1990s, over 200 papers have been published on the topic. Now our understanding of the molecular control of alternative death pathways is growing, like that of the molecular anatomy of apoptosis at the time of the discovery of caspases less than a decade ago. Here, we review recently discovered triggers and molecular regulators of alternative cell-death programs and discuss the implications of the death mode for the surrounding tissue and the potential of caspase-independent PCD signaling pathways as therapeutic targets for the treatment of cancer and neurodegenerative disorders.

2. Four patterns of death: from apoptosis to necrosis

The unclear definition of the alternative death pathways has been the major obstacle to elucidating them. If PCD is used as a synonym of apoptosis and defined by caspase activation (Samali *et al.*, 1999), alternative caspase-independent PCD pathways are evidently not possible. In contrast, the classification that we use here takes into account the implications of the death mode for the surrounding tissue and leaves space for different mechanistic observations and alternative interpretations. PCD is simply defined as cell death that is dependent on signals or activities within the dying cell (Lockshin and Zakeri, 2001). According to the morphology of dying

cells, PCD can be further divided into apoptosis, apoptosis-like, and necrosis-like PCD (Kitanaka and Kuchino, 1999; Leist and Jäättelä, 2001[**Q1**]) (*Figure 1*). Apoptosis is defined here by chromatin condensation to compact and apparently simple geometric figures (stage 2 chromatin condensation), phosphatidylserine exposure, cytoplasmic shrinkage, plasma membrane blebbing, and formation of apoptotic bodies (Kerr *et al.*, 1972; Woo *et al.*, 1998; Susin *et al.*, 2000; Leist and Jäättelä, 2001[**Q1**]). Moreover, apoptosis-like PCD is characterized by chromatin condensation and display of phagocytosis recognition molecules before the lysis of the plasma membrane. Chromatin condenses, however, to lumpy masses that are less compact than in apoptosis (stage 1 chromatin condensation) (Woo *et al.*, 1998; Susin *et al.*, 2000; Leist and Jäättelä, 2001[**Q1**]). Any degree and combination of other apoptotic features can be found. Necrosis-like PCD is used here to define PCD in the absence of chromatin condensation, or at best with chromatin clustering to loose speckles (Leist *et al.*, 1997; Vercammen *et al.*, 1998a,b; Mateo *et al.*, 1999; Holler *et al.*, 2000; Sperandio *et al.*, 2000). Varying degrees of apoptosis-like cytosolic features may occur before the lysis (Mateo *et al.*, 1999; Holler *et al.*, 2000).

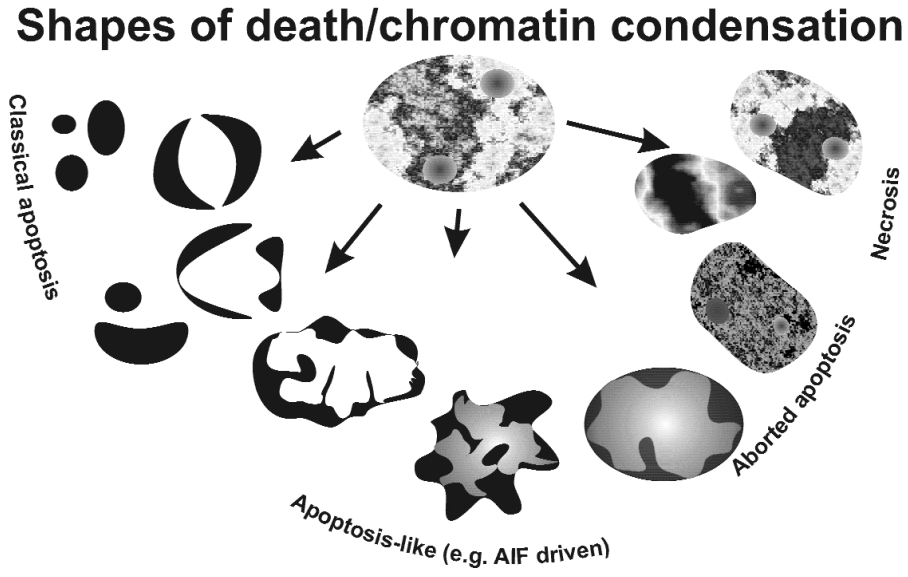

Figure 1. Nuclear alterations in different forms of PCD.

The use of chromatin condensation as a criterion to distinguish apoptosis from apoptosis-like PCD has been inconsistent in the scientific literature, and the potential for overlapping definitions and errors is large. Electron-microscopic examples of classic apoptosis and apoptosis-like PCD (Leist and Jäättelä, 2001[**Q1**]) or these schematic drawings might provide a general guideline. The control chromatin is speckled, showing areas of eu- and heterochromatin, and mostly one or several more condensed micronuclei (top middle). Caspase-dependent chromatin compaction and fragmentation to crescent- or spherical-shaped masses at the nuclear periphery is shown on the left. Caspase-independent chromatin margination triggered directly by microinjection of AIF or in a number of models of apoptosis-like death is shown at the bottom. Many intermediate forms and also transitions to necrosis are possible. Necrotic morphology is also observed in models where caspases are inhibited before apoptosis is completed (aborted apoptosis).

Unlike necrosis, necrosis-like PCD is the result of active cellular processes that can be intercepted by, for instance, oxygen-radical scavengers (Schulze-Osthoff *et al.*, 1992; Vercammen *et al.*, 1998a,b), inhibition of poly(ADP) ribose polymerase (PARP) (Ha and Snyder, 1999) or mutations in intracellular signaling molecules (Holler *et al.*, 2000). A subgroup of necrotic PCD models is often classified as 'aborted apoptosis'; that is, a standard apoptosis program is initiated, then blocked at the level of caspase activation, and finally terminated by alternative, caspase-independent routes (Nicotera *et al.*, 1999). Autophagy is characterized by the formation of large lysosome-derived cytosolic vacuoles (Bursch *et al.*, 1996; Chi *et al.*, 1999; Elliott *et al.*, 2000), and dark cell death in specialized cells, such as chondrocytes (Roach and Clarke, 2000) or neurons (Turmaine *et al.*, 2000), usually lacks chromatin condensation and can thus be classified as necrosis-like PCD. Accidental necrosis, characterized by a rapid lysis of plasma membrane and organelle swelling, is the conceptual counterpart to PCD, since it is prevented only by removal of the stimulus. It occurs after exposure to high concentrations of detergents, oxidants, and ionophores, or high intensities of pathologic insult (Nicotera *et al.*, 1999). Finally, it should be noted that in tissue-culture conditions that lack the phagocytizing cells, the plasma membrane of cells dying either by classic apoptosis or apoptosis-like PCD will eventually break. Such loss of the cellular permeability barrier followed by passive changes in cell organelles is often confusingly referred to as secondary necrosis.

3. Death programs beyond caspases

In its classic form, apoptosis occurs almost exclusively when caspases, particularly caspase-3, are activated (Woo *et al.*, 1998; Susin *et al.*, 2000). The unexpected ability of certain cells to survive the activation of proapoptotic caspases (Lacana *et al.*, 1997; Wright *et al.*, 1997; Jäättelä *et al.*, 1998; De Maria *et al.*, 1999; Zeuner *et al.*, 1999; Harvey *et al.*, 2000; Foghsgaard *et al.*, 2001; Hoeppner *et al.*, 2001; Los *et al.*, 2001; Reddien *et al.*, 2001) demonstrates, however, the remarkable plasticity of the cellular death program, and argues against the idea that caspases alone are sufficient for the induction of mammalian PCD. Furthermore, recent evidence indicates a diversification of the apoptosis program in higher eukaryotes with respect to the necessity and role of caspases. Signals emanating from the established key players of apoptosis, including death receptors and caspases themselves, may result in necrosis-like PCD (Leist *et al.*, 1997; Vercammen *et al.*, 1998a,b; Khwaja and Tatton, 1999; Leist *et al.*, 1999; Holler *et al.*, 2000), and apoptosis-like PCD characterized by chromatin condensation and phosphatidylserine exposure is not necessarily accompanied by effector caspase activation (Berndt *et al.*, 1998; Lavoie *et al.*, 1998; Monney *et al.*, 1998; Mathiasen *et al.*, 1999; Roberts *et al.*, 1999; Nylandsted *et al.*, 2000[**Q2**]; Gingras *et al.*, 2002). Other important apoptosis hallmarks, such as detachment, shrinkage, and zeiosis, can also be present in cells dying in a caspase-independent manner (McCarthy *et al.*, 1997; Berndt *et al.*, 1998; Nylandsted *et al.*, 2000[**Q2**]; Foghsgaard *et al.*, 2001; Joza *et al.*, 2001; Gingras *et al.*, 2002).

Contrary to earlier expectations, the inhibition of caspase activation does not necessarily protect against cell death stimuli. Instead, it may reveal, or even

enhance, underlying caspase-independent death programs. These programs may take the form of apoptosis-like (Deas *et al.*, 1998; Luschen *et al.*, 2000; Foghsgaard *et al.*, 2001; Joza *et al.*, 2001; Volbracht *et al.*, 2001[**Q3**]), or necrosis-like (Xiang *et al.*, 1996; Leist *et al.*, 1997; McCarthy *et al.*, 1997; Vercammen *et al.*, 1998a,b; Chautan *et al.*, 1999; Khwaja and Tatton, 1999; Xue *et al.*, 1999; Holler *et al.*, 2000; Matsumura *et al.*, 2000) PCD. In many experimental apoptosis models, including those triggered by death receptors (Vercammen *et al.*, 1998a,b; Holler *et al.*, 2000; Matsumura *et al.*, 2000), cancer drugs (Amarante-Mendes *et al.*, 1998), growth-factor deprivation (Xue *et al.*, 1999), staurosporine (Deas *et al.*, 1998), anti-CD2 (Deas *et al.*, 1998), oncogenes (McCarthy *et al.*, 1997), colchicine (Volbracht *et al.*, 2001[**Q3**]), GD3 (Simon *et al.*, 2001), or expression of Bax-related proteins (Xiang *et al.*, 1996; McCarthy *et al.*, 1997), the existence of backup death pathways has been uncovered following inhibition of caspase activity by pharmaceutical pancaspase inhibitors. However, several lines of evidence support the relevance of such 'second-line' mechanisms also for normal physiology and pathology. In addition to pharmacologic inhibitors, caspase pathways can be inactivated by other factors such as mutations (Chautan *et al.*, 1999), energy depletion (Leist *et al.*, 1997), nitrative/oxidative stress (Leist *et al.*, 1999), other proteases that are activated simultaneously (Chua *et al.*, 2000; Lankiewicz *et al.*, 2000; Reimertz *et al.*, 2001), members of the 'inhibitor of apoptosis protein' (IAP) family (Jäättelä, 1999; Strasser *et al.*, 2000), defective release of Smac/Diablo (Deng *et al.*, 2002), or an array of viral proteins that can silence caspases (Strasser *et al.*, 2000). Thus, it is not surprising that the list of model systems where PCD is not accompanied by the effector caspase activation is growing (*Table 1*). This is especially evident in cancer cells, which often harbor defects in classic apoptosis pathways (Jäättelä, 1999).

Upon caspase inhibition, the alternative death pathways surface also *in vivo*. They are involved in processes such as the negative selection of lymphocytes (Smith *et al.*, 1996; Doerfler *et al.*, 2000), cavitation of embryoid bodies (Joza *et al.*, 2001), embryonic removal of interdigital webs (Chautan *et al.*, 1999), tumor necrosis factor (TNF)-mediated liver injury (Kunstle *et al.*, 1999), and the death of chondrocytes controlling the longitudinal growth of bones (Roach and Clarke, 2000). These examples may represent just the tip of the iceberg with regard to the complexity of death signaling *in vivo*. And the overlapping death pathways initiated by a single stimulus seem rather to be the rule than the exception (Holler *et al.*, 2000; Charette *et al.*, 2001; Joza *et al.*, 2001). The examination of potential crossovers of death pathways that lead eventually to different phenotypic outcomes may offer a chance to understand which events do determine commitment to death, and which ones are instead involved in upstream signaling or downstream execution.

4. Signaling in caspase-independent PCD

Several molecular mediators of classic caspase-mediated apoptosis pathways have been characterized during the last decade (Mattson, 2000; Strasser *et al.*, 2000; Kaufmann and Hengartner, 2001), whereas the description of most alternative death routines has remained limited to the phenomenological level. But recent mechanistic findings have opened a new era in this field. Like classic apoptosis,

Table 1. PCD models not accompanied by effector caspase activation

Stimulus	Cell type	Morphology	Rescued by	Reference
Adenoviral E4orf	Fibroblast p53-null fibroblast	Apoptosis-like	Bcl-2	Lavoie *et al.*, 1998
Inhibition of the ubiquitin pathway	Fibroblast	Apoptosis-like	Bcl-2	Monney *et al.*, 1998
CD4/CXCR4 receptor antibodies	CD4+ T cells	Apoptosis-like	Stromal cell growth factor	Berndt *et al.*, 1998
Camptothecin	Liver cancer	Apoptosis-like	Cysteine cathepsin inhibitors	Roberts *et al.*, 1999
Vitamin D compounds	Breast cancer	Apoptosis-like	Bcl-2, calpain inhibitors Calbindin	Mathiasen *et al.*, 1999 I. S. Mathiasen and M. Jäättelä, unpublished
Antigen receptor cross-linking	B-cell lymphoma	Apoptosis-like	Inhibition of calpains and cysteine cathepsins	Katz *et al.*, 2001
Sigma-2 receptor agonists	Breast cancer	Apoptosis-like		Crawford and Bowen, 2002
Hsp70 depletion	Various cancers (not fibroblast or immortalized epithelium)	Apoptosis-like	Cysteine cathepsin inhibitors (not by Bcl-2/Bcl-X_L)	Nylandsted *et al*, 2000 J. Nylandsted and M. Jäättelä, unpublished
Intracellular Acidification	Bladder cancer, fibroblast, leukemia	Necrosis-like	Inhibition of SAPK (not by Bcl-2)	Zanke *et al.*, 1998
Oncogenic Ras	Glioblastoma gastric cancer	Necrosis-like autophagy	(not by Bcl-2)	Chi *et al.*, 1999
Bin1	Liver cancer (not fibroblast)	Necrosis-like autophagy	SV40 large T antigen, serine protease inhibitor (not by Bcl-2)	Elliott *et al.*, 2000 **[Q2]**
IGF1R	Kidney epithelium	Necrosis-like vacuolar degeneration	Actinomycin D Cycloheximide (not by Bcl-X_L)	Sperandio *et al.*, 2000

CXCR4, chemokine coreceptor; SAPK, stress-activated protein kinase.

alternative death programs can be mediated by proteases and switched on by mitochondrial alterations or death receptors.

4.1 Noncaspase proteases as mediators of PCD

The most extensive evidence linking noncaspase proteases with PCD originates from studies of calpains; cathepsins B, D, and L; and granzymes A and B (Kitanaka and Kuchino, 1999; Johnson, 2000; Leist and Jäättelä, 2001[**Q1**]). These proteases cooperate often with caspases in classic apoptosis, but recent data suggest that they can also trigger PCD and bring about many of the morphologic changes characteristic of apoptosis in a caspase-independent manner (Johnson, 2000; Wang, 2000; Leist and Jäättelä, 2001[**Q1**]) [**Q4**](*Table 2*). As noncaspase proteases have only recently attracted broader interest among cell-death researchers, this list is likely to present only a fraction of all PCD-mediating proteases.

Cathepsins

The cathepsin protease family comprises cysteine, aspartate, and serine proteases (Johnson, 2000; Turk *et al.*, 2001). So far, the cysteine cathepsins B and L and the aspartate cathepsin D have been most clearly linked to PCD. Most cathepsins mature in the endosomal-lysosomal compartment. They can be activated by autoproteolysis in acidic pH or proteolysis by other proteases (for example, cathepsin D can activate cathepsins B and L). Furthermore, ceramide specifically binds to and promotes the proteolytic activation of cathepsin D, possibly linking sphingomylinase-mediated ceramide production and cathepsins to a common PCD pathway (Heinrich *et al.*, 1999). Until recently, the function of cathepsins was presumed to be limited to the disposal of proteins in the lysosomal compartment and degradation of extracellular matrix once secreted. During the past few years, however, many of them have been assigned specific functions, as, for example, in bone remodeling, hair follicle morphogenesis, antigen presentation, and PCD (Reinheckel *et al.*, 2001; Turk *et al.*, 2001). Genetic evidence for the role of cysteine cathepsins in PCD is provided by studies showing resistance to TNF-induced liver apoptosis in mice that lack cathepsin B (Guicciardi *et al.*, 2001), and massive PCD in the brains of mice that lack the cysteine cathepsin inhibitor, cystatin B (Pennacchio *et al.*, 1998).

Cathepsins participate in both caspase-dependent and -independent PCD induced by a variety of stimuli, including death receptors, camptothecin, B-cell receptors, bile salt, oxidants, and retinoids (Roberts *et al.*, 1997, 1999; van Eijk and de Groot, 1999; Guicciardi *et al.*, 2000; Foghsgaard *et al.*, 2001; Katz *et al.*, 2001; Roberg, 2001; Zang *et al.*, 2001). Cathepsins translocate from lysosomes to the cytosol and/or nucleus before the appearance of gross morphologic changes indicative of PCD. Notably, the inhibition of cathepsin activity protects cells from PCD without preventing the release of cathepsins from the lysosomes (Foghsgaard *et al.*, 2001). These data indicate that the release of cathepsins is not merely a sign of final organelle disintegration in the dying cell, and suggest that cathepsins have to escape the lysosomal compartment to trigger PCD. The latter hypothesis is further supported by the data showing that microinjection of cathepsin D (K. Roberg, personal communication), as well as limited disruption of lysosomes, triggers

Table 2. Characteristics of noncaspase proteases involved in programmed cell death

Protease/antiprotease	External stimuli	Cellular localization	Substrates	Effects
Cathepsin B and L/Cystatins (stefins)-cysteine proteases	TNF, TRAIL, camptothecin, Hsp70 depletion, B-cell antigen receptor, laser-beam-triggered microcavitation	Lysosomal or extracellular, translocates to the cytosol in apoptotic cells	Bid, PARP, procasp-1, -2, -6, - 7, 11, cathepsin C Extracellular matrix	Cytochrome *c* release, chromatin condensation, blebbing, PS exposure, degradation of extracellular matrix
Cathepsin D -Aspartyl protease	TNF, interferon-γ, oxidative stress, retinoid CD437, ceramide photosensitizers	Lysosomal or extracellular translocates to the cytosol in apoptotic cells	Cystatins Procathepsins	Cytochrome *c* release, activation of cathepsin B
Calpains/Calpastatin -cysteine proteases	Irradiation, ionophores, vitamin D compounds, TGF-β, β-lapacphone, dexamethasone, etoposide neurotoxins	Mainly cytosolic	Bax, Bcl-X_L, fodrin, procasp-3, -9, and -12, gelsolin, FAK, actin, c-fos, c-jun, c-mos, p53, cyclin D, etc.	Cytochrome *c* release, PS exposure, activation of procaspase-12
Granzymes/Serpin PI-9 -Serine proteases	Cytotoxic T cells, perforin-assisted entry to target cell	Cytotoxic granules in T cells	procasp-3, -6, -7, 8, -9, ICAD, lamins, histones, Bid, SET, DNA-PK_{CS}	Cytochrome *c* release, DNA breaks and fragmentation, nuclear condensation
AP24/Serpins -Serine protease	Death receptors, UV, DNA-damaging drugs	Cytosolic, translocates into nucleus in dying cells		DNA fragmentation
Omi (htra2)/serpins -Serine protease		Mitochondrial, translocates into cytosol in dying cells		Inhibition of IAPs Cell rounding and shrinkage
Other serine proteases/ Serpins	Bin-1 tumor-suppressor Death receptors		LEI (elastase)	L-DNase II-mediated DNA fragmentation

TGF, transforming growth factor; UV, ultraviolet light; FAK, focal adhesion kinase; PARP, poly (ADP) ribose polymerase; ICAD, inhibitor of caspase-activated DNase; SET, nucleosome assembly protein; DNA-PK_{CS}, catalytic subunit of DNA-dependent protein kinase.

apoptosis dependent on cathepsin activity (Kagedal *et al.*, 2001). It should also be noted that in some cells cathepsins may be pivotal for survival, as the cysteine cathepsin inhibitor, CATI-1, kills leukemia and lymphoma cells (Zhu and Uckun, 2000).

Calpains

Calpains are cysteine proteases that reside in the cytosol in an inactive zymogen form (Johnson, 2000; Wang, 2000). Two forms of calpains, μ-calpain and m-calpain, are ubiquitously expressed in human cells and have been linked to differentiation and PCD. The active forms of the enzymes consist of a variable large subunit (80 kDa) and a common small subunit (30 kDa). Activation of calpains requires an elevation in intracellular calcium $[Ca^{2+}]_i$. Proteolytic cleavage and association with membrane phospholipids may further contribute to their activation, possibly by lowering the $[Ca^{2+}]_i$ requirement. Calpain activity is controlled by calpastatin, a natural inhibitor that can be inactivated by calpain- or caspase-mediated cleavage. Calpains are activated by various stimuli (see *Table 2*) that increase the $[Ca^{2+}]_i$ and they can participate in PCD signaling upstream or downstream of caspases (Leist *et al.*, 1998; Waterhouse *et al.*, 1998; Nakagawa and Yuan, 2000; Choi *et al.*, 2001; Varghese *et al.*, 2001). Furthermore, calpains can mediate apoptosis-like PCD, even in the absence of caspase activation (Mathiasen *et al.*, 1999; I. S. Mathiasen and M. Jäättelä, unpublished). For example, EB1089/seocalcitol, a vitamin D analog currently on phase III clinical trials for the treatment of cancer, induces calcium- and calpain-dependent apoptosis-like PCD in breast cancer cells without triggering detectable caspase activation.

Serine proteases

The most prominent components of cytotoxic granules of cytotoxic lymphocytes are the pore-forming protein, perforin, and the serine proteases, granzyme A and granzyme B (Johnson, 2000; Trapani *et al.*, 2000). Upon activation, cytotoxic lymphocytes release their granular contents. Subsequently, target cells take up granzymes by receptor-mediated endocytosis and subsequent perforin-mediated release from endosomes into the cytosol or by diffusion via perforin-generated pores in the plasma membrane. Studies employing mice lacking either granzyme A or B have demonstrated that granzyme B is required for the granule-induced rapid caspase-mediated apoptosis (Johnson, 2000). Granzyme B cleaves its substrates after aspartate residues and can thus directly activate caspases. However, in the presence of caspase inhibitors, granzyme B triggers a slower necrosis-like PCD (Talanian *et al.*, 1997). Granzyme A is a trypsin-like protease that cleaves its substrates after lysine or arginine residues. Death induced by granzyme A is associated with DNA single-strand breaks created by a granzyme A-activated DNase (Beresford *et al.*, 2001).

Other serine proteases that have been associated with cell death include apoptotic protease 24 (AP24), which mediates DNA fragmentation in TNF-, UV light-, and chemotherapy-induced PCD of some cancer cells (Wright *et al.*, 1997; 1998), and the recently identified omi/htra2 (Suzuki *et al.*, 2001), which is released from mitochondria into the cytosol during apoptosis, and can mediate caspase-independent PCD dependent on its serine protease activity and/or contribute to the

caspase activation by counteracting members of IAP family. A family of protease inhibitors called serpins inhibits the activity of serine proteases. Interestingly, the serine-protease-mediated inactivation of a serpin, leukocyte elastase inhibitor (LEI), transforms LEI into an endonuclease, L-DNase II (Torriglia *et al.*, 1998). L-Dnase II translocates to the nucleus in various PCD models; it can induce pyknosis and DNA degradation *in vitro*. Thus, the transformation of LEI to L-DNase II may act as an important switch of protease and nuclease pathways during caspase-independent PCD.

The definition of the role of the individual proteases in the complex process of PCD still requires much careful work. The dependence on certain proteases may be extremely cell-type and stimulus specific, and may depend on the relative expressions, activations, and inactivations of proteases and protease inhibitors (*Table 2* and *Figure 2*). Genetic approaches need to be combined with meticulous pharmacologic titration of inhibitors (Foghsgaard *et al.*, 2001), since it turns out that pan-caspase inhibitors, as well as many active site inhibitors of other proteases, are highly unspecific at concentrations widely used to test their role in PCD (Schotte *et al.*, 1998; Waterhouse *et al.*, 1998; Johnson, 2000; Foghsgaard *et al.*, 2001).

4.2 Death receptors as triggers of alternative PCD

The best-studied members of the death-receptor family are TNF receptor 1 (TNF-R1), Fas (also known as CD95 or Apo-1), and the receptors for TNF-related apoptosis-inducing ligand (TRAIL). Whereas it has long been known that TNF-induced death can take the shape of either apoptosis or necrosis (Laster *et al.*, 1988), the ability of the Fas ligand (FasL) and TRAIL to induce necrosis-like PCD has been

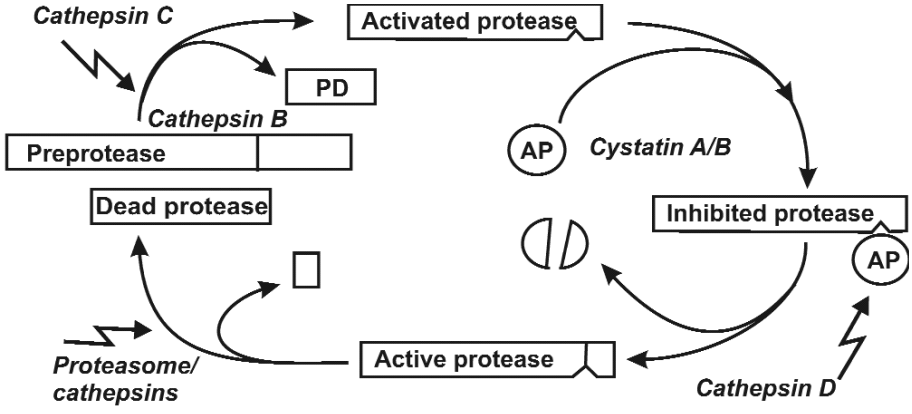

Figure 2. Interaction of proteases during PCD.

Frequently, an inactive or weakly active zymogen (preprotease) is activated by the cleaving of a prodomain (PD) (the first level of protease family interaction; examples shown in italics). Intracellular protease activity is prevented by specific antiproteases (AP), and ultimate activation requires inactivation of AP (often by proteolysis, the second level of protease family interaction). Further proteolysis can lead to inactivation of the active protease and eventually degradation (the third level of protease family interaction). The balance of all players in this circle determines which proteases dominate the death process. Pharmacologic inhibition of one protease easily shifts the balance to another pathway.

described only recently (Leist *et al.*, 1997; Kawahara *et al.*, 1998; Vercammen *et al.*, 1998a,b; Leist *et al.*, 1999; Holler *et al.*, 2000). In activated primary T cells, this caspase-independent necrosis-like PCD seems, at least in some cases, to be the dominant mode of death (Holler *et al.*, 2000). This may explain why inhibition of caspase activity in mouse T cells *in vivo* does not induce the lymphadenopathy and/or autoimmune disease usually manifested in mice with inactivating mutations in Fas or Fas ligand (Smith *et al.*, 1996).

Except for the dependence on reactive oxygen species (ROS) and, in some cases, serine protease activity, necrotic signaling pathways have remained ambiguous until recently (Denecker *et al.*, 2001). Novel data demonstrate now that TNF, FasL, and TRAIL can trigger caspase-8-independent necrosis-like PCD that is dependent on the Fas-associated death domain (FADD) protein and the kinase activity of the receptor-interacting protein (RIP) (Holler *et al.*, 2000). The dependence of RIP-mediated necrotic PCD on proteases remains to be studied. Interestingly, some TNF-resistant cells are sensitized to TNF-induced necrosis-like PCD upon inhibition of caspases, suggesting that caspases act as survival factors that directly inhibit the TNF-induced necrotic pathway (Khwaja and Tatton, 1999). Death receptors can also trigger caspase-independent apoptosis-like PCD. In immortalized epithelial cells, activated Fas has been reported to recruit Daxx from the nucleus to the receptor complex, and to trigger its binding with apoptosis signal-regulating kinase 1 (Ask1) (Charette *et al.*, 2000; Ko *et al.*, 2001). Others have, however, have failed to detect Daxx in the cytosol and have suggested that Daxx enhances Fas-induced caspase-dependent death from its nuclear localization (Torii *et al.*, 1999). Thus, Daxx may stimulate Fas-induced death by two independent mechanisms, the caspase-independent pathway being evident only when caspase activation is defective (Charette *et al.*, 2000) and enough Ask1 is available (Ko *et al.*, 2001). In addition to a caspase-dependent proapoptotic function that depends on its kinase activity, Ask1 possesses a caspase-independent killing function that is independent of its kinase activity and is activated by interaction with Daxx (Charette *et al.*, 2001). Ask1 has also been found to be essential for TNF-triggered apoptosis of primary fibroblasts, but its activation by TNF appears to require ROS (Tobiume *et al.*, 2001) instead of Daxx (Yang *et al.*, 1997). In TNF-treated fibrosarcoma cells cysteine cathepsins act as dominant execution proteases and bring about apoptosis-like morphologic changes (Foghsgaard *et al.*, 2001). Whether Ask1 and cathepsins act on the same signaling pathway is as yet unknown.

The picture described above suggests a complexity of death-receptor-induced apoptotic and necrotic signaling networks that far exceeds that of the simple linear pathway originally suggested by the discovery of the receptor-triggered caspase cascade (*Figure 3*).

4.3 Mitochondrial control of caspase-independent PCD

Analogous to classic apoptosis, mitochondrial membrane permeabilization (MMP) controlled by Bcl-2 family proteins resides at the heart of several alternative death pathways. The prevailing theory suggests that 'multidomain' proapoptotic Bcl-2 family members (such as Bax and Bak) are the actual pore-forming effector molecules required for MMP (Cheng *et al.*, 2001; Wei *et al.*, 2001). Bax

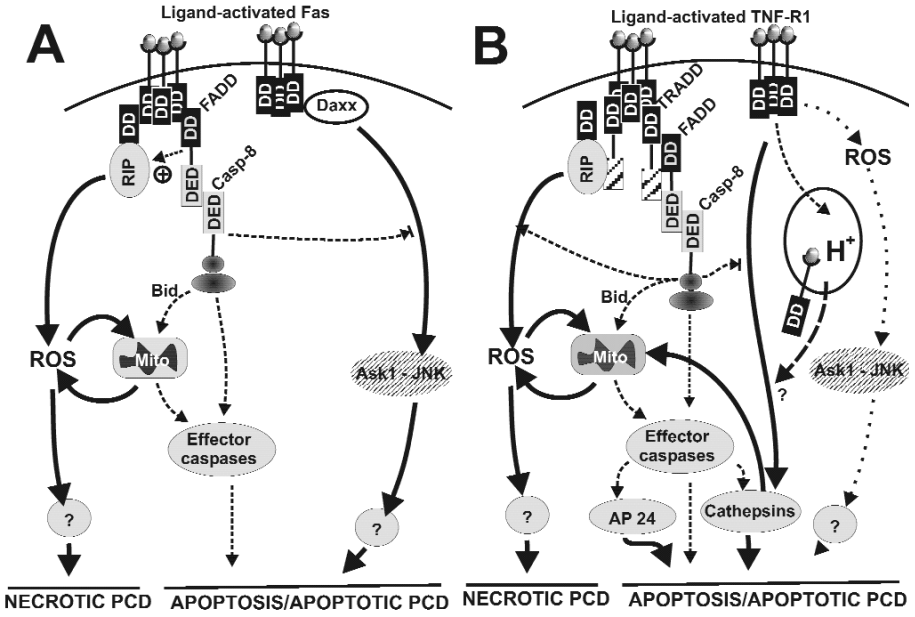

Figure 3. Multiple death pathways triggered by death receptors.

Death-receptor signaling is initiated by ligand-induced receptor trimerization. (A) Receptor death domains (DD) of Fas then recruit FADD, RIP, and/or Daxx to the receptor complex. Caspase-8 becomes activated after recruitment to FADD via death effector domain (DED) interaction, and triggers effector caspases, either directly or through a Bid-mediated mitochondrial pathway (Strasser *et al.*, 2000) (dashed lines). RIP initiates a caspase-independent (solid lines) necrotic pathway mediated by the formation of reactive oxygen species (ROS). Daxx activates the Ask1-JNK kinase pathway, leading to caspase-independent apoptosis. (B) Tumor necrosis factor receptor-1 (TNF-R1) signaling differs from that of Fas in the following steps: (i) Binding of FADD and RIP to the receptor complex requires the adapter protein TRADD. (ii) Binding of Daxx to TNF-R1 has not been demonstrated, and the Ask1-JNK pathway is activated by ROS (dotted line; caspase involvement unclear). (iii) The RIP-mediated necrotic pathway is inhibited by caspase-8. (iv) TNF-R1 can initiate a caspase-independent direct cathepsin B-mediated pathway. (v) Cathepsin B can enhance the mitochondrial death pathway. (vi) The final execution of the death – that is, phosphatidylserine exposure, chromatin condensation, and loss of viability – is brought about by effector caspases, the serine protease AP24, or cathepsin B in a cell-type-specific manner.

and/or Bak can be activated transcriptionally or by conformational change induced by cleavage or binding to an activated BH3-only Bcl-2 family member (*Figure 4*). Antiapoptotic Bcl-2 proteins (such as Bcl-2 and Bcl-X_L) oppose the MMP, probably by heterodimerization with Bax-like proteins, whereas 'BH3-only' Bcl-2 family members either oppose the inhibitory effect of Bcl-2-like proteins (Bad, Bim, Noxa, PUMA, etc.) or activate Bax-like proteins by direct binding (truncated Bid). The pathways upstream of MMP are numerous and with a few exceptions caspase-independent (Heibein *et al.*, 2000; Strasser *et al.*, 2000; Choi *et al.*, 2001; Ferri and Kroemer, 2001; Kaufmann and Hengartner, 2001; Stoka *et al.*, 2001) (*Figure 4*).

MMP does not necessarily result in an irreversible mitochondrial dysfunction and cell death. Initially, the pore-forming proapoptotic Bcl-2 family members Bax and Bak induce only outer membrane permeability but leave intact inner membrane energization, protein import function, and the ultrastructure of mitochon-

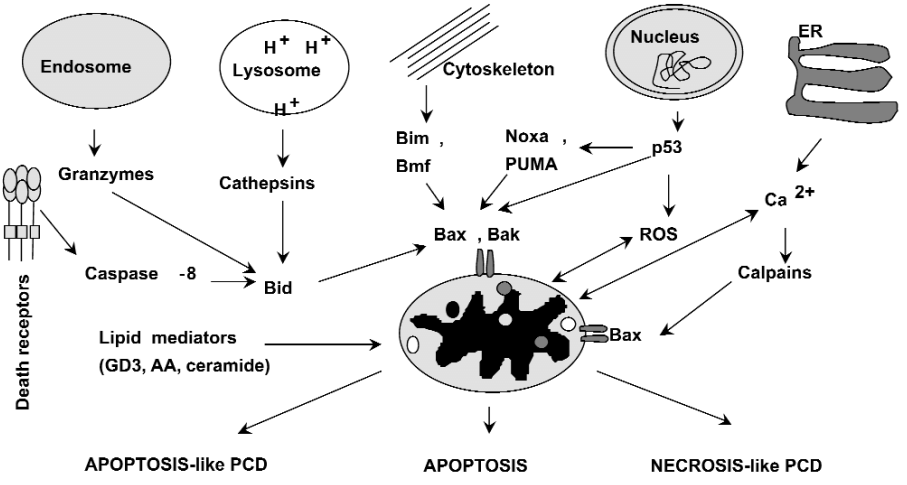

Figure 4. Caspase-independent signaling pathways leading to MMP.

The pore-forming proteins Bax and/or Bak can be activated by BH3-only proteins. Death receptors can activate caspase-8, which cleaves and activates Bid. Granzymes released from the granules of cytotoxic T cells and natural killer cells can be taken up by the target cells through perforin-assisted diffusion or endocytosis. Once released to the cytosol of the target cell by the action of perforin, granzyme B may also cleave and activate Bid. TNF and TRAIL, as well as various oxidants, detergents, and chemotherapeutic drugs, can induce the release of active cathepsins from the lysosomal compartment, and the cathepsin-mediated cleavage of Bid has been held to mediate cathepsin-induced MMP. Disruption of the cytoskeleton leads to the release of the BH3-only proteins Bim and Bmf. DNA damage induced by radiation or various chemotherapeutic drugs induces a p53-mediated transcription of genes encoding Bax, BH3-domain only proteins (Noxa or PUMA), and proteins involved in ROS generation. ER stress results in the release of Ca^{2+}, which may cause direct mitochondrial damage or activate Bax via calpain-mediated cleavage. Various death stimuli trigger the production of lipid second messengers that are involved in MMP and mitochondrial damage. Depending on the stimulus and the cell type, as well as the metabolic status of the cell, MMP leads to either caspase-mediated apoptosis or caspase-independent PCD.

dria (Von Ahsen *et al.*, 2000b). Recent data indicate that MMP prompts several caspase-dependent and -independent death pathways (Leist and Jäättelä, 2001[**Q1**]) (*Figure 5*). The apoptosome-caspase pathway leading to classic apoptosis is initiated by the MMP-dependent release of cytochrome *c* from the mitochondrial intermembrane space. Together with other essential factors (such as ATP), it triggers assembly of the apoptosome complex, which forms the template for efficient caspase processing. As a further safeguard mechanism, caspase-inhibitory factors (IAPs and XIAP) have to be removed by additional proteins (DIABLO/SMAC or Omi/HtrA2) released from mitochondria before the execution caspases can become fully active and produce the typically apoptotic morphology (Strasser *et al.*, 2000; Kaufmann and Hengartner, 2001).

The second mitochondrial death pathway leads to necrotic PCD, without necessarily activating caspases. A prominent example is TNF-induced necrosis-like PCD mediated by mitochondria-derived ROS (Schulze-Osthoff *et al.*, 1992). Intracellular control of this pathway is indicated by its susceptibility to attenuation by antioxidants (Vercammen *et al.*, 1998a,b; Schulze-Osthoff *et al.*, 1992).

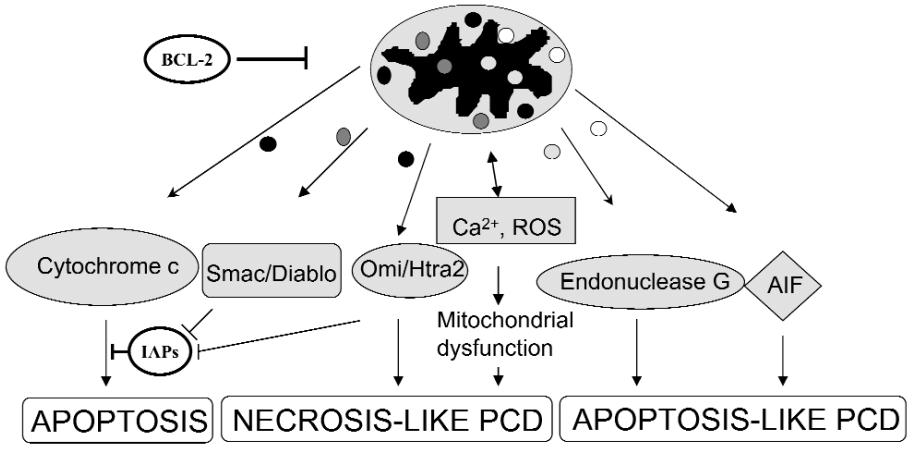

Figure 5. Death pathways downstream of MMP.

Mitochondrial damage leads to the release of numerous mitochondrial proteins that may trigger the execution of PCD. Cytochrome *c* release triggers caspase activation and classic apoptosis. Smac/Diablo and Omi/htra2 assist cytochrome *c*-induced caspase activation by counteracting IAPs. AIF triggers a caspase-independent death pathway, culminating in the DNA fragmentation and stage 1 chromatin condensation characteristic of apoptosis-like PCD. Endonuclease G cleaves DNA and induces stage 1 chromatin condensation. The serine protease activity of Omi/htra2 mediates caspase-independent cellular rounding and shrinkage without changes in the nuclear morphology. Ca^{2+} and ROS can lead to severe mitochondrial dysfunction and necrosis-like PCD. All these mitochondrial events are at least partially inhibited by Bcl-2.

A third distinct pathway from mitochondria is the release of the apoptosis-inducing factor (AIF) from the intermembrane space (Susin *et al.*, 1999; Suter *et al.*, 2000; Braun *et al.*, 2001). Recent genetic evidence indicates that this factor controls PCD in early development; that is, all the hallmarks of early morphogenetic death, including cytochrome *c* release, are prevented by deletion of AIF (Joza *et al.*, 2001). AIF induces caspase-independent formation of large (50-bp) chromatin fragments, whereas oligonucleosomal DNA fragments are generated only when caspase-activated DNase (CAD) is activated (Susin *et al.*, 1999; Strasser *et al.*, 2000). This biochemical difference is reflected by slight morphologic differences in the shape of the condensed chromatin (*Figure 1*).

Finally, endonuclease G or a serine protease, Omi/htra2, released from mitochondria during PCD can contribute to the caspase-independent death signaling downstream of MMP (Li *et al.*, 2001; Suzuki *et al.*, 2001). Extramitochondrial expression of Omi/htra2 induces caspase-independent PCD, and endonuclease G causes caspase-independent DNA fragmentation in isolated nuclei. However, direct evidence connecting endonuclease G and PCD-associated DNA fragmentation in mammalian cells is still lacking.

Often, more than one pathway seems to be activated simultaneously (Jäättelä *et al.*, 1998; Susin *et al.*, 1999; Mattson, 2000; Suter *et al.*, 2000). The cell fate and death mechanism are then determined by the relative speed of each process in a given model system, and by the antagonists of the individual pathways differentially expressed in different cell types. AIF, caspases, and ROS can feed back on

mitochondria, causing enough structural and functional damage to trigger the release of other death factors, independently of the upstream signals (Nicotera *et al.*, 1999; Susin *et al.*, 1999; Mattson, 2000; Strasser *et al.*, 2000).

Defects in any step of the cytochrome *c* or AIF pathways can switch apoptosis or apoptosis-like PCD to death with a necrotic morphology (Leist *et al.*, 1997; McCarthy *et al.*, 1997; Daugas *et al.*, 2000). This death would still fulfill the criteria of PCD, as it is blocked by the antiapoptotic oncogenes Bcl-2 or Bcr-Abl (Amarante-Mendes *et al.*, 1998; Daugas *et al.*, 2000; Single *et al.*, 2001) or by the deletion of proapoptotic Bax (Miller *et al.*, 1997). Moreover, caspase inhibition changes the mode of death, but not its extent, once the signal has arrived at mitochondria (Xiang *et al.*, 1996; Hirsch *et al.*, 1997; Leist *et al.*, 1997; McCarthy *et al.*, 1997; Miller *et al.*, 1997; Amarante-Mendes *et al.*, 1998; Nicotera *et al.*, 1999; Daugas *et al.*, 2000). Thus, it seems that in many models of cell death the master controllers of PCD operate at the mitochondrial level, while the decision on the form of death is taken at the level of caspase activation (Nicotera *et al.*, 1999).

There are, however, certain cases where Bcl-2 expression is not protective, and where mitochondria may not have a regulatory role (Chi *et al.*, 1999; Schierle *et al.*, 1999; Elliott *et al.*, 2000; Finn *et al.*, 2000; Nylandsted *et al.*, 2000[**Q2**]; Sperandio *et al.*, 2000). Although the alternative control mechanisms are not well characterized, emerging candidates include different chaperone systems, such as heat-shock proteins (Jäättelä *et al.*, 1998; Charette *et al.*, 2000; Nylandsted *et al.*, 2000[**Q2**]) or ORP150 (Tamatani *et al.*, 2001). Organelles that have not received much attention until recently, such as the endoplasmic reticulum and lysosomes, might also take an essential role in the control of death (Mattson, 2000; Ferri and Kroemer, 2001; Leist and Jäättelä, 2001[**Q1**]) (*Figure 4*).

5. The significance of the program: removal of corpses

Death is not the only important endpoint of PCD. A much less complicated machinery would be sufficient to permeabilize the plasma membrane. The classic apoptosis program is, in fact, optimized to ensure that signals for phagocytosis are displayed well before cellular constituents might be released (Savill and Fadok, 2000; Strasser *et al.*, 2000). In extreme cases, there is even a feedback control of phagocytosis on the death program itself to ensure that death occurs only when phagocytosis has been initiated (Hoeppner *et al.*, 2001; Reddien *et al.*, 2001). Does this also apply to caspase-independent programs? A dominant uptake signal in mammalian cells is the translocation of phosphatidylserine to the outer leaflet of the plasma membrane. Also this 'eat-me' indicator is uncoupled from caspase activation in many model systems (Berndt *et al.*, 1998; Mateo *et al.*, 1999; Fröhlich and Madeo, 2000; Hirt *et al.*, 2000; Foghsgaard *et al.*, 2001), and nonapoptotically dying eukaryotic cells can be efficiently phagocytized (Hirt *et al.*, 2000). Mechanisms that can lead to the translocation of phosphatidylserine and phagocytosis in cells undergoing caspase-independent death include disturbances of cellular calcium homeostasis and protein kinase C activation (Hirt *et al.*, 2000; Volbracht *et al.*, 2001[**Q3**]). Noncaspase cysteine proteases might be involved not only in the alternative death execution, but also in alternative phagocytosis signal pathways. For

instance, cathepsin B activity is required for the translocation of phosphatidylserine in TNF-challenged tumor cells (Foghsgaard *et al.*, 2001), and, in the apoptosis-like death of platelets, calpain inhibitors selectively block phagocytosis signals (Wolf *et al.*, 1999). Finally, genetic analysis in *C. elegans* has shown that the same phagocytosis-recognition molecules are involved in removing corpses produced by caspase-dependent apoptosis and caspase-independent necrosis (Chung *et al.*, 2000).

6. Complex control of tumor cell death

Paradoxically, the cell proliferation induced by enhanced activity of oncoproteins (such as Myc, E1A, E2F, and CDC25) or inactivation of tumor suppressor proteins (retinoblastoma protein, for example) is often associated with caspase activation and accelerated apoptosis (Schmitt and Lowe, 1999). The coupling of cell division to cell death has thus been proposed to act as a barrier that must be circumvented for cancer to occur (Jäättelä, 1999; Schmitt and Lowe, 1999). Indeed, high expression of the antiapoptotic proteins (Bcl-2, Bcl-x_L, survivin, and Bcr-Abl) and/or inactivation of the proapoptoic tumor-suppressor proteins (p53, $p19^{ARF}$, and PTEN) controlling caspase-dependent apoptosis pathways are often seen in human tumors (Jäättelä, 1999; Schmitt and Lowe, 1999).

6.1 Alternative death pathways in cancer

Despite showing severe defects in classic apoptosis pathways, cancer cells have not lost the ability to commit suicide. On the contrary, spontaneous apoptosis is common in aggressive tumors, and most of them respond to therapy (Kerr *et al.*, 1994). One explanation may be that defects in the signaling pathways leading to caspase activation may still allow caspase-independent death pathways to execute tumor cell death.

The alternative death pathways may also be enhanced by transformation (*Figure 6*). For example, oncogenic Ras can induce caspase- and Bcl-2-independent autophagic death (Chi *et al.*, 1999), and tumor-associated Src family kinases are involved in caspase-independent cytoplasmic execution of apoptotic programs induced by the adenovirus protein E4orf4 (Lavoie *et al.*, 2000; Gingras *et al.*, 2002). Furthermore, a transformation-associated caspase-, p53-, and Bcl-2-independent, apoptosis-like death program can be activated in tumor cell lines of different origins by depletion of a 70-kDa heat-shock protein (Hsp70) (Nylandsted *et al.*, 2000a,b). This death is preceded by a translocation of active cysteine cathepsins from lysosomes to cytosol, and inhibitors of their activity partially protect against death. Interestingly, cysteine cathepsins, as well as other noncaspase proteases, are highly expressed in aggressive tumors (Duffy, 1996).Therefore, expression of protease inhibitors may increase a cancer cell's chances of survival by impairing alternative death routes (Alexander *et al.*, 1996; Foghsgaard *et al.*, 2001; Leist and Jäättelä, 2001[**Q1**]).

Alternative death pathways can also function at an initial stage of tumorigenesis to limit tumor formation. Bin1, a tumor-suppressor protein that is often missing or functionally inactivated in human cancer, can activate a caspase-

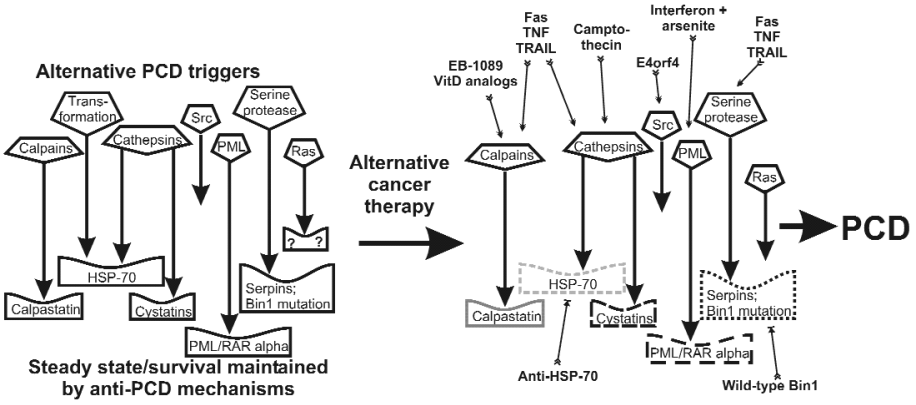

Figure 6. Alternative death pathways as regulators of tumor cell survival and as putative targets for cancer therapy.

(*Left*) Transformation is associated with upregulation of proteins that sensitize cells to caspase-independent PCD. As a defense line, death-promoting proteins are inactivated or expression of survival proteins is enhanced. Analogous changes in proteins regulating caspase-dependent apoptosis have also been demonstrated in cancer. (*Right*) Strategies of cancer therapy aimed at facilitating alternative death pathways.

independent apoptosis-like death process that is blocked by a serine protease inhibitor or the simian virus large T antigen, but not by overexpression of Bcl-2 or inactivation of p53 (Elliott *et al.*, 2000). Similarly, the promyelocytic leukemia PML/RARα oncoprotein also inhibits caspase-independent PCD induced by the PML tumor-suppressor protein (Quignon *et al.*, 1998). Interestingly, the cytoplasmic apoptotic features induced by ectopic expression of PML can even be enhanced by pancaspase inhibitors (Quignon *et al.*, 1998). It should, however, be noted that PML/RARA can also interfere with caspase activation in some death models (Wang, Z.G. *et al.*, 1998).

6.2 Designing new therapies based on alternative PCD pathways

Experimental gene-therapy approaches point to alternative death pathways as promising targets for tumor therapy. For example, the expression of Bin1 tumor suppressor or depletion of Hsp70 results in effective induction of caspase-independent apoptosis-like PCD in tumor cells (Elliott *et al.*, 2000; Nylandsted *et al.*, 2000a,b). Remarkably, adenoviral transfer of antisense Hsp70 cDNA also efficiently eradicates glioblastoma, breast-cancer, and colon-cancer xenografts in mice (J. Nylandsted and M. Jäättelä, unpublished). The ineffective delivery of viral vectors into multiple tumor sites appears to be the major limitation for the usage of this gene therapy in the treatment of human cancer. Thus, clinical applications of this approach require further development of the delivery systems or other means to activate Bin1 or to neutralize the antiapoptotic effect of Hsp70. However, in the case of local inoperable tumors, such as glioblastoma, locoregional gene therapy may prove beneficial.

Even though the signaling pathways regulating alternative PCD are only beginning to emerge, potentially cancer-relevant drugs or drug targets engaging caspase-

independent death routines already exist (*Figure 6*). For instance, the topoisomerase inhibitor, camptothecin, induces cathepsin D/B-mediated apoptosis-like PCD in hepatocellular carcinoma cells (Roberts *et al.*, 1999); activation of a thrombospondin receptor (CD47) by thrombospondin or agonistic antibodies initiates programmed necrosis in B-cell-chronic lymphoma cells (Mateo *et al.*, 1999); antibodies to CD99 trigger a rapid, apoptosis-like PCD in transformed T cells (Pettersen *et al.*, 2001); interferons and arsenite initiate a caspase-independent death pathway, possibly mediated by PML (Quignon *et al.*, 1998); EB 1089, a synthetic vitamin D analog presently in phase III trials for the treatment of cancer, kills breast-cancer cells in a caspase-independent manner (Mathiasen *et al.*, 1999); and treatment of breast-cancer cells with sigma-2 receptor agonists triggers apoptosis-like PCD independently of p53 or caspase activity (Crawford and Bowen, 2002).

7. Alternative cell death in the nervous system

Caspase-driven neuronal apoptosis strictly following the classic apoptosome pathway is best documented during development of the nervous system (Los *et al.*, 1999), where many superfluous cells are produced and turned over (Raff, 1992), and in *in vitro* cultures of cells derived from developing brain (Mattson, 2000). Evidence is scarce for adult neurons, and here caspase-dependent mechanisms may yield to alternative death pathways (Johnson *et al.*, 1999). Notably, a re-evaluation of cell death in caspase knockout mice showed that apoptosis is reduced during development, but cell death in many brain regions proceeds to the same extent in a caspase-independent necrosis-like PCD often characterized by cytoplasmic vacuoles (Oppenheim *et al.*, 2001). Cell suicide in the adult nervous system has serious implications for the whole organism, since turnover is classically very limited. Thus, a rapid caspase cascade, which is advantageous for efficient elimination of unwanted or rapidly replaceable cells, is dangerous in the developed brain and must be tightly controlled. For instance, neurons can survive cytochrome *c* release from mitochondria if they do not simultaneously receive a second signal leading to 'competence to die' (Deshmukh *et al.*, 2000). Neuronally expressed apoptosis inhibitor proteins (IAP, NAIP) buffer the caspase system, and need to be inactivated before classic apoptosis can be executed (Kaufmann and Hengartner, 2001). This buffering capacity may allow for localized caspase activation (Mattson, 2000) (within synapses or neurites, for example) or sequestration of active caspases (Stadelmann *et al.*, 1999), without a buildup of the death cascade affecting the entire neuron. Stressed neurons might also acquire a temporary resistance that allows them to withstand otherwise lethal insults, such as those by excitotoxins (Hansson *et al.*, 1999). Such circumstances favor activation of slow, caspase-independent elimination routines, where damaged organelles are digested within a stressed cell, and the chance for rescue and reversibility is maintained until the process is complete (Jellinger and Stadelmann, 2000; Yamamoto *et al.*, 2000; Xue *et al.*, 2001).

Although some caspase-dependent apoptosis might occur in adult brain (Mattson, 2000), at least part of PCD in chronic neurodegenerative disease follows alternative mechanisms and results in different morphologies (Miller *et al.*, 1997;

Colbourne *et al.*, 1999; Roy and Sapolsky, 1999; Stadelmann *et al.*, 1999; Fujikawa, 2000; Jellinger and Stadelmann, 2000; Sperandio *et al.*, 2000; Turmaine *et al.*, 2000) (*Figure 7*). Further variation is observed in acute insults, such as ischemia or traumatic brain injury. Here, neurons within one brain region are exposed to different intensities of stress that trigger different death programs. Some of the main excitotoxic processes, such as mitochondrial impairment and dissipation of cell membrane potential, differentially impair various secondary routines of PCD (Nicotera

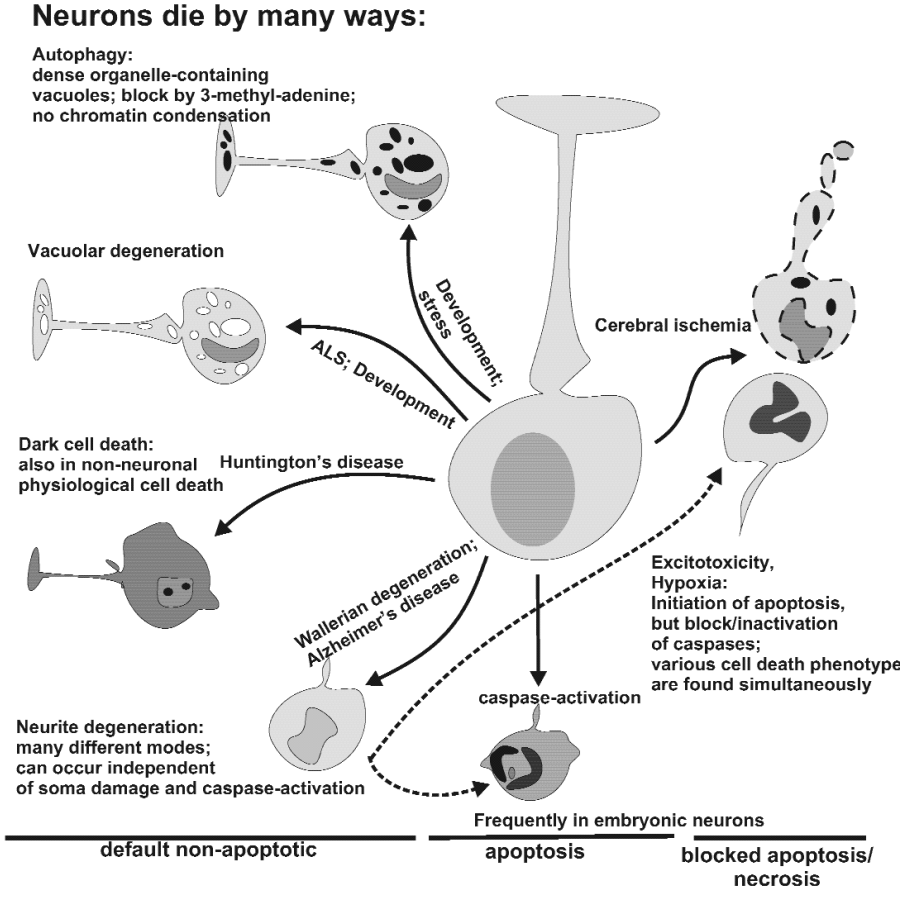

Figure 7. Developmental cell death occurs by caspase-dependent apoptosis or by morphologically and mechanistically distinct autophagy.

In various human diseases or animal models of them, the dominant form of neuronal disease is, for example, dark cell death in a Huntington's disease model, or vacuolar degeneration in a model of amyotrophic lateral sclerosis (ALS). Selective neurite degeneration occurs independently of caspase activation in different situations, and may eventually lead either to caspase-dependent apoptosis of cell bodies or to nonapoptotic death with irregular chromatin condensation. Excitotoxic death may take many forms and mechanisms depending on the intensity of insult, the age of the animal, and the brain region affected. It often results in mixed apoptotic and necrotic features, including cellular swelling, blebbing, nuclear pyknosis, display of phosphatidylserine, and some autophagic processes, such as uptake of mitochondria into lysosomes.

et al., 1999; Roy and Sapolsky, 1999; Fujikawa, 2000). For instance, rapid ATP depletion or disturbance of the intracellular ion composition impairs cytochrome *c*-induced caspase activation, and massive production of nitric oxide (NO) or calpain activation directly inactivates caspases (Nicotera *et al.*, 1999; Lankiewicz *et al.*, 2000). Accordingly, cell death has mixed features of apoptosis and necrosis, and might rely on either caspases or calpains as the dominant execution proteases (Wang, 2000; Volbracht *et al.*, 2001[**Q3**]), or the activation of PARP (Ha and Snyder, 1999) as a controller of programmed necrosis. Another group of proteases implicated as executors of ischemic death are the cysteine cathepsins (Yamashima, 2000). Possibly, they interact with calpains, and notably there is massive PCD in the brains of mice lacking the cathepsin inhibitor cystatin B (Pennacchio *et al.*, 1998).

The special shape of neurons (with projections up to 40 000 times longer than their cell bodies) allows degradative processes to be localized to a part of neurons and different death processes to be activated in different subsections of the cell (Nicotera *et al.*, 1999; Mattson, 2000). For instance, synaptic damage and neurite regression can occur by Bcl-2- and caspase-independent mechanisms (Sagot *et al.*, 1995; Finn *et al.*, 2000; Volbracht *et al.*, 2001[**Q3**]) and be initially reversible (Yamamoto *et al.*, 2000), whereas final elimination of cells may depend on caspases or proteasomal activities (Volbracht *et al.*, 2001[**Q3**]). The role of caspases as enhancers of the final phase of cell degeneration may apply to many common diseases. The longevity of neurons, combined with their dependence on effective intracellular transport, makes them sensitive to a slow form of death associated with the formation of intracellular polypeptide aggregates involving the amyloid-? precursor protein (APP), ataxins, presenilins, huntingtin, tau, and a-synuclein[**Q5**] (Mattson, 2000). As most of these proteins are caspase targets (Wellington and Hayden, 2000) and become more toxic after cleavage, caspases might contribute to the accelerated death of neurons at the end of a caspase-independent degeneration phase, or vice versa, make neurons sensitive to alternative mechanisms without directly participating in death execution (Zhang *et al.*, 2000b).

8. Evolution of cell-death principles

The driving evolutionary pressures for the development of multiple cell-death programs have increased in parallel with the increased complexity and life span of organisms (Aravind *et al.*, 2001). But when in evolution did the caspase-independent death mechanisms arise? Caspase-coding sequences are absent from the known genomes of many nonanimal species (Aravind *et al.*, 2001). Nevertheless, such organisms – including plants and a number of single-celled eukaryotes – undergo PCD under conditions of stress (Ameisen, 1996; Fröhlich and Madeo, 2000) (see also Chapter 9). For instance, in yeast, this apoptosis-like death is associated with DNA-fragmentation, plasma membrane blebbing, phosphatidylserine exposure, and chromatin condensation (Fröhlich and Madeo, 2000), and can be selectively triggered or blocked by Bax-like or ced-9-related genes, respectively (see Chapter 8). Furthermore, programmed necrosis-like death is well characterized in caspase-deficient slime molds (Wyllie and Golstein, 2001).

The introduction of the caspases, and especially of the mitochondrial Ced-

9/Bcl-2-related death switches (Ameisen, 1996; Aravind *et al.*, 2001), may represent a decisive refinement of the old caspase-independent death programs. The relative importance of different death mechanisms seems to have been optimized subsequently in various ways. One form of extreme specialization is exemplified by the somatic cell death in the nematode *Caenorhabditis elegans* (see Chapter 10). The requirements for PCD in *C. elegans* are adapted to its specific needs, and have diverged widely from those of mammals (Aravind *et al.*, 2001). Since the environmental pressure to provide a flexible death response is very low in this short-lived organism, evolutionary optimization has resulted in a single caspase-dependent apoptosis program. In contrast to mammals, control by mitochondrial proteins may play a minor role, and some degradative enzymes are supplied by the phagocytizing cell rather than by the dying cell itself (Strasser *et al.*, 2000; Hoeppner *et al.*, 2001; Kaufmann and Hengartner, 2001; Reddien *et al.*, 2001). Apoptosis in *C. elegans* is commonly cell-autonomous, that is, it is not signaled or controlled from outside, and the entire system of death receptors appears to be absent. In accordance with this minimalist program, somatic PCD is not essential for survival or development in *C. elegans* (Ellis and Horvitz, 1986). Vestiges of alternative apoptotic programs are, however, still found in the male linker cell, where a possibly Ced-3-independent PCD is triggered from outside (Ellis and Horvitz, 1986). The role of mitochondrial endonuclease G in caspase-independent degradation of DNA might also be conserved from worm to man (Li *et al.*, 2001; Parrish *et al.*, 2001).

The mammalian system of death programs could represent an opposite form of evolutionary direction, where, besides the multiple caspases, many other cysteine proteases and mitochondrial factors have taken additional roles in development and life (Los *et al.*, 1999; Strasser *et al.*, 2000). The essential nature of some factors (knockout lethality [Los *et al.*, 1999; Joza *et al.*, 2001] combined with the redundancy of others (difficulty with interpretation of knockouts [Los *et al.*, 1999]) has made the study of their specific role in PCD technically challenging. In addition, it has remained unclear which mechanisms are essential for commitment to death, and which ones merely determine the phenotypic outcome (Nicotera *et al.*, 1999).

9. Conclusion

The discovery and understanding of alternative death pathways will open new perspectives for the treatment of disease (*Figure 7*), and one of these therapies (vitamin D analogs) has already entered clinical phase III trials. New options and targets have emerged also for the prevention of death processes in neurodegenerative disease. Prominent examples of such targets that have reached the stage of clinical trials include PARP in necrosis and calpains in excitotoxicity (Ha and Snyder, 1999; Johnson *et al.*, 1999; Wang, 2000).

On a more general biologic level, the mode of cell death may differentially affect the surrounding tissue (Savill and Fadok, 2000). The important roles of caspase-independent/alternative death in the development of tumor immunity are just emerging (reviewed in Hirt *et al.*, 2000). Most recent evidence shows that the mode of cell demise controls the horizontal spread of oncogenic information (Bergsmedh

et al., 2001) and of infection (Boise and Collins, 2001). Since these processes can be favored by caspase activation, the classic apoptosis pathways can, in fact, be detrimental to the organism. This may explain the need for extremely tight control of caspase-activation by the cellular energy level (Leist *et al.*, 1997). The apparent paradox that death-bound ATP-depleted cells are not 'allowed' to activate caspases may then be explained by the fact that such cells would release activated caspases into the extracellular space upon premature lysis (Hentze *et al.*, 2001). Thus, non-apoptotic death may be not only a passive accidental event, but also, in some cases, a desirable death option for long-lived organisms having to deal with tumors, infections, and other nonlethal tissue insults throughout their life span.

Acknowledgments

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