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MULTI-AUTHOR REVIEW

Non-caspase proteases: triggers or amplifiers of apoptosis?

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Abstract Caspases are the most important effectors of apoptosis, the major form of programmed cell death (PCD) in multicellular organisms. This is best reflected by the appearance of serious development defects in mice deficient for caspase-8, -9, and -3. Meanwhile, caspase-independent PCD, mediated by other proteases or signaling components has been described in numerous publications. Although we do not doubt that such cell death exists, we propose that it has evolved later during evolution and is most likely not designed to execute, but to amplify and speed-up caspase-dependent cell death. This review shall provide evidence for such a concept.

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Introduction

Programmed cell death (PCD) is a controlled process to eliminate used-up, damaged, or misplaced cells during the embryonic development and the tissue homeostasis of multicellular organisms [1]. Aberrant PCD can lead to embryonic defects or diseases such as neurodegeneration, immunodeficiency, cancer, or autoimmunity. Apoptosis, the most abundant form of PCD, is a morphologically defined process characterized by cell shrinkage, plasma membrane blebbing, nuclear condensation and fragmentation, the formation of membrane enclosed apoptotic bodies and their selective uptake by phagocytes without provoking inflammatory responses [2]. Necrosis is another form of cell death that has also been characterized as programmed under certain circumstances (called necroptosis), but which ultimately leads to cell lysis and the triggering of an inflammatory response [3]. For a detailed comparison of molecular mechanisms that control apoptosis and necrosis/necroptosis, read Van Herreweghe et al. in this issue. In this review, we will focus on apoptotic and necrotic PCD based on the morphologies defined by Kerr and Wyllie [2].

It has become clear over the years that cysteine aspartyl proteases (caspases) are the major drivers of apoptotic PCD [4]. Some morphological features such as the fragmentation of genomic DNA into nucleosome-sized fragments strictly depend on caspases because the major endonuclease CAD is activated by caspase-mediated destruction of its inhibitor [5]. However, other aspects of apoptosis, such as cell shrinkage, membrane blebbing and the surface

exposure of phosphatidylserine, one of several possible ligands or "eat-me" signals for phagocytosis, can still occur in the apparent absence of caspase activation [6]. These aspects are clearly distinct from necrosis and may therefore be classified as caspase-independent, apoptosis-like PCD. The problem is that caspase-independence has been largely defined by the cellular treatment with so-called broad-range caspase inhibitors such as ZVAD.fmk and QVD-OPh [7]. In other words, it has been assumed that these inhibitors completely inhibit all 10–12 caspases in mammals in a way that even low residual caspase activities would not be able to trigger apoptosis. Unfortunately, with the best of our knowledge, we cannot make sure that this is the case. First, although the inhibitory constants (K_i) of Z-VAD.fmk have been determined for all recombinant caspases in vitro, they vary between the different caspases, and it is unclear if caspase inhibition is as effective inside cells as it is in vitro [8, 9]. Second, Z-VAD.fmk also inhibits other proteases, such as calpains and cathepsins, especially at the typical doses used for caspase inhibition (100 μM) [9, 10]. Third, general caspase inhibitors may get rapidly degraded inside the cells. Finally, the threshold of active effector caspase molecules still capable of executing apoptosis has not yet been determined. To definitely exclude minor caspase activities inside cells, we would have to genetically knock-out, in an inducible manner, all ten caspases in mice. Although this is a heroic approach, it is the only way to prove that caspase-independent, apoptosis-like PCD distinct from necrosis indeed exists.

Despite these uncertainties, it has become evident that the cell death observed under caspase inhibiting conditions is rather slow and inefficient [6]. This may translate into a delayed fashion to phagocytose apoptotic bodies in vivo, which of course can influence cell homeostasis and contribute to diseases. Thus, whatever mechanism is involved, PCD under low caspase-activating or caspase-inhibiting conditions may be a crucial physiological or pathological process in multicellular organisms in vivo. This is why it is important to understand the underlying mechanisms, for example if non-caspase proteases are implicated in human diseases and may hence become targets of intervention in addition to caspases.

The importance of caspases for PCD in vivo and in vitro

Lower eukaryotes are ideal model systems to study the importance of caspases in PCD. In the nematode *C. elegans* the deletion of the sole executioner caspase CED-3 results in the survival of all 131 cells, which would otherwise die during embryonic development [11]. CED-3 is also required for physiological and DNA damage-induced cell death in the worm germline [12]. Caspase-like proteins and caspase-independent mediators have been identified in worms [13], but these enzymes may amplify rather than execute CED-3 mediated death signaling as we propose for higher eukaryotes (see below). Nevertheless, *C. elegans* also exhibits a caspase-independent, necrotic program to eliminate neuronal cells by hyperactive ion channels [14]. As far as we can extract from the literature, caspase-independent PCD has been rarely described in *Drosophila melanogaster*. Here, cells perfectly survive apoptosis induction in the absence of caspase activity [15], although recent studies have demonstrated alternative cell death pathways which may be mediated via other proteases [16]. But again this might be due to residual caspase activities which are amplified by the action of other proteases. The failure of cells, in both *C. elegans* and *D. melanogaster*, to undergo caspase-independent PCD is probably due to the absence of mitochondrial outer membrane permeabilization (MOMP), a feature that has been adopted by higher eukaryotes later in evolution [7].

In contrast to worms and flies, mice and men express 10 and 12 caspases, respectively [4, 17]. They are classified into initiator caspases, acting early in the apoptotic pathway and effector caspases, performing the execution function. Initiator caspases contain large prodomains, called CARD or DED, which serve the function to bring two monomeric inactive caspases in close proximity for autoactivation when an adapter binds to the prodomain [18]. Although this close proximity already activates the initiator caspase, it can further process itself to become more active, change its localization or inactivate itself, depending on the context [19]. Effector caspases do not have large prodomains, already exist as dimers, and require proteolytic cleavage by an initiator caspase to remove an inhibitory loop covering the active site [20]. Effector caspases then can autoprocess to an active tetramer consisting of two small and two large subunits. While effector caspases can probably accept hundreds of cellular substrates whose cleavages lead to the apoptotic morphology [21], initiator caspases have a very limited substrate spectrum, predominantly effector caspases.

Two major caspase-dependent signaling pathways are known in mammals. In the extrinsic death receptor pathway, extracellular TNF-like ligands such as TNFα, FasL or TRAIL (also see Weinlich et al. in this issue) bind to cognate receptors on the cell surface, triggering receptor multimerization and a conformational change on the cytoplasmic face of the receptor [22, 23]. This allows the recruitment of the adaptor FADD which assembles the initiator pro-caspase-8 on the receptor. Proximity activated caspase-8 cleaves and activates the effector caspases-3 and -7 [18, 22]. The second signaling pathway, called the mitochondrial or intrinsic pathway (also see Pradelli et al.

in this issue), is stimulated by many apoptotic stimuli such a growth factor/cytokine removal, DNA-damaging drugs and irradiation, viruses and the detachment from the extracellular matrix (anoikis). By still unknown mechanisms these stimuli are sensed by a subgroup of Bcl-2 family members, the BH3-only proteins, which, through transcriptional upregulation, phosphorylation or proteolytic cleavage stimulate the oligomerization of Bax and Bak, another class of pro-apoptotic Bcl-2 proteins on mitochondria [24–26]. This somehow increases the permeability of the outer mitochondrial membrane (MOMP) leading to the release of apoptogenic factors from the intermembrane space. Cytochrome *c* is the major caspase-driving protein of these factors because once in the cytosol it binds the adaptor Apaf-1 which, through ATP hydrolysis and conformational change, recruits monomeric procaspase-9 to a platform, called the apoptosome [27, 28]. The apoptosome then processes effector caspases-3 and -7. Importantly, Bax/Bak induced MOMP is required for the intrinsic pathway, as Bax/Bak double knock-out (DKO) mice are embryonic lethal, and cells from these mice are highly resistant to various apoptotic stimuli [29, 30]. This is why Bax and Bak have been called the "gateway", "commitment point", or "life-or-death-decision point" of apoptosis [30].

All these findings indicate that caspases are indispensable for apoptosis induction via both the extrinsic or the intrinsic pathways. Is this really the case?

Caspase-8 and -10

Several studies demonstrated the essential role of caspase-8 in death receptor-mediated apoptosis. Caspase-8−/− mice are prenatally lethal, show impaired heart muscle development, congested accumulation of erythrocytes and circulatory failure [31, 32]. In addition, human individuals with homozygous caspase-8 reduction-of-function mutations manifest autoimmune lymphoproliferative syndrome (ALPS) [33, 34], a disease found in patients with defective Fas signaling [35].

So, is there apoptosis or cell death in the absence of caspase-8? In mouse cells caspase-dependent apoptosis in response to FasL or TNFα is blocked when caspase-8 is missing [23]. However, FasL and TNFα can also trigger necroptosis [3, 36], a necrotic cell death attributed to massive upregulation of ROS [37] and most likely mediated by receptor-interacting protein-1 or -3 [36, 38, 39]. Unfortunately, to unravel this alternative death pathway, Z-VAD.fmk was used again, and it is unclear if all intracellular caspases were blocked by this inhibitor. Moreover, humans, but not mice, express caspase-10 which can partially overtake caspase-8 function and still mediate caspase-dependent death receptor signaling in human cells. This is probably why loss-of-function mutations of caspase-8 are not as devastating in humans as in mice.

Caspase-9 and -3/-7

Caspase-9-deficient mice die perinatally due to defective brain development associated with decreased apoptosis, and MEFs derived from these animals are resistant to apoptosis induced by stimuli which use the intrinsic, mitochondrial signaling pathway [40, 41]. Most caspase-3−/− mice die peri- or postnatally in mixed 129/SvJ and C57BL/6 background but are viable in C57BL/6 background where they exhibit decreased apoptosis in the brain leading to hyperplasia [42]. Caspase-7−/− are viable and show a mild apoptosis defect, for example in endotoxin-induced lymphocyte apoptosis [43]. However, caspase-3/7 DKO mice die perinatally and MEFs derived from these mice are resistant to apoptosis [43].

Based on the knock-out phenotypes, the caspase-9/Apaf-1/caspase-3/-7 signaling pathway plays a dominant role in intrinsic apoptosis. However, some caspase-deficient cells clearly survive to birth and although cells from these knock-out strains (MEFS) show impaired apoptosis, the death is only delayed, but not abolished [40–43]. This indicates that cells can die through an intrinsic pathway independent of the apoptosome and/or caspase-3/-7 activation. Is this pathway truly caspase-independent and does it lead to necrosis or an apoptosis-like phenotype?

To answer this question one has to have a closer look at the Bax/Bak-mediated perforation of the mitochondrial outer membrane (MOMP), the "commitment point" of this pathway. It is intriguing that the phenotype of mice lacking Bax and Bak is much more severe than that unable to activate caspases downstream of MOMP (caspase-9−/− and Apaf-1−/−) [29, 30]. This indicates that Bax/Bak do not only trigger the cytochrome *c*-mediated caspase-9/Apaf-1 signaling branch but also other signaling pathways which are activated due to MOMP. These pathways are likely to be responsible for the cell death which is still observed in apoptosome- and/or caspase-3/-7-deficient cells and animals. Besides cytochrome *c*, numerous other intermembrane space proteins are released after MOMP, such as Smac/Diablo [44, 45], HtrA2/Omi [46, 47], endonuclease G [48] and apoptosis-inducing factor (AIF) [49]. Various groups have proposed that these proteins can actively induce caspase-independent PCD following mitochondrial release [50]. However, apart from Smac/Diablo and HtrA2/Omi, which facilitate caspase-dependent apoptosis by sequestering XIAP, the other apoptogenic factors have not been convincingly shown to execute caspase-independent cell death. Instead, they are needed for vital functions inside mitochondria and their lack triggers a sort of ROS-driven necrotic response.

In summary, since AIF, endonuclease G or HtrA2/Omi are not clearly involved in executing cell death in the presence of Z-VAD.fmk or in caspase-9- or caspase-3/-7-deficient cells, can other caspases take over the apoptotic job under these circumstances?

Caspases-1, -4/5/11, -12, and -14

Caspases-1, -4, -5, and -11 are enzymes mainly involved in inflammatory responses. Caspase-1 is present in both humans and mice, while caspase-4 and -5 are human enzymes replaced by caspase-11 in the mouse [51]. Caspase-5 and caspase-1 are components of the NALP1 inflammasome, a complex involved in caspase-1 activation [52]. Caspase-1 activation processes the pro-inflammatory cytokines IL-1β and IL-18 [52–54]. Both caspase-11−/− and -1−/− mice develop normally and do not show major defects in apoptosis regulation [53, 54]. A form of cell death, called pyroptosis has been described to be associated with caspase-1 activation [55]. However, apart from this, none of the inflammatory caspases seem to mimic or regulate caspases-9, -3 and -7 in the intrinsic pathway. The same is true for caspase-12 and -14. Caspase-12 negatively regulates caspase-1 activation [56], and caspase-14 is crucial for the terminal differentiation of human keratinocytes and cornification [57].

Caspases-2 and -6

Caspase-2 is thought to be the closest homolog of nematodal CED-3 [58]. Despite many reports regarding the function of caspase-2 in a variety of apoptotic processes, the precise role of caspase-2 in apoptosis remains to be verified in vivo, because caspase-2−/− mice display few, if any apoptotic defects [59, 60]. Structurally, caspase-2 is classified as initiator caspase and its activation platform seems to be the PIDDosome, a protein complex containing PIDD (p53-inducible protein with a death domain), an adaptor protein RAIDD (RIP-associated ICH-1) and caspase-2 [61–63]. Overexpression of PIDD results in spontaneous activation of caspase-2 and sensitization of the cells to genotoxic stress-induced apoptosis [61]. Substrate profiling studies revealed that caspase-2 cannot process any other member of the caspase family, but can cleave the Bcl-2 family member Bid, presumably to stimulate cytochrome *c* release [64]. In this respect, caspase-2 could replace the initiating activity of caspase-8 on the type II mitochondrial pathway, but this has not yet been tested, for example by generating caspase-2/-8 DKO mice. Caspase-2/-9 DKO mice exhibit a striking similarity to those lacking caspase-9 [65]. Moreover, caspase-9−/− cells fail to process caspase-2, and pro-caspase-2 activation was shown to be dependent on caspase-3 [65, 66]. All these findings place caspase-2 activation downstream of the apoptosome and caspase-3 activation and suggest that it is an amplifier rather than an initiator caspase.

Caspase-6 poses another puzzle in the caspase field. Due to its short pro-domain and similar substrate specificity as caspase-3 and -7, it is considered an effector caspase. However, caspase-6−/− mice are essentially normal and cells from these mice have not revealed any apoptotic defect so far [67]. But maybe caspase-6 could replace caspase-3/-7 when they are missing, a possibility that has not yet been addressed experimentally. Rather it was shown that caspase-6 is processed and activated by caspase-3 leading to an amplification loop of the caspase cascade [66, 68] In the final stage of this cascade caspase-6 catalyzes the activation of caspase-8 and -10 [69].

Non-caspase proteases as cell death regulators

Although we do not entirely know if cells deficient of particular caspases or treated with Z-VAD.fmk or QVD-OPh die by necrosis or use caspase backup systems or low residual caspase activities to die, it is clear that this cell death still displays proteolytic degradation features. This suggests the role of alternative proteolytic machineries in cell death. Many proteases, including calpains, serine proteases, metalloproteases, cathepsins and the proteasome have been found to be activated in response to apoptotic stimuli. Here, we will focus on two enzyme families, the serine proteases [70, 71] and the cathepsins [72, 73] to illustrate that they primarily act as amplifiers of caspase cascades rather than as initiators or effectors of caspase-independent cell death.

Serine proteases: triggers of MOMP and effector caspase-3 processing

Serine proteases were first implicated in apoptosis in 1987, when it was shown that serine protease inhibitors delayed apoptosis of melanoma cells [74]. By 1994, it was demonstrated that the introduction of chymotrypsin or trypsin into tumor cells led to cell death reminiscent of apoptosis [75]. Later Abate and Schroder [76] found that LPS caused a concentration-dependent toxicity in a macrophage cell line, which was inhibited by the serine protease inhibitors TPCK and TLCK. These investigators did not attempt to isolate or identify the apoptotic serine proteases. In parallel, Masson and Tschopp [77] discovered in 1987 that a family of serine proteases, the granzymes, were capable of inducing cell lysis together with perforin. The expression of granzymes was however restricted to cytotoxic T (CTL), natural killer (NK) and mast cells, and it took another 5 years until it was recognized that these serine proteases

induced classical features of apoptosis [78, 79]. In 2003, a novel trypsin-like serine protease, called EOS was isolated from macrophages [80], and we and others suggested the implication of one or several serine proteases in ER-stress and DNA damage induced apoptosis in a variety of eukaryotic cells [6, 81].

Granzymes and cell death

Granzymes (Gzm) are serine proteases that play a crucial role in eliminating virally infected and malignant cells in cooperation with the FasL-Fas signaling system [79, 82, 83]. They are present in the cytotoxic granules of CTLs and NK cells. Upon contact with target cells, these enzymes are released into the intermembrane space together with the pore-forming protein perforin and are delivered to the target cells by a still not entirely understood mechanism [79, 83]. Gzm A and B are the most abundant and extensively studied members of the granzyme family, and studies in CTLs from Gzm A−/−, Gzm B−/− or Gzm A/B DKO cells confirmed that they independently and synergistically induce cell death through distinct apoptotic pathways [84].

Gzm B cleaves substrates following aspartate residues, thus exhibiting a substrate specificity similar to that of caspase family members. It can engage multiple components of the apoptotic machinery in target cells [83, 84]. By directly processing and activating caspase-3 and -7 it majorly produces ROS, the exposure of PS and a decrease in the mitochondrial membrane potential [84, 85]. Via cleavage of the BH3-only protein Bid and the Bcl-2 survival factor Mcl-1, it triggers Bax/Bak mediated cytochrome *c* release and caspase-3 activation indirectly as well as a fall in the mitochondrial membrane potential [84, 86–88]. But even when both pathways are blocked, i.e., in Bax/Bak DKO cells in the presence of Z-VAD.fmk or QVD-OPh, the target cells still die in a so-called MOMP- and caspase-independent manner due to Gzm B action [84]. This might be due to the direct cleavage of apoptosis-relevant substrates by Gzm B [79].

Gzm A is a highly selective tryptic protease that was initially found to trigger a rapid form of cell death that exhibits all of the morphological features associated with apoptosis: membrane blebbing, chromatin condensation and nuclear fragmentation [79]. In contrast to Gzm B, Gzm A does not activate caspases and neither Bcl-2 overexpression nor Z-VAD.fmk treatment prevented GzmA induced cell death [89]. It was proposed that Gzm A may act via ROS production at mitochondrial respiration complexes [84, 89] and the induction of DNA nicks in the nucleus via the so-called SET complex [90]. However, very recently, Metkar et al. [91] convincingly showed that the killing effect of Gzm A is probably due to artificially high concentrations of the protease (micromolar) used in most in vitro experiments. Lower concentrations of human Gzm A (nanomolar) stimulate monocytic cells to secrete pro-inflammatory cytokines (IL-1β, TNFα and IL-6) and thereby provokes a pro-inflammatory response in vivo to resist LPS-induced toxicity. Thus, the Gzm A/B pair may have evolved to amplify FasL-mediated apoptosis of transformed and virally infected cells by combining Gzm B-mediated caspase activation with Gzm A-mediated ROS production and a pro-inflammatory response.

Apoptotic serine proteases in non-CTL/NK cells and their amplifying role at early and late stages of apoptosis

The essential role of granzymes in effective CTL- and NK-mediated cell killing suggests that other cell types may also exploit a serine protease-based mechanism to amplify caspase-mediated apoptosis. A few number of candidate serine proteases have so far been identified, HtrA2/Omi, AP24, a serine protease activated by Fas signaling of ca. 95 kD, three chymotrypsin-like proteases, called p16, p50 and p60 and serine proteases implicated in ER stress and DNA-damage induced apoptosis.

HtrA2/Omi, a mitochondrial survival protease

As discussed above HtrA2/Omi is a survival rather than an apoptotic serine protease. While in the intermembrane space, this protease protects cells from Bax-mediated cytochrome *c* release and apoptosis [92, 93].

AP24 and Fas-activated protease, two proteases triggering DNA fragmentation at a later step

DNA fragmentation was long thought to be a crucial, early event of apoptosis, occurring in the dying cell before it is recognized and engulfed by macrophages. However, recent data indicate that apoptotic endonucleases (CAD, DNase II) mainly act at later stages of the death process, when the apoptotic bodies are already removed by phagocytosis [94]. In addition to caspase-activated endonuclease CAD, serine proteases may be important in activating other endonucleases in order to accelerate this removal/degradation process [95, 96]. Indeed, a cytosolic serine protease activity was isolated from FasL-treated Jurkat T cells and shown to induce DNA fragmentation [97]. Moreover, during PBOX-6-induced cell death of CML cells where oligonucleosomal DNA fragmentation takes place in the absence of caspase and CAD activation, a chymotrypsin-like serine protease was found to be required for the activation of a manganese-dependent acidic endonuclease [98]. Finally, also during necrotic cell death, serine proteases have been

implicated in DNA fragmentation [96]. Unfortunately, none of these serine proteases have yet been cloned.

AP24 is a 24-kD protease with an elastase-like activity that triggers oligonucleosomal DNA fragmentation indirectly by inactivating a serpin, called LEI, through translational modification and converting it into an endonuclease (L-DNase II) [99]. The protease is activated in response to a variety of stimuli, including TNFα, UV irradiation and chemotherapeutic drugs [100, 101], and it is regulated by sphingomyelin [102], calcium/calmodulin-dependent kinase II [103] and members of the Bcl-2 family [104]. However, the exact mode of action of the protease, in particular, how it induces apoptosis, affects MOMP and amplifies caspase-3 activation has remained obscure.

Chymotrypsin-like protease, called p16, p50 and p60 and a ER-stress activated serine protease: amplifiers of apoptosis at different stages

In addition to being involved in later stages of apoptosis, such as DNA fragmentation, serine proteases have been found to act at various stages of caspase-mediated apoptotic signaling. For example, (1) apoptosome-stimulated caspase-9 processing is preventable by serine protease inhibitors [105], (2) caspase-3 processing and activation can at least partially be prevented by the inhibition of chymotrypsin-like proteases [6, 81, 106] and (3) during ER-stress one or several serine proteases are activated which act both upstream of mitochondria to stimulate cytochrome *c* release as well as downstream of mitochondria to further process and activate caspase-3 [6]. Since the cleavage fragments of caspase-3 generated by serine proteases are similar to those generated by caspase-8 or -9 (p20, p17), the serine protease(s) in question may have aspartyl activities like Gzm B.

p16 is a 16-kD chymotrypsin-like protease that is specifically activated by staurosporine in AML cells [106]. It seems to contribute to the apoptotic morphology, regulation of caspase-3, altered nuclear morphology and DNA degradation, although the amino acid sequence and exact site of action have not yet been determined. p50 and p60 were isolated from Jurkat T cells and are activated in response to staurosporine [107]. Interestingly, they have been shown to act at a late stage of apoptosis, downstream of caspase-3, probably by accelerating nuclear fragmentation and condensation as proposed above for p24 and the Fas-activated serine protease. Consistent with this notion, both serine proteases seem to be activated in the cytosol and then translocate to the nucleus as apoptosis progresses.

In 2003, we reported on the activation of one or several serine protease in response to the ER stressing drugs BFA, tunicamycin and thapsigargin in three different cellular systems, fibroblasts, HeLa cells and FDC-P1 monocytes [6]. The serine proteases exhibited three sites of action. First, they were important for the release of cytochrome *c* from mitochondria as this release was blocked with the broad range serine protease inhibitor AEBSF/Pefabloc. On the other hand they were responsible, at least in part, for caspase-3 processing as caspase-3 was still processed to the p20/p17 fragments in the presence of Z-VAD.fmk although no caspase-3 activity could be measured. Third, serine proteases seemed to contribute to phagocytosis as PS exposure and the uptake of apoptotic bodies by macrophages in vitro was still evident in Z-VAD.fmk-treated, but blocked in AEBSF-treated cells. Strikingly, while AEBSF could delay apoptosis, it could not inhibit it completely, and clonogenic survival was not possible after removing the apoptotic stimulus. This indicates that ER-stress induced apoptosis is still majorly driven by caspases, but serine proteases amplify this process by acting up- and downstream of mitochondria. We still await the isolation of the serine protease(s) in order to identify the substrate target between ER stress and MOMP.

Serpins, the endogenous inhibitors of serine proteases: can they tell us anything about apoptosis regulation by serine proteases?

Studies demonstrating the effect of the naturally occurring serine protease inhibitors (serpins) in regulating apoptosis also provide indirect evidence for the role of serine proteases in this process [108]. The viral serpin CrmA was originally discovered through its ability to prevent IL-1 processing by inhibiting caspase-1 [109]. Since then CrmA was shown to inhibit activated caspase-8 as well as Gzm B and hence prevent both Fas/TNF- and Gzm B-mediated apoptosis [110–112]. A more potent inhibition of Gzm B is achieved by the serpin PI-9 which is the only human protein with such a specificity [113]. Other members of the serpin family such as PAI-2 (plasminogen activator inhibitor 2), PN-1 (protease nexin 1) and Spi2A have also been implicated in the regulation of cell death [70, 71], and as mentioned above, AP24 induces DNA fragmentation by inactivating the serpin LEI, converting its elastase inhibiting activity to a L-DNaseII activity.

Lysosomal cathepsins: are they early triggers of PCD?

Numerous reports have implicated lysosomal cathepsins in early stages of caspase-dependent and -independent PCD [72, 73]. But are these proteases really initiators of cell death? This is a crucial issue as it has been recently proposed that cathepsins could be new therapeutic targets in immune responses by regulating the life span of inflammatory, T and B cells [72].

Cathepsins encompass three classes of lysosomal proteases, the serine proteases cathepsins A and G, the aspartic proteases cathepsins D and E and the 11, so far known human cysteine proteases cathepsins B, C, F, H, K, L, O, S, V, W and X/Z [72, 73]. Most of the cathepsins are endopeptidases, with the exception of cathepsins C and X/Z. These enzymes are all synthesized as inactive proenzymes, posttranslationally modified by glycosylations and targeted to the lumen of the lysosomes via the mannose-6-phosphate receptor sorting pathway. The processing of cathepsins from the inactive to their catalytically active form mainly occurs in the lysosome [114]. Therefore, upon lysosomal membrane permeability (LMP), which seems to widely occur in apoptotic cells, cathepsins are released in their processed form.

Cathepsins can be active in the cytosol at neutral pH, but the life-time of these enzymes is limited due to unfolding-induced inactivation [115]. In addition, most cells express in the cytosol potent cathepsin inhibitors, so called stefins or cystatins [115, 116]. So how would cathepsins then be able to trigger cellular processes in the cytosol, such as apoptosis? First, cathepsins B, D and L, the enzymes mostly implicated in apoptosis are more stable than the other isoforms. Second, stefins and cystatins may be inactivated during apoptosis. Third, the local pH around damaged lysosomes may be acidic enough to transiently stimulate cathepsin-mediated substrate cleavage and the propagation of an apoptotic signal through the cytoplasm [117].

A prerequisite for the proapoptotic function of cathepsins is that they must be released from the lysosome into the cytoplasm by lysosomal membrane permeabilization (LMP) [118]. The major problem is that the vast majority of LMP experiments have been performed with so called lysosomotropic agents such as L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) which directly perforate the lysosomal membrane [118, 119]. Any effect that is observed after this treatment is potentially non-physiological. What such experiments can tell us is how cathepsins could impart on cell survival once they appear in the cytosol.

The best characterized cytosolic substrate for cathepsins to date is the pro-apoptotic Bcl-2 family member Bid [120]. Cleavage of Bid by cathepsins B, L, K and S leads to the formation of tBid which can translocate to mitochondria to trigger Bax/Bak-induced MOMP [29, 30, 121, 122]. However, Bid is unlikely to be the only cellular substrate of cathepsins in this pathway. When mice lacking the major intracellular inhibitor of cysteine cathepsins, stefin B, were crossed with Bid−/− mice, no rescue of spontaneous cathepins-dependent neuronal apoptosis was observed [123]. Similarly, Bid−/− MEFs were not protected against MOMP induced by lysosomotropic photosensitizers [118]. Indeed, very recently, the group of Boris Turk identified pro-apoptotic Bak and Bim_{EL} and anti-apoptotic Bcl-2, Bcl-x_L and Mcl-1 as additional cysteine cathepsin substrates [124]. However, since Leu-Leu-OMe was used again in these studies, it is unclear if these cleavages also occur in response to apoptotic stimuli. Cathepsins can also directly process and activate caspases such as pro-caspase-3 and -7 [120, 125, 126]. Moreover, in vitro cleavage of pro-caspase-2 by cathepsin B generated a fragment showing cytochrome *c* releasing activity [127]. Also, during spontaneous apoptosis of neutrophils cathepsin D is released from azurophilic granules, leading to a direct and death receptor-independent activation of caspase-8 [128]. Therefore, direction activation of caspases by cathepsins may have physiological roles in some cells, such as neutrophils.

Whereas the findings described above point towards a rapid, early induction of LMP by apoptotic stimuli, several other reports propose that lysosomes are not the primary target of these stimuli. For example, in response to TNFα, FasL or TRAIL, LMP was found to be downstream of caspase-8 activation suggesting that caspases are responsible for the release of cathepsins B and D into the cytosol [129–133]. Specifically, caspase-8 appeared to activate caspase-9, and caspase-9, but no Apaf-1 was required for LMP induced by TNFα [134]. Unfortunately, in most of these experiments, cause and effect between LMP and caspase activation and/or MOMP have not been accurately dissected due to the lack of appropriate knock-out systems and the use of rather non-specific caspase-8 and -2 inhibitors.

If LMP is a decisive, early event in the signaling cascade of apoptosis, cathepsin release and action must also be critical for physiology and pathology in vivo. A variety of cathepsin deficient mouse strains have been generated. Depending on the cathepsin deletion, these mice exhibit aberrancies in bone remodeling, keratinocyte differentiation, carcinogenesis, antigen processing and immune regulation in general [135]. These findings indicate that cathepsins are not just boring enzymes which degrade bulk proteins in lysosomes but may have specific cellular functions. However, characteristic apoptosis defects are rarely seen in cathepsin knock-out animals, with the exception of a lack of resolution of inflammation due to neutrophil accumulation in cathepsin D knock-outs [128], reduced keratinocyte apoptosis resulting in periodic hair loss in cathepsin L deficient mice [136, 137] and diminished liver damage upon TNFα challenge in mice deficient of cathepsin B [130]. Of course this may be due to redundancies in the system, so that only double or triple cathepsin KO mice would reveal gross abnormalities in vivo. But we rather speculate that LMP and the subsequent release of cathepsins into the cytosol are late events that amplify rather than trigger apoptosis. In some cases such as

the resolution from inflammation, this amplification may be crucial to rapidly kill neutrophils [128], but in other cases, it may not be relevant because MOMP and caspase activation can already do the job effectively.

Why has no one attempted to dissect the order of this signaling pathway, for example by investigating if LMP and cathepsin release still occur in cells deficient for Bax/Bak, components of the apoptosome (Apaf-1 or caspase-9) or the major effectors caspase-3 and -7? The only data pointing towards a function of LMP and cathepsin release downstream of Bax/Bak was published by the group of Gregory Gores, where they found that Bax can translocate to a fraction enriched in lysosomes during staurosporine induced apoptosis [138]. However, it was not shown if it was really the lysosomally associated, and not the mitochondrial Bax, which mediated this effect. Moreover, it is evident from the literature that Bax−/− cells are not particularly resistant to staurosporine-induced cell death [29, 30]. By contrast, cells deficient in apoptosomal components are resistant to apoptosis induced by staurosporine and other stimuli [40, 41], and hence LMP could be the result of apoptosome formation after Bax/Bak-mediated MOMP. By using two different cell systems (monocytes and fibroblasts) deficient in Bax and/or Bak, caspases or Apaf-1, exposed to four different apoptotic stimuli (FasL, etoposide, UV and IL-3 removal), we could recently show that LMP and cathepsin release required Bax/Bak and the apoptosome and therefore occurred downstream of MOMP [139].

Conclusions

There is no doubt that serine proteases, cathepsins and other non-caspase proteases are emerging as key players of PCD. However, we think that instead of initiating/triggering an apoptotic response, they rather support or propagate caspase-mediated apoptosis signaling upstream, downstream or aside of mitochondria, provoke late events such as oligonucleosomal or nicked DNA fragmentation or phagocytosis or regulate caspase-independent death processes such as necrosis. What is now required is a more accurate, unbiased analysis of where exactly these proteases act in the apoptotic signaling pathway and what steps they indeed control. In addition, for serine proteases it will be important to develop methods and approaches that will permit the precise identification of serine proteases involved in cell death (apoptosis and necrosis). Only then will we be capable of judging if novel therapeutic drugs directed against these proteases are worth being developed in order to cure degenerative diseases.

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