Cell Death and Differentiation (2005) 12, 1219–1224

www.nature.com/cdd

News and Commentary

Serine proteases and calpains fulfill important supporting roles in the apoptotic tragedy of the cellular opera

P Vandenabeele[1], S Orrenius[2] and B Zhivotovsky*,[2]

[1] Molecular Signalling and Cell Death Unit, Department for Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology (VIB) and Ghent University, B9052 Ghent, Belgium
[2] Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden
* Corresponding author: B Zhivotovsky, Department of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden. Tel: +46 8 5248 7588; Fax: +46 8 32 90 41; E-mail: Boris.Zhivotovsky@imm.ki.se

Cell Death and Differentiation (2005) **12,** 1219–1224.
doi:10.1038/sj.cdd.4401719

The cellular proteolytic machinery includes numerous proteases localized in different intracellular compartments. This machinery functions to maintain fundamental cellular processes and to remove denatured or misfolded proteins. The requirement for proteolysis during apoptosis is well-documented. During the last decade, the 'main players' in the induction and execution steps of the apoptotic process were identified as a family of aspartic acid-specific cysteine proteases, the caspases. However, the involvement of additional proteolytic activities, such as granzymes, lysosomal cathepsins, calpains, proteasomes and serine proteases, was also described in different experimental models of cell death, although their role in the regulation of apoptosis has not yet been clarified in terms of defined molecular pathways.

Serine Protease Inhibitors Modulate the Cell Death Cascade

The serine proteases are characterized by the presence of a serine residue in the active centre of the enzyme implicated in the covalent acyl-enzyme transient complex between the substrate and protease. In mammals, serine proteases perform many important functions, notably in the digestion of dietary protein, in the blood-clotting cascade, in the complement system and in several pathways of differentiation and development. The family members have been classified according to their substrate specificity and can be divided into three main groups, of which the prototypes are chymotrypsin, trypsin and elastase. Although all three of these enzymes are similar in structure, they exhibit differential endopeptidase specificity based on the amino-acid residues and side chains surrounding the site of cleavage. Involvement of serine proteases in apoptosis was first documented during the attempt to inhibit TNF-mediated cytotoxicity of melanoma cells by both irreversible (*N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*-tosyl-L-lysine chloromethyl ketone (TLCK)) and reversible (*N*-acetyl-DL-phenylalanine-β-naphtyl ester (APNE) and *N*-tosyl-L-arginine methyl ester (TAME)) inhibitors of chymotrypsin- and trypsin-like proteases, respectively.[1] Later it was shown that TLCK also inhibited apoptosis induced by etoposide in HL-60 cells and by bile acids in hepatocytes.[2,3] Likewise, thymocyte apoptosis was retarded by pretreatment with TPCK, dichloroisocoumarine (DCI) and phenylmethylsulphonyl fluoride (PMSF).[4,5] More recent data using the serine protease inhibitor, Pefabloc, showed that serine protease(s) might mediate apoptosis-like cell death induced by DNA damage and ER stress.[6,7] Although the specific serine proteases involved in these processes remain unidentified, and their precise mechanisms of action are unclear, it appears that in some experimental systems these enzymes might be acting upstream of the caspase cascade, while in others they act in a caspase-independent manner. Importantly, Bcl-2 was able to inhibit not only caspase-dependent, but also caspase-independent, serine protease-mediated death signalling.[7] TNF-induced necrosis, a caspase-independent type of cell death characterized by osmotic swelling, is also efficiently blocked by the chymotrypsin-like inhibitor TPCK, and to a much lesser extent by TLCK.[8] Similar to apoptosis, overexpression of Bcl-2 retarded serine protease-mediated necrotic cell death.[9]

It is known that the plasminogen activator system is an important proteolytic complex responsible for the breakdown of the extracellular matrix, and that plasminogen activator inhibitor type 2 (PAI-2), an inhibitor of serine protease activity, is a major product of monocytes and macrophages in response to inflammatory mediators. Long ago, it was shown that expression of PAI-2 protects from TNF-induced apoptosis in HeLa cells, a cell line that does not express PAI-2, or significant levels of urokinase plasminogen activator (uPA).[10] The precise mechanisms by which PAI-2 confers resistance of cells to apoptosis are still not clear. In spite of the high degree of structural similarity between PAI-2 and CrmA, it is unlikely that PAI-2 represents the mammalian homologue of CrmA. While PAI-2 contains Arg in the P1 position, CrmA contains an Asp residue, which is covalently bound by the catalytic cysteine of inflammatory caspases and apoptotic initiator caspases.[11] A PAI-2 proteolytic cleavage product has been linked to apoptosis in myeloid leukemic cells, suggesting that a loss of the apoptotic inhibitory activity of PAI-2 is associated with proteolytic cleavage of the PAI-2 molecule. Intracellular PAI-2 might be an important factor in regulating apoptosis in certain systems through the inhibition of an as yet unidentified cell death protease.

Several years ago, it was shown that the 24 kDa apoptotic protease (AP24) is a serine protease with elastase-like activity that is activated during TNF- or UV light-induced apoptosis.[12]

In vitro, it stimulates internucleosomal DNA fragmentation in isolated nuclei. This distinguishes AP24 from several other proteases, such as caspases and granzyme B, which require the presence of cytosolic components to activate nuclear DNA fragmentation in a cell-free system. This finding led to the hypothesis that AP24 might play a role in transmitting apoptotic signals from the cytosol to the nucleus, where it may directly or indirectly activate endogenous endonucleases, resulting in DNA fragmentation. Recently, it was found that AP24-mediated DNA fragmentation indeed is indirect, the missing link being leukocyte elastase inhibitor (LEI), which is a precursor of endonuclease L-DNase II. AP24 interacts with LEI and, as a result of cleavage, transforms it into L-DNase II.[13] It appears that the interaction between AP24 and LEI might be serpin-like, as for elastases. Activation of AP24 requires the presence of sphingomyelin and calcium/calmodulin-dependent kinase, and can be inhibited by overexpression of Bcl-2, although the precise mechanism of this inhibition is unclear. It also seems that the activation of AP24/L-DNase II is tissue- and trigger-specific. Like the couple ICAD/CAD, the couple AP24/L-DNase II provides an example of DNase activation by proteases. In normal cells, AP24 is present in a complex with LEI, which, upon stimulation, releases AP24 to cleave LEI and convert it to L-DNase II. Future experiments should clarify how general this mechanism is, as well as its importance for the cell death process.

Trypsin-like proteases have also been implicated in apoptosis. Hence, it has been shown that thrombin, a 37 kDa trypsin-like protease well known for its role in the blood coagulation cascade, might either inhibit or accelerate death in a variety of cell types, including fibroblasts and neuronal, endothelial, epithelial and tumor cells.[14] Both effects rely on thrombin action as a serine protease, which cleaves protease-activated receptor PAR-1. The signalling pathways involved in induction or prevention of cell death by PAR-1 are diverse, and include JAK/STAT, RhoA, myosin light chain kinase, ERK1/2 and various Bcl-2 family members, suggesting that both pathways may share initial signalling proteins, but that differences in the concentrations used and in the amplitude as well as the duration of the signal may eventually result in different outcomes.[15] However, PAR-1 cleavage is not always required for effects of thrombin on cell viability. Hence, it has recently been shown that thrombin can stimulate survival of PAR-1-null cells,[16] and a paradoxical proapoptotic PAR-1-independent effect of thrombin on smooth muscle cells has been reported.[17] Both effects were found to depend on thrombin serine protease activity. Thus, it appears that thrombin can exert multiple effects on cell viability, and that further work is required to better define both the targets and signals by which these effects are mediated.

Activated protein C (APC) is a systemic anticoagulant and anti-inflammatory factor that protects ischemic human brain endothelium from injury by a mechanism that involves PAR-1 cleavage.[18] A comparison of the kinetics of PAR-1 cleavage in endothelial cells by APC *versus* thrombin showed that APC was 10 000-fold less potent than thrombin in this setting. Hence, the physiological significance of PAR-1 activation by APC is unclear.[19] Finally, cross-talk between two serine protease activities was recently demonstrated when it was shown that leukocyte elastase mediates a proapoptotic effect in human airway and alveolar epithelial cells via activation of thrombin, followed by cleavage of PAR-1. This sequence of events was largely prevented by a specific PAR-1 antagonist and by PAR-1 siRNA.[20] However, it is still unclear how general this cross-talk is.

Granzymes and Their Role in Cell Death

Granzymes constitute another group of exogenous serine proteases, which are located in cytoplasmic granules of cytotoxic T-lymphocytes and natural killer (NK) cells, from which they are released together with another protein, termed perforin, to attack and kill target cells. Granzymes A and B are the most abundant granzymes in cytotoxic lymphocytes. Their function, as is the case for all proteases, is defined by specificity and tempero-spatial aspects of substrate recognition and cleavage. Physiological substrates of a protease are usually rapidly recognized and cleaved by the enzyme. This correlation is observed, for example, during granzyme B cleavage of several pro-caspases.[21]

Granzyme A is a tryptase that cleaves substrates at lysine or arginine residues. It induces a type of cell death that only superficially resembles apoptosis via a mechanism that does not require activation of any known caspases and is not inhibited by antiapoptotic members of the Bcl-2 family of proteins. Within the target cell, granzyme A activity seems to be directed towards nuclear events. This process involves a SET complex (SET, HMG2, Ape-1, GAAD and pp32), which is associated with the endoplasmic reticulum and upon activation translocates to the nucleus, where it induces the characteristic single-strand DNA nicks.[22] Interestingly, granzyme A-mediated cleavage of Ape-1 was able to prevent cellular repair and recovery from the death stimulus. It seems that Ape-1 cleavage by granzyme A may serve a function similar to that of PARP cleavage during caspase-mediated apoptosis. PARP is an important participant in base excision repair as a sensor of single-stranded DNA damage. Caspase cleavage of PARP, like granzyme A cleavage of Ape-1, blocks DNA repair and thereby facilitates apoptosis.[23] More recently, it was shown that, although granzyme A does not cleave Bid or permeabilize the mitochondrial outer membrane to cause release of factors required for caspase activation, it causes a rapid increase in reactive oxygen species and loss of mitochondrial transmembrane potential in a caspase-independent manner that is not blocked by Bcl-2 overexpression. Moreover, mitochondrial damage is key to nuclear translocation of the SET complex and granzyme A induction of apoptosis. Further, peroxide treatment causes some of the SET complex to move rapidly into the nucleus, whereas treatment of cells with superoxide scavengers blocks nuclear translocation of SET complex and protects cells from granzyme A-mediated cell death.

Granzyme B is an aspartase that cleaves substrates at asparagine or glutamine residues and can efficiently induce cell death. Granzyme B can directly trigger a caspase cascade by cleaving endogenous pro-caspases in target cells. Studies carried out *in vitro* have shown that granzyme B is capable of cleaving pro-caspase-3, -6, -7, -8, -9 and -10.[24] In the case of pro-caspase-3, -7 and -9, granzyme B-mediated

processing has been shown to generate active caspase enzymes.[25] More importantly, studies with intact cells have demonstrated that caspases are activated in target cells following co-incubation with granzyme B and perforin. Hence, it seems that granzyme B may promote apoptosis simply by cleaving and activating endogenous caspases in target cells, although it should be noted that this is not the only way by which granzyme B can kill cells. It can also act indirectly via two pathways of mitochondrial membrane permeabilization. One of these involves cleavage of the proapoptotic Bcl-2 family protein, Bid, to a truncated form that targets the mitochondrial membrane. The second involves cleavage of Mcl-1, which normally binds to and neutralizes Bim, another Bcl-2 family protein that also attacks the mitochondrial outer membrane. Thus, there are several pathways by which granzyme B might kill cells, although it is unclear in which situations each pathway is preferentially activated.

While the functions of granzymes A and B have been studied extensively, the role of other highly expressed granzymes in the promotion of cell death has not yet been explored in detail. Recently, the cytotoxicity of murine granzyme C, which is most closely related to human granzyme H, was characterized.[26] The induction of cell death requires its protease activity and is associated with the rapid externalization of phosphatidylserine, nuclear condensation and collapse, and single-stranded DNA nicking. The kinetics of these events are similar to those triggered by granzyme B, and the potency of both granzymes is equivalent. The induction of cell death by murine granzyme C did not involve the activation of caspases, the cleavage of Bid, or the activation of caspase-dependent DNase. However, granzyme C did cause rapid mitochondrial swelling and depolarization in intact cells and in isolated mitochondria, and this mitochondrial damage was not prevented by cyclosporine A. This suggests that granzyme C induces cell death by attacking nuclear and mitochondrial targets, and that these targets are distinct from those used by granzyme B to cause apoptosis.

HtraA2/Omi, a Mitochondrial Serine Protease with Vital and Apoptogenic Functions

Many cytotoxic stresses, including most chemotherapeutics, induce the release of mitochondrial factors from the intermembrane space into the soluble cytoplasm.[27] The mitochondrial serine protease HtrA2/OMI belongs to the HtrA (high-temperature requirement) protein family, which is evolutionarily conserved from bacteria to man.[28] HtrA family members possess a central trypsin-like catalytic domain and one or more C-terminal PDZ domains, which are involved in the recognition of C-terminal regions of receptors. At least some members of the HtrA family are indispensable for the viability of prokaryotic and eukaryotic cells. Prokaryotic HtrA, also known as DegP, exerts a dual function: it acts as a chaperone at normal temperature, but becomes an endoprotease involved in the proteolysis of denatured proteins at elevated temperatures.[29] Four HtrA homologs are found in mammalian cells, HtrA1, -2, -3 and -4, but only HtrA2 resides in the intermembrane space of the mitochondria (IMS), whereas the others are secreted.

HtrA2/OMI is expressed from a nuclear gene as an immature 49 kDa protein with an N-terminal MLS that mediates its translocation into the mitochondrial IMS. Hence, HtrA2/OMI is processed into the 37 kDa mature form, putting the IAP-binding motif (IBM; AVPS in human; AVPA in mouse) at its N-terminus. Although recombinant HtrA2/OMI can catalyse its own maturation *in vitro*, the protease responsible for its maturation in cells remains unknown. The three-dimensional structure of HtrA2/OMI reveals the formation of a pyramid-shaped homotrimer, with the N-terminal IBMs on top, the serine protease domains in the centre and the PDZ domains at the bottom.[30] The C-terminal PDZ domain restricts access to the active site by packing against the protease domain, thereby exerting an intermolecular inhibitory effect on HtrA2/OMI proteolytic activity.[30,31] From a combinatorial peptide library screen, GQYYFV turned out to be the preferred binding sequence of the PDZ domain of HtrA2/OMI. In addition, binding of this peptide to the PDZ domain of HtrA2/OMI leads to a major increase in protease activity by permitting better access to the catalytic site.[31]

HtrA2/OMI exerts an essential function in mitochondrial homeostasis, but the molecular targets and interaction partners of HtrA2/OMI in the mitochondrion are as yet undefined. This essential function requires the proteolytic activity of HtrA2/OMI. By tracing the genetic locus responsible for early-onset motor neuron degeneration in the mouse mutant *mnd2*, Jones *et al*. identified a missense mutation (Ser276Cys) in HtrA2/OMI as the genetic cause of this disease. The *mnd2* phenotype is associated with selective loss of a population of striatal neurons, resulting in a neurodegenerative disorder, muscle wasting and involution of vital organs, culminating in juvenile death of the mice.[32] The Ser276Cys mutation in HtrA2/OMI strongly impairs the proteolytic activity of HtrA2/OMI, but leaves the IBM intact (HtrA2/OMI maturation is normal in *mnd2*). As a result, *mnd2* MEFs are very susceptible to etoposide, tunicamycin, antimycin, H_2O_2 and calcium pulsing, agents that impinge on the mitochondria. Targeted disruption of HtrA2/OMI results in a similar 'parkinsonian' phenotype in mice.[33] HtrA2/OMI$^{-/-}$ primary cortical neurons are much more sensitive to glutamate-mediated excitotoxicity. On the other hand, HtrA2/OMI$^{-/-}$ thymocytes are as sensitive as wild-type thymocytes to anti-Fas treatment. Loss of HtrA2/OMI did not affect the activity of mitochondrial complex I, II, III or IV, but decreased mitochondrial density in tissues and increased the number of mitochondria with abnormal shape and ballooning cristae in knockout cells. Clearly, mammalian HtrA2/OMI functions like its bacterial counterpart HtrA, contributing to cellular survival rather than to cell death, possibly by degrading noxious proteins accumulating in the mitochondria as a result of stress or even normal mitochondrial metabolism. In accordance with this stress-related function, HtrA2/OMI expression is induced by heat shock, the ER stress inducer tunicamycin, and the chemotherapeutic agent cisplatin.[34]

HtrA2/OMI released from the mitochondrial IMS contributes to cell death in a caspase-dependent way by antagonizing IAPs, and in a caspase-independent way as a protease.[35–39] HtrA2/OMI blocks IAPs through its N-terminal IBM, presented

in a trimeric configuration. The IBM of HtrA2/OMI has a higher affinity for the BIR2 domain than for the BIR3 domain.[35–37] Furthermore, *in vitro* data using high concentrations of HtrA2/Omi revealed the degradation of XIAP, cIAP1, cIAP2 and Apollon by the protease activity of HtrA2/OMI.[40–43] *Vice versa*, Apollon ubiquitinates HtrA2/OMI, targeting it for proteasomal destruction. However, a role for IAP-binding and degradation by HtrA2/OMI *in vivo* is unclear. Recently, the group of Emad Alnemri suggested a pathogenic role for extramitochondrial HtrA2/Omi using the synthetic HtrA2/Omi inhibitor ECF101 in a model for myocardial ischemia-reperfusion damage.[44]

Next to the IAPs other interaction partners and substrates of HtrA2/OMI have been identified. The antiapoptotic Ped (phosphoprotein enriched in diabetes)/Pea-15 (phosphoprotein enriched in astrocytes-15 kDa) interacts with, and is degraded by, cytosolic HtrA2/OMI.[45] HS1-Associated Protein X-1 (HAX-1) was also identified as an interaction partner and degradation target of HtrA2/OMI. HAX-1 degradation during apoptosis is absent in *mnd2* MEFs.[46] Of note, HAX-1 is associated with the mitochondria and has antiapoptotic properties. Furthermore, the Alzheimer's disease-associated presenilin-1 can regulate the proteolytic activity of HtrA2/OMI through direct interaction with the regulatory PDZ domain.[47] However, the functional relevance of the interaction between HtrA2/OMI and the above-mentioned proteins is still unclear and needs further investigation.

Overexpression as well as RNA interference studies have indicated a clear contribution of HtrA2/OMI to cell death. Expression of mature, proteolytically active HtrA2/OMI results in cell death that is characterised by a relatively long period of cell rounding before the typical apoptotic morphological changes become visible.[35] Whether or not caspase activity is associated with this form of HtrA2/OMI-induced cell death is controversial.[35–37] Conversely, decreasing the levels of HtrA2/OMI in cells by antisense or RNA interference lowers the susceptibility of different cell lines (MCF-7, HeLa, U2OS) to cell death induced by staurosporine, Fas, UV or cisplatin.[37,38,40] These data are apparently at odds with the increased sensitivity of HtrA2/OMI$^{-/-}$ MEFs to death stimuli. However, the complete absence of HtrA2/OMI in knockout cells is associated with perturbed mitochondrial function imposing a constant cellular stress, whereas temporary knocked down levels of this protein may allow the unveiling of its extramitochondrial apoptogenic function. The serine protease HtrA2/Omi is not released from mitochondria during necrotic cell death.[39]

Based on experiments with synthetic and natural inhibitors it is obvious that several serine protease activities are implicated in cell death; however, in the majority of cases neither the protease nor its specific substrates have yet been identified. At present a clear role for only a few serine proteases have been described, such as granzymes A and B as well as HtrA2/Omi. While granzymes A and B might play a role in induction of caspase-dependent and -independent apoptosis, the role of HtrA2/Omi consists of signal amplification downstream of mitochondria during apoptotic cell death by binding and degrading caspase inhibitors, and by other not yet defined pathways. However, as discussed above, it should be emphasized that the role of Omi/HtrA2 serine protease is mainly pro-apoptotic.

Ca^{2+}-Dependent Proteases in Cell Death

Calpains are cytosolic proteases that are closely related to the calcium metabolism of the ER-mitochondrial organelle system.[48] Calpains belong together with caspases to the family of cysteine proteases, which play important roles in regulating cell death, and accumulating data suggest that cross-talk between calpains and caspases are important in the regulation of this process. Proteins that can be cleaved by both calpains and caspases during apoptosis include pro-caspases-3, and -7, Bcl-2 family proteins (Bid, Bcl-X_L, Bcl-2), structural proteins (actin, α-fodrin, cytokeratin, focal adhesion kinase (FAK)), neural cell adhesion molecules (NCAM)), signal transduction proteins (calcium/calmodulin dependent protein kinase, PKC α and δ), DNA repair and cell cycle regulatory proteins (PARP, DNA polymerase ε, cyclin D, p53), glutamate receptors (AMPA, NMDA), and several unclassified proteins (Ins(1,4,5)P_3R, calcineurin, amyloid precursor protein (APP)).

Although both calpains and caspases are cysteine proteases, their functions and cleavage specificity are different. Thus, all caspases contain a conserved QACXG pentapeptide with the active-site cysteine. They have a unique, strong preference for cleavage of the peptide bond C-terminal to aspartate residues. Calpains are Ca^{2+}-activated proteases, which include several tissue-specific isoforms (n-calpains) and two ubiquitous isozymes (μ-calpain and m-calpain). For activation, μ- and m-calpains require micromolar or millimolar Ca^{2+} concentrations, respectively. Compared to caspases, calpains do not exhibit strict cleavage specificity.[49] Calpain activity is regulated by the endogenous inhibitor, calpastatin. Although calpastatin is normally cleaved by calpain, in several experimental models of apoptosis it has been shown that calpastatin might also be cleaved by caspase-3, and that this cleavage is essential for regulation of calpain activity during cell death. Calpain activation can lead to cleavage of several pro-caspases, which may either activate or inactivate their function.[50]

One mechanism contributing to the maintenance of p53 stability in cells is inhibition of calpain activity by calpastatin. However, recently it has been shown that Gas2, which plays a role in regulating microfilament and cell shape changes during apoptosis, also interacts with m-calpain and inhibits calpain-dependent degradation of p53. Like calpastatin, Gas2 is cleaved by caspase-3 during apoptosis, and released m-calpain decreases the susceptibility of cells to p53-mediated apoptosis.[51]

As mentioned above, calpain can also cleave caspases. Most often this cleavage inactivates caspase function. For example, calpain can cleave caspase-7 at sites distinct from those cleaved by the upstream caspases, generating proteolytically inactive fragments. Pro-caspases-8 and -9 can also be cleaved by calpains, and truncated caspase-9 is unable to activate pro-caspase-3. Finally, pro-caspase-3 cleavage by calpain was early reported to generate a 29 kDa fragment, although it is still unclear whether such cleavage results in activation or inactivation of caspase-3 function.[52,53] The best known example of caspase activation by calpain is the cleavage of pro-caspase-12 during ER stress-mediated cell death.[54] Although the precise mechanism of this activation is unclear, it appears that the extent

of calpain-mediated activation of caspase-12 depends on the time of exposure. Prolonged exposure to calpains results in degradation of caspase-12 (Kalai, Lamkanfi and Vandenabeele, unpublished results).

Several cell survival proteins, including Bcl-2 and Bcl-X_L are cleaved by both caspases and calpains. Although the cleavage sites are different, the consequences of cleavage catalysed by the two protease families are usually similar; the generated fragments promote cell death rather than survival. In contrast, an 18 kDa product of Bax cleavage by calpain or caspases is a more potent inducer of apoptosis than uncleaved Bax.[55] Similarly, the pro-apoptotic protein Bid can be cleaved by calpain as well as by caspase-8. Calpain cleaves Bid between Gly70 and Arg71, while caspase-8 cleaves between Asp59 and Gly60 as well as between Asp75 and Ser76.[56] Importantly, in both situations the BH3 domain of Bid remains intact, and the pro-apoptotic activity is maintained. Thus, it is clear that calpains and caspases interact, and that cleavage of the same proteins by two (or more) proteases allows either protease alone, or both in combination, to activate the apoptotic process.

During apoptosis, calpains might operate also independently of caspases, or in combination with other enzymes. Recently, it was shown that both the precursor and mature forms of recombinant AIF were cleaved near the amino-terminus by calpain *in vitro*.[57] Mitochondrial outer membrane permeabilization by truncated Bid induced AIF release only in the presence of active calpain. Importantly, inhibition of calpain by calpeptin precluded AIF release, demonstrating that proteolytic activity was required for release. Both calpeptin and cyclosporine A also inhibited calcium-induced AIF release from isolated liver mitochondria, implicating the involvement of endogenous mitochondrial calpain activity in release of AIF during the permeability transition. Cleavage of AIF was also found to decrease its association with pure lipid vesicles of mitochondrial inner membrane composition. Finally, studies in transgenic mice lacking or overexpressing calpastatin showed that calpastatin deficiency augmented kainate-evoked exitotoxicity in the hippocampus and resulted in mitochondrial changes associated with AIF release. Consistently, calpastatin overexpression suppressed these effects. These results define a novel mechanism of calpain involvement in cell death through mobilization of proapoptotic factors in a caspase-independent manner.

More recently, calpains were identified as being responsible for cleavage of the Na^{2+}/Ca^{2+} exchanger (NCX), in particular the NCX3 isoform, in brain regions affected by a simulated stroke.[58] Overexpression of calpastatin in granule neurons was able to block glutamate-induced NCX3 cleavage and reduced neuronal death. Interestingly, this calpain-induced NCX cleavage has an intriguing parallel in the caspase-mediated cleavage of another Ca^{2+}-extrusion pump, PMCA.[59] It is likely that both of these cleavage events can promote cell death by impairing cellular defense mechanisms, although under different circumstances. Further, sustained calpain activation associated with lysosomal rupture was shown to execute cell death in postischemic CA1 neurons. In this case, activated calpain was responsible for the spillage of hydrolytic cathepsins as well as lysosome-associated membrane protein-1 (LAMP-1) from lysosomes, leading to cell death.[60]

Finally, another Ca^{2+}-dependent protease, termed CRP, has also been implicated in apoptosis.[5] This protease is associated with the nuclear matrix (and endoplasmic reticulum), where it catalyses the cleavage of proteins in response to certain apoptotic triggers. Like caspase-6, CRP can cleave lamin. Whether the activation of this protease is under the control of caspases (or *vice versa*) is unclear, although the nuclear localization of CRP makes it an attractive candidate for activation by caspases.

Conclusions

From the discussion above, it is clear that, in addition to caspases, several other proteolytic systems are critically involved in the regulation of cell death. In many cases, the sequential activation of different protease families leads to the killing and swift demolition of cells. For example, activation of initiator caspases or granzyme B leads to caspase-dependent apoptosis through the specific (alternative) cleavage of caspase substrates, such as Bid, PARP and ICAD, whereas granzyme A activates a caspase-independent pathway of cell death. Some enzymes (e.g. caspase-3) require proteolytic processing for activation, while others (e.g. HtrA2/Omi) depend on protein–protein or protein–lipid interactions. It is increasingly clear that cross-talk between different proteases might amplify and accelerate the cell death process. The identification and characterization of these synergizing protease cascades might also provide novel therapeutic targets for the modulation of the cell death process, positively or negatively, depending on the disease.

1. Ruggiero V *et al.* (1987) Cell. Immunol. 107: 317–325
2. Kaufmann SH *et al.* (1993) Cancer Res. 53: 3976–3985
3. Kwo P *et al.* (1995) Am. J. Physiol. 268: G613–G621
4. Walker PR *et al.* (1993) Biotechniques 15: 1032–1040
5. Zhivotovsky B *et al.* (1995) Exp. Cell. Res. 221: 404–412
6. de Bruin EC *et al.* (2003) Cell Death Differ. 10: 1204–1212
7. Egger L *et al.* (2003) Cell Death Differ. 10: 1188–1203
8. Vercammen D *et al.* (1997) Cytokine 9: 801–808
9. Denecker G *et al.* (2001) Cell Death Differ. 8: 829–840
10. Dickinson JL *et al.* (1995) J. Biol. Chem. 270: 27894–27904
11. Garcia-Calvo M *et al.* (1998) J. Biol. Chem. 273: 32608–32613
12. Wright SC *et al.* (1994) J. Exp. Med. 180: 2113–2123
13. Altairac S *et al.* (2003) Cell Death Differ. 10: 1109–1111
14. Ahmad R *et al.* (2000) Blood 96: 4001
15. Donovan FM and Cunningham DD (1998) J. Biol. Chem. 273: 12746–12752
16. Song SJ *et al.* (2005) Am. J. Pathol. 166: 857–868
17. Rossignol P *et al.* (2004) Exp. Cell. Res. 299: 279–285
18. Cheng T *et al.* (2003) Nat. Med. 9: 338–342
19. Ludeman MJ *et al.* (2005) J. Biol. Chem. 280: 13122–13128
20. Suzuki T *et al.* (2005) Am. J. Respir. Cell. Mol. Biol., May 12 (Epub ahead print), doi: 10.1165/rcmb.2005-0109OC
21. Trapani JA and Sutton VR (2003) Curr. Opin. Immunol. 15: 533–543
22. Fan Z *et al.* (2003) Cell 112: 659–672
23. Martinvalet D *et al.* (2005) Immunity 22: 355–370
24. Van de Craen M *et al.* (1997) Eur. J. Immunol. 27: 1296–1299
25. Darmon AJ *et al.* (1995) Nature 377: 446–448
26. Johnson H *et al.* (2003) Blood 101: 3093–3101
27. Saelens X *et al.* (2004) Oncogene 23: 2861–2874
28. Clausen T *et al.* (2002) Mol. Cell 10: 443–455
29. Spiess C *et al.* (1999) Cell 97: 339–347
30. Li W *et al.* (2002) Nat. Struct. Biol. 9: 436–441
31. Martins LM *et al.* (2003) J. Biol. Chem. 278: 49417–49427
32. Jones JM *et al.* (2003) Nature 425: 721–727

33. Martins LM *et al.* (2004) Mol. Cell. Biol. 24: 9848–9862
34. Gray CW *et al.* (2000) Eur. J. Biochem. 267: 5699–5710
35. Suzuki Y *et al.* (2001) Mol. Cell 8: 613–621
36. Verhagen AM *et al.* (2002) J. Biol. Chem. 277: 445–454
37. Martins LM *et al.* (2002) J. Biol. Chem. 277: 439–444
38. Hegde R *et al.* (2002) J. Biol. Chem. 277: 432–438
39. van Loo G *et al.* (2002) Cell Death Differ. 9: 20–26
40. Yang QH *et al.* (2003) Genes Dev. 17: 1487–1496
41. Srinivasula SM *et al.* (2003) J. Biol. Chem. 278: 31469–31472
42. Suzuki Y *et al.* (2004) Cell Death Differ. 11: 208–216
43. Sekine K *et al.* (2005) Biochem. Biophys. Res. Commun. 330: 279–285
44. Liu HR *et al.* (2005) Circulation 111: 90–96
45. Trencia A *et al.* (2004) J. Biol. Chem. 279: 46566–46572
46. Cilenti L *et al.* (2004) J. Biol. Chem. 279: 50295–50301
47. Gupta S *et al.* (2004) J. Biol. Chem. 279: 45844–45854
48. Orrenius S *et al.* (2003) Nat. Rev. Mol. Cell. Biol. 4: 552–565
49. Goll DE *et al.* (2003) Physiol. Rev. 83: 731–801
50. Porn-Ares MI *et al.* (1998) Cell Death Differ. 5: 1028–1033
51. Benetti R *et al.* (2001) EMBO J. 20: 2702–2714
52. Waterhouse NJ *et al.* (1998) Cell Death Differ. 5: 1051–1061
53. Chua BT *et al.* (2000) J. Biol. Chem. 275: 5131–5135
54. Nakagawa T and Yuan J (2000) J. Cell. Biol. 150: 887–894
55. Choi WS *et al.* (2001) J. Neurochem. 77: 1531–1541
56. Mandic A *et al.* (2002) Mol. Cell. Biol. 22: 3003–3013
57. Polster BM *et al.* (2005) J. Biol. Chem. 280: 6447–6454
58. Bano D *et al.* (2005) Cell 120: 275–285
59. Schwab BL *et al.* (2002) Cell Death Differ. 9: 818–831
60. Yamashima T *et al.* (2003) Hippocampus 13: 791–800