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In the cut and thrust of apoptosis, serine proteases come of age

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Abstract

Proteolysis is central to the systematic cellular degradation that occurs during apoptosis. Predominantly, caspases have been studied in this regard. However, increasing evidence suggests that certain serine proteases may also play a significant role in apoptosis. Not only are these serine proteases involved in apoptosis signalling pathways independently, but they may also interact with more classical mediators of apoptosis such as the caspases or Bcl-2 family proteins. Isolation of apoptosis-associated serine proteases and the use of specific inhibitors have helped to shed light on potential pathways in which they are involved. Despite the recent developments in the field, knowledge regarding the role of serine proteases in apoptosis remains limited, but it is clear that investigations are gathering momentum and such studies may herald a new and exciting departure in apoptosis research.

Keywords: Apoptosis; Serine proteases; Omi; AP24; ASP-1 and -2; Caspases

1. Introduction

Apoptosis is a physiological form of cell death, fundamental to multicellular animal development and the maintenance of tissue homeostasis. Dysfunctional apoptosis, leading to too much or too little apoptosis, contributes to the development of a broad spectrum of diseases, including Alzheimer's disease and cancer [1,2]. Selective, tightly controlled proteolysis is a defining characteristic of apoptosis resulting in the ordered disassembly of the cell [3]. Early evidence suggested that many types of proteases, differing in their structure, substrate specificity and mode of action were involved in this disassembly. However, a particular family of conserved cysteine-dependent aspartic acid-specific proteases, the caspases, rose quickly to the forefront of apoptosis research. Caspases are critical mediators of several pro-apoptotic signalling pathways [4]. These include the mitochondrial pathway in which pro-caspase-9 is activated [5], the death receptor pathway, leading to pro-caspase-8 activation [6,7] and the endoplasmic reticulum (ER) stress-mediated pathway, resulting in pro-caspase-12 processing [8]. These pathways converge upon the activation of effector caspases like caspase-3, resulting in the cleavage of several substrates, including PARP and DNA Fragmentation Factor 45 (DFF45)/ICAD, with consequent cellular disassembly. Recent studies in a variety of cell systems, have demonstrated that inhibition of caspase activation *in vitro* is not always accompanied by prevention of cell death [9–12]. Furthermore, caspase-independent cell death has also been observed *in vivo*, where thymocytes and splenocytes from caspase-3 and -9 knockout mice underwent apoptosis in response to a variety of stimuli [13,14]. The form of cell death observed in caspase-independent models usually exhibits morphological features characteristic of apoptosis, such that caspase-independence does not normally define a different type of cell death [9,13]. This has led to renewed interest in the identification of alternate apoptosis-associated proteases [9,10,12,15]. However, in comparison to caspases, knowledge regarding the participation of other proteases in the death process is relatively limited [16,17]. Some of the proteases implicated in the process include calpains, lysosomal proteases, and serine proteases. The calcium-activated cysteine protease calpain, is activated in response

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Abbreviations: AP24, apoptotic factor 24; ASP-1 and -2, apoptosis-associated serine proteases 1 and 2; Bcl-2, b-cell lymphoma-2; IAP, inhibitor of apoptosis protein; LEI, leukocyte elastase inhibitor; PARP, poly(ADP-ribose) polymerase; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TNF, tumour necrosis factor; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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to the accumulation of cytosolic calcium ions, leading to the cleavage of multiple substrates including pro-caspase-12 [8]. In addition, the binding of death receptors like the TNFα receptor can induce the release of cathepsin B (a cysteine protease) from the lysosomal compartment, leading to the activation of caspase-dependent and independent cell death [18]. Finally, cytotoxic T lymphocytes employ pore-forming perforins and the granzyme family of proteases to induce death in neighbouring cells. Granzyme B is a serine protease that cleaves at aspartate residues and can activate the caspase cascade [19]. It can also cleave caspase substrates directly, e.g. PARP [20] and Bid [21].

Here, we focus on the apoptotic roles for serine proteases described in experimental mammalian systems. We highlight current understanding of the role of serine proteases in apoptosis, drawing on research carried out by our own group.

2. Serine proteases and apoptosis

The serine proteases are a family of proteins involved in diverse biological processes, ranging from digestion, blood-clotting and inflammation, to development. Protein members are defined by a shared catalytic mechanism, requiring a highly reactive serine residue that participates in the formation of a transient acyl-enzyme complex. This complex is stabilised by the presence of histidine and aspartate residues within the active site, promoting cleavage of target substrates. Individual family members have been classified according to their substrate specificity with chymotrypsin, tryspin and elastase being proto-typical of three subgroups so far defined. Chymotrypsin preferentially cleaves at the carboxyl side of aromatic side chains such as phenylalanine. Trypsin cleaves at basic residues such as lysine, while elastase prefers smaller uncharged residues such as glycine [22].

Serine protease involvement in apoptosis was suggested in early investigations into the mechanism of TNF-mediated cytotoxicity, where addition of serine protease inhibitors (SPI) prevented apoptosis induced in SK-MEL-109 melanoma cells [23]. Later, it was demonstrated that overexpression of plasminogen activator inhibitor type-2 (PAI-2), an inhibitor of serine protease activity, prevented TNF-induced apoptosis in HT-180 fibrosarcoma and HeLa cells [24]. In this instance, the serine protease urokinase-type plasminogen activator was not the target of PAI-2 [25]. The specific serine protease involved remains unidentified. A link between serine proteases and apoptosis was further established when the loading of active chymotrypsin and trypsin into tumour cells was shown to activate a cell death programme that bore the hallmarks of apoptosis [26].

Despite the fact that these early studies demonstrated a clear role for serine proteases in apoptosis, the number of specific serine proteases with apoptotic function since identified, remains limited. These include Omi/HtrA2 (high temperature requirement protein A2) [27–30], AP24 [31,32] and thrombin [33–35].

Omi, a mammalian homologue of the bacterial HtrA2, was first discovered as a protein released from mitochondria during the course of apoptosis. It exists as an inactive 50 kDa precursor protein. Recently, Omi has been described within the ER and the nucleus, indicating that it may function in more than one apoptotic pathway [27,36]. Omi contains a tryspin-like protease domain and in response to an apoptotic insult undergoes autocatalytic processing into an active 36 kDa form. It then translocates to the cytosol upon mitochondrial permeabilisation [27–30]. In the cytosol, Omi abrogates the anti-apoptotic function of XIAP (X chromosome-linked inhibitor of apoptosis protein) by directly binding Baculovirus IAP repeat (BIR) domains, through an N-terminal reaper motif [30,37]. Mutational studies have shown that deletion of the catalytic serine protease moiety results in partial inhibition of cell death, indicating a role in apoptosis in addition to its IAP-binding ability [29]. The exact mechanism through which the serine protease activity of Omi impinges on apoptosis remains to be elucidated and this may be aided by the recent development of an Omi-specific inhibitor that interferes with its proteolytic function [38]. Cell death induced by overexpression of Omi is not prevented by general caspase inhibition and can occur in apoptosis protease-activating factor 1 (Apaf-1) and caspase-9 null cells [27,30]. These data indicate that Omi can induce death via a caspase-independent mechanism and may also enhance caspase-mediated cell death through inhibition of XIAP activity, as illustrated in Fig. 1. It is worth noting that Omi may also participate in the general cellular response to stress, as indicated when kidney ischemia led to raised levels of Omi activity [39].

The apoptotic protein AP24 is a 24 kDa chymotrypsin-like protease that causes DNA fragmentation. This activity has been observed in the cytosol and nucleus of tumour cell lines undergoing apoptosis, from which it has been partially purified. AP24 is activated in response to a variety of stimuli including TNF, UV irradiation and chemotherapeutic agents [31,40]. Inhibition of protein synthesis does not affect AP24 activation indicating that the protein is expressed constitutively in either an active sequestered, or an inactive form, prior to exposure to an apoptotic stimulus [31,32,40]. AP24 induces DNA fragmentation indirectly by inactivating the SPI, leukocyte elastase inhibitor (LEI), through translational modification. This converts its inhibitor function to that of an endonuclease (L-DNase II). The resultant DNase II translocates to the nucleus and subsequently induces DNA degradation (see Fig. 1) [31,41]. Activation of AP24 is influenced by multiple signals. For example, overexpression of Bcl-2 inhibits AP24 activity in HL-60 cells. In addition, it requires both sphingomyelin and calcium/calmodulin-dependent kinase II (CaMKII) [31,32,40].

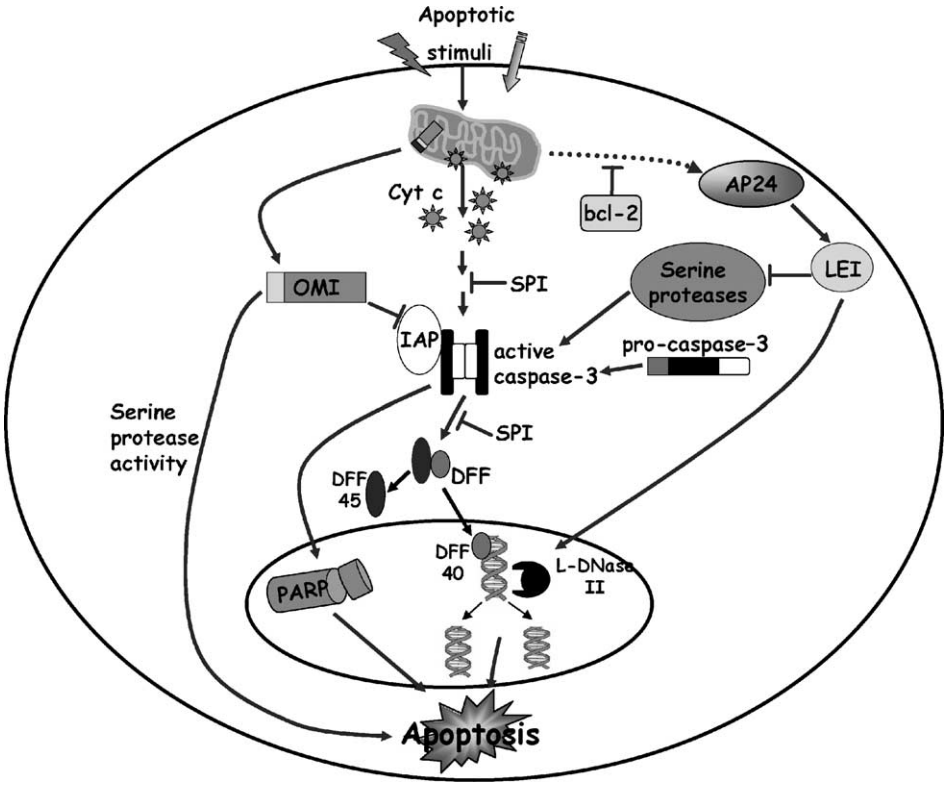

Fig. 1. Serine proteases implicated in apoptosis exert their effect at various stages of mitochondrial-mediated cell death. Omi, following release from mitochondria promotes apoptosis, by inhibiting the function of IAPs, or by directly cleaving downstream targets. AP24, by interfering with LEI-mediated inhibition of serine proteases, may promote caspase-3 activation. Alternatively, LEI, following interaction with AP24, participates in DNA degradation via its L-DNAse II activity. SPIs interfere with events downstream of mitochondrial release of cytochrome *c*, both prior to and post-caspase-3 activation, potentially inhibiting DFF45 and/or PARP cleavage. Dotted arrow indicates a suggested mode of activation of AP24.

Thrombin is a 37 kDa tryspin-like serine protease that is best known for its role in the blood coagulation cascade. More recently, it has been shown to exert a concentration-dependent dual effect on the apoptotic or mitogenic signalling pathways of a wide variety of tumour cell lines [34,35,42]. High concentrations of thrombin impair tumour cell growth *in vitro*, associated with cell cycle arrest and caspase-dependent apoptosis. Normal cells are less susceptible to thrombin than tumour cells giving thrombin, or its mimetics, potential as anticancer tools. In particular, neurons and astrocytes require 200-fold greater concentrations to induce death when compared to tumour cells [43,44] and low doses of thrombin protects them from stressors such as oxidative stress [45,46].

Roles for other serine proteases that are potentially involved in cell death are being investigated. They include the nuclear matrix-associated histone H1-specific enzyme that is induced by DNA damage and whose activity can be blocked by an inhibitor of chymotrypsin-like proteases in isolated thymocyte nuclei [47,48]. The mechanism appears to be sensitive to Bcl-2 overexpression and does not require caspase activation. A potential anti-apoptotic role for the serine protease myeloblastin has been indicated by the growth arrest of HL-60 cells following its down-regulation [49].

Our current understanding of the mechanism of action of individual apoptotis-associated serine proteases is still primitive. This is exacerbated by a lack of appropriate tools, such as antibodies, with which to investigate the role of specific serine proteases. However, increased understanding of the role of serine proteases in apoptosis has been aided through the production of inhibitors that target specific subfamilies of serine protease (see Fig. 1).

3. Catching the killer: use of SPIs to elucidate serine protease-mediated mechanisms of apoptosis

SPIs are substrate analogues that contain a reactive chloromethylketone group that covalently binds His 57 within the active site, thereby causing enzymatic inactivation. Their specificity comes from their resemblance to the preferred enzyme substrate. Commonly used inhibitors of serine proteases include chymotrypsin-like *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), the trypsin-like *N*-tosyl-L-lysine chloromethyl ketone (TLCK) and 3,4-dichloroisocoumarine (DCI). More recently, fluorochrome-labelled affinity ligands of serine proteases (FLISP) have been developed, including a fluorescent analog of TPCK, fluoresceinyl-L-phenylalanine chloromethyl ketone (FFCK), that we have used in our studies.

This ligand binds to the enzymatic centres of active chymotrypsin-like serine proteases [50,51]. In a manner similar to caspase inhibitors, the use of SPIs has highlighted the importance of serine proteases in apoptosis. Work carried out by Fearnhead *et al.* [52] implied that multiple serine proteases can function at different stages of an apoptotic programme [49]. TLCK prevented all features of apoptosis in rat thymocyte death induced by a range of agents, whereas, TPCK only prevented the terminal biochemical and morphological features of apoptosis, suggesting a nuclear target for some serine proteases [52]. Using the TPCK and TLCK inhibitors, we have deduced that staurosporine-induced apoptotic morphology and DNA fragmentation in HL-60 and Jurkat cells is a chymotrypsin-like protease-mediated event, while tryspin-like proteases do not appear to play a role.[1] FFCK, in conjunction with antibodies to fluorescein was then used to identify two putative apoptosis-associated serine proteases, termed ASP-1 and -2, activated in response to staurosporine. ASP-1 is a 60-kDa constitutively active protein that is further stimulated following staurosporine treatment, while ASP-2 (50 kDa) activation is detected only in staurosporine-treated cultures (see footnote 1). The existence of constitutively active serine proteases is not without precedent, as a tryspin-like protease has been shown to be active in rat thymocytes under normal conditions [52].

The relationship between serine proteases and caspases appears to be cell- and stimulus type-dependent. Serine proteases may act upstream or downstream of caspases, or alternatively in parallel signalling pathways. For example, hypoxia-induced caspase activation in rat kidney proximal tubule cells is suppressed by SPIs [53] indicating that caspases may act as downstream targets and/or mediators of serine proteases. In HL-60 cells treated with TNF, the converse has been shown, where caspases activate serine proteases [54]. In our studies of sts-induced apoptosis in Jurkat cells, we have proposed a model where caspases act upstream of serine proteases (see footnote 1). Serine protease and caspase-mediated apoptotic mechanisms can be activated simultaneously within the cell, but may have fundamentally different roles. For example, staurosporine-induced apoptotic features in HL-60 cells and buprenorphine-induced apoptosis in nerve cells can be prevented by administration of TPCK, but not by a pan-specific caspase inhibitor [10,15].

Serine proteases most likely function at intermediate and/or late stages of apoptosis. In our studies of HL-60 cells, we have observed that TPCK does not prevent staurosporine-induced cytochrome *c* release (see footnote 1). In agreement with this, TPCK has been shown not to affect hypoxia-induced cytochrome *c* release in proximal tubule cells [53]. Taken together TPCK is likely to affect events downstream of the mitochondria, such as cytochrome *c*-mediated procaspase-9 activation.

Given the pan-specificity of the SPIs being used in present studies, it is possible that several proteases may be targeted simultaneously. However, preliminary results from our laboratory indicate that ASP-1 and -2 are activated during the staurosporine-induced cell death programme, and further work is underway in an effort to identify and characterise these proteins.

4. Conclusions

Development of novel and effective therapeutic agents depends on a clear understanding of the nature of all apoptosis programmes. The body of evidence supporting a function for serine proteases in caspase-dependent and -independent apoptotic pathways is growing. However, further clarification of their sites of action in apoptotic cascades is required. The cloning and isolation of apoptotic-associated serine proteases will contribute significantly to the resolution of apoptotic pathways, not least because of the ability to generate useful tools like antibodies, or short interference RNAs. We focused on the involvement of serine proteases in staurosporine-induced apoptosis. Given that analogues of staurosporine are currently being investigated as potential chemotherapeutic agents, the additional data provided will aid the development of novel therapeutic targets or points of intervention.

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