Lysosomes in cell death

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For many years apoptosis research has focused on caspases and their putative role as sole executioners of programmed cell death. Accumulating information now suggests that lysosomal cathepsins are also pivotally involved in this process, especially in pathological conditions. In particular, the role of lysosomes and lysosomal enzymes in initiation and execution of the apoptotic program has become clear in several models, to the point that the existence of a 'lysosomal pathway of apoptosis' is now generally accepted. This pathway of apoptosis can be activated by death receptors, lipid mediators, and photodamage. Lysosomal proteases can be released from the lysosomes into the cytosol, where they contribute to the apoptotic cascade upstream of mitochondria. This review focuses on the players and the molecular mechanisms involved in the lysosomal pathway of apoptosis as well as on the importance of this pathway in development and pathology.

Keywords: cathepsins; Spi2A; TNFα

Introduction

For many years, lysosomes have been thought to be solely involved in necrotic and autophagic cell death, with their role in apoptosis being limited to the digestion of engulfed apoptotic bodies (Ferri and Kroemer, 2001a; Leist and Jaattela, 2001a). Likewise, lysosomal protease functions were believed to be limited to nonspecific intracellular protein degradation occurring within the lysosome. These concepts now seem to be outdated by a growing body of evidence that strongly points to a role of lysosomes in apoptosis that goes beyond that of simple 'garbage disposals'. Indeed, partial lysosomal permeabilization with subsequent release of proteolytic enzymes into the cytosol, and their active contribution to the signaling pathways, has been recently described in several models of apoptosis. This phenomenon is so widely recognized that the 'lysosomal pathway of apoptosis' is now accepted terminology. The key-factor in determining the type of cell death (necrosis vs apoptosis) mediated by lysosomal enzymes seems to be the magnitude of lysosomal permeabilization, and, consequently, the amount of proteolytic enzymes released into the cytosol (Li *et al*., 2000). A complete breakdown of the organelle with release of high concentrations of lysosomal enzymes into the cytosol results in unregulated necrosis, whereas partial, selective permeabilization triggers apoptosis (Bursch, 2001; Turk *et al*., 2002). In both instances, the amount of released enzymes is sufficient to overcome the protective barrier of the endogenous inhibitors present in the cytosol (i.e. cystatins), which usually prevent potential damage from the spontaneous leakage of lysosomal enzymes naturally occurring through the lysosomal membrane (Berg *et al*., 1995; Claus *et al*., 1998). Once in the cytosol, lysosomal enzymes can contribute to the execution of the apoptotic program either by direct cleavage of key cellular substrates, or by acting in concert with the caspases in the signaling pathway (Leist and Jaattela, 2001a, b). However, the precise mechanisms by which lysosomes are involved in apoptosis are still largely unknown and currently under intense investigation. The most recent findings regarding the role of lysosomal proteases in apoptosis are described below.

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When does the lysosomal pathway of cell death occur?

Lysosomal enzymes were among the first proteases to be associated with programmed cell death, for example, in the tadpole tail during the 1960s. However, this line of research has not been actively perpetuated into the modern age of apoptosis. While it is firmly established that autophagy, and herewith lysosomes, plays a role in developmental cell death, the evidence for a pivotal role of individual cathepsins in developmental apoptosis is poor or negative. Unlike many other pathways of apoptosis, lysosomal cathepsins do not appear to contribute to developmental or physiologic apoptosis of the central nervous system (CNS), heart, liver, and limbs. In contrast, various caspase knockout mice show developmental defects in these tissues (Los *et al*., 1999). The lysosomal pathway of apoptosis has been identified primarily in pathological situations. For instance, cathepsins have been implicated in CNS apoptosis following ischemia and during neurodegenerative processes (Yamashima *et al*., 1998; Tsuchiya *et al*., 1999; Lieuallen *et al*., 2001; Houseweart *et al*., 2003a). In the liver, a lysosomal-mediated pathway of apoptosis has been found to contribute to TNF-α signaling, liver

damage following bile duct obstruction, and cold-ischemia/warm-reperfusion injury (Guicciardi *et al*., 2000; Guicciardi *et al*., 2001; Canbay *et al*., 2003). Another pathological situation favoring the activation of the lysosomal pathway is tumorigenic transformation of cells. Many tumors express considerably increased amounts of cathepsins and the TNF-dependent apoptosis pathway in tumor cells is often particularly cathepsin B dependent (Foghsgaard *et al*., 2002; Fehrenbacher *et al*., unpublished).

Photodynamic therapy, a process employing UV-light and photosensitizers to kill cancer cells, as well as accumulation of lysosomotropic agents within the organelle, also triggers the lysosomal pathway of cell death (Boya *et al*., 2003a, b; Cirman *et al*., 2004; Li *et al*., 2003). As this pathway becomes better understood, its role in additional pathologies will likely become apparent.

Role of the lysosomal pathway of cell death in cancer biology

Many lysosomal enzymes, including cathepsins, are overexpressed in cancer (Koblinski *et al*., 2000). For example, increased expression and activity of cathepsin B and/or cathepsin D have been observed in breast (Castiglioni *et al*., 1994), colorectal (Campo *et al*., 1994; Meyer *et al*., 1997), gastric (Watanabe *et al*., 1989), lung (Sukoh *et al*., 1994), prostate (Sinha *et al*., 1995), and thyroid cancers (Shuja and Murnane, 1996), as well as in different brain tumors, including gliomas and meningiomas (Rempel *et al*., 1994; Levicar *et al*., 2003), and the magnitude of expression has often prognostic significance. Many authors have suggested that enhanced secretion of these proteases by exocytosis promotes cancer growth, invasion, and metastasis by promoting degradation of extracellular connective matrices and angiogenesis (Koblinski *et al*., 2000). Indeed, inhibition of cathepsin B by synthetic cysteine protease inhibitors has been shown to effectively reduce the invasiveness of glioblastoma cells (Demchik *et al*., 1999) and breast cancer cell lines (Xing *et al*., 1998). However, the role of the lysosomal pathway of cell death in transformed cells remains largely unexplored. Tumor cell death is often caspase-independent (Foghsgaard *et al*., 2001; Leist and Jaattela, 2001a), due to acquired defects in the classic caspase-dependent pathways of apoptosis during the malignant transformation, and these alternative pathways may represent potential targets for cancer therapy (Jaattela, 2002). For example, enhancing the lysosomal cell death pathway may be a therapeutic strategy to overcome the blocks in caspase-dependent cell death. Indeed, the topoisomerase inhibitor camptothecin induces apoptosis in hepatocellular carcinoma cells via a cathepsin D/B-mediated pathway (Roberts *et al*., 1999). Moreover, cathepsin B has been shown to play a dominant role in executing the apoptotic program in several tumor cell lines (Foghsgaard *et al*., 2001). Therefore, it seems that cathepsin B may play two opposing roles in malignancy: as an executioner of apoptosis in cytotoxic signaling cascades and as a mediator of tumor invasion.

Oncogenic activation sensitizes cancer cells to various apoptotic stimuli. Transformation of cells, for example by Ras, strongly affects processing, trafficking and subcellular localization of lysosomal enzymes (Nishimura *et al*., 1998; Demoz *et al*., 1999), and it is conceivable that cathepsins might take part in the transformation-induced death of tumor cells. Indeed, Ras triggers cell death of human cancer cells by activation of a caspase-independent cell death program (Chi *et al*., 1999), which may involve lysosomal proteases.

The overexpression of cathepsins often observed in tumors should render cancer cells more susceptible to cell death via the lysosomal pathway. It should be possible to exploit this pathway to differentially modulate susceptibility of cancer cells to cell death. For example, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) preferentially kills tumor cells both *in vitro* and *in vivo* (Ashkenazi *et al*., 1999; Walczak *et al*., 1999). Although the underlying mechanism of TRAIL selectivity toward tumor cells remains unclear, TRAIL, which can trigger lysosomal cell death (Foghsgaard *et al*., 2001), might induce apoptosis in transformed cells via the lysosomal pathway. These concepts merit further investigation.

Mechanisms of lysosomal permeabilization

Vesicle permeabilization in apoptosis is selective. Whereas lysosomes are permeabilized, endocytic vesicles are not, as the absence of total vesicular breakdown implies the existence of a regulatable process. Consistent with these observations, even lysosomal breakdown is partial and/or selective in this apoptotic pathway. Indeed, two manuscripts have reported that in cells with lysosomes labeled with LysoTracker red, a fluorescent dye that loads predominantly into lysosomes, release of the dye appears to occur in only a subpopulation of lysosomes (Werneburg *et al*., 2002; Madge *et al*., 2003). Whether these observations represent a time-dependent phenomenon, partial release, or targeting of mediators to a distinct subpopulation of 'killer lysosomes' remains unclear.

It is unquestionable that lysosomal enzymes, in order to participate to the apoptotic process, need to be translocated to the cytosol (and, possibly, to the nucleus) where most of the cellular proteins degraded during apoptosis are found. Therefore, the first logical question regards the mechanism(s) leading to lysosomal permeabilization. How is it possible to achieve a partial, selective permeabilization that allows the translocation of only a moderate amount of lysosomal enzyme to the cytosol without risking a complete breakdown of the organelle and the induction of a necrotic process? And what are the molecular pathways signaling lysosomal permeabilization after DNA damage or the engagement of death receptors, both models of apoptosis often associated with lysosomal dysfunction? Several studies have tried to answer these questions, generating numerous intriguing hypotheses. One of the mechanisms recently proposed suggests that intracellular sphingosine

may be responsible for lysosomal dysfunction. Indeed, sphingosine generation is increased in certain models of apoptosis, such as after TNF-α treatment (Schutze *et al*., 1999), which has been associated with lysosomal permeabilization (Guicciardi *et al*., 2000). Owing to its lysosomotropic properties, sphingosine accumulates within the lysosomes, where it can permeabilize the membrane via a detergent mechanism, and facilitate the relocation of lysosomal enzymes to the cytosol (Kagedal *et al*., 2001b). When Jurkat cells and J774 cells were incubated with low concentrations of sphingosine, partial lysosomal rupture was observed in a dose-dependent manner, which preceded caspase activation and mitochondrial dysfunctions. In contrast, high concentrations of sphingosine caused extensive lysosomal rupture and necrosis, without involvement of caspases (Kagedal *et al*., 2001b). Consistently, sphingosine has been found to induce lysosomal dysfunction in primary mouse hepatocytes as well as to permeabilize isolated hepatic lysosomes *in vitro* (Werneburg *et al*., 2002). Moreover, reduction of sphingosine generation by overexpression of a dominant-negative factor associated with neutral sphingomyelinase (FAN), an adaptor protein involved in TNF-R1-mediated activation of neutral sphingomyelinase, prevents lysosomal destabilization (Werneburg *et al*., unpublished). In the cell, sphingosine is generated from the processing of ceramide, which has also been proposed as a mediator of lysosomal permeabilization. Although exogenous C6 ceramide does not permeabilize lysosomes *in vitro* (Werneburg *et al*., 2002), ceramide generated by the endolysosomal acid sphingomyelinase has been shown to bind to and activate the lysosomal aspartate protease cathepsin D. The direct binding of ceramide to cathepsin D results in the processing of the preprocathepsin D to the enzymatically active form of the protease, which, in turn, is responsible for the proteolytic activation of other lysosomal proteins (Heinrich *et al*., 1999).

Another possible mechanism of lysosomal permeabilization involves the generation of reactive oxygen species (ROS). Lysosomal destabilization has been recognized as a feature of oxidative stress-induced cell damage (Zdolsek *et al*., 1993), and ROS can induce lysosomal leakage, possibly as a consequence of intralysosomal iron-catalysed oxidative processes (Antunes *et al*., 2001; Dare *et al*., 2001; Persson *et al*., 2003). Many apoptotic stimuli that induce lysosomal permeabilization, such as TNF-α (Manna *et al*., 1998) and lipopolysaccharide (Wang *et al*., 1997), as well as light-activated photosensitizing lysosomotropic agents (Ferri and Kroemer, 2001b), also induce generation of intracellular H_2O_2 during apoptosis. Moreover, ROS contribute to neuronal apoptosis during aging, which seems to be associated with lysosomal dysfunction (Bahr and Bendiske, 2002; Raha and Robinson, 2000). Experimental evidence suggests that ROS-induced lysosomal permeabilization usually precedes mitochondrial dysfunction (Roberg and Ollinger, 1998; Roberg *et al*., 1999; Guicciardi *et al*., 2000); however, lysosomal enzymes have been found to act on mitochondria and promote mitochondrial ROS generation, therefore, creating a feedback loop that can lead to more lysosomal permeabilization (Zhao *et al*., 2003). Finally, recent studies suggest that short-term exposure to low concentrations of H_2O_2 may induce lysosomal rupture indirectly by activation of phospholipase A2 (PLA2), which would cause a progressive destabilization of the membranes of intracellular organelles, including lysosomes and mitochondria, by degradation of the membrane phospholipids (Zhao *et al*., 2001a). Consistently, activation of PLA2 has been reported during apoptosis triggered by TNF-α and oxidative stress (Jaattela *et al*., 1995; Suzuki *et al*., 1997). ROS-stimulation of PLA2 may also depend on the oxidative stress-mediated activation of Ca^{2+} signaling and/or protein phosphorylation. (Suzuki *et al*., 1997). Several oxidants have been shown to increase cytosolic Ca^{2+} by mobilizing it from the endoplasmic reticulum, the extracellular space, or the mitochondria, in response to oxidative stress influence on Ca^{2+} pumps, channels, and transporters. Indeed, oxidant-induced lysosomal permeabilization can be mediated by an increase in intracellular free Ca^{2+} (Smolen *et al*., 1986).

An intriguing explanation on how lysosomes are permeabilized would be the translocation of proapoptotic members of the Bcl-2 family to the lysosomal membrane after apoptotic stimuli, where they would induce the formation of pores with a mechanism similar to what observed in the outer mitochondrial membrane. Although no direct evidence supporting this hypothesis are currently available, recent studies have shown that the phosphorylated, active form of Bcl-2 blocks oxidant-induced apoptosis, at least in part, by stabilizing lysosomes (Zhao *et al*., 2000, 2001b). The authors conclude that Bcl-2 may prevent activation of PLA2, but it is certainly tempting to speculate that Bcl-2 may also exert its protective effect by antagonizing proapoptotic Bcl-2-like proteins (i.e. Bax or Bid) that might translocate to the lysosomal membrane to induce membrane permeabilization.

Besides extralysosomal signals, it is clear that many events occurring within the lysosome (i.e. iron-catalysed oxidative reactions) are important to promote lysosomal permeabilization. For example, in a model of TNF-α-induced hepatocyte apoptosis, the absence of the lysosomal cysteine protease, cathepsin B, confers increased resistance to lysosomal permeabilization, suggesting that lysosomal enzymes are not only passively released but can also participate to the process of membrane destabilization from within the lysosome (Werneburg *et al*., 2002). These findings are supported by the evidence that the antichymotripsin-like serpin, serine protease inhibitor 2A (Spi2A), which effectively inhibits cathepsin B, also partially reduces lysosomal breakdown (Liu *et al*., 2003). This latter study also demonstrates that NF-κB inhibits the lysosomal pathway of cell death by inducing Spi2A. Finally, an increase in lysosomal size often observed during the early stages of apoptosis (i.e. after TNF-α-treatment), may determine their susceptibility to rupture; indeed, larger lysosomes may breakdown more efficiently with stress (Ono *et al*., 2003). These observations suggest that the

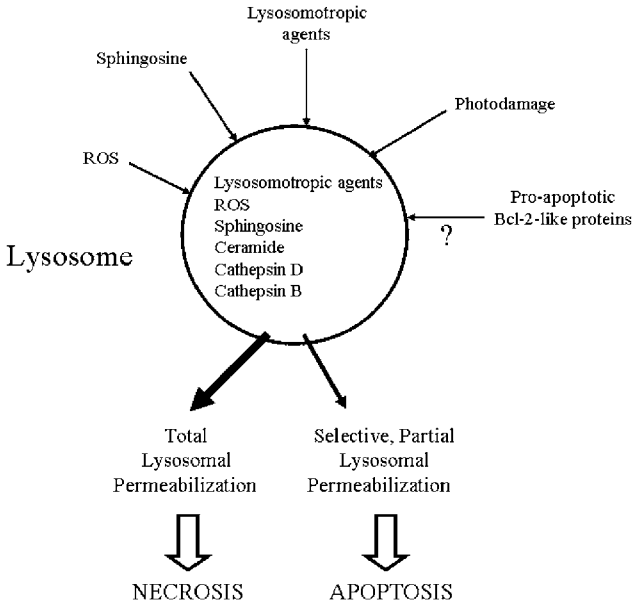

Figure 1 Mechanisms of lysosomal permeabilization. Schematic representation of the current concepts of lysosomal permeabilization. Lysosomes undergo membrane permeabilization in response to different stimuli including lysosomotropic agents photodamage ROS and lipid-mediators such as sphingosine. Intralysosomal generation of ceramide from activated acid sphingomyelinase has also been associated with activation of cathepsin D and lysosomal permeabilization. The accumulation of these agents within the lysosome, and/or intralysosomal proteolytic reactions mediated by enzymes such as cathepsin B and L, result in membrane damage and release of lysosomal proteases into the cytosol. Massive lysosomal breakdown results in cellular necrosis, whereas a partial and selective lysosomal permeabilization is associated with induction of apoptosis. See text for details

role of oncotic processes also warrants study as a mechanism of lysosomal destabilization.

In conclusion, it appears that multiple mechanisms can contribute to lysosomal permeabilization, likely in a stimulus- and cell type-dependent fashion (Figure 1). However, some common findings can be recognized in each instance, such as that the release of lysosomal enzymes does not appear to be mediated by specific transporters, and it is not selective for specific proteases. Therefore, it is conceivable that all lysosomal proteases, especially small-size cathepsins, if not membrane-bound, will be released into the cytosol after lysosomal permeabilization, the amount of which will be proportional to the extent of the membrane damage.

Lysosomal proteases implicated in the apoptotic pathway

The lysosomal cysteine proteases, also called cathepsins, represent the largest group of proteolytic enzymes in the lysosomes. Among the 11 human cathepsins known (cathepsin B, H, L, S, F, K, C, W, X, V, and O), cathepsin B and L are ubiquitously expressed and are the most abundant in the lysosomes, together with cathepsin D, the only lysosomal aspartic protease (Turk *et al*., 2002). The processing of the proenzymes into the catalytically active form usually occurs within the lysosome (Ishidoh and Kominami, 2002); therefore, the enzymes escaping from the lysosome are in their active, monomeric form and generally do not require any other conformational change. Besides their role in intralysosomal protein degradation, cathepsins also have other physiologic functions in the cells (Chapman *et al*., 1997). Cathepsin B has been involved in processing and release of other proteins (Turk *et al*., 2000; Foghsgaard *et al*., 2002), as well as in the response to exogenous antigens (Katunuma *et al*., 2003). Both cathepsin L and S play an important role in antigen processing and presentation (Chapman *et al*., 1997), and cathepsin L is also involved in keratinocyte and melanocyte differentiation during hair follicle morphogenesis (Reinheckel *et al*., 2001; Tobin *et al*., 2002). Cathepsin K contributes to bone remodeling (Chapman *et al*., 1997) and cathepsin D is known to participate in protein targeting (Cantor and Kornfeld, 1992). Despite their high concentrations and their fundamental role in protein turnover, none of these proteases seem to be essential for an effective intralysosomal protein degradation, as demonstrated by the absence of a substantial phenotype at this regard in single knockout mice lacking either cathepsin B, or L, or D (Saftig *et al*., 1995; Reinheckel *et al*., 2001).

Following lysosomal permeabilization, several proteases are released into the cytosol, some of which have been implicated in apoptotic processes (Roberts *et al*., 1999; Turk *et al*., 2000, 2002). One of the most controversial issues regarding the role of lysosomal proteases outside the lysosomes is their actual ability to function at neutral pH. A series of elegant studies showed that, although lysosomal enzymes have an activity optimum at acidic pH, lysosomal cysteine proteases are stable and active at neutral pH for a time that ranges from a few minutes to an hour or more, confirming their destructive potential in cellular compartments other than the lysosomes (Turk *et al*., 1993, 1995, 2000). Cathepsin B, which is one of the most stable proteases at physiologic pH, is essential in different models of apoptosis, including bile acid-induced hepatocyte apoptosis (Roberts *et al*., 1997; Jones *et al*., 1998; Faubion *et al*., 1999; Canbay *et al*., 2003), TNF-α-induced apoptosis of primary hepatocytes and tumor cells (Guicciardi *et al*., 2000, 2001; Foghsgaard *et al*., 2001), neuronal apoptosis in Unverricht-Lundborg progressive myoclonus epilepsy (EPM1) (Houseweart *et al*., 2003a) and after brain ischemia (Yamashima *et al*., 1998; Tsuchiya *et al*., 1999), and PC12 cell apoptosis after serum deprivation (Shibata *et al*., 1998). Cathepsin D has been implicated in apoptosis induced by staurosporine (Bidere *et al*., 2003; Johansson *et al*., 2003), interferon-γ, Fas/CD95/APO-1 and TNF-α (Deiss *et al*., 1996; Demoz *et al*., 2002), oxidative stress (Roberg and Ollinger, 1998; Roberg *et al*., 1999; Ollinger, 2000; Kagedal *et al*., 2001a), sphingosine (Kagedal *et al*., 2001b), and p53 (Wu *et al*., 1998). Finally, cathepsin L, the least stable of the lysosomal cysteine proteases at neutral pH, is an important

regulator of ultraviolet-induced apoptosis of keratinocytes (Tobin *et al*., 2002; Welss *et al*., 2003), and etoposide-induced apoptosis of P39 cells (Hishita *et al*., 2001). Much less is known about the role of other cathepsins in apoptosis.

Mechanisms of lysosome-mediated apoptosis

At this time, an impressive body of evidence supports a key role of lysosomes in apoptosis, but the mechanisms by which this occurs, and the possible, functional relationships and/or cross-talks with other known apoptotic pathways remain largely unclear. In many instances, lysosomal permeabilization appears to be an early event in the apoptotic cascade, preceding other hallmarks of apoptosis like destabilization of mitochondria and caspase activation (Roberg *et al*., 1999; Guicciardi *et al*., 2000; Li *et al*., 2000; Bidere *et al*., 2003; Boya *et al*., 2003a, b; Liu *et al*., 2003). Thus, proteases released from the lysosomal compartment seem to be able to trigger initiating events of apoptosis. It is not clear what determines the relative contribution of lysosomal enzymes vs caspases in initiation and execution of apoptosis. Some models of apoptosis appear to be dependent exclusively on cathepsins (Foghsgaard *et al*., 2001; Leist and Jaattela, 2001a; Boya *et al*., 2003a), whereas others rely on both caspases and cathepsins for their initiation and execution (Faubion *et al*., 1999; Guicciardi *et al*., 2000). The latter might be the case also for a number of models believed to be solely caspase-dependent. Indeed, misleading data have been generated from studies utilizing pharmacological inhibitors of caspases, which, at the concentrations generally used, are now known to inhibit also other proteases, including cathepsins (Faubion *et al*., 1999; Foghsgaard *et al*., 2001).

But how do lysosomal proteases promote apoptosis after being released in the cytosol? A possible mechanism would be via direct cleavage and activation of caspases. Indeed, cathepsin B has been shown to process procaspase-1 and -11 *in vitro* (Schotte *et al*., 1998; Vancompernolle *et al*., 1998). However, both caspase-1 and -11 are mainly involved in inflammatory responses and have a very limited, if any, role in the apoptotic process. On the other hand, main effector caspases, such as procaspase-3, -6, -7, as well as caspase-2, -9, -12, and -14, have been proven to be poor substrates for various cathepsins *in vitro*, including cathepsin B, H, K, L, S, and, X (Vancompernolle *et al*., 1998; Stoka *et al*., 2001). Nonetheless, a human cathepsin L-like protease (Ishisaka *et al*., 1999, 2001) and the trypanosomal cysteine cathepsin, cruzipain (Stoka *et al*., 2001), structurally related to cathepsin L, have been shown to cleave procaspase-3/-7 in cell-free systems and cell culture. Cathepsin L has also been proposed to trigger activation of procaspase-3 indirectly, via activation of another lysosomal, membrane-bound protease, which has not been identified in the study (Katunuma *et al*., 2001). Moreover, although cathepsin B cleavage of procaspase-2 *in vitro* is less effective than the one by active caspase-2 during the auto-activation process, yet the generated fragment shows significant mitochondrial cytochrome *c*-releasing activity, suggesting that this pathway may have biological importance (Guicciardi *et al*., unpublished). Thus, direct activation of caspases by cathepsins cannot be ruled out, at least in some models. However, a growing body of evidence suggests that lysosomal proteases rather promote apoptosis by acting on mitochondria to induce mitochondrial dysfunction, associated with release of proapoptogenic factors (Guicciardi *et al*., 2000; Bidere *et al*., 2003; Boya *et al*., 2003a, b; Cirman *et al*., 2004; Zhao *et al*., 2003). ROS generated following the mitochondrial damage, and possibly other factors of mitochondrial origin, could also feedback to the lysosome, resulting in further lysosomal breakdown and exacerbation of the apoptotic cascade (Zhao *et al*., 2003). The release of lysosomal proteases may cause mitochondrial dysfunction directly (Zhao *et al*., 2003) or indirectly (Bidere *et al*., 2003; Boya *et al*., 2003a, b; Cirman *et al*., 2004; Guicciardi *et al*., 2000; Stoka *et al*., 2001). Addition of purified cathepsin B or D to mitochondria *in vitro* results in substantial ROS generation (Zhao *et al*., 2003), although cathepsin B *per se* induces only minimal release of cytochrome *c* from the mitochondria (Guicciardi *et al*., 2000). In contrast, when isolated mitochondria are incubated with purified cathepsin B in the presence of cytolic extracts, a substantial release of cytochrome *c* is detected, suggesting that one or more cytosolic factor(s) activated by cathepsin B mediate mitochondrial dysfunction (Guicciardi *et al*., 2000). A very plausible candidate would be a cytosolic member of the Bcl-2 family, which is known to control the mitochondrial checkpoint of apoptosis in different models. Bid is cleaved and translocated to the mitochondria following lysosomal disruption by lysosomotropic agents (Cirman *et al*., 2004). Moreover, lysosomal extracts have been shown to cleave Bid *in vitro* to generate a fragment with cytochrome *c*-releasing activity (Stoka *et al*., 2001). The cleavage site of lysosomal enzymes within Bid has been mapped at Arg^{65}, a different site compared to the one cleaved by caspase-8 (Asp^{59}) or granzyme B (Asp^{75}), but located in the same flexible loop (Stoka *et al*., 2001). Further studies confirmed that several purified cathepsins, such as B, K, L, H, and S, but not cathepsin D, C, and X, cleave Bid at Arg^{65}, with the exception of cathepsin H, which has a unique cleavage site at Arg^{71} (Cirman *et al*., 2004). However, proteolytic cleavage at Arg^{71} is not observed with lysosomal extracts, thus a role of cathepsin H in Bid cleavage *in vivo* seems unlikely (Stoka *et al*., 2001; Cirman *et al*., 2004). Considering the higher concentration of cathepsin B and L compared to other lysosomal cysteine proteases, and the fact that cathepsin B is more stable than cathepsin L at physiologic pH, and is less effectively inhibited by cystatin B, the major endogenous inhibitor of cysteine cathepsins (Turk *et al*., 1997), cathepsin B seems to play a key role in lysosome-mediated Bid cleavage. Nevertheless, data are still controversial at this regard, as Bid is cleaved to the

same extent in wild-type and cathepsin B-deficient hepatocytes *in vitro* and *in vivo* after TNF-α-treatment, suggesting that Bid is cleaved either by multiple lysosomal enzymes or by non-lysosomal enzymes (i.e. caspase-8) in this apoptotic pathway (Guicciardi *et al*., unpublished). The data are consistent with a report showing that pharmacological inhibition of cathepsin B, L, and D activities does not suppress Bid cleavage, or procaspase-9 and -3 activation following lysosomal photodamage in murine hepatoma 1c1c7 cells, suggesting that other lysosomal proteases might be responsible for Bid cleavage (Reiners *et al*., 2002). Moreover, a study employing a model of cystatin B/Bid double knockout mice showed that the absence of Bid did not rescue the neurological phenotype caused by constitutional activation of cathepsins (due to the lack of the endogenous inhibitor cystatin B), suggesting that Bid is not required for cathepsin-mediated apoptosis in this *in vivo* model of cell death (Houseweart *et al*., 2003b). Finally, Bid does not contribute to apoptosis induced by the lysosomotropic photosensitizers ciprofloxacin or norfloxacin, as Bid-deficient mouse embryonic fibroblasts are not protected against mitochondrial membrane permeabilization (Boya *et al*., 2003a). Recent studies demonstrated that lysosomal enzymes can also cleave and activate another cytosolic member of the Bcl-2 family involved in mitochondrial destabilization, Bax. A study in human lymphocytes showed that lysosomal proteases translocate into the cytosol during the early phase of staurosporine-induced apoptosis preceding mitochondrial permeabilization. Using specific pharmacological inhibitors and the siRNA technique, the authors demonstrated that cytosolic cathepsin D, but not cathepsin B or L, triggers Bax activation and relocation to the mitochondria, resulting in release of the proapoptogenic factor apoptosis-inducing factor (AIF), and the apoptotic phenotype (Bidere *et al*., 2003). The requirement of Bax and/or Bak in lysosome-mediated apoptosis has been further confirmed by demonstrating that mouse embryonic fibroblasts from Bax/Bak double knockout are resistant against mitochondrial membrane permeabilization induced by the lysosomotropic agents ciprofloxacin, norfloxacin, and hydroxychloroquine (Boya *et al*., 2003a, b). However, the absence of Bax and Bak in the double knockout cells does not impair lysosomal permeabilization, suggesting that lysosomal enzymes are not able to directly induce mitochondria dysfunction, but require the activation of a Bax/Bak-dependent pathway (Boya *et al*., 2003b).

In conclusion, it appears that, consistently with their relatively low substrate specificity, lysosomal proteases trigger apoptosis not via a single specific pathway, but rather multiple molecular pathways, which often integrate with the ones controlled by traditional mediators of apoptosis, like caspases and Bcl-2 family proteins (Figure 2). As the key role of lysosomes in apoptosis is becoming increasingly evident, many other molecular pathways mediated by lysosomal enzymes are likely to be described in the years to come.

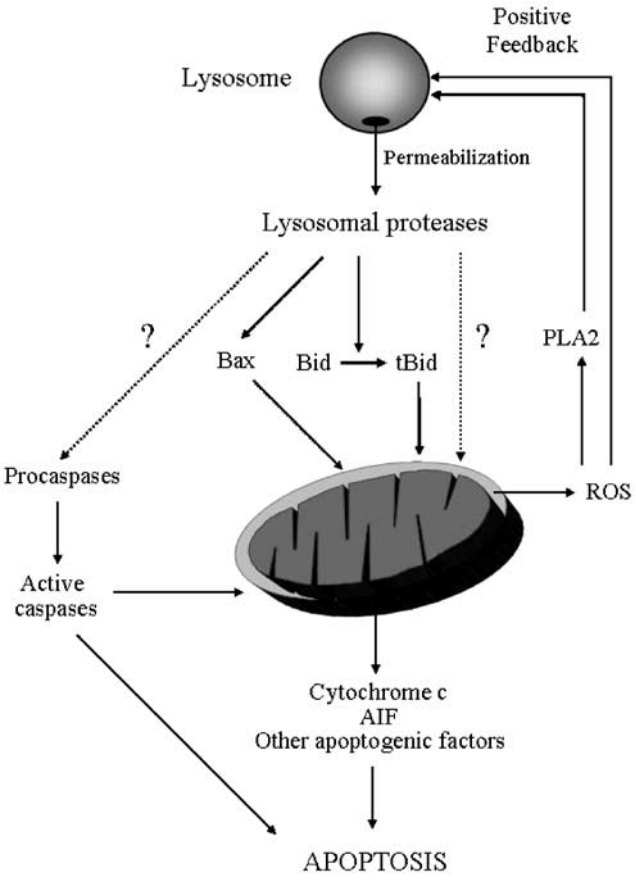

Figure 2 Lysosome-mediated apoptotic signaling pathways. Schematic representation of proposed apoptotic signaling pathways activated by lysosomal proteases. Lysosomal extracts, as well as selected purified cathepsins, have been shown to cleave and activate the proapoptotic Bcl-2-like protein, Bid. Cathepsin D can trigger Bax activation and translocation to the mitochondria, where it induces the opening of pores on the outer mitochondrial membrane. Direct induction of mitochondrial damage as well as direct activation of procaspases by lysosomal enzymes have also been described, but the results are controversial and need further elucidation. Generation of ROS following mitochondrial dysfunction can feedback to the lysosome (directly or indirectly via activation of PLA2) to maximize lysosomal permeabilization and, in turn, mitochondrial damage. See text for details

Role of the lysosomal pathway of apoptosis in animal development and pathogenesis of diseases: lessons from knockout mice

The role of lysosomes in apoptosis merits attention for the possible implications in senescence and storage diseases (Cuervo and Dice, 2000; Bahr and Bendiske, 2002). As previously mentioned in this review, lysosomal cathepsins seem not to be essential during embryonic development. Generally knockout mice for single cathepsins develop normally and do not have any manifest phenotype at the time of birth that distinguish them from their wild-type littermates. The explanation for the lack of an abnormal phenotype is likely in the redundancy of the cathepsin pathways in the lysosomes and in their functional overlap, which ensure that the

proper intralysosomal protein degradation and protein turnover during embryonic development occur even in the absence of one protease. However, these mice can develop abnormalities later in life, suggesting that cathepsins play an important role in postnatal tissue homeostasis and pathophysiology. The most striking example comes from the cathepsin D knockout mice. These mice manifest a normal phenotype at birth, but die at postnatal day 26 ± 1 in a state of anorexia because of massive intestinal necrosis, thromboembolia, and lymphopenia (Saftig *et al*., 1995). They also manifest seizures and become blind near the terminal stage, with the histopathological appearance of storage of autophagosome/autolysosome-like bodies containing ceroid lipofuscin in the CNS neurons (Koike *et al*., 2000). However, whether the impairment of cathepsin D-mediated apoptotic pathways contributes somehow to the development of both the gastrointestinal and the neuronal pathologic phenotypes is not known, although it seems rather unlikely. Indeed, cathepsin D has been found to play an important role in various pathological conditions, the majority of which affects the CNS, where its contribution seems to be limited to alterations of normal proteolytic processes rather than induction of cell death (Banay-Schwartz *et al*., 1987; Matus and Green, 1987; Nakanishi *et al*., 1997). Nonetheless, studies employing cathepsin D-deficient cells, as well as specific pharmacological inhibitors, have demonstrated that cathepsin D is a central mediator of apoptosis induced by several stimuli, such as staurosporine, death receptor-activation, growth factor-deprivation, and oxidative stress (Roberg and Ollinger, 1998; Brunk and Svensson, 1999; Kagedal *et al*., 2001a; Johansson *et al*., 2003).

The cathepsin L knockout mice do not show any difference from their wild-type littemates, except for a slightly higher mortality rate upon weaning. However, they start to lose their fur at postnatal day 21, they regrow it thereafter, and continue to undergo periodic cycles of hair loss and re-growth throughout their life (Reinheckel *et al*., 2001). This phenomenon has been related to an impaired cathepsin L function in the skin, which results in alteration of hair follicle physiology, as well as in marked thickening of the interfollicular epidermis. The latter, however, appears to be due to the hyperproliferation of the cells in the basal layer of the epidermis, rather than to a reduced rate of cell death, suggesting that the role of cathepsin L in apoptosis is not relevant in this model (Roth *et al*., 2000). Moreover, cathepsin L knockout mice show a marked depletion of $CD4^+$ T lymphocytes likely due to an impaired positive selection of T cells in the thymus (Nakagawa *et al*., 1998), but once again the possible relationship between this phenotype and an impairment in cathepsin L-mediated apoptosis has not been verified. Indeed, although cathepsin L has been implicated in some experimental models of apoptosis (Hishita *et al*., 2001; Tobin *et al*., 2002; Welss *et al*., 2003), the importance of this phenomenon in the pathogenesis of human and animal diseases *in vivo* has never been established.

Similar to other cathepsins, cathepsin B is not essential for embryonic development, as cathepsin B knockout mice do not manifest any morphological, functional, or behavioral alteration (Reinheckel *et al*., 2001). However, cathepsin B is perhaps the only lysosomal protease whose role in apoptosis has been associated to pathological processes. Inhibition of cathepsin B significantly reduces hepatocyte apoptosis and liver injury in cholestasis (Canbay *et al*., 2003), TNF-α-mediated acute liver failure (Guicciardi *et al*., 2001), and nonalcoholic steatohepatitis (Feldstein *et al*., unpublished), strongly suggesting that cathepsin B-mediated apoptosis is a key player in the induction and progression of liver damage of different etiologies. Moreover, transformed fibroblasts derived from cathepsin B deficient mice are resistant to TNF-α-triggered apoptosis, while cathepsin B plays a relatively minor role in primary fibroblasts (Fehrenbacher *et al*., unpublished). This underlines the more dominant role of the lysosomal pathway in the pathological tumor situation. Cathepsin B has been implicated in other pathophysiological conditions (Chapman *et al*., 1997; Koblinski *et al*., 2000), including the onset of acute pancreatitis due to cathepsin B-mediated premature intracellular trypsinogen activation and acinar cell necrosis (Halangk *et al*., 2000). The contribution of cathepsin B-mediated apoptosis in these diseases, however, remains to be elucidated.

Whereas cathepsin L and cathepsin B single knockout mice manifest a rather mild phenotype, the simultaneous deficiency of these two functionally related proteases results in profound abnormalities. Combined deficiency of cathepsins B and L in mice is lethal during the second to fourth week of life, due to massive apoptosis of select neurons in the cerebral cortex, the cerebellar Purkinje and granule cell layers, resulting in severe brain atrophy (Felbor *et al*., 2002). Neurodegeneration is accompanied by accumulation of unique lysosomal bodies in large cortical neurons and by axonal enlargements, suggestive of impaired proteolysis (Felbor *et al*., 2002). It is not clear whether the increased neuronal apoptosis is directly caused by the absence of the two proteases, but, considering their proapoptotic role in other models, it appears that apoptosis might be a consequence of the intracellular proteolytic imbalance rather than a primary event. Regardless of the mechanisms involved, these data demonstrate the crucial role of cathepsins L and/or B in maintenance of the CNS.

Conclusions

Apoptosis is a crucial process in embryonic development, as well as in adult tissue homestasis, and uncontrolled increase or suppression of apoptosis always leads to disease pathogenesis. During apoptosis, activation of specific proteases results in degradation of cellular components and, ultimately, in the appearance of the characteristic morphological and biochemical

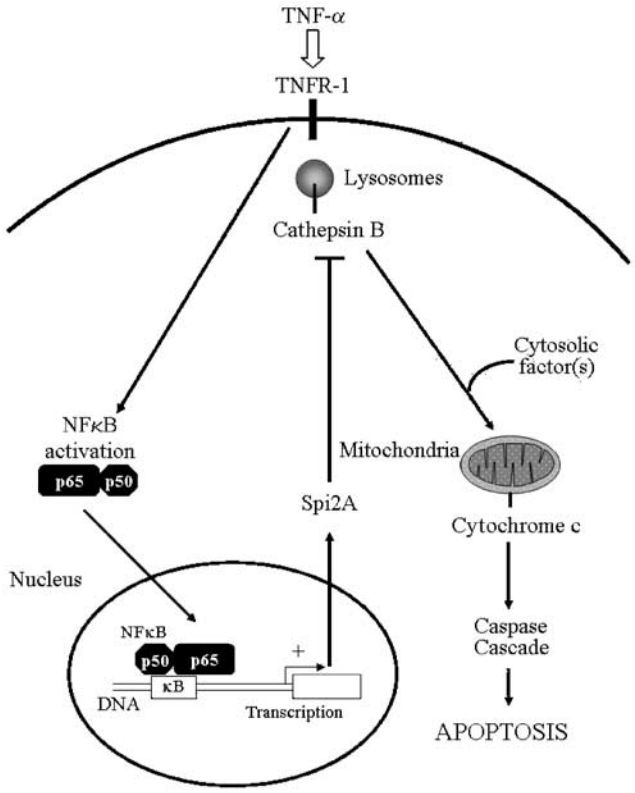

Figure 3 Inhibition of the TNF-α-induced lysosomal pathway of apoptosis by NF-κB. Engagement of TNF-R1 by TNF-α simultaneously activates: (i) a death pathway mediated by lysosomal permeabilization, release of cathepsin B into the cytosol, and mitochondrial dysfunction, and (ii) a survival pathway, mediated by the activation of the transcription factor NF-κB. NF-κB translocates to the nucleus, where it promotes the transcription of several antiapoptotic genes, including the serine protease inhibitor Spi2A, which potently inhibits cathepsin B activity, preventing mitochondrial dysfunction

features of the apoptotic cell. For many years it was believed that caspases were the only enzymes responsible for the proteolytic cascade in apoptosis, and an impressive amount of time and resources were employed in order to define their biochemistry and biological effects. While caspases certainly continue to play a central role in apoptosis, there is now a large body of evidence suggesting that other proteases, such as lysosomal proteases, calpains, and granzyme B, may be involved in the initiation and/or execution of the apoptotic program. Lysosomal permeabilization and release of proteolytic enzymes into the cytosol and nucleus have been described in several models of apoptosis, and many data support the hypothesis that this is generally a primary, early event, and not part of the late degenerative stage of cell death. These findings also suggest that proteases released from the lysosome are capable of triggering mitochondrial dysfunction with subsequent caspase activation and cellular demise. Interestingly, this pathway is inhibited by the transcription factor NF-κB, which strongly induces the cathepsin B inhibitor Spi2A (Liu *et al*., 2003). The inhibition of this pathway by NF-κB implies that this is a key pathway for death receptor-mediated apoptosis (Figure 3). The identification of the cellular substrates specifically cleaved by lysosomal proteases during apoptosis, as well as the mechanisms and signaling pathways that mediate lysosomal permeabilization, will represent an enormous advancement in the study of the apoptotic pathways and will have great potential for therapeutic application in diseases linked to lysosomal dysfunction.

Abbreviations

CNS, central nervous system; EPM1, progressive myoclonus epilepsy; FAN, factor associated with neutral sphingomyelinase; PLA2, phospholipase A2; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; TNF-R1, tumor necrosis factor-receptor 1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

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