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REVIEW

Lysosomal membrane permeabilization in cell death

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Mitochondrial outer membrane permeabilization (MOMP) constitutes one of the major checkpoint(s) of apoptotic and necrotic cell death. Recently, the permeabilization of yet another organelle, the lysosome, has been shown to initiate a cell death pathway, in specific circumstances. Lysosomal membrane permeabilization (LMP) causes the release of cathepsins and other hydrolases from the lysosomal lumen to the cytosol. LMP is induced by a plethora of distinct stimuli including reactive oxygen species, lysosomotropic compounds with detergent activity, as well as some endogenous cell death effectors such as Bax. LMP is a potentially lethal event because the ectopic presence of lysosomal proteases in the cytosol causes digestion of vital proteins and the activation of additional hydrolases including caspases. This latter process is usually mediated indirectly, through a cascade in which LMP causes the proteolytic activation of Bid (which is cleaved by the two lysosomal cathepsins B and D), which then induces MOMP, resulting in cytochrome *c* release and apoptosome-dependent caspase activation. However, massive LMP often results in cell death without caspase activation; this cell death may adopt a subapoptotic or necrotic appearance. The regulation of LMP is perturbed in cancer cells, suggesting that specific strategies for LMP induction might lead to novel therapeutic avenues.
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Introduction

Physiological and pathological cell death have been classified according to morphological criteria into at least three categories: type I cell death or apoptosis; type II cell death or autophagic cell death; and type III cell death or necrosis (Clarke, 1990; Kroemer *et al*., 2005; Galluzzi *et al*., 2007). Apoptosis or type I programmed cell death is characterized by cell rounding and nuclear condensation. The cell diminishes in size, the plasma membrane starts to bleb and the cell finally fragments into apoptotic bodies that are engulfed by neighboring cells where they are degraded within phagolysosomes. This type of cell death has been extensively studied and (some of) the molecular pathways have now been elucidated. Among these, mitochondrial outer membrane permeabilization (MOMP) and caspase activation have prominent functions: the former determines the point of no return of the lethal process; the latter orchestrates the morphological and biochemical changes that characterize apoptosis (Taylor *et al*., 2008; Youle and Strasser, 2008). MOMP is regulated by the Bcl-2 family of proteins, which act as inducers or blockers of the process. As a result of MOMP, proapoptotic molecules such as cytochrome *c* are released into the cytosol and contribute to the activation of caspases (Kroemer *et al*., 2007; Taylor *et al*., 2008; Youle and Strasser, 2008). Caspases are aspartic proteases that are responsible for the degradation of hundreds of cytoplasmic and nuclear proteins in the apoptotic cell. Caspases can be activated after MOMP or independently of MOMP (Taylor *et al*., 2008). Other mitochondrial proteins such as the apoptosis-inducing factor (AIF) are also released from the mitochondria during apoptosis and activate caspase-independent cell death pathways (Modjtahedi *et al*., 2006).

Autophagy is a lysosomal degradative pathway by which cells degrade and recycle macromolecules and organelles (Mizushima, 2007). As a result of the discovery of the proteins responsible for this phenomenon—the autophagy (Atg) proteins—and the implication of autophagy in a plethora of pathological situations, this process has received great attention (Klionsky, 2007; Levine and Kroemer, 2008). Although autophagy was initially described as a type of cellular demise, it remains unclear whether autophagy represents an independent mode of programmed cell death, a backup mechanism of cell death when apoptosis is inhibited or simply a stress response that is activated in damaged cells (Maiuri *et al*., 2007). Indeed, one possible explanation for the frequent presence of autophagosomes in dying cells is that cells upregulate autophagy in an attempt to eliminate toxic molecules or damaged organelles and that cells die from apoptosis or necrosis only once the autophagic system of defense has been

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overwhelmed. Necrosis has been defined as a type of cell death that lacks the features of apoptosis and autophagy, and is often considered to be an uncontrolled process. However, recent research suggests that its occurrence and course might be regulated. Following a signaling- or damage-induced lesion, necrosis can include signs of controlled processes such as mitochondrial dysfunction, enhanced generation of reactive oxygen species, ATP depletion, proteolysis by calpains and cathepsins, and early plasma membrane rupture. In addition, the inhibition of specific proteins involved in regulating apoptosis or autophagy can change the morphological appearance of cell death to necrosis (Festjens *et al*., 2006; Golstein and Kroemer, 2007).

Cell death can be triggered by toxic insults from the extracellular or intracellular milieu. Thus, the damage of nuclear DNA or the endoplasmic reticulum both induce apoptotic cell death that is dependent on MOMP and caspase activation (Ferri and Kroemer, 2001; Boya *et al*., 2002). In addition, lysosomal damage can induce MOMP-dependent cell death and lysosomal proteases released into the cytosol have been implicated in apoptotic cell death. In this review, we will focus on recent discoveries that implicate lysosomal membrane permeabilization (LMP) in cell death.

Lysosomes and LMP

Lysosomes are cytoplasmic membrane-enclosed organelles that contain hydrolytic enzymes and that control the intracellular turnover of macromolecules (Luzio *et al*., 2007). Lysosomes contain many different types of hydrolytic enzymes including proteases, lipases, nucleases, glycosidases, phospholipases, phosphatases and sulfatases that usually exert their maximal enzymatic activity at low pH. The acidic milieu of lysosomes ($pH \leqslant 5$) is maintained by a vacuolar ATPase that pumps protons from the cytosol into the lysosomal lumen (Luzio *et al*., 2007). The lysosomal membrane is protected from the acidic hydrolases by lysosome-specific expression of membrane proteins such as Lamp-1 and Lamp-2, which are heavily glysosylated and hence resist digestion (Eskelinen, 2006). Several degradation pathways converge at the level of lysosomes. This applies to the endocytotic degradation of plasma membrane receptors and proteins from the extracellular matrix, as well as the phagocytotic degradation of bacteria and apoptotic cells. If the material that is degraded derives from the cell itself, the process of lysosome-dependent degradation is called autophagy (Mizushima, 2007). During macroautophagy, cytoplasmic components such as long-lived and damaged proteins, as well as entire organelles, are degraded and recycled to generate ATP as well as new building blocks for anabolic processes (Levine and Kroemer, 2008). Indeed, autophagy protects cells under conditions of nutrient starvation and, in some circumstances of cellular stress, cells die by apoptosis when autophagy is inhibited either pharmacologically or by depletion of essential *atg* gene products (Boya *et al*., 2005; Gonzalez-Polo *et al*., 2005).

The high content of hydrolytic enzymes in lysosomes makes them potentially harmful to the cell. If the lysosomal membrane is damaged, lysosomes release their contents into the cytosol, setting off indiscriminate degradation of cellular components. In addition, massive lysosomal breakdown may induce cytosolic acidification, which in turn can induce cell death by necrosis. This concept was first appreciated by de Duve (1959), the discoverer of lysosomes, who termed them 'suicide bags'. However, as we will discuss, partial and selective LMP induces cell death by apoptosis. Under these circumstances, proteases liberated from the lysosomal lumen activate a controlled mode of cell death (for earlier reviews see Guicciardi *et al*., 2004; Kroemer and Jaattela, 2005; Tardy *et al*., 2006; Terman *et al*., 2006). We will refer to this process as LMP.

Lysosomal proteases that have been implicated in cell death are those cathepsins that remain active at neutral pH, such as cathepsin B (CB), cathepsin D (CD) and cathepsin L (CL). These proteases activate apoptotic effectors such as mitochondria and/or caspases. In spite of the extensive characterization of apoptosis mechanisms, the involvement of lysosomes in cell death has been neglected for several reasons. First, at the level of transmission electron microscopy, the ultrastructure of lysosomes appears intact during apoptosis, even when LMP can be detected by immunofluorescence techniques. Second, several pharmacological inhibitors of apoptosis (caspase inhibitors) also block lysosomal proteases when they are used at high concentrations (Foghsgaard *et al*., 2001). Thus, the cytoprotective effects of inhibitors such as Z-VAD-fmk may well include hitherto neglected effects on lysosomal proteases (Turk and Stoka, 2007).

'Lysosomotropic agents' accumulate within lysosomes. This term was coined by de Duve *et al*. (1974) to describe weakly basic amines that attain concentrations several 100-fold higher within the lysosomes than in the cytosol. Lysosomotropic amines freely diffuse across membranes in their uncharged form but become trapped in their protonated (non-diffusible) form when they are localized in acidic vesicles. In the 1980s, several lysosomotropic compounds were synthesized that were capable of lysing lysosomal membranes. Accumulation of the protonated form above a certain threshold concentration results in their acquisition of detergent-like properties, which induce lethal lysosomal destabilization. As a proof of principle for this concept, lysosomotropic detergents cannot lyse red blood cells, which do not possess lysosomes (Firestone *et al*., 1982; Miller *et al*., 1983).

Lysosomal membrane permeabilization: its detection

The distinctive sign of LMP is the translocation of soluble lysosomal components (including enzymes) from the lysosomal lumen to the cytosol. Accordingly, LMP can be measured by a variety of simple techniques.

Immunofluorescence techniques using antibodies against cathepsins such as CB and CD reveal the redistribution of these proteases from lysosomes to the cytosol (Figure 1a). In healthy cells, CB- or CD-specific immunostainings reveal cytoplasmic punctate structures that are surrounded by lysosomal membrane proteins such as Lamp-1 and Lamp-2. After LMP, the immunofluorescence detection of CB or CD reveals a diffuse staining throughout the entire cell (Boya *et al*., 2003a). The translocation of CB or CD from lysosomes to the cytosol can also be detected by immunoblots of subcellular fractions, typically cytosolic versus heavy membrane fractions (which include mitochondria and lysosomes) (Michallet *et al*., 2004). Cytosolic protease activity can be measured using lysosome-impermeable cathepsin substrates such as pepstatin BODIPY (Yin *et al*., 2005). Alternatively, protease activities can be monitored in cytosolic extracts with cathepsin-specific substrates such as zFR-amino-trifluoromethylcoumarin (Groth-Pedersen *et al*., 2007). Cytosolic cathepsin translocation can also be visualized by transfecting cells with fusion constructs encoding cathepsins that have been coupled to fluorescent proteins (Werneburg *et al*., 2002, 2007; Broker *et al*., 2005). Furthermore, lysosomal destabilization can be determined by pulse-chase experiments, in particular by loading lysosomes with

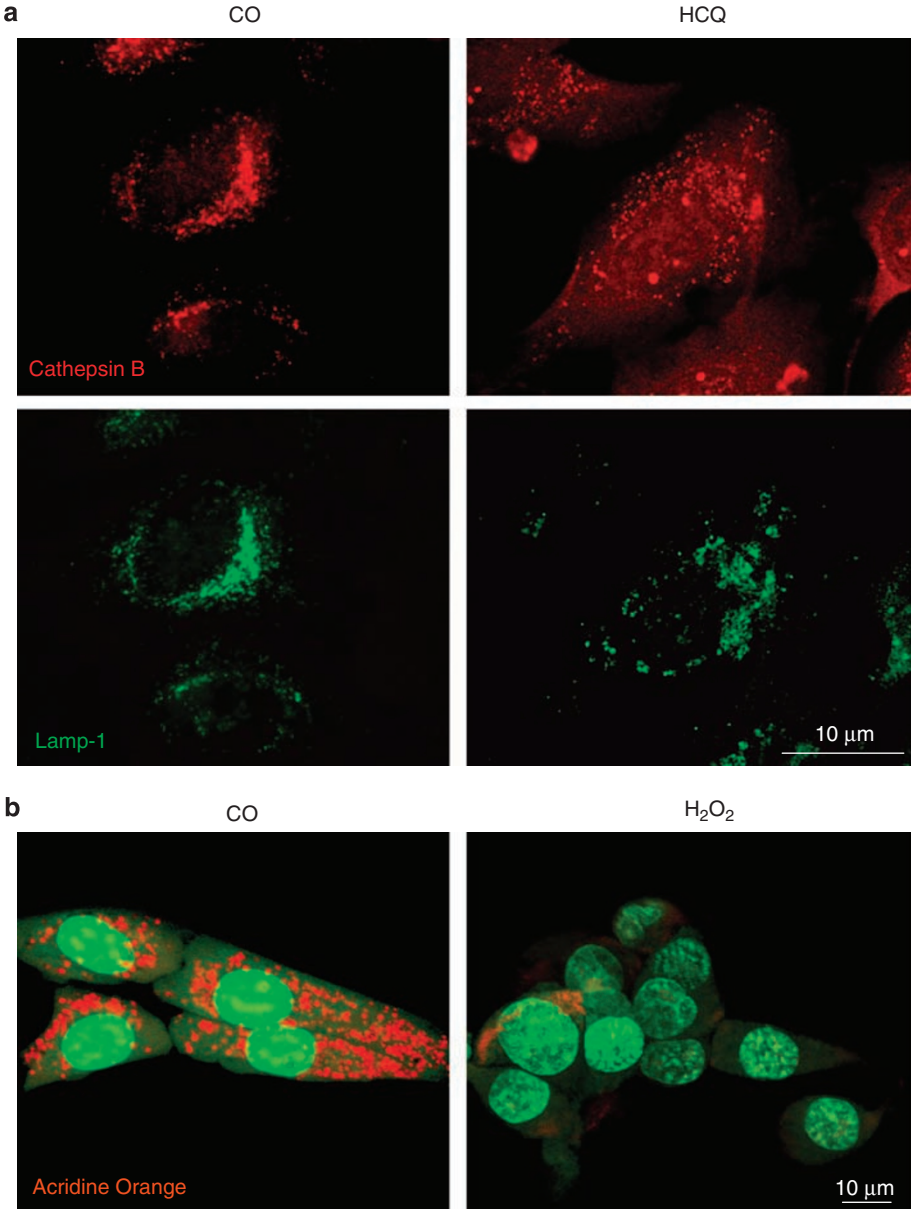

Figure 1 LMP detection by immunofluorescence in cell culture. (**a**) HeLa cells were treated with 30 μg/ml hydroxychloroquine (HCQ) for 3 h, fixed and stained for immunofluorescence detection of cathepsin B (red) and Lamp1 (green). Note that after LMP, cathepsin B diffuses throughout the cytosol. (**b**) 3T3 fibroblasts were cultured for 3 h with 150 μM H_2O_2 and stained for 15 min with 10 μg/ml acridine orange. Note the loss of the punctate red staining of acidic organelles after H_2O_2 treatment. LMP, lysosomal membrane permeabilization.

fluorescent dextrans and observing the cytosolic release of the dye after lysosomal insult. Dextrans of different molecular weights can be used to assess the selectivity of lysosomal permeabilization (Bidere *et al*., 2003).

Other methods to assess LMP are based on the use of lysosomotropic fluorochromes. Lysosomotropic probes such as the Lysotracker dyes from Invitrogen (Carlsbad, CA, USA) accumulate inside acidic organelles (which include lysosomes and late endosomes) and can be visualized by fluorescence microscopy or flow cytometry (De Milito *et al*., 2007). Decreased Lysotracker fluorescence may reflect LMP and/or an increase in lysosomal pH, meaning that this method is not absolutely specific for LMP. Following LMP, cells manifest less cytosolic puncta by fluorescence microscopy and appear weakly fluorescent when analysed by flow cytometry (Boya *et al*., 2003b). Some Lysotracker probes are compatible with aldehyde fixation and thus may be combined with the immunofluorescence detection of proteins.

Another probe used to measure the functional state of lysosomes is acridine orange (AO). AO is a lysosomotropic metachromatic fluorochrome. When excited with blue light, AO emits red fluorescence at high concentrations (when it is present in lysosomes) and green fluorescence at low concentrations (when it is present in the cytosol and the nucleus) (Figure 1b). AO-loaded cells manifest reduced red fluorescence and increased green fluorescence after LMP (Antunes *et al*., 2001). AO may be used either in live fluorescence microscopy or flow cytometry (Boya *et al*., 2003a; Michallet *et al*., 2004; Trincheri *et al*., 2007).

Lysosomal membrane permeabilization: complete or partial

Lysosomal membrane permeabilization is one mechanism for the induction of cell death. As mentioned above, complete disruption of lysosomes provokes uncontrolled cell death by necrosis. In contrast, partial and selective LMP induces the controlled dismantling of the cell by apoptosis. Several reports indicate that, in response to some lethal stimuli, not all lysosomes are permeabilized at the same time, although the mechanisms explaining this heterogeneity remain elusive. Large lysosomes seem to be particularly susceptible to the action of LMP-inducing agents (Ono *et al*., 2003). In addition, the production of intracellular mediators such as reactive oxygen species (ROS), which have a spatially limited range of activity, may induce the permeabilization of lysosomes only in those subcellular regions that are near to mitochondria, the major ROS-generating organelles. Therefore, lysosomes that are localized in the proximity of uncoupled mitochondria are more likely to suffer damage to their membranes than distant lysosomes. Moreover, lysosomes constitute the principal reservoir of chelatable iron, which accumulates upon the autophagic degradation of iron-containing proteins including mitochondrial cytochromes and ferritin. Iron-catalysed Fenton reactions, which produce highly reactive pro-oxidants, thus may damage lysosomal membranes. Iron overload, as it occurs in some pathologies and aging processes, can increase the susceptibility of lysosomes to LMP (Mak and Weglicki, 1985; Link *et al*., 1993).

A second important question regarding LMP is whether it occurs through non-selective rupture of the membrane or whether specific pores are formed that allow for the selective translocation of molecules up to a certain size through the partially permeable lysosomal membrane. Through the use of fluorescently labeled dextran molecules of different sizes (Bidere *et al*., 2003), the induction of LMP by staurosporine was shown to release low-molecular weight dextrans into the cytosol (10 and 40 kDa), but not larger molecules (dextrans of 70 and 250 kDa). Accordingly, the hexosaminidase enzyme (a 250 kDa protein normally resident in lysosomes) was retained within these organelles (Bidere *et al*., 2003), in conditions in which CB, CL (both $\sim$37 kDa) and CD (44 kDa) were released. Nevertheless, it is currently unclear whether such a molecular sieve effect can be mediated by proteinaceous pores present in lysosomal membranes. A number of reports indicate that different cathepsins (CB, CL and CD) simultaneously translocate after LMP is induced (Kagedal *et al*., 2001a; Bidere *et al*., 2003; Zhao *et al*., 2003; Michallet *et al*., 2004; Chen *et al*., 2005; Laforge *et al*., 2007), although in most of the studies the inhibition or genetic ablation of a single cathepsin isoform was sufficient to retard cell death (Kagedal *et al*., 2001a; Bidere *et al*., 2003; Laforge *et al*., 2007). Moreover, in some cases in which cathepsins were detected in the cytosol, lysosomes still could be labeled with lysosomotropic fluorochromes, indicating that the cytosol–lysosome pH gradient was maintained (Bidere *et al*., 2003) and that LMP may occur selectively in a subset of lysosomes. It remains a matter of speculation whether special transporters are responsible for the selective relocation of lysosomal proteins or whether transient pores are formed that lead to the release of proteins up to a maximum size, then reclose and facilitate rebuilding of a proton gradient. In the outer mitochondrial membrane, proapoptotic proteins Bax and Bak may form transient pores that allow for the translocation of a number of molecules larger than 100 kDa without provoking a rupture of the membrane (Kuwana *et al*., 2005). According to some reports, Bax is also capable of inducing LMP by acting directly on the lysosomal membrane (Kagedal *et al*., 2005; Feldstein *et al*., 2006). Nonetheless, the implication of proteins from the Bcl-2 family in LMP has not been studied extensively thus far.

Inducers of LMP

A large panel of distinct agents and molecules can induce LMP (Table 1 and Figure 2), although the best-studied mechanism of LMP is ROS-mediated lysosomal destabilization (Terman *et al*., 2006). Indeed, enhanced ROS generation precedes LMP and cell death in many settings. Indirect damage to the lysosomal membrane by ROS is mediated by the intralysosomal accumulation of

Table 1 Inducers of LMP

Inducer	*Example*	*Mechanisms*	*Reference*
ROS	H_2O_2, napthazarine, redox cycling quinones, fenretinide	Chemical modification of lysosomal membrane lipids?	Terman *et al*. (2006)
Lysosomotropic agents	Sphingosine, hydroxychloroquine, MDL-72,527, LCL204, BPC, *N*-dodecyl-imidazole, 3-aminopropanal	LMP due to detergent-like effects.	Kagedal *et al*. (2001a); Yu *et al*. (2003); Boya *et al*. (2003b)
	Detergents (MSDH, siramesine)		Li *et al*. (2000); Ostenfeld *et al*. (2005)
	Antibiotics (norfloxacin, cyprofloxacin)		Boya *et al*. (2003a)
Lipids	Fatty acids, bile salts, cholesterol oxidation products, palmitate, HePC		Feldstein *et al*. (2006); Paris *et al*. (2007)
Bcl-2 family proteins	Bax	Formation of proteaceous pores within lysosomal membranes?	Kagedal *et al*. (2005); Feldstein *et al*. (2006)
Caspases	Caspase-9, caspase-8	Digestion of lysosomal proteins or indirect effects?	Werneburg *et al*. (2004); Gyrd-Hansen *et al*. (2006)
Cathepsins	Cathepsin B	Digestion of lysosomal proteins?	Werneburg *et al*. (2002)
Microtubule toxins	Vincristine, vinorelbine, vinblastine, epothilone B, paclitaxel	Unknown	Groth-Pedersen *et al*. (2007)
Photodamage	NPe6, ATXs10	Lysosomal membranes	Xue *et al*. (2003); Ichinose *et al*. (2006)
Poyphenols	Resveratrol	Unknown	Trincheri *et al*. (2007)
Receptors	CD3, PHA, TRAIL, TNF-α	Unknown	Guicciardi *et al*. (2000); Heinrich *et al*. (2004)
Lysosomal proteins	LAPF	Pores?	Chen *et al*. (2005); Li *et al*. (2007)
DNA damage	p53, camptothecin, etoposide	Unknown	Emert-Sedlak *et al*. (2005); Paquet *et al*. (2005)
Silica	ROS?	Unknown	Thibodeau *et al*. (2004); Hamilton *et al*. (2007)
Toxins	Cobra venom, yesotoxin, crotoxin	Direct effect on lysosomal membranes?	Feofanov *et al*. (2005); Malagoli *et al*. (2006); Yan *et al*. (2006)

Abbreviations: LMP, lysosomal membrane permeabilization; ROS, reactive oxygen species; TNF-α, tumour necrosis factor alpha.

iron (Eaton and Qian, 2002). Free iron catalyses the conversion of hydrogen peroxide (which freely crosses membranes) into hydroxyl radicals. Accordingly, iron loading increases the fragility of lysosomal membranes (Mak and Weglicki, 1985; Link *et al*., 1993). Conversely, desferrioxamine mesylate, an iron chelator that specifically accumulates inside lysosomes by fluid-phase endocytosis, can inhibit lysosomal-mediated cell death induced by oxidant challenge (Doulias *et al*., 2003; Yu *et al*., 2003; Kurz *et al*., 2006). A positive feedback loop between LMP and mitochondrial damage exists during oxidation-induced cell death, suggesting that both iron chelation in lysosomes and inhibition of ROS production by mitochondria may have cytoprotective effects (Terman *et al*., 2006).

Another putative mediator of LMP is the sphingolipid sphingosine. As a lipophilic weak base, sphingosine is lysosomotropic. Upon protonation, sphingosine accumulates within the acidic compartment where it may act as a detergent. Limited doses of sphingosine induce a cascade of lethal events including LMP, caspase activation, as well as the dissipation of the mitochondrial inner transmembrane potential (Kagedal *et al*., 2001a). High doses of sphingosine rapidly cause extensive lysosomal rupture culminating in rapid necrosis (Kagedal *et al*., 2001b). The intracellular levels of sphingosine increase after TNF-α (tumor necrosis factor alpha) treatment, particularly in the liver where sphingosine has been implicated in LMP. TNF-α or sphingosine induces LMP in $CB^{+/+}$ but not $CB^{-/-}$ hepatocytes *in vitro*, and sphingosine permeabilizes hepatocyte lysosomes isolated from $CB^{+/+}$ but not $CB^{-/-}$ livers as assessed by Lysotracker (Werneburg *et al*., 2002). In addition, the overexpression of a dominant-negative factor associated with neutral sphingomyelinase activation (FAN) reduces LMP, CB translocation and diminishes apoptosis in a rat hepatoma cell line (Werneburg *et al*., 2004).

Free fatty acids and bile salts can also induce LMP-dependent cell death (Roberts *et al*., 1997; Feldstein *et al*., 2004, 2006). Glycochenodeoxycholate is the primary bile salt induced during cholestasis. When added to a rat hepatoma cell line, glycochenodeoxycholate induces LMP, CB translocation, caspase activation and cell death, which can be effectively blocked by caspase inhibition and overexpression of the cathepsin inhibitor cystatin A (Jones *et al*., 1998). This pathway seems to play a prominent role in animal models of

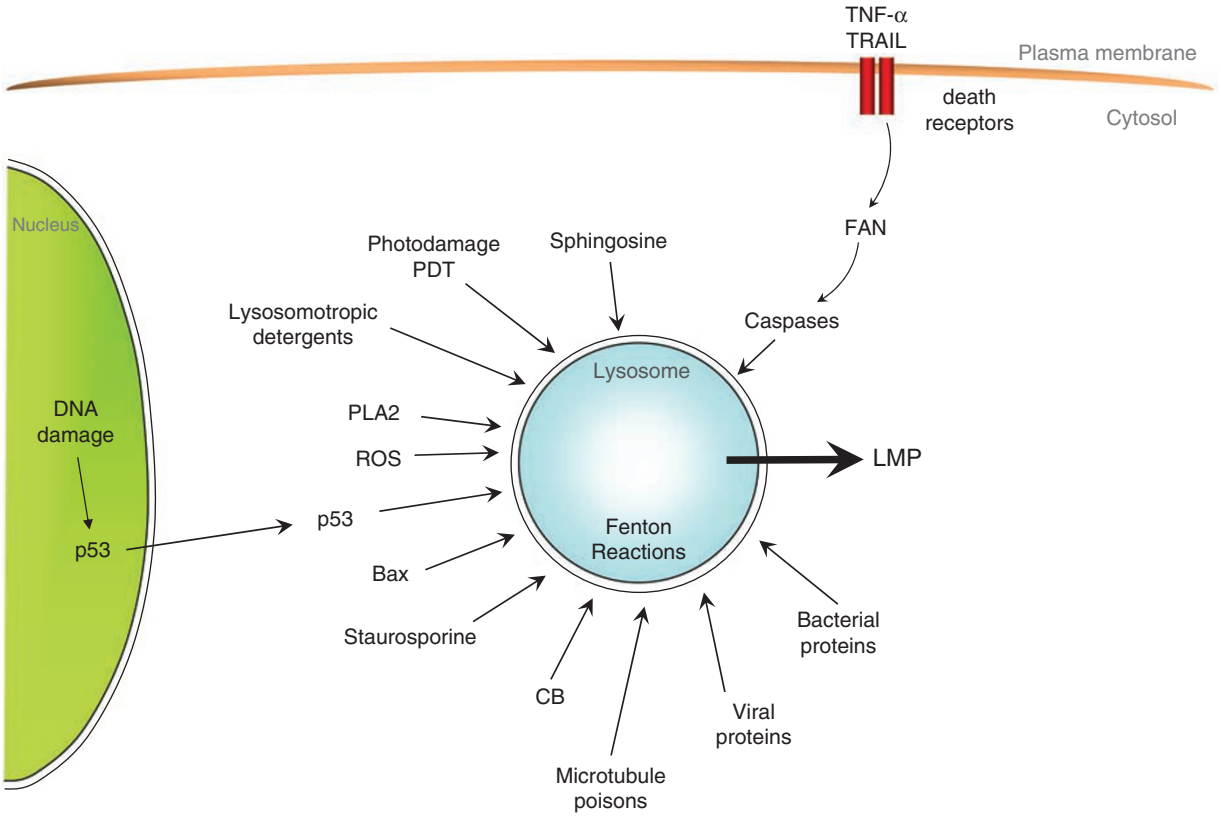

Figure 2 Pathways leading to the induction of LMP. For details see text. CB, cathepsin B; FAN, factor associated with neutral sphingomyelinase activation; LMP, lysosomal membrane permeabilization; PDT, photodynamic therapy; TNF-α, tumor necrosis factor alpha.

cholestasis where CB inactivation attenuates liver injury (Roberts *et al*., 1997; Canbay *et al*., 2003).

Hexadecylphosphocholine is an experimental anticancer agent that induces cell death through a lysosomal pathway characterized by partial lysosomal rupture and CB relocation. In this model, the overexpression of Bcl-2 and the addition of the pan caspase inhibitor Q-VD-OPH inhibit lysosomal rupture, but only at early time points, suggesting that lysosomal rupture is amplified by caspases and proapoptotic Bcl-2 family proteins, yet does not entirely rely on them in this system (Paris *et al*., 2007). Thus, depending on the specific lipid, LMP induction may be a primary event (upstream of caspase activation) or occur downstream of the initiation of the apoptotic cascade.

The oxidation products of cholesterol are capable of inducing LMP, CD translocation and apoptotic cell death in macrophages (Yuan *et al*., 2000). Several reports indicate that lysosomes are destabilized in macrophages exposed to oxidized low-density lipoprotein or 7β-hydroxycholesterol, which produces leakage of lysosomal enzymes into the cytosol (CB, CD and CL) and apoptotic cell death. These effects are reversed by pre-exposing cells to the iron chelator, desferrioxamine (Li *et al*., 1998; Yuan *et al*., 2000; Li and Yuan, 2004). The cell death pathway following exposure to 7β-hydroxycholesterol or 7β-hydroxysitoesterol, the major oxidation products from cholesterol and plant sterols, is associated with LMP (Roussi *et al*., 2007). However, the cell death mechanism induced by each compound differs insofar as 7β-hydroxycholesterol induces ROS accumulation, extensive MOMP and endonuclease G expression, whereas 7β-hydroxysitoesterol does not induce any of these events (Roussi *et al*., 2007). LMP occurs after exposing cells to these lipid oxidation products, more so in response to 7β-hydroxysitoesterol than in response to 7β-hydroxycholesterol. These two hydroxyphenols exhibit different lipophilic properties despite their very similar structure (Roussi *et al*., 2007), indicating that slight variations in the hydrophobicity of lipid derivatives may have a strong influence on the cell death pathway that they activate.

Other examples of lysosomotropic detergents are *N*-dodecylimidazole and *O*-methyl-serine dodecylamide hydrochloride, which both induce LMP followed by caspase-3-dependent apoptosis at low doses (Li *et al*., 2000; Zhao *et al*., 2003) and necrosis (without caspase activation) at high doses (Li *et al*., 2000). Siramesine is a sigma-2 receptor ligand that was initially developed to treat anxiety and depression. Its structure involves an amphiphilic amine, suggesting that it can act as lysosomotropic detergent. Indeed, siramesine induces a rise in lysosomal pH followed by LMP, CD release and caspase-independent cell death, which can be blocked by antioxidants and CB inhibitors yet does not require sigma-2 receptors (Ostenfeld *et al*., 2005). L-leucyl-L-

leucine-methyl ester is taken up by cells through receptor-mediated endocytosis and accumulates inside lysosomes, thus inducing LMP, cathepsin release, cleavage of Bid, MOMP- and caspase-dependent apoptosis (Uchimoto *et al*., 1999; Cirman *et al*., 2004).

The activation of phospholipase A2 also induces LMP as the phospholipase A2 inhibitor 4-bromophenacyl bromide diminishes lysosomal rupture and apoptosis in a lymphoma cell line (Zhao *et al*., 2001a, 2003; Marchi *et al*., 2004). Interestingly, lysosomal enzymes activate phospholipase A2, thereby providing a positive feedback loop (Zhao *et al*., 2001a). Phospholipase A2 also induces MOMP, ROS production and cytochrome *c* release, providing an efficient means to induce cell death by acting directly on lysosomes and mitochondria.

The Bcl-2 family of proteins are important apoptotic regulators as they are able to control MOMP (Youle and Strasser, 2008). After their activation and translocation to mitochondria, proapoptotic members of the family such as Bax and Bak induce MOMP, and this process is blocked by antiapoptotic members of the family such as Bcl-2 and Bcl-X_L. Moreover, when proteins from the BH3-only family, such as Bid, are activated, they interact with multidomain proteins of the Bcl-2 family, as well as with other mitochondrial proteins to promote apoptosis (Zamzami *et al*., 2000; Youle and Strasser, 2008). The effects of Bcl-2 proteins are not confined to the regulation of MOMP; they also affect calcium homeostasis of the endoplasmic reticulum, autophagy and the unfolded protein response (Hetz and Glimcher, 2008). Recently, the proapoptotic Bcl-2 family member Bax has been shown to exert a direct effect on lysosomes. Thus, Bax can translocate from the cytosol to lysosomal membranes and induce LMP (Kagedal *et al*., 2005). Fibroblasts cultured in the presence of the proapoptotic pan-tyrosine kinase inhibitor staurosporine exhibit a conformational change in Bax and its translocation to lysosomes, resulting in LMP and CD translocation to the cytosol (Kagedal *et al*., 2005). The requirement of Bax for LMP has been demonstrated by comparing $Bax^{+/+}$ and $Bax^{-/-}$ hepatocytes exposed to long-chain fatty acids (Feldstein *et al*., 2006). Thus, palmitate induces a conformational change in Bax that is typically linked to apoptosis (and that is detected by means of a monoclonal antibody that interacts with Bax only in its 'apoptotic' conformation, when the N terminus is exposed), resulting in the translocation of Bax to lysosomes. Indeed, genetic ablation of Bax expression and increased Bcl-X_L expression protect cells from palmitate-induced LMP and death (Feldstein *et al*., 2004, 2006). Moreover, TRAIL has been recently shown to induce cell death in several cell lines following a pathway that involves the apical caspase-8-dependent JNK activation, which then induces the translocation of Bax and Bim (a BH3-only protein from the Bcl-2 family) to lysosomal membranes followed by LMP (Werneburg *et al*., 2002). All these events occur upstream of mitochondrial changes (such as cytochrome *c* release and loss of the mitochondrial transmembrane potential), suggesting that the two proapoptotic Bcl-2 family members, Bim and Bax, can induce LMP independently from and upstream of MOMP. siRNA-mediated downregulation of Bax expression has also been shown to protect cells from LMP and cell death after a hydrogen peroxide insult (Castino *et al*., 2007). From studies of $Bid^{-/-}$ cells, it appears clear that the TNF-α-mediated LMP and cell death of hepatocytes is Bid dependent (Guicciardi *et al*., 2005). All these data suggest that Bcl-2 family members might modulate LMP in a similar way to their regulation of MOMP.

Other more recently identified LMP regulators are caspases, the activation of which has been shown to induce LMP-dependent apoptosis in response to some stimuli. For instance, TNF-α can induce LMP through caspase-8 and FAN, and the resulting CB translocation then accounts for cell death. Reportedly, FAN acts upstream of caspase-8 and Bid in a signaling cascade that culminates in lysosomal permeabilization (Werneburg *et al*., 2004; Nagaraj *et al*., 2006). Moreover, FAN has recently been shown to regulate the size of lysosomes (Mohlig *et al*., 2007). Interestingly, FAN has striking structural homology with Lyst (lysosomal trafficking regulator), a BEACH protein mutated in Chediak-Higashi syndrome, which is a lysosomal storage disease characterized by severe immunological defects and peripheral neuropathies, and that is often diagnosed because of the presence of large lysosomes (Ward *et al*., 2000). Caspases are activated upstream of LMP after TNF-α treatment of ME-180 cells but not after an identical treatment of WEHI-S cells, indicating that the relative hierarchical position of LMP and caspase activation depends on the cell type (Foghsgaard *et al*., 2001). Caspases are activated in a mitochondria-dependent manner in many cell settings, as the final result of cytochrome *c* release from mitochondria and the formation of a multiprotein complex called the apoptosome. This complex, which is composed of cytochrome *c* as well as Apaf-1, results in the recruitment and allosteric activation of caspase-9, which then activates caspase-3 (Riedl and Salvesen, 2007). When mouse embryonic fibroblasts are challenged with TNF-α, caspase-9 can be activated through another Apaf-1-independent cell death cascade that involves LMP-dependent cathepsin activation, as well as caspase-8-dependent caspase-9 activation (Gyrd-Hansen *et al*., 2006). In this cell type, caspase-9 has been identified as a new molecular link between the receptor-proximal caspase-8 activation and LMP. In contrast, proteins such as Bid and Bax do not seem to have any major function in the induction of LMP in TNF-α-stimulated mouse embryonic fibroblasts (Gyrd-Hansen *et al*., 2006). Thus, TNF-α can induce different effectors to induce cell death depending on the cell type, as seen in combination treatments with IFN-γ (Li and Pober, 2005).

Interestingly, cathepsins have also been implicated as inducers of LMP. Hepatocytes from $CB^{-/-}$ mice display less LMP after exposure to TNF-α, which may reflect the direct induction of LMP by CB (either acting from inside or outside the lysosome) or the existence of an amplification loop in which LMP induces CB activation, and CB then triggers further LMP (Werneburg *et al*.,

2002). Indeed, in an *in vitro* assay using isolated lysosomes, sphingosine was capable of permeabilizing liver lysosomes that had been isolated from $CB^{+/+}$ livers but not from $CB^{-/-}$ hepatocytes (Werneburg *et al*., 2002). Similarly, the transfection-enforced overexpression of an endogenous CB inhibitory protein, Spi2a, can reduce LMP in TNF-α-treated cells (Liu *et al*., 2003). In a model of acute H_2O_2 stress affecting primary alveolar type II cells, cathepsin inhibitors also reduce the extent of LMP (Yin *et al*., 2005). Altogether, these data suggest that the lysosomal enzymes (CB and CD) may directly attack lysosomes (from inside or outside?) and facilitate LMP-mediated cell death. In addition, other proteases like calpains can induce lysosomal destabilization and LMP-dependent cell death, as this is suggested by the use of calpain inhibitors in stressed cells (Yap *et al*., 2006; Cheung *et al*., 2007).

There is some evidence that p53 can induce LMP upstream of MOMP, as indicated by experiments in cells expressing a thermolabile form of p53. When such cells are shifted from 37 °C (when p53 is destroyed) to 32 °C (when p53 accumulates), they manifest sequential LMP and MOMP (Yuan *et al*., 2002). Recently, a new adaptor protein capable of translocating to the lysosomal membranes and inducing LMP has been described (Chen *et al*., 2005). This protein, which is called LAPF (lysosome-associated protein containing PH and FYVE domains), belongs to the family of phafins and induces LMP-, MOMP- and caspase-independent cell death (Chen *et al*., 2005). After TNF-α exposure and ionizing irradiation, LAPF specifically interacts with phosphorylated p53 and induces LMP. Silencing LAPF expression abrogates the lysosomal translocation of phosphorylated p53, whereas silencing p53 has no effect on the lysosomal translocation of LAPF (Li and Baker, 2007). Like LAPF silencing, silencing endogenous p53 expression in L929 cells can significantly impair TNF-α-induced LMP and apoptosis. Hence, phosphorylated p53 might translocate to the lysosome while forming a complex with the LAPF adaptor protein, locally stimulate LMP and finally induce apoptosis in a transcription-independent manner (Chen *et al*., 2005; Li and Baker, 2007). However, p53 can also translocate to mitochondria and induce permeabilization of the outer mitochondrial membrane by forming complexes with the multidomain proteins from the Bcl-2 family, resulting in cytochrome *c* release (Mihara *et al*., 2003; Chipuk *et al*., 2004). Thus, it is tempting to speculate that p53 can recruit LAPF, and perhaps Bcl-2 family members, to induce LMP in a manner similar to that in which it triggers MOMP.

The apoptotic response of T lymphocytes stimulated by phytohemagglutinin and anti-CD3 in T lymphocytes is thought to mimic peripheral T-cell deletion induced by high-dose immune tolerance. This model of T-cell death has been shown to involve rapid LMP with CB and CL translocation that occurs before phosphatidylserine exposure on the outer leaflet of the plasma membrane and nuclear chromatin condensation (Michallet *et al*., 2004). Caspase inhibition blocked nuclear pyknosis but had no effect on phosphatidylserine exposure. In addition, cell death was only prevented by incubating the cells in the presence of CB inhibitors but not with inhibitors of CD or calpains. The mitochondrial release of cytochrome *c* was observed at a late stage, suggesting that MOMP was not required for inducing cell death in this model (Michallet *et al*., 2004). However, this speculation awaits experimental verification.

A range of different viral proteins kill infected cells by facilitating the induction of MOMP (Boya *et al*., 2004). In addition, several viral proteins such as Nef from human immunodeficiency virus-1, protein U7 from human papillomavirus type-16, parvovirus H1 and herpes simplex virus infection induce LMP- and cathepsin-dependent cell death (Kaznelson *et al*., 2004; Di Piazza *et al*., 2007; Laforge *et al*., 2007). Cystatin C can block the enhanced CB activity observed in herpes simplex virus type-1-infected cells and concomitantly inhibits herpes simplex virus type-1 replication and host cell apoptosis (Peri *et al*., 2007). The protein E7 encoded by human papillomavirus type-16 can induce caspase-dependent apoptosis in different cell lines. However, in U2OS cells, this protein alone does not induce apoptosis unless the cell-cycle regulator p21 is overexpressed, in which case E7 triggers CB-dependent cell death. This cell death is characterized by LMP and CB translocation, which is completely inhibited by the CB inhibitor CA074Me (Kaznelson *et al*., 2004).

Mycobacterium tuberculosis-infected macrophages undergo cathepsin-dependent caspase-independent cell death (Lee *et al*., 2006), and infection with Bacillus Calmette-Guérin also induces LMP and cell death (Sandes *et al*., 2007). A recent report indicates that virulent strains of *M. tuberculosis* and *M. leprae* (but not *M. bovis* and Bacillus Calmette-Guérin) translocate from lysosomes to the cytosol, which then can cause apoptotic cell death within a week (van der Wel *et al*., 2007). Thus, it is tempting to speculate that LMP may proceed in this setting and that intracellular pathogens have evolved mechanisms to exploit the lysosomal pathway for cell death induction.

Drugs that act on microtubules can also induce LMP- and caspase-independent cell death. This applies to microtubule destabilizing vinca alkaloids such as vincristine, vinorelbine and vinblastine (Groth-Pedersen *et al*., 2007), as well as to compounds that stabilize microtubules such as paclitaxel, epothilone B and dicodermoliede. All these agents reportedly induce LMP- and CB-dependent apoptosis (Broker *et al*., 2004; Groth-Pedersen *et al*., 2007). The general applicability of these findings, however, remains to be confirmed.

Proton pumps like the vacuolar-type H ATP-ase are involved in the control of cellular pH in normal and tumor cells, and they are targeted for the treatment of gastroesophageal acid-related diseases. Proton pump inhibitors, such as ompeprazole, are weak bases that accumulate inside lysosomes (De Milito *et al*., 2007). Proton pump inhibitors induce the early production of ROS that precedes the alkalinization of lysosomal pH, LMP and cytosol acidification, as well as caspase-

Table 2 Inhibitors of LMP or post-LMP effector mechanisms

Name	*Target*	*Type*	*Reference*
Hsp70 family of proteins	LMP	Chaperone	Gyrd-Hansen *et al.* (2004); Daugaard *et al.* (2007b)
Deferioxamine	ROS, LMP	Iron chelator	Kurz *et al.* (2006)
α-tocopherol	ROS, LMP	Antioxidant	Bivik *et al.* (2007)
N-acetyl cysteine	ROS, LMP	Antioxidant	De Milito *et al.* (2007)
Vitamin C	ROS, LMP	Antioxidant	Roussi *et al.* (2007)
Bcl-2	Bax, Bid, LMP	Antiapopotic Bcl-2 family protein	Zhao *et al.* (2001b)
3-*O*-methylsphingomyelin	Neutral sphingomyelinase	Lipid	Caruso *et al.* (2005)
Cystatins	Cystein proteases	Protease inhibitors	Jones *et al.* (1998); Peri *et al.* (2007)
Serpins	Serin proteases (caspases)	Protease inhibitors	Liu *et al.* (2004c)
E64d, CA074Me, R3032	Cathepsin B	Protease inhibitors	Werneburg *et al.* (2002); Michallet *et al.* (2004); Nagaraj *et al.* (2006)
Pepstatin A	Cathepsin D	Protease inhibitor	Roberg *et al.* (1999)
Aryl hyrdrocarbon receptor	Lysosomal membrane?	Steroid receptor	Caruso *et al.* (2006)

Abbreviations: LMP, lysosomal membrane permeabilization; ROS, reactive oxygen species.

independent cell death in B cells. Interestingly, cathepsin translocation occurred very late in ompeprazole-treated cells, and cathepsin inhibitors failed to protect from apoptosis, whereas the antioxidant *N*-acetylcysteine did protect the cells from LMP and cell death (De Milito *et al.*, 2007). Thus, the participation of LMP in lethal signaling remains to be demonstrated in this model.

Endogenous inhibitors of lysosome-mediated cell death

Heat-shock (Hsp)70 proteins function as ATP-dependent chaperones that regulate the folding of newly synthesized proteins, the assembly of multiprotein complexes and the transport of proteins across cellular membranes (Daugaard *et al.*, 2007b). Hsp70 is frequently overexpressed in malignant tumors, and this overexpression is associated with a poor therapeutic outcome in human breast cancer (Daugaard *et al.*, 2007b). Hsp70 has a marked cytoprotective effect and inhibits apoptotic signaling by inhibiting MOMP, by reducing caspase activation or by neutralizing AIF (Garrido *et al.*, 2006). Hsp70 also localizes to lysosomal membranes (Rohde *et al.*, 2005) and can protect lysosomal membranes against LMP induced by different stimuli such as etoposide, TNF-α and oxidative stress (Nylandsted *et al.*, 2004) (Table 2). In addition, Hsp70 depletion mediated by antisense constructs or small interfering RNAs induces caspase-independent cell death of cancer cells but not of non-tumorigenic cells (Nylandsted *et al.*, 2000). The specific depletion of one protein of the Hsp70 family, Hsp70-2, triggers LMP- and cathepsin-dependent cell death (Daugaard *et al.*, 2007a). This pathway seems to be mediated by the lens epithelium-derived growth factor, which is a transcriptional co-activator that regulates the transcription of several stress genes including Hsp70-2 and that confers protection against several proapoptotic stimuli (Ganapathy *et al.*, 2003). Knockdown of lens epithelium-derived growth factor induces destabilization of lysosomal membranes and caspase-independent cell death that cannot be avoided by Bcl-2 overexpression. Conversely, lens epithelium-derived growth factor upregulation protects lysosomal membranes and inhibits cell death induced by agents like siramesine, etoposide and TNF-α. Thus, lens epithelium-derived growth factor regulates LMP and cell death, presumably by regulating Hsp70-2 expression (Groth-Pedersen *et al.*, 2007).

Sphingomyelin and some of its derivatives (for example, 3-*O*-methylsphingomyelin, an inhibitor of neutral sphingomyelinase) can stabilize lysosomes against the lysosomal photosensitizer, *N*-aspartyl chlorin e6, which causes rapid loss of AO staining of acidic organelles, release of CD from late endosomes/lysosomes and the activation of procaspase-3, as well as TNF-α-induced LMP and cell death (Caruso *et al.*, 2005). These results point to the possibility that proteins, as well as non-proteinaceous molecules, act as endogenous regulators of LMP.

A host of proteins act as endogenous inhibitors of cathepsins and hence may avoid cell death after LMP, by neutralizing cathepsins that have been released into the cytosol. Cystatins are endogenous inhibitors of lysosomal cysteine proteases and they act as endogenous protectors of leakage after LMP by trapping and inhibiting cathepsins, protecting the cell from their protease activity. Cystatins are not very selective and inhibit endopeptidases in the picomolar range and exopeptidases in the nanomolar range (Turk *et al.*, 2001). Serpins (serine protease inhibitors) are a family of protease inhibitors that act either at the extracellular or at the intracellular levels (Silverman *et al.*, 2001; Law *et al.*, 2006). The majority of serpins inhibit serine proteases, although some of them can inhibit caspases and cysteine proteases from the papain family, such as cathepsins B, L and K (Law *et al.*, 2006). Some intracellular members of the serpin family are able to block LMP-dependent cell death. Serpin 2A has been shown to block caspase-independent, LMP- and CB-dependent cell death triggered by TNF-α (Liu *et al.*,

2004c). Moreover, serpin 2A, a transcriptional nuclear factor-κB target, is upregulated in memory T-cell precursors, facilitating memory T-cell development by blocking CB-dependent cell death (Liu *et al*., 2004b). Another member of the serpin family, hurpin, blocks CL but not CB and confers resistance to ultraviolet light-induced apoptosis in human keratinocytes (Welss *et al*., 2003). Recently, an intracellular serpin from *C. elegans*, SRP-6, has been shown to protect against cell death induced by hypotonic shock, hypoxia, heat shock and paraquat, which are all stimuli that reportedly induce lysosomal rupture and cysteine protease-dependent cell death in worms (Abraham and Shaham, 2007; Luke *et al*., 2007). Thus, serpins act as physiologically relevant inhibitors of LMP-mediated cell death.

Recently, overexpression of cFLIP has been shown to stimulate the p42/44 MAP-kinase pathway and to protect hepatocellular carcinoma cells from TRAIL-induced LMP and apoptosis (Guicciardi *et al*., 2007). Moreover, pharmacological inhibition of p38, transfection with a p38α dominant-negative mutant or knockout of the p38α gene (in mouse embryonic fibroblasts) protects cells from apoptosis induced by the lysosomotropic photosensitizing compound, tetrakis-meso-(4-ethyleneglycol-2,3,5,6-tetrafluorophenyl) porphyrin (Kralova *et al*., 2007). The molecular mechanism by which p38 MAPk regulates LMP and cell death remains elusive, although it may be related to p38 binding to lysosomes and endosomes through an interaction with p14 and MP1 proteins (Kolch, 2005).

Downstream signals leading to cell death after LMP

Depending on the lethal stimulus, the extent of LMP, the amount and type of cathepsins released into the cytoplasm, as well as the abundance of cathepsin inhibitors, LMP can trigger a variety of death-associated morphologies ranging from classical apoptosis to necrosis. Thus, LMP associated with cathepsin translocation may directly activate calpains and caspases, but LMP may also trigger the classical MOMP–caspase pathway, as well as MOMP- and caspase-independent apoptosis. Which lethal pathways are stimulated by LMP is influenced by the cellular context, including the cell type, the immortalization status and the genetic background. The main downstream signaling pathways leading to cell death after LMP are summarized in Figure 3.

As stated above, ROS are among the principal inducers of LMP. ROS-dependent LMP often initiates a cell death pathway that involves sequential CD translocation, MOMP with cytochrome *c* release and caspase-dependent apoptosis. Antioxidants, such as desferrioxamine mesylate, *N*-acetylcysteine and α-tocopherol, block LMP and hence prevent all subsequent steps of the apoptotic cascade (Zang *et al*., 2001). Endogenous CB inhibitors such as cystatins block CB protease activity in paradigms of ROS-induced LMP-dependent cell death, which points to the involvement of CD (rather than CB) in this pathway. Thus, cystatin C upregulation has been observed in PC12 cells following 6-hydroxydopamine exposure, a classical model of

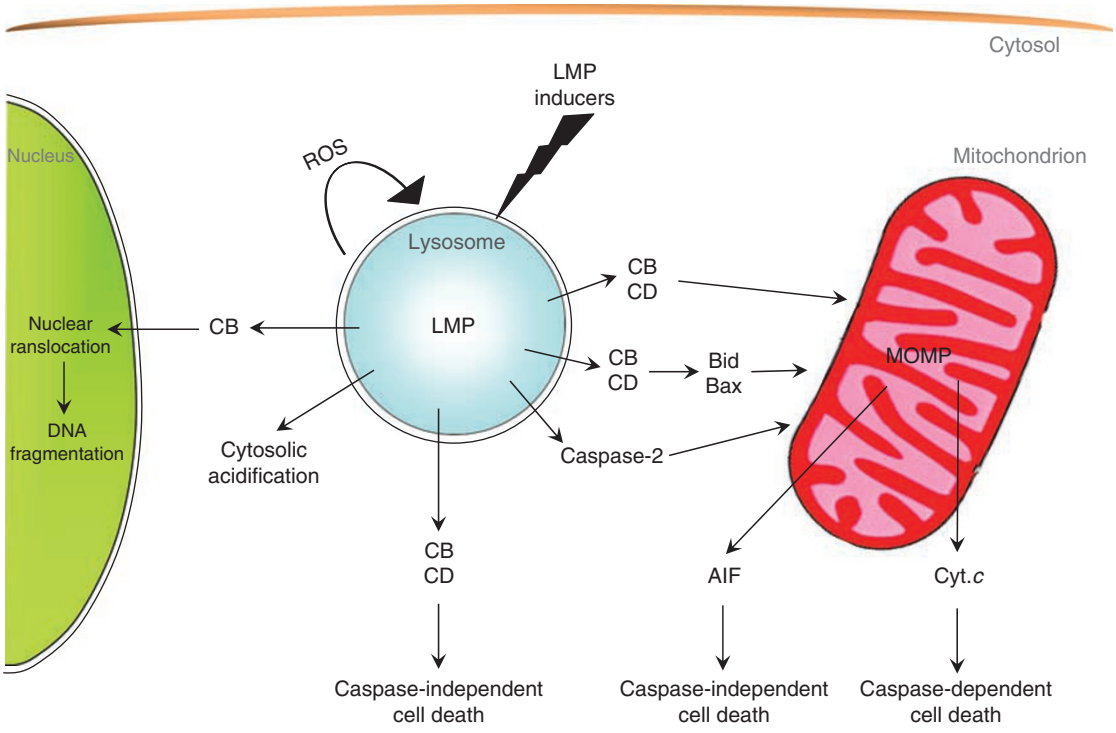

Figure 3 Lethal pathways activated after LMP. Note that a variety of different effector molecules can trigger a range of distinct modalities of cellular dismantling. The dominant pathway depends on the intensity of LMP, on the expression level of lysosomal hydrolases, the cytosolic concentrations of cathepsin inhibitors, the functional state of mitochondria, the concentration of caspases and their antagonists, as well as multiple additional factors. AIF, apoptosis-inducing factor; CB, cathepsin B; CD, cathepsin D; Cyt. *c*, cytochrome *c*; LMP, lysosomal membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization.

ROS-mediated toxicity in which CD translocates to the cytosol and induces caspase-dependent cell death (Lee *et al*., 2006). However, in other paradigms, CB does play a role in linking LMP to cell death.

What are then the signals that link LMP to MOMP? One prominent MOMP inducer is the BH3-only protein Bid, which requires proteolytic cleavage to become active. *In vitro* experiments performed at neutral pH showed that the papain-like cathepsins B, H, L, S and K cleave Bid predominantly at Arg(65) or Arg(71), whereas cathepsins C and X were unable to cleave Bid (Cirman *et al*., 2004). In addition, when full-length Bid was treated with cathepsins B, H, L and S, the resulting cleavage product was fully capable of releasing cytochrome *c* from isolated mitochondria (Cirman *et al*., 2004). CB-dependent Bid cleavage has been observed in various cell lines such as glioma cells killed by interleukin-24 (Yacoub *et al*., 2008), TTC bladder cancer cells killed by Bacillus Calmette-Guérin (Sandes *et al*., 2007), U2O2 osteosarcoma cells and oral squamous cell carcinoma with TRAIL (Garnett *et al*., 2007) (Nagaraj *et al*., 2006) and MCF-7 breast cancer cells exposed to camptothecin (Lamparska-Przybysz *et al*., 2006). Bid also represents a direct downstream target of CD. Indeed, in cathepsin-deficient fibroblasts, the TNF-mediated apoptotic response was blocked at the level of Bid cleavage (Heinrich *et al*., 2004). The CD inhibitor pepstatin A suppresses the cleavage of Bid by lysosomal extracts from a murine hepatoma in a cell-free system. Pepstatin A also delayed the induction of apoptosis in cell cultures treated with TNF-α and cycloheximide, which correlated with reduced Bid cleavage and reduced activation of pro-caspases-3/7 (Caruso *et al*., 2006). In human neutrophils, fimbriated *Escherichia coli* bacteria can induce LMP- and CD-dependent Bid cleavage (Blomgran *et al*., 2007). Thus, CB- or CD-mediated Bid cleavage emerges as a key connection between LMP and MOMP.

Several reports indicate that LMP can initiate a caspase-independent cell death pathway, as caspase inhibitors fail to block cell death. For example, microtubule-stabilizing agents such as epothilone B, discodermolide and paclitaxel reportedly induce CB-dependent and caspase-independent cell death, as evidenced by the significant cytoprotection conferred by the CB inhibitor CA074-Me (but not the broad-spectrum caspase inhibitor Z-VAD-fmk) (Broker *et al*., 2004). However, microtubule poisons have been shown to induce either caspase-dependent or caspase-independent cell death, depending on the cell type. Similarly, TRAIL can induce LMP- and caspase-dependent apoptosis (Werneburg *et al*., 2007) or LMP- and caspase-8-independent cell death (Garnett *et al*., 2007), depending on the cell type. Downregulation of Hsp70, which protects lysosomal membranes from LMP-inducing stimuli, induces caspase-independent cell death in breast cancer cells (Nylandsted *et al*., 2000). Macrophages infected with high multiplicities of infection of *M. tuberculosis* induce LMP and cell death that cannot be blocked by caspase inhibitors (Lee *et al*., 2006). The distal death effectors in these systems have not been characterized in detail, although AIF has been shown to be one of the main effectors of caspase-independent cell death (Modjtahedi *et al*., 2006). During apoptosis, AIF translocates from mitochondria to the nucleus where it induces DNA degradation (Modjtahedi *et al*., 2006). AIF has proapoptotic activity after LMP in activated T lymphocytes following exposure to staurosporine (Bidere *et al*., 2003) and after LAPF overexpression, a lysosomal protein capable of inducing LMP-dependent and caspase-independent apoptosis in mouse fibrosarcoma cells (Chen *et al*., 2005). Thus, AIF may be responsible for promoting caspase-independent cell death after LMP. Last, as a high level of oxidative stress is a common occurrence during LMP induction, caspases may become inactivated because of ROS-mediated oxidation (Mannick *et al*., 2001).

In some cases, LMP and subsequent MOMP are linked with each other by the activation of caspase-2. In mouse hepatocytes, TNF-α triggers Bid-dependent lysosomal permeabilization, followed by the release of CB into the cytosol and the activation of caspase-2. Caspase-2 then facilitates efficient mitochondrial cytochrome *c* release and apoptosis (Guicciardi *et al*., 2005). In addition, the proteasome inhibitor bortezomid induces CB release from lysosomes, activation of caspase-2 and cell death (Guicciardi *et al*., 2005). Caspase-2 activation is blocked when CB is inhibited, suggesting that CB release occurs upstream of caspase-2. Moreover, Z-VDVAD-FMK (a chemical inhibitor of caspase-2) or depletion of caspase-2 with an siRNA attenuated MOMP. Thus, caspase-2 emerges as a new link between lysosomal and mitochondrial permeabilization (Yeung *et al*., 2006). It remains to be determined in which specific circumstances Bid and caspase-2 play predominant roles in mediating post-LMP events such as MOMP.

Cathepsin B has been shown to translocate to the nucleus and induce cell death upon exposure of SHSY-5Y cells to 3-aminopropanal, mouse hepatocytes to TNF-α or rat hepatocytes to glycochenodeoxycholate (Roberts *et al*., 1997, 2000; Li *et al*., 2003). There is some evidence that CB can induce direct nuclear damage from studies in which purified CB induces rapid chromatin condensation in isolated HeLa nuclei (Vancompernolle *et al*., 1998). Interestingly, this effect was abolished when CB was preincubated with 2 μM zVAD-fmk, a concentration at which only caspase activity is inhibited. Thus, the proapoptotic effect of CB may depend on secondary caspase activity.

The most common cathepsins implicated in cell death are CB and CD, although CL can also induce caspase-3 activation (Ishisaka *et al*., 1999; Hishita *et al*., 2001). In addition, CL has an important function in the cell death associated with the involution of the mammary gland after weaning, as CL inhibitors reduce cell death in this system (Burke *et al*., 2003). Cathepsin G is normally expressed in macrophages and other myeloid cells, and it has been implicated in the antibacterial effect of polymorphonuclear leukocytes. Cathepsin G staining becomes diffuse during ultraviolet light-induced apoptosis of leukemia cells, and cathepsin G may be

implicated in the proteolytic degradation of the Hbrm protein, which is implicated in chromatin remodeling (Biggs *et al*., 2001). Chymotrypsin B is a digestive protease secreted by the exocrine pancreas that has recently been implicated in apoptotic cell death of hepatocytes treated with TNF-α. Chymotrypsin B translocates from lysosomes to the cytosol and induces a mitochondrial pathway of apoptosis that is significantly blocked by RNA interference of chymotrypsin B or by pretreatment with the chymotrypsin B inhibitor, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone. These results demonstrate that chymotrypsin B can function as a proapoptotic protease in the LMP-initiated cell death pathway (Miao *et al*., 2008).

As previously stated, many factors can influence cell fate during LMP-induced apoptosis, including cell type, the intensity of the cell death stimulus, the state of cellular transformation, the amount of endogenous antioxidants and the levels of endogenous cathepsin inhibitors. In addition, in a given experimental system, very similar compounds may trigger multiple signaling pathways that lead to cell death. For example, ultraviolet C irradiation of HeLa cells treated with two very similar fluoroquinonole antibiotics, norfloxacin and ciprofloxacin, converts cell death from an ROS-dependent, caspase-independent pathway to an ROS-independent, caspase-dependent pathway (Boya *et al*., 2003a).

LMP in cancer cells

Cathepsins are often upregulated in human cancers and their high levels of expression have been correlated with an increased risk of relapse and poor prognosis (Gyrd-Hansen *et al*., 2004; Palermo and Joyce, 2008). Cathepsins contribute to distinct tumorigenic processes such as angiogenesis, metastasis and invasion, and these functions are attributed to the extracellular actions of cathepsins once they have been released from secretory lysosomes (Palermo and Joyce, 2008). Thus, cathepsins participate in the degradation of basement membranes and extracellular matrix, thereby facilitating tumor invasion and metastasis (Tardy *et al*., 2006). Interestingly, each cathepsin participates in a different process during tumorigenesis, as revealed by specific cathepsin knockout mice (Vasiljeva and Turk, 2008). In addition to these extracellular effects of cathepsins in matrix remodeling, intracellular lysosome-dependent degradation also has an important function in tumorigenesis, as suggested by the observation that collagen degradation can be blocked by means of specific intracellular cathepsin inhibitors (Sameni *et al*., 2000). In addition to these tumor-promoting effects of cathepsins, evidence is emerging that proteases, and in particular cathepsins, may have a function as tumor suppressors as well (Lopez-Otin and Matrisian, 2007).

During cellular transformation, cancer cells undergo profound changes in their lysosomal compartment, which affect their size, intracellular localization, cathepsin expression and enzymatic activity. Immortalization and transformation have been shown to increase the susceptibility of mouse embryonic fibroblasts to lysosome-dependent cell death induced by anticancer agents (Fehrenbacher *et al*., 2004). This effect is mediated through CB overexpression and increased cathepsin-dependent cell death. Lysosomal maturation, size and activity are tightly regulated by phosphatidylinositol-3 kinase (PI3K) (Mousavi *et al*., 2003), an enzyme that is activated in many cancers. Inhibition of PI3K induces the translocation of CB to the cytosol and may sensitize endothelial cells to TNF-α-induced apoptosis (Madge *et al*., 2003; Li and Pober, 2005). Hsp70 may promote tumorigenesis by stabilizing lysosomal membranes and by protecting cells against LMP induced by hypoxia, non-inflammatory cytokines, oxidative stress, irradiation or anticancer drugs (Rohde *et al*., 2005). The reasons for the increased susceptibility of cancer cell lysosomes to LMP are not understood. As one possibility, relatively large lysosomes, as found in cancer cells (Glunde *et al*., 2003), may be more fragile than normal-sized lysosomes (Ono *et al*., 2003). Moreover, cancer cells exhibit higher metabolic rates and an increased turnover of iron-containing proteins, leading to the lysosomal accumulation of iron, with consequent iron-mediated sensitization to ROS-induced LMP (Eaton and Qian, 2002). Cancer cells often produce elevated ROS levels, and the associated higher rate of spontaneous cathepsin release from lysosomes may facilitate cell death induction (Gyrd-Hansen *et al*., 2004). On theoretical grounds, all these factors render lysosomes from cancer cells particularly susceptible to the therapeutic induction of LMP. However, this speculation awaits experimental verification.

Recently, parvoviruses have been shown to induce the death of glioma cells by inducing LMP with consequent accumulation of CB and CL in the cytosol, while reducing the intracellular levels of cystatins B and C. In addition, intracerebral injection of H1-parvovirus induced tumor regression in a rat glioma model, which was associated with increased CB activity (Di Piazza *et al*., 2007). These results provide circumstantial evidence that LMP may represent a new therapeutic strategy for the treatment of brain tumors.

Cancer cells often exhibit a reduced expression of proapoptotic signaling or effector molecules and/or overexpress antiapoptotic proteins. The discovery of alternative non-apoptotic cell death pathways might stimulate the development of novel therapeutic strategies (Fehrenbacher and Jaattela, 2005). In this regard, new therapeutic approaches to treat cancer through lysosomal destablization are emerging and several experimental anticancer agents developed by the NIH seem to kill cells through p53-independent LMP (Erdal *et al*., 2005). Indeed, the lysosomotropic detergent siramesine has an antitumorigenic effect *in vivo* in mouse models of fibrosarcoma and breast cancer when administered concomitantly with tumor induction (Ostenfeld *et al*., 2005).

Photodynamic therapy combines a drug (a photosensitizing agent) with a specific wavelength of light to induce the generation of ROS that kill cancer cells (Buytaert *et al*., 2007). The subcellular localization of

the photosensitizer is of special importance, as it determines the localization of the primary damage. Many photosensitizers accumulate in mitochondria or lysosomes. Whereas photosensitizers that accumulate in mitochondria tend to induce rapid apoptosis, photosensitizers that enrich in lysosomes may induce either apoptosis or necrosis (Moor, 2000). Photodynamic therapy with photosensitizers that accumulate inside lysosomes may induce cell death dependent on LMP and cathepsins (Buytaert *et al*., 2007), as has been shown for ATX-s10 and *N*-aspartyl chlorin e6 (Caruso *et al*., 2004; Ichinose *et al*., 2006). In addition, antiapoptotic members of the Bcl-2 family of proteins are often degraded after photodynamic therapy, making cells more sensitive to apoptotic cell death (Xue *et al*., 2003; Ichinose *et al*., 2006).

LMP during development and aging or other diseases

The role of LMP-dependent cell death has not been extensively investigated in mammalian organogenesis. CD appears to be overexpressed in areas of prominent cell death during limb development such as in the interdigital areas (Zuzarte-Luis *et al*., 2007b). Similar observations have been reported in heart development (Zuzarte-Luis *et al*., 2007a). CD is expressed during insect metamorphosis in a tissue- and developmental stage-specific manner, and CD expression is transcriptionally regulated by the hormone ecdysone. Depletion of CD by RNAi arrests larval–pupal metamorphosis and abolishes DNA fragmentation (Gui *et al*., 2006). The role of cathepsin during insect development may be related to the role of autophagy and lysosome degradation during *Drosophila* metamorphosis, where both autophagy and caspases have prominent functions (Lee *et al*., 2002). Caspase knockout animals possess supernumerary neurons and the knockout of lysosomal proteases can exacerbate cell death in different tissues and notably in the brain (Boya and de la Rosa, 2005).

The combined deficiency of CB and CL results in early-onset neurodegeneration in mice that is reminiscent of neuronal ceroid lipofuscinoses (a hereditary neurodegenerative disease in humans), and cathepsin $B^{-/-}/L^{-/-}$ mice exhibit a major degree of cerebral atrophy (Felbor *et al*., 2002). This atrophy is due to the massive apoptosis of selected neurons in the cerebral cortex and in the cerebellar Purkinje and granule cell layers. Similarly, central nervous system neurons in $CD^{-/-}$ mice display a new form of lysosome accumulation with a neuropathological phenotype that resembles neuronal ceroid lipofuscinosis (Koike *et al*., 2000). Batten disease manifests as massive neuronal degeneration with the accumulation of undigested material inside lysosomes. Niemann-Pick type C (NPC) disease is an autosomal recessive disorder caused by mutations of NPC1 and NPC2 genes. The progressive neurodegeneration that accompanies NPC is fatal, and an association has been established between autophagic-lysosomal dysfunction and cholesterol accumulation in $Npc1^{-/-}$mice. Brain levels of lysosomal CD were significantly higher in $Npc1^{-/-}$ than in wild-type mice (Stahl *et al*., 2007).

Relatively few *in vivo* studies have addressed the relationship between lysosomal membrane destabilization and pathogenic cell death. LMP likewise has a major function in hepatic dysfunctions like cholestasis, where glycochenodeoxycholate induces caspase activation, LMP, CB translocation and cell death, whereas CB inactivation attenuates liver injury (Canbay *et al*., 2003). Moreover, in a dietary murine model of non-alcoholic fatty liver disease, either genetic or pharmacological inactivation of CB protects against the development of hepatic steatosis, liver injury and insulin resistance with its associated 'dysmetabolic syndrome' (Feldstein *et al*., 2004). In animal models of iron overload, iron-mediated lysosomal instability and enhanced lipid peroxidation occur (Mak and Weglicki, 1985).

Atherosclerosis is an inflammatory disease characterized by extensive remodeling of the extracellular matrix (Liu *et al*., 2004a). It has recently been reported that atherosclerotic lesions in both humans and mice are associated with the expression of several lysosomal proteases, including cathepsins B, D, L and S, which may affect plaque development and stability. Oxidized low-density lipoprotein may favor LMP, inducing the death of phagocytic cells, as this has been shown in cultured cells (Li and Yuan, 2004; Liu *et al*., 2004a). *In vivo* knockout studies revealed that the deficiency of cathepsin K or S attenuates atherosclerosis (Sukhova *et al*., 2003). Another pathogenic situation in which macrophages have been implicated is lung damage induced by silica exposure. Silica particles enter cells by phagocytosis and their reactive surface may interact with phagolysosomal membranes, thus triggering the translocation of lysosomal enzymes to the cytosol and consequent cell death (Thibodeau *et al*., 2004).

Conclusion

A plethora of cell death-relevant molecules can operate upstream or downstream of LMP. Although the number of papers dealing with LMP is finite, the regulation of LMP appears rather complex, and the precise sequence of events that trigger LMP or emanate from LMP has not been established. In an apparently confusing fashion, it appears that multiple molecular players can act both upstream and downstream of LMP. This has been reported for caspases, ROS, Bax and Bid. Depending on the stimulus, the cell type and the transformation status, cells may use the same elements to fine-tune lethal signaling. In addition, the same building blocks can control cell death pathways at many different levels, thus further increasing the perplexing complexity of lethal signal transduction cascades.

The exact molecular mechanisms that mediate LMP remain enigmatic. Bcl-2 family members may have similar permeabilizing properties for lysosomal membranes as they have for mitochondrial membranes, but

this has not yet been demonstrated in a convincing fashion in cell-free systems. In this regard, it appears intriguing that apolipoprotein L is structurally and functionally similar to proteins of the Bcl-2 family, insofar that it possesses pore-forming domains and displays the ability to induce apoptosis in several cell types (Vanhollebeke and Pays, 2006). Using a bioinformatics approach, apolipoprotein L6 has been identified as a BH3-only protein, and its MOMP-inducing and cell-killing activities are both lost upon deletion of the BH3 domain (Liu *et al*., 2005). Whether apolipoprotein can induce LMP-dependent cell death is currently unknown. In this context, it appears most intriguing that apolipoprotein L1 is the major serum factor endowed with the capacity of lysing *Trypanosoma cruzi* and that its trypanolytic effect is correlated with its capacity to form pores in the lysosomal membrane of *Trypanosoma* (Perez-Morga *et al*., 2005). Thus, one of the major challenges for further investigation remains the elucidation of LMP in molecular terms.

For theoretical reasons, it will be of utmost importance to determine which endogenous inhibitors prevent LMP, both in normal and in cancer cells, and which mechanisms mediate the suppression of lysosomal hydrolases in the cytosol, either by inhibiting their enzymatic activity or by targeting them to autophagic or proteasomal destruction. Disinhibiting LMP-dependent death might unleash self-destructive processes in cancer cells, especially if the cancer cells were 'addicted' to such inhibitors. One example for such endogenous inhibitors of LMP is HSP70, a class of HSP70 for which thus far no specific chemical inhibitors have been developed. Future investigations will clarify whether antagonists of HSP70 or other yet-to-be-discovered LMP inducers may constitute a useful addition to the clinical oncologist's armamentarium.

Abbreviations

AIF, apoptosis-inducing factor; AO, acridine orange; CB, cathepsin B; CD, cathepsin D; CL, cathepsin L; FAN, factor associated with neutral sphingomyelinase activation; LAPF, lysosome-associated protein containing PH and FYVE domains; LMP, lysosomal membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization; PDT, photodynamic therapy; TNF-α, tumor necrosis factor alpha.

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