Lysosomes as "Suicide Bags" in Cell Death: Myth or Reality?[*][S]

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50 years ago, Christian de Duve introduced the term "suicide bags" to describe lysosomes (1), the organelles containing numerous hydrolases, which were, until the discovery of the ubiquitin-proteasome system, thought to be responsible for the major part of the intracellular turnover of proteins and other macromolecules. It is well established now that the endosomal/lysosomal system has numerous other functions, including survival functions. Recently, lysosomes and lysosomal proteases were found to participate in cell death pathways, which, at least during apoptosis, are suicidal for cells. It seems timely therefore to discuss whether or not lysosomes in fact play suicidal roles in cellular processes.

Lysosomes and Endolysosomal System

Lysosomes are single membrane-bound cytoplasmic organelles present in almost all eukaryotic cells. They are the major degradative compartment of the endosomal/lysosomal system and the terminal part of the endocytic pathway, where a variety of macromolecules, such as proteins, glycoconjugates, lipids, and nucleic acids, are degraded to their building blocks (2). They are extremely well suited for this function, as they contain over 50 different hydrolases. They are further characterized by low pH (3.8 –5.0) and by the absence of mannose 6-phosphate receptors. Among the hydrolases, the proteases, which are responsible for protein degradation, are considered to be highly important (3, 4). Although lysosomes were long considered to be responsible primarily for the nonspecific degradation of organelles and of the long-lived proteins, it is now clear that they have a number of other functions, including selective degradation of proteins, repair of the plasma membrane, release of endocytosed material, and removal of certain pathogens (2, 4, 5).

Proteins destined for degradation enter lysosomes via endocytosis (extracellular proteins), phagocytosis (pathogens and cellular debris), and micro- and macroautophagy (intracellular proteins). Cytosolic proteins can also enter lysosomes via chaperone-mediated autophagy across the lysosomal membrane using heat shock proteins as chaperones and LAMP-2 protein as the receptor that recognizes specific sequences on the target proteins (2, 4). With the exception of microautophagy and chaperone-mediated autophagy, all other pathways were shown to involve fusion of lysosomes with other vacuoles (phagosomes or autophagosomes) or organelles (late endosomes), indicating that lysosomes are very dynamic organelles.

Lysosomal Cysteine Cathepsins

A special place among the lysosomal proteases is held by the cathepsins, which include the cysteine cathepsins and the aspartic protease cathepsin D, another very abundant lysosomal protease (3). In humans, 11 cysteine cathepsins have been found, including cathepsins B, C, F, H, K, L, O, S, V, W, and X. They all share the same core structure and are all monomers of ~30 kDa with the exception of the tetrameric cathepsin C. In the active form, the catalytic Cys and His residues form a thiolate-imidazolium ion pair with pK_a values ~3.5 and ~8.0, which is essential for the activity of the enzymes. The enzymes do not exhibit high specificity, consistent with their role of recycling enzymes, and they cleave their substrates primarily after hydrophobic residues, but they will also accept basic residues in the P_1 position. Although the majority of cathepsins are endopeptidases, some are exopeptidases, which is a consequence of the additional structural features protruding into the active-site cleft (6). Collectively, these mixed specificities provide an excellent arsenal of weapons for dismantling the proteins to small peptides, before the job is finished by dipeptidases and oligopeptidases. There is also a lot of redundancy among the cathepsins, as none of the single cathepsin knock-outs that have been performed showed any defects in intracellular protein turnover (supplemental Table 1) (7, 8).

Cell Death and Lysosomes

Any description of lysosomes as suicide bags requires them to be linked to the death of a cell. There are three major morphologically distinct pathways of cell death, and lysosomes have been found to be linked with all of them. The first one is apoptosis, which is characterized by specific biochemical and morphological changes within the affected cell, for a number of which the caspases, a group of cysteine proteases, are responsible. Apoptosis is the major way by which eukaryotes remove superfluous, damaged, and other potentially dangerous cells. The process is especially important during development and homeostasis and is remarkably conserved through evolution. A critical step in apoptosis is caspase activation, which can be achieved in several ways, including death receptor activation and mitochondrial permeabilization or through the action of granzymes, which are released from secretory granules of NK[2] and T cells. A number of signals converge at the level of the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.
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[2] The abbreviations used are: NK, natural killer; ROS, reactive oxygen species; LMP, lysosomal membrane permeabilization; XIAP, X-linked inhibitor of apoptosis.
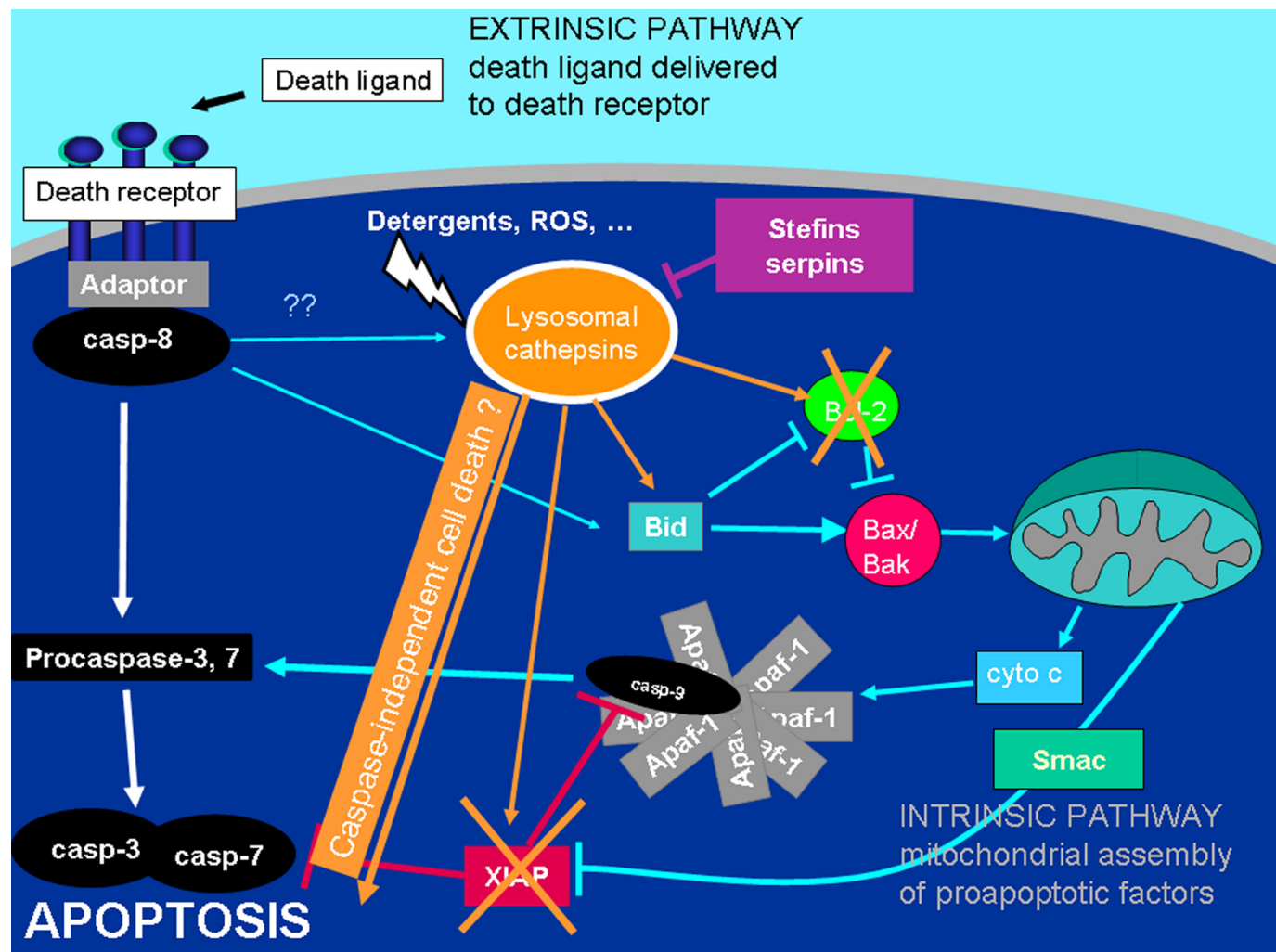
FIGURE 1. **Cysteine cathepsin pathways to apoptosis.** Lysosome disruption results in release of cathepsins to the cytosol. Once in the cytosol, cathepsins process Bid and degrade the anti-apoptotic Bcl-2 homologs as well as XIAP. Proteolytic removal of anti-apoptotic Bcl-2 family members, similarly to their sequestration by BH3-only proteins, results in cytochrome *c* (*cyto c*) release from mitochondria, followed by executioner caspase (*casp*) activation and apoptosis. The possible link between the death receptor pathway and the lysosomes is also marked, as well as the possible caspase-independent cell death pathway. Intracellular cathepsin inhibitors serpins and stefins serve as cellular guardians, blocking escaped cathepsins.

mitochondria, which are the central organelles in apoptosis (9, 10). Although lysosomes and lysosomal cathepsins were also suggested to play a role in apoptosis (3, 11), their involvement seems to be of lesser importance for apoptotic progression and limited to specific stimuli, such as lysosomal detergent action, ROS linked with redox-active iron, and several anticancer drugs (12–14). Nevertheless, lysosomes and lysosomal cathepsins might play an important role in neurodegeneration and aging, mediated primarily by oxidative stress (13, 15). Based on cathepsin knock-out studies and cathepsin silencing, cathepsins were also suggested to be implicated in the death receptor pathway (16), a model that is, however, still under debate (Fig. 1) but was suggested to play an important role in liver homeostasis and cancer cell apoptosis (11, 16).

The second pathway is often referred to as type II programmed cell death or autophagic cell death. In general, autophagy (to eat oneself) is the main process for recycling of organelles and parts of the cytoplasm. In simple unicellular organisms such as yeast, this self-digestion was found to be triggered as a response to primarily starvation-induced stress.

Removal of superfluous or damaged organelles provides nutrient supplies to the cell to help it to survive. The process is highly conserved in eukaryotes and provides protection to the organism against various kinds of stress and pathologies, whereas a basal level of autophagy is critical for homeostasis of the organism (17, 18). In support of the pro-survival origin of autophagy is also the finding that autophagy exists in unicellular trypanosomatid parasites, where in addition to its role during starvation, it clearly plays a pro-survival role during differentiation, which is known to be critical for parasite survival and infectivity (19). However, under certain conditions, autophagy was suggested to be detrimental to the cell. Several examples exist, including *Drosophila* salivary gland cell degradation, where cells were observed to undergo substantial autophagy (20). Nevertheless, blocking autophagy only delayed cell death in this system but could not prevent it, suggesting that autophagy is dispensable for this type of cell death. These results suggest either that the autophagic system can be activated under presumptively non-stress conditions or, more likely, that the affected cells are subject to currently undetected stress or star-

vation. Moreover, it has been recently suggested that essentially all of the findings where autophagy was found to accompany cell death can be ascribed to cell death with autophagy and not to cell death due to autophagy largely because of the lack of selectivity of methods and reagents used, arguing against the role of autophagy as a cell death pathway (21).

The molecular mechanism of autophagy and the double membrane autophagosome formation, which has been largely unraveled only during the last decade, is highly conserved (22). The whole process thus seems to serve simply to pack the cargo and deliver it to the lysosomes for a final meal for lysosomal hydrolases. Lysosomes, which fuse with autophagosomes into autolysosomes, are clearly of major importance for proper clearance of autophagosomes. Cathepsin D-deficient or cathepsin B and L double-deficient mice developed neuronal ceroid lipofuscinosis characterized by accumulation of LC3-II positive autophagosomes in the brain (23). Late onset neuronal ceroid lipofuscinosis was also observed in cathepsin F knock-out mice; however, autophagosome accumulation was not followed (24). Moreover, it has been recently shown that restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function, further showing the importance of lysosomes in cellular homeostasis (25).

The third pathway, which is largely triggered in a more catastrophic situation as a consequence of physicochemical stress such as heat shock or ROS and when the damage is too severe to be kept under control within the cell, is called necrosis. Necrosis is often associated with various human pathologies caused by local inflammation triggered by the release of content of dead or dying cells, triggering the innate immunity response. However, there is increasing evidence that even necrosis may be a controlled process. Based on findings in *Caenorhabditis elegans*, it was suggested that the major step in the necrotic cell death of neurons was an increase in cytosolic Ca^{2+} concentration, which was suggested to lead to an imbalance of proteolysis, in particular activation of calpains and aspartic lysosomal cathepsins due to LMP (26). Moreover, later studies in the worm demonstrated that calpain-mediated LMP was also a critical step in hypotonic shock, heat shock, oxidative stress, hypoxia, and cation channel hyperactivity, with cysteine cathepsins found to be the major downstream regulators (27). In contrast, in death receptor-mediated necrotic cell death (necroptosis), the serine/threonine kinase RIP1 (receptor-interacting protein 1) was suggested to be one of the major molecular players (28). Despite the fact that few of the molecular players have been identified, the molecular mechanism(s) of necrosis are much less clear than in apoptosis or autophagy, and it is much more likely that necrosis is a result of the interplay of several signaling pathways. Few attempts have been made to delineate the sequence of events in necrosis. Dysfunction of mitochondria linked with ATP depletion was thus suggested to be an early event, whereas LMP seems to be a late event just preceding the plasma membrane rupture. This was recently confirmed in the protist *Dictyostelium discoideum*, where lysosome permeabilization was found to be the critical irreversible step, whereas mitochondrial uncoupling showed reversibility for a long time (29). The main messengers of the signal were found to be Ca^{2+} and ROS, which likely results in greatly increased proteolysis, in particular in calpain activation and in cytosolic proteolysis due to liberated lysosomal cathepsins (30).

The three cell death pathways seem to be largely interconnected. However, this has been the subject of a number of excellent reviews (31, 32) and will not be further discussed here.

Differential Roles of Lysosomal Cathepsins in Cell Death

Localization—Clearly, lysosomes and lysosomal proteases are implicated in all three death pathways; however, their contributions in the three pathways differ significantly. Lysosomes and cathepsins are absolutely required for autophagy, and they were also found to be highly important in necrosis, whereas their importance for apoptotic progression seems to be more limited (see above).

So what are the major differences between the roles of lysosomes and lysosomal proteases in these three pathways? One of them is in the localization of lysosomal proteases. During autophagy, lysosomes fuse with autophagosomes into autolysosomes, where nonselective recycling and degradation of engulfed material occur (18). Under these conditions, lysosomal proteases are held within the membrane and cannot affect other cytosolic components. In contrast, in both apoptosis and necrosis, lysosomes leak their contents, including the cathepsins, into the cytosol (3, 30). However, lysosomes also seem to be well protected against external damage that could lead to LMP, with Hsp70 being one of the important guardians against different types of cellular stress (33), thus arguing against their role as suicide bags. There is, however, an important difference between the roles of lysosomes and lysosomal cathepsins in apoptosis and necrosis. Whereas a massive burst of lysosomes and release of cathepsins are often a critical step for necrosis, there are only a few situations known where limited damage of lysosomes linked with the release of cathepsins into the cytosol is critical for apoptotic progression. Currently, very little is known about the molecular mechanisms of lysosomal membrane breakdown and the release of cathepsins. Although it is extremely difficult to study it under necrosis conditions, where LMP is rapidly followed by loss of plasma membrane integrity, it seems that release of the cathepsins and other hydrolases is a rather nonselective process in apoptosis, as a number of them have been observed in the cytosol following lysosome disruption (3, 16, 32). The major criterion seems to be the size, as smaller proteins were found to translocate faster and more efficiently than larger proteins (34).

Cathepsin Substrates and Regulation—Despite decades of work on the cathepsins, little is known about their physiological substrates (6–8). Most of the early evidence came from *in vitro* experiments performed at acidic pH, where cathepsins are optimally active and which probably affected the structure of the proteins. Under these conditions, which we find in lysosomes during autophagy, cathepsins largely degrade their substrates. Such conditions are the most optimal ones, as they provide a good compromise between the activity and the stability of cathepsins and, in addition, provide optimal working conditions due to partial denaturation of the substrates. As extralysosomal proteins enter lysosomes nonselectively during autophagy, one can assume that all of them are also degraded in autolysosomes.

During apoptosis and necrosis, however, cathepsins are released into the cytosol, where they face unfavorable conditions due to higher pH. Therefore, not every protein that is cleaved/degraded *in vitro* at acidic pH is a cathepsin substrate in cells or *in vivo*, following the release of cathepsins into the cytosol. There are several possible reasons for this, the most likely being instability of cysteine cathepsins and almost zero activity of cathepsin D at the neutral pH of the cytosol. Whereas cysteine cathepsins lose their activity due to irreversible unfolding (35), cathepsin D activity is lost reversibly due to deprotonation of both active-site Asp residues. Additional reasons probably lie with the substrates: (i) their structures are probably more compact at neutral pH and thereby less accessible to proteolysis, and (ii) it is likely that some proteins are inaccessible to proteolysis due to their involvement in complex formation and/or membrane attachment. Two such examples are the pro-apoptotic Bcl-2 family members Bak and Bim, which are good substrates of various cysteine cathepsins *in vitro* even at neutral pH, but were not found to be cleaved by cathepsins in a cellular model of apoptosis following lysosome destabilization and subsequent release of cathepsins into the cytosol (36). Nevertheless, acidification of the cytosol, which has been observed during tumor necrosis factor-α-induced apoptosis (37), may help stabilize the cathepsins and destabilize at least some of their substrates.

There are no real data concerning cathepsin substrates during necrosis. The major reason is probably related to the very short duration and complexity of the process and to the fact that this problem has not been examined in any detail. An assumption can be made that a significant number of proteins are cleaved during necrosis, as blocking the cathepsins has been shown to prevent necrosis induced by a number of stimuli in *C. elegans* (27).

Apoptosis, which is a considerably slower process than necrosis and more controllable, thus remains the only process where at least some cathepsin substrates have been identified. Currently, the only well established apoptotic substrate is Bid, which was initially identified in a cell-free study (38) and later confirmed in several cellular models using different means of lysosome disruption (36, 39–41). Cathepsins exhibit a great deal of redundancy, as several of them, including cathepsins B, L, S, and K, were shown to be capable of cleaving Bid at the same site *in vitro*. Moreover, they were also capable of releasing cytochrome *c* from mitochondria in the presence of Bid in cell-free extracts (40). In addition to Bid, cathepsins were found to degrade the anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-x$_L$, and Mcl-1 and XIAP (36). This is in agreement with previous data demonstrating that knocking out Bid simultaneously with the ablation of stefin B (see also above) did not rescue cerebellar apoptosis, which is largely cathepsin-dependent (42). This implies that cathepsins can trigger the mitochondrial pathway of apoptosis and therefore are dependent on caspases to finish the job and kill the cell, in agreement with findings in several cellular models (43). Although cathepsins were also found to participate in the death receptor pathway, no substrates have been conclusively identified. However, knocking out the cathepsins failed to completely block apoptosis but led only to its suppression (44–47), arguing against an essential role of cathepsins in this pathway. This is also more consistent with recent data on Fas-induced apoptosis, where lysosomes were indeed observed to be broken but substantially later than mitochondria (48, 49). These latter findings are more consistent with a bystander role of cathepsins, although their role in an amplification loop through mitochondria cannot be excluded. At this stage, no more cathepsin substrates have been identified, more in line with the concept of caspases as the major cell death executioners and cathepsins as the helper molecules (Fig. 1). In any event, because the cathepsin substrates Bcl-2, Mcl-1 and IAPs were found to be up-regulated in a number of cancers (10, 36), LMP-inducing drugs have a major potential in anticancer treatment (14). Although cathepsins have often been linked with caspase-independent pathways to cell death (11, 16, 32), the substrates have essentially remained unidentified. A similar problem concerns the involvement of cathepsin D in various cell death pathways, as no substrates except Bid (50) have really been identified, and all the current models are based on *in vitro* studies carried out at pH ~4 (51). Moreover, when overexpressed in cells, even catalytically inactive cathepsin D was able to mediate apoptosis, a finding that has not helped to clarify the issue (52).

The list of cathepsin substrates during apoptosis is definitely far from complete. When a more systematic approach (such as a proteomics-based one) is applied, more substrates are expected to be identified. This should also help to answer the question as to whether cathepsins can kill cells independently of caspases, as suggested previously (11).

However, despite all their stability problems, cysteine cathepsins can be active at neutral pH, at least for a short time, which is sufficient to enable cleavage of a limited subset of substrates, allowing them to participate in signal transduction (43). Moreover, this activity time can be prolonged by binding to various ligands, including substrates. Cathepsins therefore pose a permanent latent threat to cells in cases where there is significant damage to lysosomes. Control of this threat is maintained by their intracellular inhibitors, stefins and serpins, which serve as cellular guardians, playing a more efficient role than just carrying out simple pH-induced, irreversible inactivation (35, 53). This activity became even more evident when the major intracellular inhibitor of the cathepsins, stefin B, was ablated, and stefin B-deficient mice were observed to spontaneously develop cerebellar apoptosis (42). Similarly, removal of the intracellular serpin SRP6 in *C. elegans* sensitized the worms to necrosis (27).

Lytic Granules from NK Cells: The True Suicide Bags

In addition to lysosomes, a number of specialized cells contain unique organelles that resemble endosomes/lysosomes by virtue of sharing some of their common features. Collectively, they are called lysosome-related organelles or secretory lysosomes, usually contain a number of lysosomal hydrolases and membrane proteins, and are characterized by low luminal pH. The best characterized lysosome-related organelles are lytic granules from cytotoxic T cells and NK cells and azurophilic granules from neutrophils and eosinophils. Lytic granules are somewhat exceptional, as cytotoxic T lymphocytes do not contain the conventional lysosome. The granules thus serve two roles, one for intracellular digestion and one to destroy other cells (5). Nevertheless, they appear to be the closest cellular structures to the concept of real suicide bags. Following NK or T cell activation and during temporary fusion with their target

cells, the lytic granules release their contents, including a number of hydrolases such as granzymes, into the target cells, which then undergo apoptosis (54). However, the differences between these organelles and conventional lysosomes are quite considerable, so they should not be confused.

Conclusion

In conclusion, the major function of lysosomes and lysosomal proteases is not to kill the cell but to take care of cellular homeostasis and possibly differentiation by recycling cellular components. Even minor damage of lysosomes does not seem to be sufficient to kill the cell; cells survive possibly through engagement of autophagy, thereby entering into a cycle where lysosomes take care of lysosomes. Following considerable lysosomal damage, cells can also undergo apoptosis or, more commonly, if damage is more severe, necrosis. However, despite their devastating potential, it would be inaccurate to call them suicide bags because, at least based on current knowledge, such cell death happens primarily during pathologies. Although our current level of knowledge suggests that lysosomal breakdown seldom represents the boundary between life and death, it is likely that future studies will show that this may be the case in far more physiological situations.

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