ELSEVIER

Death by design: apoptosis, necrosis and autophagy

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Apoptosis is the principal mechanism by which cells are physiologically eliminated in metazoan organisms. During apoptotic death, cells are neatly carved up by caspases and packaged into apoptotic bodies as a mechanism to avoid immune activation. Recently, necrosis, once thought of as simply a passive, unorganized way to die, has emerged as an alternate form of programmed cell death whose activation might have important biological consequences, including the induction of an inflammatory response. Autophagy has also been suggested as a possible mechanism for non-apoptotic death despite evidence from many species that autophagy represents a survival strategy in times of stress. Recent advances have helped to define the function of and mechanism for programmed necrosis and the role of autophagy in cell survival and suicide.

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Abbreviations

PARP	poly(ADP-ribose) polymerase
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand

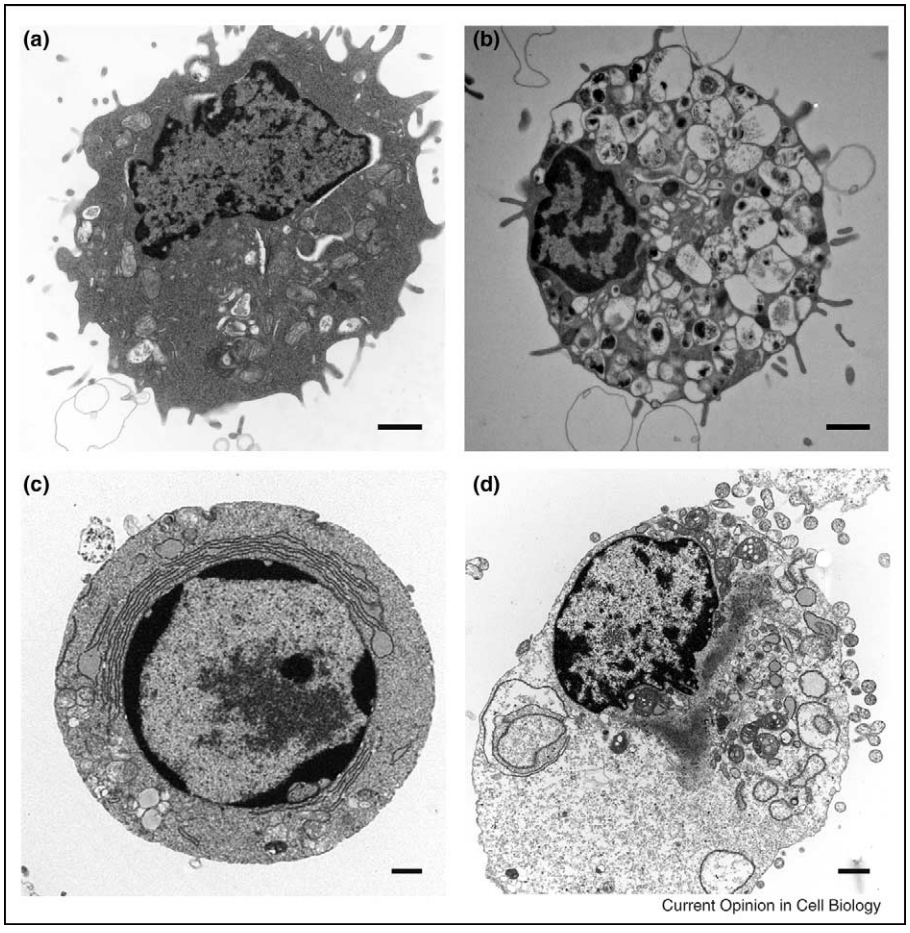
Introduction

Cell death can be divided into two classes, apoptosis and necrosis. Apoptosis has come to be used synonymously with the phrase 'programmed cell death' as it is a cell-intrinsic mechanism for suicide that is regulated by a variety of cellular signaling pathways (for a recent review see [1]). For cell death to be classified as apoptotic, nuclear condensation and fragmentation, cleavage of chromosomal DNA into internucleosomal fragments and packaging of the deceased cell into apoptotic bodies without plasma membrane breakdown must be observed. Apoptotic bodies are recognized and removed by phagocytic cells and thus apoptosis is also notable for the absence of inflammation around the dying cell. The morphologic features of apoptosis result from the activation of caspases (cysteine proteases) by either death receptor ligation or the release of apoptotic mediators from the mitochondria. Dying by apoptosis requires energy in the form of ATP; a tidy, regulated death does not come for free.

In contrast to apoptosis, necrosis has been traditionally thought to be a passive form of cell death with more similarities to a train wreck than a suicide. Necrosis is the end result of a bioenergetic catastrophe resulting from ATP depletion to a level incompatible with cell survival and was thought to be initiated mainly by cellular 'accidents' such as toxic insults or physical damage. Necrosis is characterized morphologically by vacuolation of the cytoplasm, breakdown of the plasma membrane and an induction of inflammation around the dying cell attributable to the release of cellular contents and proinflammatory molecules. Cells that die by necrosis frequently exhibit changes in nuclear morphology but not the organized chromatin condensation and fragmentation of DNA into 200 bp fragments that is characteristic of apoptotic cell death. Figure 1 illustrates the morphological differences between cells dying by apoptosis and necrosis.

Over the past several years the idea that cells can commit suicide by mechanisms other than apoptosis has been gaining momentum [2–6]. Alternative, non-apoptotic forms of programmed cell death have been described and classified as programmed necrosis or autophagic cell death. Linking the term 'programmed' to the word 'necrosis' implies that cellular signaling pathways initiate necrosis in response to specific cues rather than 'by accident'. The concept of programmed necrosis has existed in the literature for several years, but several recent papers have added significantly to our understanding of the mechanism behind, and the regulation of, this process. Although there are morphologic similarities between the two processes (Figure 1), autophagic cell death has traditionally been classified as a distinct form of non-apoptotic death that is separate from necrosis. Autophagy means, literally, to eat oneself. In a strategy conserved across taxa, cells switch to a catabolic metabolic program in which cellular constituents are degraded for energy production as a survival mechanism during periods of nutrient stress. The process of autophagy has been extensively reviewed elsewhere ([7] and references therein). Briefly, a double membrane vesicle forms in the cytosol that encapsulates whole organelles and bulk cytoplasm. This autophagosome then fuses with the lysosome where the contents are degraded and recycled. Autophagy plays a degradative role by providing a mechanism for the turnover of both

Figure 1



Morphological features of autophagic, apoptotic and necrotic cells. **(a)** Normal, **(b)** autophagic, **(c)** apoptotic **(d)** and necrotic cells. Whereas the morphologic features of apoptosis are well defined, the distinction between necrotic and autophagic death is less clear. The bioenergetic catastrophe that culminates in cellular necrosis also stimulates autophagy as the cell tries to correct the decline in ATP levels by catabolizing its constituent molecules. Thus, vacuolation of the cytoplasm is observed in both autophagic cells (b) and in cells stimulated to undergo programmed necrosis (d). By contrast, ATP levels are maintained in normal (a) and apoptotic cells (c) consistent with the limited number of autophagic vacuoles in their cytoplasm. The scale bar represents 1 μm.

damaged organelles and long-lived proteins in addition to its catabolic role providing energy in times of famine. It has been suggested that cells can die a 'programmed' death by autophagy in which cells digest themselves to death as a suicide strategy (reviewed in [8–10]). This idea is in direct contradiction with the proven role of autophagy in many different organisms as a survival mechanism during lean times. Thus, the question emerges, is autophagy a survival pathway, a suicide pathway or both in mammalian cells? This review will discuss recent advances in our understanding of the biological relevance of programmed necrosis and the role of autophagy in determining cell fate and will explore the idea that necrosis and autophagic death are really two sides of the same coin.

Necrosis: not just an accident?

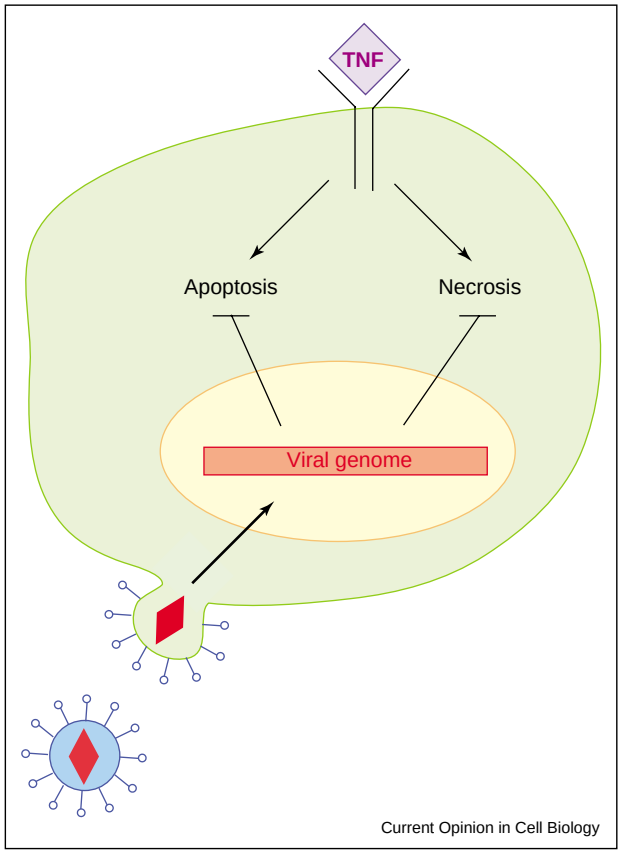
When caspases were originally identified as the mediators of apoptosis, it was hypothesized that many of their substrates were essential proteins whose destruction ensured the inevitability of cell death. However, caspase-independent cell death is observed in many systems indicating that cells still die even if the executioner is absent. Following an apoptotic stimulus, such as the expression of Bax or treatment with tumor necrosis factor (TNF) or Fas ligand, cells will die even in the presence of non-specific caspase inhibitors such as zVAD-fmk or anti-apoptotic molecules like Bcl-XL that prevent caspase activation [2,4]. Under these conditions, cells that would normally die by apoptosis exhibit all the hallmarks of necrosis. In some cases, caspase-independent necrotic cell death can be forestalled by treatment with antioxidants or by eliminating the activity of the protein kinase RIP. These results led to the idea that necrosis could be 'programmed' — cellular signaling events initiated necrotic destruction that could be blocked by inhibiting discrete cellular processes. A criticism of this concept of

necrosis as a programmed event has been that this form of cell death is only observed under conditions in which apoptosis is inhibited either chemically or genetically. In fact, recent evidence suggests that the initiation of apoptosis might actively suppress necrosis because activated caspases cleave and inactivate proteins required for programmed necrosis (e.g. RIP and poly(ADP-ribose) polymerase [PARP]) [11–13,14••]. Furthermore, the lack of understanding of the actual mechanism(s) for necrotic cell death has helped to condemn the concept of programmed necrosis to designation as a tissue culture phenomenon. However, recent studies have shed light on the mechanism by which programmed necrosis kills cells, highlighted the important ramifications of programmed necrosis in patients treated with cancer chemotherapeutics and suggested an important physiologic role for programmed necrosis in response to viral infection.

Is programmed necrosis a physiologically relevant process?

Although there have been many reports of programmed necrosis following death receptor (TNFR, Fas, TRAIL) ligation when apoptosis is blocked, it has not been observed in a physiologic setting. A recent paper by Chan *et al.* makes progress towards demonstrating a biologically relevant role for this alternative strategy of cell death [15••]. In these studies, cells infected with vaccinia virus are used to illustrate a natural condition in which apoptosis is inhibited and necrosis takes over (like many other viruses, vaccinia encodes an anti-apoptotic protein). When the inflammatory cytokine TNF is added, a molecule likely to be present during an anti-viral immune response, it induces necrotic rather than apoptotic death in virally infected cells by initiating signal transduction cascades downstream from its receptor. Although it has long been known that viruses encode anti-apoptotic proteins to prevent their host cells from killing themselves apoptotically, this work also demonstrates that viruses harbor proteins that suppress programmed necrosis. Although it remains to be established whether these anti-necrotic proteins are important for viral pathogenicity, proteins that interfere with TNF-mediated signal transduction have been identified in viruses of different classes. In addition, a beneficial role for programmed necrosis during viral infection is suggested by the observation that mice lacking the TNFR2 protein (and therefore deficient in the necrotic response to vaccinia infection) exhibited both reduced inflammation in response to infection and decreased clearance of the virus. Thus, programmed necrosis might not simply be a backup when apoptosis fails, but might serve an important function in fighting microbial infection (Figure 2).

Figure 2

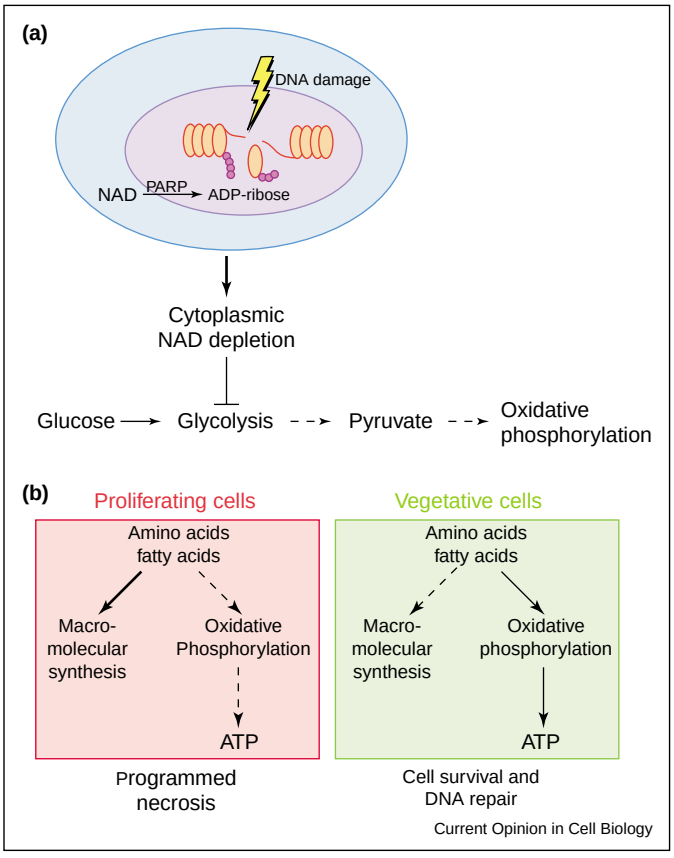


Viral infection can induce programmed necrosis. Binding of the inflammatory cytokine, TNF, to its receptor results in either apoptosis or in programmed necrosis in cells in which the apoptotic pathway is disabled. Many viruses carry genes that inhibit apoptosis thereby prolonging the life of their host cell and foiling the cell's attempt to limit its use as a virus-producing factory by initiating apoptosis. A recent study [15••] indicates that viruses also carry genes to suppress programmed necrosis, the back-up mechanism that infected cells use for suicide. These observations establish a physiologic role for programmed necrosis in mammalian cells.

DNA damage can initiate programmed necrosis in proliferating cells

Programmed necrosis might also be important in protecting metazoan organisms from accumulating cells that have sustained DNA damage. Although DNA damage can initiate apoptosis, it appears that an intact apoptotic pathway is not required for the elimination of proliferating cells that acquire DNA damage [14••]. Thus, even cells that have an impaired apoptotic response can still be removed when placed at risk of acquiring fixed mutations. Programmed necrosis in response to DNA damage was found to be initiated by the DNA repair protein PARP, but surprisingly this form of cell death only occurred in cells that were actively proliferating. This selectivity was attributable to the fact that PARP activation leads to the rapid depletion of nuclear and cytoplasmic NAD and thus the inhibition of glycolysis. As a result, cells dependent on glycolysis for ATP production quickly become depleted of ATP following PARP activation and die by necrosis.

Figure 3



DNA damage and PARP activation runs a metabolic test on cells resulting in programmed necrosis selectively in proliferating cells. **(a)** DNA damage results in the depletion of cytoplasmic NAD owing to the PARP-dependent modification of nucleosomal proteins with ADP-ribose chains enzymatically derived from NAD. These modifications expose the damaged DNA and assist in the targeting of DNA repair complexes to the site of the damage. The depletion of cytoplasmic NAD results in the inhibition of glycolysis as NAD is required in the glycolytic pathway for the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. Thus, following PARP activation and NAD depletion glucose can no longer be converted to the pyruvate needed to fuel oxidative phosphorylation in the mitochondrion. **(b)** The loss of the ability to oxidize glucose for energy creates a situation in which cells must oxidize alternative substrates such as amino acids and fatty acids. Proliferating cells are committed to using amino acids and lipids to building new proteins and membranes respectively, and do not have the metabolic programs in place to switch to using these substrates to fuel ATP production. Thus, ATP levels decline below the level compatible with the operation of plasma membrane ion transporters and the cell dies by necrosis. Vegetative cells, in contrast, are not committed to a high rate of macromolecular synthesis and are able to divert amino acids and fatty acids into pathways leading to their oxidation in the mitochondrion. This maintains intracellular ATP levels and allows DNA repair.

Growing cells, particularly transformed cells, are dependent on glucose metabolism for ATP production because they utilize amino acids and lipids for the synthesis of proteins and membranes, respectively. Vegetative cells, by contrast, are able to maintain ATP levels through oxidative phosphorylation and by catabolizing amino acids and lipids. In such cells, PARP activation can promote DNA repair and correction of the damage without lethally depleting cellular ATP. Thus, PARP activation might run a metabolic test on cells that have sustained DNA damage to determine the risk that mutations will be amplified by replication without repair (rate of cellular proliferation) and the degree of DNA damage (severity of NAD reduction) (Figure 3).

Harnessing programmed necrosis for cancer chemotherapy

Many if not all human tumors carry mutations that inactivate apoptotic pathways allowing tumor cells to persist despite growing beyond normal homeostatic limits. Thus, the idea that tumor cells might be particularly sensitive to programmed necrosis could help to explain how some targeted chemotherapeutic agents induce tumor cell death. For example, Okada *et al.* [16•] demonstrate that BCR-ABL-positive leukemia cells can exhibit caspase-independent cell death in response to treatment with the Abl kinase inhibitor, Gleevec (imatinib). This is a drug that has received a great deal of attention as it is the first rationally designed, effective cancer chemotherapeutic.

Imatinib-induced necrotic cell death correlates with the release of HtrA2/Omi, a serine protease known to be a potential mediator of caspase-independent necrotic cell death [17], from the mitochondria. This death was blocked by treatment with serine protease inhibitors. The inflammatory component of necrotic death has the potential advantage of stimulating an immune response that could increase the efficacy of Gleevec therapy. Whether Gleevec's effectiveness is enhanced *in vivo* by an inflammatory response remains to be tested. By adjusting the balance between apoptotic and necrotic cell death, one could hope not only to kill tumor cells, but to modulate the patient's immune response to the tumor.

Is there really autophagic death?

It has been demonstrated in yeast, Dictyostelium, *C. elegans* and plants that autophagy represents a survival mechanism employed to allow these organisms to survive times of famine [7]. Autophagy can be stimulated by nutrient deprivation in mammalian cells as well, although cultured mammalian cells that are subjected to nutrient deprivation rapidly undergo apoptosis rather than switch to the stationary phase that allows survival in less complex organisms. Nevertheless, in certain disease states, including neurodegenerative disorders such as Alzheimer's and Parkinson's disease, cells in an advanced state of autophagy are frequently observed. This led to the idea that autophagy is not only an adaptive response to nutrient limitation but also a mechanism for cell suicide [8–10]. This 'autophagic death' has been classified as distinct from necrotic cell death. The data supporting the hypothesis that autophagy can be used as a suicide program are largely correlative. It is important to consider that necrosis occurs in cells that are undergoing severe bioenergetic stress, the same conditions that would stimulate autophagy as a mechanism for boosting ATP levels. Thus, it is likely that autophagy and necrosis often occur in parallel, initiated in response to the same stimuli but with completely opposite objectives. Deciphering the truth about the relationship between autophagy and necrosis is further complicated by the manner in which these processes are studied. For example, zVAD is frequently employed in the initiation of 'programmed necrosis'. However, this drug can inhibit cathepsins at the same concentrations at which caspases are inhibited and thus might affect lysosomal degradation and have additional non-specific effects on the cell [18]. In addition, 3-methyladenine, frequently employed as a 'specific' inhibitor of autophagy, is a general inhibitor of phosphatidyl-inositol 3-kinases and thus interferes with numerous cellular processes besides autophagy [19]. Now that we are beginning to understand the molecules involved in these alternative death signaling pathways, the ability to design experiments that do not depend upon these non-specific chemical inhibitors should help to separate the roles of necrosis and autophagy in cell death.

Autophagy is a strategy for survival, not death, in mammalian cells

Several recent papers address the function of autophagy in mammalian cells. Beclin 1 is the mammalian homolog of the yeast Atg6 protein that is involved in the early steps of autophagic vesicle formation. Two groups generated Beclin 1 deficient mice and showed that Beclin 1 is a haplo-insufficient tumor suppressor gene [20•,21•]. The observation that the loss of one copy of Beclin 1 promotes transformation seems on the surface to be most consistent with the model that autophagy is a cell death pathway that suppresses tumorigenesis. Upon further consideration, however, these studies do not support this initial conclusion. Embryonic stem cells genetically null for Beclin 1 were generated and then tested for their resistance to cell death initiated either by UV irradiation or following serum withdrawal [20•]. No differences in the induction of cell death were found in *beclin* $1^{-/-}$ cells compared to the control wild-type cells. If autophagy were a cell suicide mechanism, then Beclin-1-null cells should have been resistant to cell death. In addition, the wild-type allele was never found to be deleted in any of the tumors in *beclin* $1^{+/-}$ mice despite the fact that Beclin 1 is not an essential protein, at least under tissue culture conditions [20•,21•]. These results are most consistent with the idea that autophagy is a survival not a death pathway. In fact, the retention of the wild-type allele is consistent with the idea that a low level of autophagy might actually be required for tumor initiation. Tumor cells are exposed to limiting nutrient conditions as they grow beyond their blood supply. This means that transformed cells might use autophagy as a temporary survival strategy until new blood vessels are formed.

If disabling an autophagic cell death pathway is not the mechanism by which haplosufficiency for *beclin 1* stimulates tumor formation, what is the mechanism? One possibility is that it is Beclin 1's role in regulated degradation that is important for tumor suppression. Although autophagy represents an important mechanism for producing ATP catabolically in times of nutrient stress, the autophagic pathway also plays an important role in the turnover of proteins and organelles under nutrient-replete conditions [7,22]. No mechanism for specificity has yet been described for autophagic degradation, but it is likely that specific proteins or damaged organelles are somehow targeted for turnover. Some evidence already exists demonstrating that Beclin 1 can direct the degradation of specific proteins [23]. It could be that Beclin 1 is involved in the degradation of proteins that promote cell growth and survival and that it is the maintenance of these signaling pathways and not the general decrease in autophagy that promotes transformation in the absence of Beclin 1. Another possibility is that as autophagy is involved in the degradation of damaged organelles, interfering with the turnover of sources of free radicals, like damaged mitochondria

and endoplasmic reticulum, might increase the level of genotoxic stress and thus the likelihood of oncogenic mutations.

In certain populations of apoptosis-resistant cells such as neurons autophagy can be observed following growth factor withdrawal, a condition where nutrient uptake declines [24,25]. When neurons are cultured for long periods in the absence of growth factors, they will eventually die even when caspases are inhibited. Although to date there is no proof that the autophagy observed is required to maintain cell survival, autophagy is necessarily a self-limited process and cells dependent on autophagy to maintain bioenergetics will ultimately die of necrosis when their internal resources are exhausted.

Can autophagy also be a part of a cell death program?

Other recent studies present data that are more in keeping with the idea that autophagy is negative regulator of cell survival. Yu *et al.* examined zVAD-induced cell necrosis in the murine fibroblast line L929 [26••]. These authors found that autophagy and programmed necrosis were stimulated by zVAD treatment, probably as a result of the inhibition of caspase 8-mediated cleavage of the kinase RIP. Intriguingly, by using RNAi to decrease the levels of both Beclin 1 and Atg7, it was shown that these proteins, which are known to regulate autophagy, are required for zVAD-mediated death. These studies suggest that autophagy is required to induce cell death in this system. However, other interpretations should be considered. It must be taken into account that zVAD-fmk is not a specific inhibitor of caspases and that the lysosomal degradative pathway is probably affected by zVAD-fmk as well, confounding the interpretation of the results. It is possible that autophagic vacuoles merely become more prominent following zVAD treatment as lysosomal degradation of their contents is disrupted. Now that conditions have been established to suppress Beclin 1 and Atg7 by the RNAi pathway, the role of these proteins in the induction of cell death in response to physiologic stimuli can be tested.

Another instance in which autophagy might kill cells is during receptor-induced tissue remodeling. Mills *et al.* have shown that death receptor ligation stimulates autophagy and suggest that this function is important in concert with apoptosis for acinar morphogenesis [27•]. In this case, TRAIL was shown to positively regulate lumen formation in acini formed *in vitro* by the breast cancer cell line MCF-10. In previous studies, these authors showed that MCF-10A cells expressing the anti-apoptotic protein Bcl-XL were capable of lumen formation although with delayed kinetics [28]. Autophagy occurred in both the presence and absence of apoptosis, and cell death in the absence of apoptosis was hypothesized to result from autophagy. In this new report, it was observed that TRAIL treatment stimulated autophagy [27•]. In addition, eliminating both TRAIL signaling and apoptosis resulted in luminal filling. These data are consistent with the idea that autophagy represents an alternative death pathway when apoptosis is disabled. Although blocking TRAIL inhibited autophagy, these studies remain correlative. If autophagic death is responsible for TRAIL-induced lumen morphogenesis in the absence of apoptosis, blocking autophagy in the presence of TRAIL signaling should lead to luminal filling.

Conclusions

In conclusion, the available evidence is consistent with two forms of regulated cell death, apoptosis and necrosis. Programmed necrosis might have a biological function under conditions where an immune reaction to the dying cell is desirable such as in microbial infection. Based on abundant evidence from yeast, slime molds, nematodes and plants, autophagy seems more likely to represent a survival strategy than a mechanism for cell death. Several papers cited above, however, indicate that this hypothesis requires further testing. Separating autophagy and cell death is confounded by the fact that the stimuli for both processes often overlap. A demonstration that autophagic cell death occurs in cells that are not bioenergetically compromised would go a long way towards establishing that autophagy and necrosis represent distinct forms of cell death. A better understanding of the genes involved in autophagy and necrosis should allow future studies to dissect the role of autophagy in cell death and survival more thoroughly.

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