

REVIEW

Caspase-independent cell death?

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Many cells die with apoptotic morphology and with documented activation of an effector caspase, but there are also many exceptions. Cells frequently display activation of other proteases, including granzymes, lysosomal cathepsins, matrix metalloproteinases, and proteasomal proteases, and others display morphologies that are not fully consistent with classical apoptosis. In some experimental situations, evidence of caspase-dependent death is indirect, demonstrating that the cell can activate caspases rather than that it does. In other situations, such as involution of mammary or prostate tissue, many cells display autophagic or other morphology different from apoptosis, and there is considerable evidence for the activation of a lysosomal system. Prior to total collapse and necrosis, cells that are in trouble can activate numerous physiological pathways toward self-destruction. Intrinsic or extrinsic routes to effector caspase activation are frequently the most rapid and efficient. If neither of these routes is immediately available, owing to mutation, genetic manipulation, inhibitor, or the biology of the cell, other routes may be followed, leading to variant forms of cell death that may display one or more characteristics of apoptosis. Experimental and therapeutic procedures must account for this possibility.

Oncogene (2004) 0, 000–000. doi:10.1038/sj.onc.1207514

Keywords: caspase; caspase-independent; apoptosis; programmed cell death; lysosome; autophagocytosis; protease

Introduction

Apoptotic cell death has been a very powerful and fruitful paradigm. The original generalization was that most cell deaths displayed a characteristic and common morphology (Kerr, 1971; Kerr *et al.*, 1972). Exploration of this generalization, coupled with the brilliant genetic analysis of programmed cell death in the nematode *Caenorhabditis elegans* (Horvitz *et al.*, 1983; Yuan and Horvitz, 1990; Horvitz *et al.*, 1994; Xue *et al.*, 1996), led to a molecular basis of the common morphology, heavily deriving from what are essentially highly conserved restriction endopeptidases, the caspase

family of proteases, and thence to an equally exciting analysis of the control, activation, and inhibition of these enzymes. Most of this story is discussed elsewhere in this issue and will not be further explored here.

The clarity of the caspase story has rendered it an outstanding paradigm that has now achieved the hoary authority of being diagrammed or outlined in textbooks. However, it is far from the only story in cell death. As other papers in this issue argue, at least one other prominent form of cell death exists: autophagic cell death. Many authors have attempted to define the several morphological forms of physiological cell death and necrosis. Among the clearest definitions are those of Schweichel and Merker (1973), Clarke and Clarke (Clarke, 1990), and, most recently for lymphocytes, Jäättelä and Tschopp (2003). These reviews should be consulted. Here, we attempt to address some generalizations, most importantly the issues that, first, the presence of a caspase does not prove that cell death is caspase dependent; second, a lack of phenotype resulting from knockout of a caspase or caspase regulator does not eliminate the possibility of compensation by other caspases; and, third, caspase activation does not always mean death (Perfettini and Kroemer, 2003) and does not preclude the activity of other lethal mechanisms. Often, caspase activation appears to be the preferred mode of execution, but in its absence or failure, there are many other default pathways.

Cell death is not synonymous with the activation of hydrolytic enzymes

Proteolytic damage by any of several proteases, not uniquely caspases, can trigger cell death. Thus, there are examples of death associated with the activation of lysosomal and proteasomal proteases, as well as granzyme B and matrix metalloproteinases. In some instances, lysosomal or proteasomal proteases trigger apoptosis by proteolytically activating caspases (Boya *et al.*, 2003), but in other cases they act by undermining primary cell structure. Cleavage of cytoskeletal proteins or necessary enzymes such as PARP may be highly destructive in the absence of caspase activation. Also, activation of a protease is not necessarily synonymous with cell death. Caspase inhibitors and caspase knock-outs often produce phenotypes that cannot be explained by the absence of cell death. Autophagy is more often

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associated with reduction of cell volume than cell death, and autophagy may even help protect a cell against death by sequestering mitochondria and preventing the release of cytochrome *c* (Bauvy *et al.*, 2001; Elmore *et al.*, 2001). Third, there is sporadic evidence for caspase function in various situations not related to cell death, such as mitosis. Fourth, very sick cells will die by one means or another. A severely injured cell in which, for instance, protein synthesis or respiration is blocked simultaneously with the blockage or absence of an initiator caspase, will still fail. Most typically, its death is slightly delayed and may follow a route such as autophagic or another type of death. Fifth, there is far more inter-relationship among the types of cell death than is generally acknowledged. For instance, cells can be protected from necrosis by bcl-2 (Kane *et al.*, 1995; Vaux *et al.*, 1996; Zamzami *et al.*, 1997; Kroemer *et al.*, 1998). Either bcl-2 is a more general protectant, maintaining reduction potential, or necrosis and apoptosis have some pathways in common. Finally, recognition of apoptosis depends to a great deal on the environment of a cell. This idea is best illustrated by the statement that, *in vitro*, the common fate of a cell is ultimately necrosis. Apoptotic cells *in vivo* are generally rapidly phagocytosed. *In vitro*, where phagocytes may not be found, an apoptotic cell will persist until its ion pumps have completely failed. At this time it is likely to swell, rupture, or show signs of a rise in $[Ca^{2+}]_i$, leading to the precipitation of proteins in the cell. We assume that we can quantify apoptosis as opposed to necrosis *in vivo*, but the assumption depends on the ability of phagocytes to reach the scene. In many instances, access of phagocytes is severely limited. For instance, a thrombus can limit access. In bone and a few other restricted sites, very limited circulation delays the arrival of phagocytes. Drugs or pathologies that directly affect phagocytes, or massive, overwhelming cell death (as perhaps in chemotherapy), can also limit the arrival of phagocytes or overwhelm them. Dying cells may well initiate apoptosis but ultimately succumb to alternative pathways to death.

Physiological cell deaths that are not clearly apoptotic

There are many programmed or at least physiological cell deaths exist that do not appear to be classical apoptosis or dependent on the activity of caspases (Clarke and Clarke, 1996; see Figure 2). These include the maturation death of osteoblasts (Manolagas, 2000) in maturing bone, the differentiation of keratinocytes (Norsgaard *et al.*, 1994; Polakowska *et al.*, 1994; Tamada *et al.*, 1994; Mammone *et al.*, 1998), and the more equivocal, death-like processes of organelle elimination in cells such as lens fibers (Dahm *et al.*, 1997; Counis *et al.*, 1998; Ishizaki *et al.*, 1998; Wride *et al.*, 1999) and erythrocytes (Sasaki *et al.*, 1993; Bratosin *et al.*, 2001b). The death of plant cells in many physiological situations is also not apoptotic or caspase dependent, but will not be discussed in a journal devoted

to understanding cancer. Likewise, caspases may have roles other than in cell death, for instance, in the differentiation of osteoblasts (Mogi and Togari, 2003) or of monocytes into macrophages (Sordet *et al.*, 2002). There is some evidence that caspase activity down-regulates epidermal growth factor receptor signaling (Zhuang *et al.*, 2003), and cells such as muscle fibers or some hormone-dependent glands or tissues can atrophy, using autophagic, proteasomal, or other means of mass reduction, without dying. These atrophic tissues may regenerate at a later date.

It is also not often recognized that some markers of apoptosis are not uniquely diagnostic: cells dying by autophagy and necrosis also display mitochondrial permeability transition (Lemasters *et al.*, 1998, 2002) and in Alzheimer's disease and others, the number of TUNEL-positive cells is far higher than the number of dying cells (Cotman *et al.*, 1998; Jellinger and Stadelmann, 2001). In cleaving mammalian embryos, caspase inhibitors cause abnormalities long before the first cell deaths are expected (Zakeri and Lockshin, in preparation). Finally, one point is often overlooked in the effort to have clear, linear, comprehensible sequences of biological processes: it requires no specific skill or mechanism to die. Phrased differently and in a manner more suggestive of research direction, even if caspase activity is for some reason blocked, a cell that is in deep trouble for any reason can still fail. Blocking caspases will not preserve the cell. For instance, death of T-lymphocytes is very important during the resolution of an immune response and in eliminating autoreactive lymphocytes. This death is often classical apoptosis, with the activation of caspases. However, as described in an excellent review by Jäättelä and Tschopp (2003), many deaths can be defined as apoptosis-like programmed cell death (an active process dependent on signaling events in the dying cell, not necessarily driven by caspase activation and often lysosomal) or necrosis (in which loss of control occurs before proteases such as caspases or cathepsins have organized the death of the cell). The interest of the researcher is to find and relieve the stress on the cell, and to determine how the threshold at which it commits to death is established.

Most studies that analyse the importance of caspases rest on one or more experimental paradigms and assumptions, the validity of which may be questioned. Some studies measure or force the upregulation of caspase transcripts, although in the acute situation caspase activation is not a matter of transcription. Others rely on the use of caspase inhibitors, often at concentrations in which they are relatively nonspecific (Schotte *et al.*, 1999). Finally, although the assumption is probably valid that activation of effector caspase-3 or -7 by activator caspase-8 or -9 is the major pathway, knockout, knockin, or upregulation of experiments typically address one caspase only and do not address the possibility that other caspases or caspase-like enzymes can act instead of the affected enzyme.

The issues that should be raised are therefore the following: For each, there is some evidence but not full experimental or intellectual comprehension.

1. How do specialized cells such as osteoblasts and keratinocytes die?
2. What is the relationship of organelle elimination in lens and erythrocytes to apoptosis?
3. What determines when a cell should activate caspases or other death mechanisms?
4. How is autophagy as a protective mechanism different from autophagic cell death?
5. At what point is a cell's loss of physiological control such that the cell is condemned?

Terminal differentiation is often described as a form of apoptosis, but frequently the defense of the argument seems a bit forced. Most typically, the fact that a cell can undergo apoptosis is used to argue that it does. A literature is available for many instances of terminal differentiation, and it illustrates both that caspase activity may result in a morphology different from classical apoptotic morphology as described by Kerr and Wyllie, and that many factors other than caspase activity determine the fate of the cell. Each is discussed in turn below.

Early vertebrate embryos

Early vertebrate embryos, prior to the maternal–zygotic transition (MZT), putatively cannot undergo apoptosis, and this failure has been variously attributed to the presence of antiapoptotic molecules or the absence of proapoptotic molecules (Hensey and Gautier, 1999). Surely, although it is possible to kill an embryo prior to the MZT, in our laboratory, we find that if early zebrafish embryos are subjected to toxins such as cycloheximide or staurosporine, they activate apoptotic machinery such as caspase-3, but the cells fail and lyse approximately 1 h before they would be expected to undergo apoptosis. Thus they die by necrosis, and the distinction seems to be a matter of timing and physiology. The situation highlights the arbitrariness of the choice of routes to death that a cell takes (Negrón and Lockshin, submitted for publication).

With most caspase knockouts in mammals, there appears to be either no effect on embryonic cell death or only a very belated effect. For instance, knockout of caspase-3 or -9 yields a catastrophic (but in developmental terms modest and late – after embryonic day 10 in mouse) overgrowth of the brain (Kuida *et al.*, 1996; Kuida *et al.*, 1998) and even this teratology may derive from factors other than cell death (Oppenheim *et al.*, 2001). Even in these animals most other cell deaths, such as those defining the hand palette, or during forebrain enclosure at EDs 7–8, are normal. There are also cell deaths in early preimplantation embryos, ED3, or between eight-cell stage and blastula. These are unaffected by caspase inhibitors, and caspase knockout typically does not affect them. Thus, if caspases are involved, there is redundancy to assure the deaths of these cells. Overall, embryos seem well equipped to guarantee most cell deaths by alternate routes if necessary.

Lens

Lens fibers are living cells that are free of organelles. The loss of organelles – nuclei and mitochondria have been most closely studied – appears by most criteria to be a process related to apoptosis. For instance, upregulation of bcl-2 can delay enucleation in chick lenses and in lentoids (small lenses differentiating from lens epithelial cells in culture) (Sanders and Parker, 2003). Lens epithelial cells can undergo apoptosis and they contain caspase-3 (Ishizaki *et al.*, 1998; Yao *et al.*, 2003). Surprisingly, caspase-3 is present in lenses, and cataract formation may be associated with a decrease in caspase-3, suggesting either that even the differentiated lens fiber can undergo a form of apoptosis or that caspase-3 has functions other than apoptosis (Andersson *et al.*, 2003).

The loss of the nucleus from the lens is characterized by shrinkage of the nucleus and margination of the chromatin as in apoptosis, followed by clustering of nuclear pores (Dahm and Prescott, 2002). A lysosomal DNase, similar to DNase II, is created by proteolysis from a different enzyme (Counis *et al.*, 1998; Torriglia *et al.*, 2001). Overexpression of bcl-2 slows denucleation (Fromm and Overbeek, 1997; Dahm, 1999; Sanders and Parker, 2003), and caspase inhibitors inhibit denucleation but do not reduce TUNEL-positive cells (Wride *et al.*, 1999; Wride, 2000). As in many apoptotic cells, p35 is cleaved and Cdk5 is activated (Gao *et al.*, 1997). Thus lens denucleation is an attenuated form of apoptosis. One interpretation suggests that caspase-9 migrates into the mitochondria after MPT and can form an apoptosome in the cytosol, but the difference is that there is no translocation of molecules between the cytoplasmic compartments and the nucleus (Sanders and Parker, 2002). However, in living zebrafish embryos, caspase-3/7 is not detected during denucleation, although it is readily detected during stalk separation (Abraham and Lockshin, in preparation). Thus it is harder to document the direct activity of caspase-3/7, at least, than to argue indirectly that lens differentiation is apoptotic or caspase dependent.

Erythrocytes

Erythrocytes are of interest at two stages of their life cycle: during differentiation, when they shed their organelles, and at the end of their lives, when they are cleared from the bloodstream in the spleen. During erythropoiesis, nucleated precursors of erythrocytes can clearly undergo apoptosis, as can nucleated erythrocytes of non-mammalian vertebrates and mammalian fetuses. Erythrocyte precursors can undergo apoptosis and upregulate caspases. Bcl-xL can be upregulated to protect them (Mori *et al.*, 2003). Interestingly, tissue inhibitor of metalloproteinases (TIMP)-1 has an antiapoptotic effect in an erythroleukemic cell line, indicating that proteases other than caspases can be used to activate apoptosis (Lambert *et al.*, 2003). The role of caspases in these processes is complex. *In vitro*, the

pancaspase inhibitor zVAD.fmk can block erythropoiesis. Caspase-3, -6, and -7 are activated and degrade lamin B and acinus, but they do not degrade GATA-1, and phosphatidylserine asymmetry is not disturbed. Erythropoiesis is normal in caspase-3 knockout mice. In nucleated erythrocytes, experimental induction of apoptosis does not necessarily activate caspases and is not necessarily prevented by zVAD.fmk (Daugas *et al.*, 2001). Mature, senescing erythrocytes undergo at least those aspects of an apoptotic program that mark them for clearance by phagocytes: increase in intracellular Ca^{2+} , leading to exposure of phosphatidylserine, loss of K_i^+ , and cell shrinkage (Lang *et al.*, 2003a–c; see also, Macho *et al.*, 1997). This process is at least partially mimicked by hyperosmotic shock, which likewise leads to phosphatidylserine exposure. In this case, both increase in intracellular ceramide and influx of Ca^{2+} participate (Lang *et al.*, 2003a–c).

Bratosin *et al.* have argued that at least a partial explanation for the conflicting data concerning mature erythrocytes resides in the activity of cysteine proteases other than caspases. Cysteine protease inhibitors protected erythrocytes even though caspases were not activated (Berg *et al.*, 2001; Bratosin *et al.*, 2001a). Many researchers rely heavily on caspase inhibitors to distinguish among proteases participating in cell death, but some of these inhibitors are relatively nonspecific at the concentrations used (Schotte *et al.*, 1999). One thought-provoking suggestion (Daugas *et al.*, 2001) is that the mature erythrocyte is a postapoptotic mummy. In this scenario, caspases were activated during erythropoiesis, leading to the destruction of some nuclear proteins, and elimination of nucleus and mitochondria. However, the terminal phases of apoptosis, which would be characterized by exposure of phosphatidylserine, for instance, have been delayed. Daugas *et al.* interpret these observations to suggest that the final death may not be truly apoptotic but rather a common consequence of an increase in entropy of a cell that can no longer maintain itself. Alternatively, it may represent the end of an apoptotic pathway in which a hiatus has been interpolated between the initial events and the final collapse. By either interpretation, the maturing and senescing erythrocyte raises several very provocative questions, including how it is possible to create a partial apoptosis during erythropoiesis, how the completion is held in abeyance, and how the final steps are finally activated. A similar argument, suggesting a possible mechanism of constraint of caspase activity, has been raised for the slightly less spectacular situation of neurons degenerating in the presence of caspase inhibitors: they selectively eliminate all mitochondria, thereby committing themselves to eventual death (Xue *et al.*, 1999, 2001; Tolkovsky *et al.*, 2002).

Osteoblasts

Osteoblasts die when cartilage ossifies (most commonly recognized as epiphyseal closure) and this death is most

commonly described as not appearing to be apoptotic in nature. However, cultured osteoblasts show some indication of being able to undergo apoptosis: dexamethasone activates caspases and leads to the release of cytochrome *c* from mitochondria, a process that can be prevented by Bcl-xL (Chua *et al.*, 2003). Also, one can find cells that appear apoptotic that have been phagocytosed by osteoclasts (Cerri *et al.*, 2003), and by electron microscopy osteoclasts can appear apoptotic (Sun *et al.*, 2003). In differentiating bone, osteoblasts can die a very lonely death, with phagocytes having some difficulty in reaching them. Since at some point apoptotic cells or cell fragments must lose control of their ionic balance, the fate of an unphagocytosed cell will ultimately be necrosis. In this situation as well, there is far more indirect than direct evidence that the death of the osteoblast is apoptotic or caspase dependent.

Keratinocytes

The normal destiny of a keratinocyte is to differentiate into squamous epithelium, a dead cell lacking organelles and filled with keratin. Keratinocytes, mostly represented by HaCAT cells (a spontaneously immortalized aneuploid human keratinocyte cell line capable of differentiation) or primary cultures from the human foreskin, undergo apoptosis when subjected to high levels of UV-B. These types of cells are frequently subjected to apoptosis-inducing regimens such as UV-B and exposure to free radicals or other reactive oxygen species. In these conditions, produced to mimic sunburn or potential generation of melanomas, keratinocytes clearly undergo apoptosis, as documented by positive TUNEL assays, a defensive increase in transcription from antiapoptotic genes, morphology, and activation of one or more caspases. However, the issue is more complex. First, apoptosis is produced at only low doses of UV, 5–20 mJ/cm², and this level permits differentiation of the cells into mature keratinocytes; but above 20 mJ/cm², both differentiation and apoptosis are inhibited and the cells undergo necrosis (Gandarillas *et al.*, 1999; Mammone *et al.*, 2000). Some of the confusion concerning the effect of UV-B derives from failure to compare doses. The situation for differentiation of keratinocytes is even more equivocal. Earlier literature reported that during differentiation, the cytoskeleton and nuclear matrix were rearranged; that caspases were activated during normal differentiation; and that inhibition of caspases resulted in retention of the nucleus (Norsgaard *et al.*, 1994; Bolognia, 1995; Weil *et al.*, 1999; Gniadecki *et al.*, 2001; Weisfelner and Gottlieb, 2003). However, it is difficult to conclude from current evidence that the normal differentiation of keratinocytes is a form or variant of apoptosis. Differentiating keratinocytes do not appear apoptotic by morphology; TUNEL assays are subject to high background; and DNA ladders, complicated by the presence of keratin, are not very clean (Gandarillas *et al.*, 1999). Furthermore, p53-null mice, which might be

expected to show decreased apoptosis, have normal skin (Sanchez-Prieto *et al.*, 1995; Weinberg *et al.*, 1995).

More recent information suggests that, as for erythrocytes, keratinocyte differentiation may be an example of a constrained apoptosis. Differentiating keratinocytes express and process caspase-14 but not caspase-3, -6, or -7, whereas the opposite is true for experimentally provoked apoptosis (Lippens *et al.*, 2000). Thus differentiation as opposed to induced apoptosis of keratinocytes appears to involve a selection of which caspases to activate. Allombert-Blaise *et al.* detect activation of caspase-3, -8, and -9 in differentiating skin and found that, in a skin-like model, zVAD.fmk prevented normal differentiation of the stratum corneum (Allombert-Blaise *et al.*, 2003). They argue that caspase activation occurs in only a few cells at a time and therefore may be missed. Intriguingly, they present evidence that, in the large, relatively flat differentiating epidermal cells, mitochondrial depolarization may not be simultaneous: within a single cell, some mitochondria may depolarize and release cytochrome *c* before others. This selective depolarization may be spatially controlled, and caspase activation follows at a substantially later time. These authors query the prolonged, slow progress toward apoptosis and speculate that the upregulation of antiapoptotic factors in differentiating keratinocytes may stretch out what is an inherently apoptotic pathway. Like erythrocytes, keratinocytes may represent a highly restricted and constrained form of apoptosis, worthy of further investigation. A similar situation may be obtained for neurons, in which there is evidence for at least initial confinement of apoptotic processes to specific neurites, axons, or dendrites. This 'partial apoptosis' may protect the cell body from complete collapse (Jellinger and Stadelmann, 2001; Raff *et al.*, 2002).

Metamorphosing tadpole tissues

The destruction of a tadpole tail was examined many years ago as an indication of lysosomal activity (Lockshin, 1969; Weber, 1969), but later the lysosomes appeared to be confined to phagocytes. Kerr and his co-workers identified many situations in tadpole metamorphosis that were clearly apoptotic (Kerr, 1971; Kerr *et al.*, 1972, 1974). Tadpole metamorphosis was one of the examples used to define apoptosis as a general phenomenon. However, the tail muscle fragments into large 'sarcolytes', presumably by isolation via the T system or sarcoplasmic reticulum, which are ultimately consumed by phagocytes and overall the destruction of the muscle involves considerably more than classical apoptosis.

Metamorphosing insect tissues

Caspases and caspase-like molecules have been identified in insects (Dorstyn *et al.*, 1999a,b; Lee and

Baehrecke, 2000; Harvey *et al.*, 2001; Cakouros *et al.*, 2002; Baehrecke, 2003) and, in culture at least, insect cells can be forced to undergo apoptosis. There is good evidence for apoptotic death in cells in the central nervous system (Gorski *et al.*, 2003; Lee *et al.*, 2003). However, in metamorphosing *Manduca* muscle and labial gland, and in *Calliphora* salivary gland, death does not appear to be apoptotic (Bowen *et al.*, 1993; Jochová *et al.*, 1997a,b). In muscle, by far the greatest enzymatic activity detected is proteasomal (ubiquitin-driven) proteolysis (Haas *et al.*, 1995), closely followed by the activity of lysosomal cathepsins (Halaby *et al.*, 1994; Jochová *et al.*, 1997a,b). Nuclear condensation occurs very late, long after all cytoplasmic organelles have disappeared into autophagosomes and myofibrils have been destroyed by other proteases (Zakeri *et al.*, 1990; Halaby *et al.*, 1994; Jochová *et al.*, 1997a,b). In *Manduca* labial gland and the homologous *Drosophila* salivary gland, the most prominent early activity is rearrangement of the cytoskeleton and autophagic destruction of organelles. Only extremely late in the process can one detect any indicator of apoptosis, such as positive TUNEL reaction, formation of DNA ladders, nuclear condensation, or phosphatidylserine exposure (Jochová *et al.*, 1997a,b). Caspase activity (as measured by digestion of fluorogenic substrates) is virtually undetectable or unchanged, while that of cathepsins and proteasomal proteases rises during the period of degeneration (Facey and Lockshin, in preparation, Figure 1). Interestingly, matrix metalloproteinases are upregulated in both autophagic cell death and apoptosis, suggesting that liberation of the cell from its environment is a key issue (Gorski *et al.*, 2003; Lee *et al.*, 2003). Thus we raise the question of whether or not caspases play significant roles in metamorphosis, and if not, what role they play in the life of the cell.

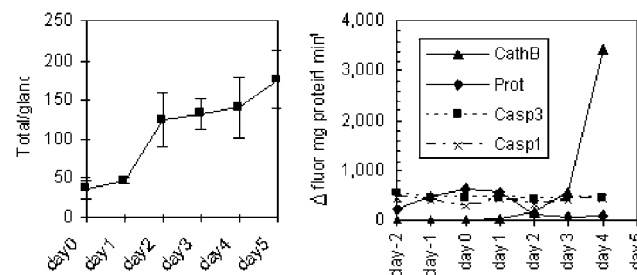


Figure 1 Comparison of proteolytic activities of cathepsin B (left) and several proteases (right) in the metamorphosing labial gland of *Manduca sexta*. Metamorphosis starts at day 0; the period of major collapse is day 2; and the gland is virtually destroyed by day 5. All assays were conducted using fluorogenic substrates. During this autophagic type involution, activities of caspase-1 and -3 were virtually undetectable and unchanging; proteasomal protease fell during the most active phase of involution; and only cathepsin B increased in both total amount (left) and relative amount (right). By comparison, using the same substrates, caspase-3 activity in embryos of the zebrafish *Danio rerio* exposed to cycloheximide reaches approximately 9000 U/mg/protein (not shown)

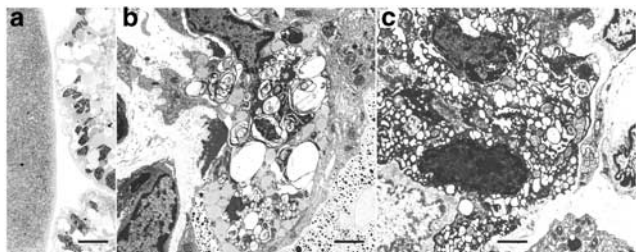


Figure 2 Caspase-independent cell death? (a) Involuting labial gland of the tobacco hornworm, *Manduca sexta*. Although caspases have been documented in insects, there is little evidence for activation or upregulation of caspases during the death of this tissue (Figure 1) and substantial evidence for high lysosomal activity. The segment of gland on the right is at a considerably more advanced stage of degeneration than the segment on the left. The tissue vacuolates (many of the vacuoles are lysosomes, beginning on the left and advanced on the right) and nuclear condensation are very late, although they finally do occur, and, later than the stage illustrated, positive TUNEL reactions and DNA laddering are observed (Zakeri *et al.*, 1993). (b) Postlactational involution of mouse mammary gland, 4 days after weaning. This involution is usually considered to be apoptotic, but was once known as an autophagic involution (Helminen *et al.*, 1968). Many cells are heavily vacuolated and filled with late autophagic vacuoles, and DNA condensation is very late. (c) Hormonoprivic involution of prostate, 2 days after castration. Often considered to be an apoptotic death, cells in the prostatic epithelium have substantially increased lysosomal activity. Many of the cells are vacuolated, and nuclei condense but do not fragment in a classically apoptotic manner. For earlier literature on (b) and (c), see Lockshin and Zakeri (2001)

Spermatogenesis

In insects, caspases are activated during sperm terminal individualization. Caspase inhibitors prevent the removal of bulk cytoplasm and block sperm maturation. The sperm nucleus is protected by a ubiquitin-conjugating enzyme. Thus an apoptosis-like mechanism is required to protect the sperm nucleus against hypercondensation and degeneration (Arama *et al.*, 2003). In mammalian spermatogenesis, apoptosis is common, and genetic disruptions of the Bax-Bcl-2 and other regula-

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tory pathways distort spermatogenesis in usually predictable ways. However, fertility is often not completely disrupted, and spermatogenesis occasionally continues in the face of presumptive catastrophic interference with apoptosis, indicating that the decision-making regulation still functions and that alternative pathways can be found (Smith *et al.*, 1992; Zakeri *et al.*, 1992; Knudson *et al.*, 1995; Miller *et al.*, 1997; Print and Loveland, 2000; Kierszenbaum, 2001; Sinha Hikim *et al.*, 2003).

These considerations have profound implications for the assessment and management of tumors, and must be considered.

Conclusions

The assumption that all programmed or physiological deaths are apoptotic or caspase dependent is often a bit facile. Evidence for the assumption is often indirect: documentation that a cell can die by caspase-dependent apoptosis does not prove that it normally follows that route, and apoptosis-controlling molecules such as bcl-2, Bcl-xL, or bax can influence, or change in response to, other types of death. Also, in embryos certainly and most likely in many other instances, cells that are under pressure to die have many options and routes to death. If a route involving caspases is blocked, the cell can activate other mechanisms or routes. Researchers and clinicians expecting straightforward results from blocking a single enzyme need to be aware of the adaptability of cells to accomplish their own imperatives.

Acknowledgements

The electron micrographs were taken in collaborative research with Daniela Quaglini (Figure 2a) and Martin Tenniswood (Figures 2b and c). The research quoted of Javier Negrón, Caroline Facey, and Nathaniel Abraham was carried out as part of their research, supported respectively by GANN, IMSD from the National Institutes of Health, and St John's University.

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