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Cell death during development

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Abstract

There are many ways to measure apoptosis and other forms of programmed cell death in development. Once nonmammalian embryos have passed the midblastula transition, or much earlier in mammalian embryos, apoptosis is similar to that seen in adult organisms, and is used to sculpt the animal, fuse bilateral tissues, and establish the structure of the nervous system and the immune system. Embryos present unique problems in that, in naturally occurring cell deaths, few cells are involved and they are frequently in very restricted regions. Thus, identification of apoptotic or other dying cells is more effectively achieved by microscopy-based techniques than by electrophoretic or cell-sorting techniques. Since embryos have many mitotic cells and are frequently more difficult to fix than adult tissues, it is best to confirm interpretations by the use of two or more independent techniques. Although natural embryonic deaths are frequently programmed and require protein synthesis, activation of a cell death pathway is often post-translational and assays for transcriptional or translational changes—as opposed to changes in aggregation of death-related molecules or proteolytic activation of enzymes—is likely to be uninformative. Also, embryos can frequently exploit partially redundant pathways, such that the phenotype of a knockout or upregulated death-related gene is often rather modest, even though the adult may develop response or regulation problems. For these reasons, the study of cell death in embryos is fascinating but researchers should be cautious in their analyses. © 2002 Published by Elsevier Science B.V.

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1. Origins of the concept of cell death

As is thoroughly discussed by Clarke and Clarke (1996), the concept of cell death arose from the study of developing systems as developmental biologists noticed the presence of cells with unusual morphology. Metamorphosis of insects and amphibia had, of course, been known since classical times, Vesalius had recognized the transient ductus venosus in the 16th century, and Harvey had observed the remodeling of the embryonic heart in the 17th century. However, a true recognition of the significance of cell death required the discovery of microscopic lenses by van Leeuwenhoek in the late 17th century, followed by the development of cell theory in the mid 19th century, and the development of sectioning and staining technology during the same period. Observation of cell death as a naturally occurring phenomenon followed remarkably quickly, with Vogt in 1842 recognizing the disappearance of the notochord of the midwife toad during metamorphosis (cited in Clarke and Clarke, 1996). From this point through the middle of the 20th century, developmental biologists comfortably lived with the recognition that in metamorphosis

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and in embryonic development, tissues and organs were formed that did not persist through the life of the organism. However, the philosophical and emotional orientation of researchers was more directed toward apparently constructive processes, and there was little experimental interest in how tissues were lost: the appearance of a luminous butterfly was far more fascinating than the loss of the dumpy larva. Similarly, there was much fascination with mitosis, but if there was any recognition of the requirement for an equivalent number of cell deaths, scientists assumed that these deaths were accidental, followed by homeostatic sensing and compensatory replacement of cells. By the 1950s, some change in attitude became apparent. Hamburger and Levi-Montalcini (1949), in the quest that ultimately led to the discovery of nerve growth factor, recognized that many neurons were lost in the course of normal neurogenesis, and Saunders (1948), noting specific patches of dead cells in the developing limbs of chicks, began a quest that led to the recognition of controlled cell death in the embryo. Lockshin and Williams (1965a,b,c), working with insect metamorphosis, extended the concept and described these deaths as ''programmed''. By the 1970s, Kerr et al. (1972) had established the similarities of many cell deaths in many organisms and had amalgamated these descriptions under the rubric ''apoptosis''. By the 1980s, the existence of specific cell death genes was demonstrated for the nematode worm *Caenorhabditis*, and between 1989 and 1991, cell death was identified as an important component in lymphomas, in AIDS, and in autoimmune disease and differentiation of the immunological system in general. The discovery of the clinical relevance of cell death launched the frenzy that we are now experiencing, including a revisiting of the role of cell death in developing systems.

To present a complete review of all the developing systems in which cell death plays a role would be an impossible task. In this review, we will examine the importance of cell death in a few developing systems with emphasis on some of the methodology used. However, the reader should understand that some of the most valuable techniques for the study of apoptosis in maturing or mature immune systems or in cultured cells of immunological origin, such as flow cytometry, are rarely applicable to embryos. For these latter, the small number of cells and the importance of temporal and spatial distribution of cell death necessitate the use of techniques such as immunocytochemistry and in situ hybridization.

2. Cell death in the dynamic embryo

Embryonic development is dynamic, encompassing a continuous progression of cell division, movement, differentiation, and death. These well-orchestrated cellular events render the investigation of the underlying mechanisms both possible and challenging. In a developing embryo, a dying cell is often surrounded by many dividing and or differentiating cells, making its identification difficult. In addition to the fact that rarely does a group of cells die en masse, the onset of cell death is usually not synchronous, making any study of the kinetics of cell death more difficult. The prominence of cell death in development has resulted in the origin of most of the theoretical bases of cell death research in developmental biology, including the concepts of programming, identification of specific cell death genes, and discovery of the caspases. One of the most important facets of the developing tissues is the crosstalk and interaction of the cells that are constantly changing from a stage of totipotency to a stage of determination. Developmental biologists have been studying the fate of the cells within this ever-changing milieu and have understood that cells act in response to their microenvironment. This not only affects and regulates the behavior of the cells during differentiation and cell division, it also plays an important role on the regulation of cell death during development. The classical studies of Saunders and Fallon (1966) and Saunders (1966) using transplantation studies, brilliantly demonstrated this fact. These studies were further supported by the discovery that in mutant animals, in which the defect represents an aberrant pattern of cell death, this effect is under local regulation (Zakeri et al., 1994). Much is known about the different genes and signaling molecules that set up specific patterns of overlapping expression that can result in local differences, ultimately accounting for the local and specific regulation of cell death in developing systems (Singh Ahuja et al., 1997a). Today, we recognize that this idea is generally applicable and that cells die in the context of their environment (Lockshin et al., in press) or, as Raff (1992) described it, there is a ''social control'' of apoptosis.

Although cell death in developing systems can be used to understand the mechanisms of cell death in other systems, it is also important to keep in mind that this type of cell death may differ in its regulation and manner of execution. Such a conclusion is supported by the observation that knockout of many genes influencing cell death results in no specific embryonic phenotype. For instance, p53 knockout mice, which are deficient in the ability of their precancerous cells to undergo apoptosis, present no abnormal phenotype of cell death in the embryo (Donehower et al., 1992), although they do develop spontaneous tumors (Jacks et al., 1994; Williams et al., 1994).

Studies such as these also document functional redundancy for cell death processes in the embryo. Such redundancy has been shown many times for other genes not related to cell death as well. Cdk5 $-/-$ embryos were normal and present at the expected frequency (24%) as late as gestation day 16.5 (Ohshima et al., 1996). p35 $-/-$ embryos develop normally, with no gross anatomical defects found in somatic organs or tissues (Chae et al., 1997). Mice deficient for $p21^{CIP/WAF1}$ develop normally, but are defective in G1 checkpoint control (Deng et al., 1995). Bak $-/-$ mice are developmentally normal and reproductively fit and do not develop any age-related disorders (Lindsten et al., 2000). Bcl-2 knockouts showed minor abnormalities: at E14 in vivo, the number of trigeminal neurons undergoing apoptosis was significantly greater in bcl-2($-/-$) embryos, and there were significantly fewer neurons in the trigeminal ganglia of bcl-2($-/-$) embryos at E16 and E18 (Pinon et al., 1997).

Early observations were essentially entirely morphological, leading to the recognition that patterns of death could be predicted and identified in most embryos, and therefore, to the conclusion that cell death is as much a part of normal development as proliferation or differentiation (Glücksmann, 1951; Saunders, 1966; Hinchliffe, 1981; Coucouvanis et al., 1995). Glücksmann also importantly recognized that cell death continued throughout life, including cell turnover and even pathology, and did not distinguish any significant distinction in the mechanisms of these deaths, thus presaging a conflation of all physiological cell deaths.

Cell death in mammalian embryogenesis occurs as early as inner cell mass differentiation (Fig. 2a; Hardy et al., 1989; Zakeri et al., in preparation). Cell death plays an important role in the development of both embryonic and extraembryonic tissues. Apoptosis has been demonstrated in fetal membranes (Nijhawan et al., 2000). Studies using the electron microscope have revealed changes consistent with apoptosis in amniotic epithelium and chorionic trophoblast cells, such as the condensation of chromatin along the periphery of the nucleus and nuclear shrinkage (Wiley et al., 1992). Later, cell death in several organs molds the shape and function of the differentiating organs. Cell death removes interdigital webs during limb development. One of the most dramatic features of limb development is cell death, which shapes and contours the digital palettes (Saunders and Fallon, 1966; Hinchliffe, 1981; Hurle et al., 1996). The specific spatial and temporal pattern of cell death is species-dependent. During limb formation, cell death is first observed in the anterior and posterior marginal zones (AMZ and PMZ) of the developing limb bud. In later stages, there is massive cell death in almost all of the interdigital mesenchymal tissue (IMT) located between the chondrifying digits (Hurle et al., 1996). The ability of the IMT to undergo cell death is dependent upon the overlying ectoderm as well as the presence of adjacent digits. Cell death is also detected in the developing AER; this death is thought to limit the size of the AER and subridge and therefore prevent polydactyly (Hurle et al., 1996). Thus, cell death accompanies the formation of free and independent digits of birds (Saunders and Fallon, 1966) and mammals (Ballard and Holt, 1968).

In the developing nervous system, a definitive pattern of neuronal connections and major axonal pathways is established (Hamburger and Oppenheim, 1967). During vertebrate development, more nerve cells are produced than are needed, and the death of 20–80% of neurons is a regular feature (Gordon, 1995; Clarke et al., 1998). In this case, fetal neurons compete for limited amounts of nerve growth factor (NGF), a survival factor produced by other cells including neurons. Only some cells get enough NGF to survive. Thus, apoptosis induced by growth factor withdrawal adjusts the numbers of developing neurons to the number of target cells (Jacobson et al., 1997). Secondary palate formation occurs by the fusion of two opposing palatal shelves in which the midline epithelium undergoes degeneration and death

(Shapiro and Sweney, 1969). Likewise, immunological tolerance is defined by the destruction of cells bearing potential anti-self immunoglobulins. In addition, "neglected" cells bearing nonfunctional immunoglobulins also die (Osborne, 1998). In the developing lens, the nuclei of lens fiber cells undergo nuclear apoptosis, but the cytoplasm survives and the cells remain as normal functional ghost cells after day 18 of mice embryonic gestation (Appleby and Modak, 1977; Wyllie et al., 1980; Gao et al., 1997; Nijhawan et al., 2000).

Cell death during embryogenesis is not restricted to the mammals. For example, neuronal, muscle, epithelial, intestinal, and gonadal cells undergo cell death in the developing nematode, *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994; Sulston et al., 1977). Muscle cell death (Lockshin and Beaulaton, 1981; Schwartz and Truman, 1982; Haas et al., 1995) and neuronal cell death (Truman, 1984; Fahrbach et al., 1994) have been detected in moths as well. In fact, in the higher insects, almost all larval tissues are destroyed at metamorphosis, and in anurans, the tail and gills of the tadpole are wholly destroyed and in many other tissues, there is substantial apoptosis during remodeling (Weber, 1969; Fox, 1973).

In plants, cell death plays an essential part in embryogenesis from fertilization through development as well as in response to environmental stimuli such as cold and attacks by bacteria and viruses. A major role for cell death in plants is the formation of the sexual organs, in essence much like the mammals (Pennell and Lamb, 2001). Although death in plant cells sometimes resembles apoptosis, there are also unique types of cell death seen in plants in which the vacuoles play a critical role (Fukuda, 2001). Much of the methodology used to study animal embryos has also been used to study cell death in plants.

From a functional or evolutionary standpoint, dying cells have been divided into several categories (Glücksmann, 1951; Ellis et al., 1991). Some cells may have served some evolutionary purpose but provide no current function and undergo a phylogenetic cell death (Fallon and Simandl, 1978). Morphogenetic cell death may modify the tissue to permit differentiation of its final form (Glücksmann, 1951; Hinchliffe, 1981). Histogenic cell death can modify a tissue so that it is able to function or to function differently from a similar tissue type (Glücksmann, 1951). For example, three different patterns of cell death occur in the somites of the developing embryo. These variations are dependent on the stage of the embryo and on the path that migrating neural crest cells will take (Jeffs and Osmond, 1992; Coucouvanis et al., 1995). Cell death may also occur in one sex as a means of differentiating sexually dimorphic traits. For example, sexual differentiation in vertebrates involves hormonal control of cell death of reproductive structures, such as the müllerian and wolffian ducts (Scheib, 1963). In developing neurons, cell numbers may exceed that required and therefore, some can be removed (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984; O'Leary, 1987). Cells may be necessary at one stage of development but are no longer required such as the tadpole tail during metamorphosis (Kerr et al., 1974; Lockshin, 1981). Some cells may be defective in shape or function such as lymphocytes that have failed to produce functional antigen specific receptors (Cohen, 1991; Golstein et al., 1991). Other cells may yield an autoimmune attack on the organism such as thymocytes that carry self-recognizing T-cell receptors (Cohen, 1991; Golstein et al., 1991; McCarthy et al., 1992; Osborne, 1995). Therefore, cell death accounts for the deletion of cells that takes place in normal tissues. Where it occurs pathologically, it may serve an adaptive or homeostatic role (Walker et al., 1988).

2.1. Types of cell death

Various types of cell death had been seen by many workers and were considered to be related, but issues became clearer with the definition of apoptosis by Kerr et al. (1972). Following a period of acrimonious dispute over a binary distinction (between apoptosis and necrosis), Schweichel and Merker (1973) attempted to classify different types of cell death into at least three types, using electron microscopy to evaluate cell death in developing embryos. They described three types of "necrosis", which we today recognize as apoptosis (Type I necrosis), lysosomal cell death (Type II), and necrosis (Type III). These three forms of death were identified by morphological criteria, as is described immediately below. The term "programmed cell death" originally referred to the existence of reversible steps clearly leading to the

destruction of the cell, operationally defined by experiments. Today, this latter term more specifically refers to defined genetic pathways in cells but more loosely to apoptotic or other physiological cell deaths. Likewise, the morphological appearance of the apoptotic cell is now understood in more biochemical terms.

Apoptosis defines a situation in which a dying cell loses its adherence for neighboring cells or extracellular matrix, rounds up, and condenses. The chromatin in its nucleus coalesces into one or a few masses along the nuclear membrane, while the cytoplasm, dense by staining with dyes for light or electron microscopy, fragments as the cell forms blebs that are ultimately taken up by phagocytes (Figs. 1 and 2). Mitochondria appear to be intact—that is, they are normal to shrunken in appearance rather than dilated or swollen—but have depolarized and permitted the escape of cytochrome *c* and a few other components. The characteristic appearance of the cytoplasm and nucleus results from activation of pre-existing caspases, site-restriction proteases that can cleave important components of the cytoskeleton, and nuclear matrix. Perhaps because of the destruction of the nuclear matrix, DNase II-type endonucleases are activated or gain access to the DNA and cut it between nucleosomes, producing fragments that are identified as a ladder when the DNA is electrophoresed. This pattern of DNA fragmentation is characteristic of apoptosis, but it may reflect failure to degrade fragments further or failure to activate exonucleases rather than specific activation of the endonuclease. Phagocytosis by professional phagocytes or neighboring cells occurs quickly, and the identifiably apoptotic cell has often disappeared within 1–2 h. The phagocytosis is mediated by the appearance on the external face of the cell membrane of phosphatidylserine, extruded from the internal face of the dying cell by an active process. As enunciated by Kerr et al., apoptosis is a very common process and is seen in the majority of forms of cell death, but is most typified by dying cells that derive from mitotic cell lines and have relatively little cytoplasm, such as lymphocytes and thymocytes. This situation obtains in most embryonic situations—that is, most embryonic deaths are apoptotic—but it is important to realize that both the conversion to apoptotic morphology and the translocation of phosphatidylserine require energy (Schlegel et al., 2000). In situations in which energy (ATP) is compromised, there are severe osmotic considerations (as in the eggs of freshwater organisms), or cell death is so massive that phagocytes cannot rapidly remove all the dying cells (toxicity or genetic problems, perhaps also in regions of poor circulation such as bone). Cells may begin to die by an apoptotic mechanism but fail to complete it. In these cases, the cells when recognized may have a very different appearance and the mechanism can be misinterpreted.

Lysosomal (Type II) cell death is more characteristic of large, quiescent or postmitotic cells that have massive cytoplasm, such as glandular tissues (insect glands at metamorphosis and mammary epithelium), muscle, and differentiated neurons. In these cells, destruction of DNA is not an imperative as it might be for, e.g., a potentially mutated or virus- or plasmid-carrying lymphocyte. On the other hand, cytoplasm in these cells is a bulky mass that cannot be simply shed to be phagocytosed. In these cells, although many of the characteristic changes of apoptosis eventually develop, they are markedly delayed, and the cell undergoes substantial alteration well before this stage. The most prominent characteristic is the appearance of large autophagic vacuoles, lysosomal derivatives that consume the bulk of the cytoplasm. These appear while the cell remains functional and in the case of muscle, can retain its resting potential (Lockshin and Beaulaton, 1979). Other lytic mechanisms operate as well: erosion of myofilaments in muscle can occur in these cells external to autophagic vacuoles, most likely in proteasomes (Haas et al., 1995). In essence, the function of the phagocyte is conducted within the cell. Finally, when approximately 80% of the cytoplasm has been destroyed, the condensation of the cytoplasm and coalescence and margination of the chromatin become apparent, electrophoresis reveals the appearance of a DNA ladder, and the remnants of the cell are phagocytosed as in classic apoptosis (Zakeri et al., 1993). Nevertheless, judging from the few reports extant, the apoptotic phase of death in these circumstances amounts to approximately 10% of the period in which the dying cell is identifiable, and most of the tests for cell death based on characteristics of apoptosis will produce negative results (Jochová et al., 1997).

Necrosis is usually considered to be the antithesis of the physiological cell deaths described above. In

essence, necrosis is an uncontrolled death. If a cell encounters such severe insult or sustains such severe injury that either its ATP-generating mechanisms or the integrity of its permeability barriers is compromised—as might occur, for instance, in infarct, sudden change in pH or osmolarity, or in the presence of several toxins or poisons—then the cell is likely to lyse. The usual scenario is that in the absence of adequate mitochondrial function and effective ion pumps, the cell switches to glycolysis and accumulates lactic acid. If the cell is not yet severely acidotic, the lactate is trapped within the cell and acts as an osmotic attractant, pulling in water and causing the cell to lyse. This lysis releases into the surrounding tissues many components, including pyrogens and other elements that will attract mast cells, leading to inflammation. Necrosis is frequently described as a multicell phenomenon, as opposed to apoptosis as a single-cell phenomenon. However, this description fails to address the situation in which a cell attempts to undergo a dignified and self-effacing apoptosis, but because of the circumstances cannot complete it before succumbing and reverting to necrotic morphology. For instance, in cases of acute liver toxicity, cells most exposed to the toxin (closest to the central vein) undergo necrosis. The more peripheral cells may undergo apoptosis, or they may begin to undergo apoptosis but finally convert to necrosis. During maturation of bone, resident chondrocytes appear to undergo necrosis, but this may be the outcome of failure of phagocytes to reach them. In plants, there are several types of programmed cell death, including spontaneous deaths, for instance of flowers; deaths activated through oxidative mechanisms or other stresses; deaths induced by infectious agents, apparently as a defense mechanism; and deaths induced by hypersensitivity of this defense mechanism. In some deaths for instance, the maturation of tracheal elements, the expansion and finally, collapse of the central vacuole triggers the prompt death of the cell, perhaps by releasing lytic enzymes into the cytoplasm (Fukuda, 1996). There is some evidence for internucleosomal cleavage of double-stranded DNA (Danon et al., 2000) as well as the existence of potentially caspase-like proteases (Lam et al., 1999). However, other researchers have failed to document these animal-like properties and the morphologies in general do not resemble either apoptosis or autophagic cell death.

3. Methods useful to study cell death in development

The dynamic of the embryo presents an intriguing situation for the analysis of cell death. The cell population changes constantly and the cells are at different stages of the cell cycle. Therefore, many of the methods used in the determination and analysis of cell death for cultured cells, or normally suspended cells such as lymphocytes, are not useful or are difficult to interpret. The aim in studying cell death in the embryo is also to identify the deaths in their spatial and temporal context. For this reason, a number of methods used deal with in situ examination of either complete embryos or embryonic sections on slides. In this paper, we attempt to introduce several of the most commonly used approaches with some degree of detail. Since there are many methods, we cannot give specific details of the procedures; these details are found in the references. The reader should bear in mind that individual methods might not be useful in all situations. These limitations will be noted when possible. Images of results using these several techniques are illustrated in Figs. 1 and 2.

3.1. Detection of cell death by morphology

Because of the evanescence and spatial restriction of deaths of interest in embryos, one of the most useful methods is microscopy. Since for apoptosis at least the morphology is frequently unequivocal, standard techniques for the preparations generally suffice.

3.2. Detection of cell death by light microscopy

One approach for detecting cell death has been the use of light microscopy and staining. A common stain used is hematoxylin (Fig. 1a). In this case, the dead cells, both the cytoplasm and the condensed nuclei, are more darkly stained than the live cells. The sensitivity of this method is rather low and it works for sectioned tissues and embryos. There are a few variations on the theme for the specific method. Basically, frozen sections are brought to room temperature from $-70\ ^{\circ}C$ or paraffin embedded tissue sections are deparaffinized and stained in filtered Harris Hematoxylin (Sigma) for 3 min. Slides are then washed in tap water three times for 1 min each

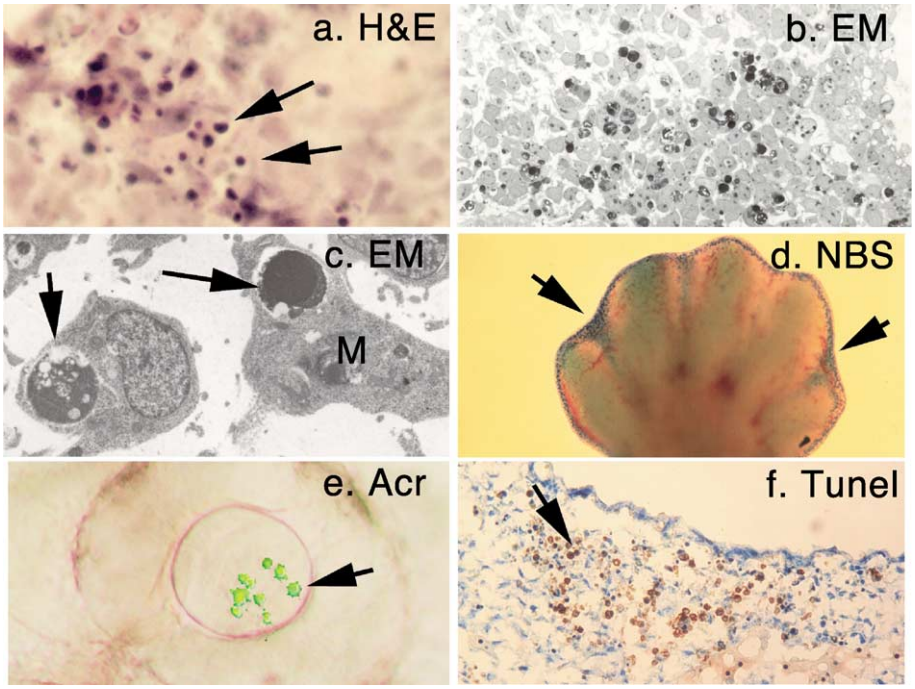

Fig. 1. Examples of several techniques to illustrate cell death or apoptosis. (a) Haematoxylin and eosin. The dark dead cells (arrows) lying in the interdigital region of a day 13.5 embryonic mouse hand palette are easily distinguished but must be examined at high magnification to confirm that they are not metaphase cells. (b) Semi-thin plastic section showing apoptotic cells (dark) scattered throughout the interdigital region of a day 13.5 embryonic mouse hand palette. (c) Apoptotic nuclei (arrows) are readily recognized in the vacuoles of phagocytes (M) in this electron micrograph. (d) Nile blue sulfate is readily taken up into dead cells (actually, vacuoles of phagocytes) in the interdigital regions of a day 12.5 embryonic mouse hand palette. Some of the most prominent regions are indicated by arrows. This technique, though effective, is heavily dependent on time and conditions, including the ability of the dye to penetrate into deeper layers of larger specimens. (e) Acridine orange penetrates small embryos well. Here, dead cells in the lens are easily identified by green fluorescence (arrow) in this composite picture of a stage 17 zebrafish eye. The phase and fluorescence images were overlaid. (f) The TUNEL technique, here, using the DAB-peroxidase reaction rather than fluorescent substrates, reveals dead cells, one of which is marked by an arrow, in the interdigital regions of a day 13.5 mouse hand palette, in a section similar to that shown in (a).

and mounted with Crystal Mount (Fisher) (Zakeri and Ahuja, 1994).

3.3. Electron microscopy

Light microscopy can be used, preferably high-resolution light microscopy such as aldehyde fixation and embedding in plastic. Standard histological clinical techniques can be used if nothing else is available, but in this situation, distortion of tissues and cells makes it much more difficult to distinguish between apoptotic cells and, for instance, early metaphase cells or a small, protein-rich cell among larger, less dense cells. Also, since embryos contain substantially more water than adult tissues, adequate preservation requires close attention to osmolarity and salt balance. The major limitations of electron microscopy as an analytical technique, in addition to the tediousness and expense of the preparation, are of course sampling size and the transience of the apoptotic cell. An experiment that interferes with phagocytosis and hence extends clearance time, for instance, might readily result in an apparent increase in the number of apoptotic cells. For these reasons, more experimental or analytical techniques should be applied in conjunction with microscopic observations. There are a number of published methods in use. In brief, samples are fixed in 2.5% glutaraldehyde in 1 × PBS at 4 °C for several days, and postfixed in 1% osmium tetroxide in 1 × PBS at 4 °C for 1 h. They are then

dehydrated in graded ethanols, in propylene oxide, and finally embedded in Spurr resin. Semi-thin sections are cut with an ultramicrotome and can then be stained with toluidine blue and observed by light microscopy. This semi-thin section provides an excellent view of cell death in the tissue. Selected areas cut in thin sections are collected on copper grids and stained with uranyl acetate (5% in 70% ethanol) for 15 min and then in lead citrate for 10 min. The stained sections are examined with an electron microscope (Zakeri et al., 1993). Phagocytosed apoptotic cells are illustrated in Fig. 1b and c.

4. Specific staining techniques applicable to embryos

Whether one uses light or electron microscopy, identification of an apoptotic cell in a field is a somewhat risky affair. Tidball has calculated that the entire liver could disappear in 1 month with only one or two apoptotic cells visible per field at any point (Tidball and Albrecht, 1998). Thus, techniques that highlight apoptotic cells are preferable to those attempting to locate apoptotic cells by appearance alone. The methods most likely to succeed pinpoint the molecular, physiological alteration within the dead or dying cells. These techniques can be categorized as follows: in vivo staining of the live embryo by vital dyes; techniques detecting fragmented DNA; detection of phosphatidylserine; detection of certain molecules associated with cell death; detection of lysosomal activity; and detection of phagocytes. Some recent techniques show promise, such as antibody-based or fluorogenic substrate-based techniques to detect activated proteases as opposed to inactive proenzymes. For embryos at least, mixed-cell analyses such as flow cytometry and electrophoresis of DNA are of restricted interest unless they can be scaled down and used for specific tissues. Similarly, most components of the apoptotic machinery are present in normal cells and their detection in dying cells, other than in transgenic animals, is not revelatory. In all instances, developing systems are by definition rapidly changing and all considerations concerning dynamics (baselines and cell types shifting during the course of the experiment, relative rates of appearance, and clearance of apoptotic cells) must be evaluated.

4.1. Detection of cell death by vital staining

The changes that allow the exposure of phosphatidylserine are also reflected in a modestly increased permeability of apoptotic cells, allowing the use of vital dyes to identify apoptosis. Vital stains have been useful for many developing amniotes such as chicks and mice. The advantage of this method is that it is fast and one can examine cell death in three dimensions. For this method, one needs a living embryo, which adds to the limitation of the technique since, as the embryo dies, the number of cell deaths imposed by the condition increases. However, it is a useful method for the detection of cell death if one very carefully regulates the time and temperature. For the assessment of the extent of cell death, mouse fetuses are freed of extraembryonic membranes and stained for 30 min at 37 °C, with a 0.01% solution of Nile blue sulfate (NBS, Sigma) in 1 × PBS with 5% CO_2. The dead or dying cells stain dark blue when observed under the microscope, as is illustrated in Fig. 1d. For deep layer observation, the embryo can be frozen in OTC® after staining and then sectioned and observed under the microscope. One can still detect the deep layer cell death with the Nile blue staining that remains in the cells. This stain indicates the acidic compartments of the cells such as lysosomes, which may be activated in dying cells. This method has been used for chick and mouse embryos (Alles and Sulik, 1989; Zakeri et al., 1994). Alternatively, the acridine orange technique when properly used can be very effective. Acridine orange is classified as cell permeant but it is only weakly so. Thus, living cells fluoresce a pale green, reflecting the 460 excitation and 650 emission peaks for RNA, while at low magnification, dead cells fluoresce more brightly, depending on the amount of RNA in the cell, and reflecting the higher penetrance of the dye and its 500 excitation and 526 emission peaks for DNA. With appropriate optics, acridine orange staining can be easily seen, as is illustrated in Fig. 1e. At higher magnification, the shape of the nuclei can be readily seen. This method is most effective for small embryos or embryos at early stages of development, as penetration of the dye may be a problem. For zebrafish embryos from cleavage through segmentation, the embryos are submerged in acridine orange (15 μg/ml) solution in water for 1 h at 30 °C and rinsed well.

The fluorescence can be seen using general-purpose filters in a fluorescence microscope. Commercial kits exploit similar possibilities. For instance, several dyes such as calcein AM are cell-permeable and not fluorescent, but inside the cell are cleaved to fluorescent ionic compounds, which are therefore trapped within living cells. Ethidium homodimer I, on the other hand, penetrates living cells poorly but penetrates apoptotic or necrotic cells and stains nucleic acids. Live cells are therefore fluoresce green, and dead ones red-orange. These commercial kits, such as the Live–Dead® kit from Molecular Probes, do not distinguish between apoptotic and necrotic cells. They also may not penetrate whole embryos well.

5. Measurement of fragmented DNA

5.1. Gel electrophoresis

One of the most used methods for the detection of fragmented DNA has been the use of gel electrophoresis to separate the fragmented DNA. This method works extremely well when cultured cells are used. However, this is not the method of choice for the study of cell death in most developing systems. It can be used, for example, to examine cell death during metamorphosis of insects or frogs when an entire tissue undergoes cell death in a more or less synchronous way. One can also use this method to examine cell death in mammalian or chick embryos if one can carefully isolate the area undergoing massive cell death. However, the efficiency of detection of the signal is not very good—one needs minimally 3% of the cells at the appropriate stage of apoptosis (Zakeri et al., 1993). For DNA analysis by gel electrophoresis, the tissue is dissected and immediately digested with 0.6 μg/μl proteinase-K (Jersey Lab and Glove Supply) in 50 mM Tris buffer with 100 mM EDTA and 0.5% SDS overnight at 37 °C. The mixture is treated with 33 μg/ml RNase A (Jersey Lab and Glove Supply), extracted with saturated phenol (Boehringer Mannheim), and loaded onto 2% agarose gels together with ethidium bromide. To increase the sensitivity of the detection, one can enrich for the small molecular weight DNA by lysing the tissue in lysis buffer (0.2% Triton X-100, 10 mM Tris–HCl, and 10 mM EDTA, pH 7.5). The cell lysate is then held on ice for 15 min, and centrifuged at 4 °C at 12,000 × *g* for 20 min. The supernatant contains the low molecular weight DNA and the pellet contains the high molecular weight DNA. The supernatant is incubated for 1 h with RNase and then extracted twice with phenol/chloroform/isoamyl alcohol (24:24:1) and once with chloroform/isoamyl alcohol (24:1). The DNA is precipitated with 300 mM NaCl and 2.5 volumes ethanol at − 20 °C overnight. The fragmented DNA is visualized by gel electrophoresis. After electrophoresis, the gels are viewed and photographed under UV light (Karasavvas et al., 1996).

5.2. The TUNEL technique

TUNEL is an acronym for terminal deoxyuridine nucleotide end labeling and as one might expect, in principle, any free 3′ end of DNA could be labeled. Indeed, it is possible to see TUNEL labeling in necrotic cells (Frankfurt et al., 1996) and in S-phase mitotic cells, particularly those of high chromosome number (Halaby et al., 1994). The distinction appears to be that most DNA fragments are extracted from necrotic cells and the number of Okazaki fragments in S-phase cells is much less than the number of free ends in an apoptotic cell. Thus, TUNEL labeling is a relative affair, though usually sufficiently differential to be reliable as an assay. Thorough positive and negative controls are a must. This technique, which relies on the application of exogenous terminal transferase to endogenous fragmented DNA, can be used for frozen sections and paraffin sections, fixed by a variety of techniques including ethanol/acetic acid. There are several kits that are available for this type of detection. The basic idea is to identify cells with fragmented DNA. We have used a nonisotopic DNA end labeling in situ technique, employing digoxigenin-11-dUTP and terminal transferase (ApopTag™ Peroxidase Kit, Oncor, Gaithersburg MD, Zakeri et al., 1994) or TUNEL POD (Oncogene Roche Molecular Biochemical, Germany). Briefly, sections are postfixed in ethanol/acetic acid (2:1) for 5 min at − 20 °C and washed in 1 × PBS twice for 5 min each. Endogenous peroxidase is quenched with 0.1% hydrogen peroxide in 1 × PBS for 20 min and then rinsed in 1 × PBS twice for 5 min. Sections are equilibrated in equilibration buffer for 20 min before the addition of reaction buffer containing terminal

deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-11-dUTP or FITC-dUTP. The reaction is stopped and the label is detected using the appropriate detection system. Slides are counterstained and mounted with Permount® for visualization. The fragmented DNA within the dying cell is usually visualized as a dark red to brown staining in the cell, as is illustrated in Fig. 1f. The staining sometimes appears on the surface of the cell rather than within the nuclei. Also, only the cells that are in late apoptosis, when the DNA fragments, can be visualized. Cells at early stage of cell death or cells that do not have the nuclei in the plane of section may not be identified.

5.3. Comet assays

In situations where few cells are available but they can be suspended or otherwise freed from the matrix, the comet assay is worth exploring. Like the TUNEL assay, it depends on the retention by apoptotic cells of fragmented DNA, but in this instance, individual cells are embedded in agar and subjected to electric fields. Fragmented DNA can be forced from the cell and when stained with acridine orange or Hoechst dye, appears as comet tail to the cell's comet head. Intact DNA moves much more slowly, if at all, and the much smaller fragments from necrotic cells diffuse rapidly from the cell (Benitez-Bribiesca, 1998). This technique is useful where small but adequate numbers of cells can be isolated for study.

5.4. Detection of exposed phosphatidylserine

The use of annexin V labeled with a fluor is usually described as a technique best suited to flow cytometry, but if living cells or embryos can be obtained and examined with a good fluorescence microscope or preferably a confocal microscope, one can detect apoptotic cells as brilliantly fluorescent cells in a dark background. We have had excellent results with both mammalian (mouse) embryos and zebrafish embryos. The penetration of the protein into a whole embryo can be a problem, and false negatives are possible even where some positive cells are detected. Alternatively, annexin V can theoretically penetrate and stain ruptured cells to produce false positives, though in practice, this is either uncommon enough or so obvious (i.e., the entire embryo is stained) that it is not a major problem. As is the case for vital staining, the embryo needs to be alive when using this method. The embryo is placed in a diluted mixture of annexin V as described by the vendor for 10 min. The staining is visualized by confocal microscopy, as is the case for Fig. 2a. This method is also described in detail in this issue.

5.5. Detection of lysosomes and autophagic vacuoles

Most differentiating cells have relatively low cytoplasmic volume and are either still mitotic or have recently exited cell cycle. Their mode of death is predominantly apoptotic. Even in this situation, cytochemical examination of lysosomal enzymes highlights phagocytes, which can, by counterstaining, be identified as containing apoptotic fragments. In other situations, autophagic cells are highlighted by the density and size of their autophagic vacuoles. Acid phosphatase is a simple and reliable screening enzyme that survives cryostat sectioning and even, with care, paraffin embedding. It is readily assayed by use of naphthol-based substrates that couple with fast garnet to form insoluble tetrahydrofuran compounds. Other lysosomal enzymes can be assayed using comparable substrates. However, not all phagocytes or phagocytic or autophagic vacuoles test positive for acid phosphatase, and other lysosomal enzymes, such as β-glucuronidase, could be tested. Positive acid phosphatase results are illustrated in Fig. 2b.

This assay is useful in many situations, as the role of autophagy is frequently seriously underestimated. To give three examples: involution of mammary epithelium is often used as an example of apoptosis, but autophagy is well documented as a major process in these cells (Helminen et al., 1968, Helminen and Ericsson, 1970, 1971); likewise, involuting prostate epithelium is a classic example of apoptosis, but a major upregulated gene in both involuting prostate and involuting mammary gland is the lysosomal enzyme cathepsin B (Guenette and Tenniswood, 1994); and lastly, although caspase-like enzymes are highly conserved, have been identified in *Drosophila*, and are considered to be major players in apoptosis in *Drosophila* embryos (Dorstyn et al., 1998, 1999a,b), the death at metamorphosis of the labial glands of the hawk moth *Manduca sexta* and apparently of the salivary glands of *Drosophila* is mediated by pro-

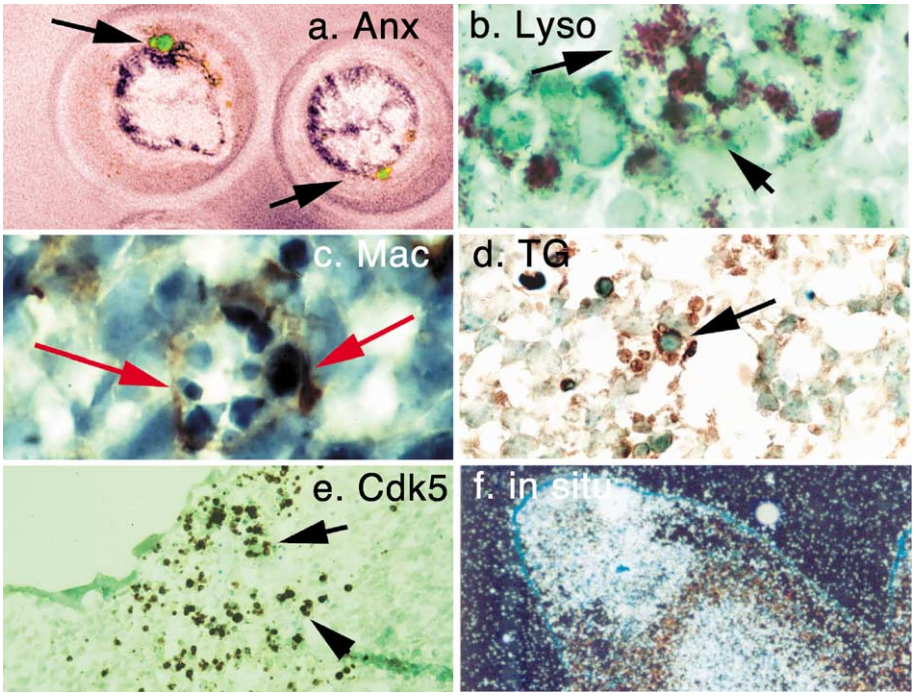

Fig. 2. Further examples of techniques to illustrate cell death or apoptosis continued. (a) Annexin V coupled to a fluor marks the exposed phosphatidylserine on the polar bodies of a mouse blastocyst, as is readily seen by fluorescence (arrows) or confocal microscopy. (b) Although lysosomes are found in virtually all cells, phagocytes or cells dying by autophagy contain high densities of the organelles and are easily distinguishable (arrows). Here, cells in the interdigital region of the mouse hand palette have been incubated in the presence of a naphthol AS-BT phosphate substrate. (c) F4/80 antibody identifies the cell membrane of a macrophage, as indicated by the arrows, in a mouse embryo hand palette. (d) Immunohistochemistry reveals up-regulation of transglutaminase (arrows) in dying cells in a mouse hand palette. (e) Although most proteins do not change much in amount, immunohistochemistry can reveal activation of enzymes such as Cdk5, illustrated here (arrows), from a day 13.5 mouse embryo hand palette. (f) In situ hybridization for bcl-2 message in a developing mouse digit reveals substantial labeling over the cartilaginous areas, but there is no difference in the level of labeling in the interdigital region in which cell death is present compared to the adjacent regions.

teases other than caspases (Facey and Lockshin, in preparation). Autophagy is prominent in other situations as well (Klionsky and Emr, 2000; Stoka et al., 2001).

To measure lysosomal activity, one can analyze acid phosphatase by use of a kit (Sigma, 180-A) among other lysosomal enzymes (Zakeri et al., 1994). Frozen sections are fixed in formaldehyde, postfixed with citrate (pH 3.6)—acetone—37% formaldehyde (13:33:4) for 30 s, washed in dH_2O, and treated with naphthol AS-BI phosphate and fast garnet stain for 1 h at 37 °C. Slides are washed, air-dried, counterstained with methylene blue, and mounted with Crystal Mount (Fisher). The acid phosphatase activity is detected as a distinct red focal precipitate. Paraffin embedding is also satisfactory, though the activity of acid phosphatase, measured in deparaffinized sections brought to H_2O, is substantially lower.

5.6. Detection of phagocytes

Apoptotic cells persist very transiently, primarily because, characteristic of the process, they are readily identified as prey for professional or amateur phagocytes. Happily, both types of phagocytes may be readily identified by a cell surface marker recognized by a commercially available antibody. Although this technique detects phagocytes, not apoptotic cells, the phagocytes are large and their vacuoles can be readily seen at higher magnifications. The cell boundaries of a macrophage are labeled in Fig. 2c. Counterstaining or dual labeling frequently reveals, within the vacuole,

dense blebs of cytoplasm or dense nuclear fragments containing highly condensed and marginated chromatin. Both types of inclusion reveal the fate of an apoptotic cell, though it is usually not possible to make any assumption about the number of cells consumed by a phagocyte or the number of phagocytes containing parts of a single cell. However, one can use this marker to assess the level of cell death and or the fate of dead cells in the developing systems. Although, most of the time, one finds positive reactivity associated with dead or dying cells that can also be identified by other methods, it is also possible to see situations in which the dead or dying cell is not engulfed by the phagocytic cells. This can be seen in some abnormal situations such as that caused by an insult in which the level of cell death is very high i.e., cyclophosphamide-treated embryos (Zhu et al., in press (Cell Death and Differentiation)).

For detection of phagocytic cells, basic immunohistochemistry can be used. Frozen sections are brought to room temperature and rehydrated. Endogenous peroxidase is inactivated by treating sections with hydrogen peroxide ($1H_2O_2$ (30%, Sigma):$2H_2O$). Nonspecific binding sites are blocked with 5% dry milk and primary antibody F4/80 (Serotec, Hume et al., 1984, diluted at 1/10 in 1 × PBST-gelatin) is applied for overnight incubation at 4 °C. Secondary antibody: peroxidase labeled $F(ab')_2$ fragment of goat anti-rat IgG (H + L) at dilution of 1/50 (Jackson Immunological) in 1 × PBST-gelatin is applied and slides are incubated for 2 h at room temperature (RT). After washes, immunoreactivity is visualized by DAB staining. Slides are counterstained with hematoxylin (Sigma) and mounted with Crystal Mount® (Zakeri et al., 1994).

6. Detection of deregulation of certain gene products during cell death

A characteristic of truly programmed cell death, defined in 1966 for metamorphosing tadpoles (Tata, 1966), in 1969 for insects (Lockshin, 1969), and later for glucocorticoid-treated thymocytes (Makman et al., 1966; Munck, 1971) and trophin-deprived neurons (Oppenheim et al., 1990), is a requirement for protein synthesis. The required proteins are not yet known and are therefore, not identified as members of any specific pathway of apoptosis. This situation is common in embryonic or developmental situations. Species differences, as between web-footed and other birds and abnormalities such as cleft palate or hammertoe, also indicate that cell death is under genetic regulation (Singh Ahuja et al., 1997a) and of course, studies on nematodes documented several genes both responsible for cell death and for preventing it. Genetic regulation can theoretically be manifested in the differential expression of new gene product, differential expression of already present gene products or differential activation or silencing of gene products. To examine these possibilities, during cell death in developing systems, efforts focused on the regulation of known cell death related genes as well as unknown genes. Techniques such as in situ hybridization and immunocytochemistry are routinely applicable to embryonic development. In situ hybridization can be done but often does not identify significant changes, as is illustrated in Fig. 2f. The methodology for immunocytochemistry has been described above and can be found in Zhu et al. (2002). It is possible that the changed level of transcription can be observed by in situ hybridization (see Ahuja et al., 1996 for detailed methodology).

It is, nevertheless, important to understand that in many instances, the commitment to die is not accompanied by a transcriptional or translational change, but rather by the activation of a proenzyme or otherwise dormant enzymatic activity. In fact, in many situations, particularly those not involving physiological cell death mechanisms in embryos, the program is preinstalled in the cell: protein synthesis is not required and apoptosis may even be induced by treatment with the protein synthesis inhibitor, cycloheximide. Given this situation, any measurement purporting to demonstrate an increase or decrease of a component of an apoptotic pathway, whether message or protein, must be considered likely dubious and at best regarded with skepticism. Most changes, if they involve changes in the machinery as opposed to changes in environment (pH and ionic composition) that alter activity of enzymes, must result from activation or inactivation of enzymes. Some of these changes are known and should be investigated, as quite often specification or activation of a cell death mechanism does not involve transcriptional or translational changes.

For many genes and gene families that have been associated with cell death, the association has been confirmed by knockout or transgenic or viral up-regulation. The most well known of these include the following: the bcl-2 family, in which the eponymous bcl-2 is strongly antiapoptotic but related molecules, such as bax, are pro-apoptotic; Fas and Fas ligand, which represent a generic type of interaction in the tumor necrosis gene family, in which binding of ligand to receptor triggers activation of cell death pathways; cytochrome *c* and other mitochondrial proteins, which when released into the cytoplasm can activate proteases; and the caspase (cysteinyl protease cleaving C-terminal to an aspartic acid) family of proteases, which are central to the generation of an apoptosis phenotype. New members of these and other families are being discovered on a daily basis. Quite frequently, experimental up- or down-regulation of these genes will affect cell death, though usually by changing the threshold rather than the machinery of cell death. For instance, the hammertoe mouse is characterized by loss of cell death only between digits 2 and 5, and not between 1 and 2 or on the peripheries, and this regional failure can be repaired by retinoic acid (Zakeri et al., 1994). In this and many other situations, the subtleties that determine the thresholds are not well understood. Other publications note the relative abundance of, for instance, bcl-2 or bax, in healthy and dying cells.

6.1. Detection of mRNA and protein(s) associated with apoptosis

Most techniques used to measure apoptosis-related proteins such as bcl-2, bax, release of cytochrome *c*, and other apoptosis-related proteins include flow cytometry, yeast 2-hybrid evaluation, or immunocytochemistry. Techniques such as in situ hybridization and immunocytochemistry have been most routinely applicable to embryonic development.

6.2. Detection of changes in activation of enzymes

Many changes that occur in dying cells involve post-translational changes such as aggregation or proteolytic cleavage. These changes have been detected electrophoretically as a decrease in molecular weight, for instance as in the conversion of a procaspase to an active caspase. Such procedures are typically not applicable to normally developing embryos. However, immunohistochemistry has often proved useful in revealing changes in amounts of active forms of several apoptosis-related proteins such transglutaminase (Fig. 2d) or Cdk5 (Fig. 2e). Another example is an antibody, which recently became available through commercial sources (Cell Signaling Technology, New England Biolabs), that is purported specifically to recognize activated caspase 3. Another antibody is purported to identify free nucleosomes and can be used in an ELISA procedure, again on the assumption that there are sufficient numbers of cell deaths to detect. Immunohistochemical techniques have also demonstrated an association of proteins previously associated with apoptosis, such as cyclin-dependent kinase 5 (Qi et al., 1995). The association, detected primarily by immunohistochemistry, is a post-translational change.

Another technique of some interest is detection of proteolytic activity in living cells by use of cell-permeant fluorogenic substrates. The fluorescent cleavage product is ionic and remains trapped at least through the early phases of apoptosis (Komoriya et al., 2000). The one most commonly used is a substrate for caspase 3. Caspase 3 is the major effector caspase, meaning that it is activated by an initiator caspase (caspase 8 or 9, themselves resident in cells as proenzymes) and digests major intracellular components of the cytoskeleton and nuclear matrix. Though they have not yet been used extensively for embryos, these cell-penetrant fluorogenic substrates are promising. In preliminary experiments, we have seen that the fluorogenic substrate can identify in situ cells dying in zebrafish embryos.

These techniques can be used to assess apoptosis in abnormal situations, whether locally as a result of mutation (e.g., hammertoe mutation in mice—Zakeri et al., 1994) or massive as a result of treatment with a strong teratogen such as camptothecin (Mirkes, 1985) or an inducer of apoptosis such as cycloheximide (Hensey and Gautier, 1997). One may also learn in a generic manner from knockouts, as for instance in the case of a caspase 9 knockout in mice. Homozygous knockouts die from massive overgrowth of the brain, suggesting either that far too many neurons survive in the knockout mice, or that caspase 9 has a specific function in neurons other than its role in cell death.

6.3. Immunohistochemistry and DNA fragmentation double labeling

Fluorescence detection of DNA fragmentation and Cdk5 expression can be used as in Singh Ahuja et al. (1997b) and Zhang et al. (1997). Sections are processed according to the instructions for the Apoptag® kit, Intergen, Purchase, NY, with FITC fluorescein labeling, followed by two washes with PBST. We have used primary anti-Cdk5 antibody at a final concentration of 1 μg/ml with secondary biotinylated antibody and finally, incubating the slides with cy3-conjugated IgG mouse antibiotin (Jackson Immuno Research Laboratory, West Grove, PA) for 30 min. The slides are mounted with 90% glycerol. DNA fragmentation is seen with FITC as green and the Cdk5 as detected by cy3 is red.

6.4. In situ hybridization

The in situ hybridization is described in the same reference. Paraformaldehyde postfixed sections are incubated with proteinase K in PK buffer (1 M Tris, 0.5 M EDTA). After dehydration in graded ethanol, sections are hybridized with hybridization buffer and either the Cdk5 or p35 probe in 10 mM DTT. Sections are then washed in 5 × SSC with 10 mM DTT, in 50% formamide in 2 × SSC, in 1 × washing solution (23.4 g NaCl, 10 ml 1 M Tris (pH 7.5)) with 5 mM EDTA, in 20 mg/ml RNase A in 1 × washing solution, in 2 × SSC, and 0.1 × SSC. Finally, the dehydrated sections are dipped in photographic NTB-2 emulsion (Kodak) and exposed for 1–2 weeks, after which they are developed, counterstained with 0.2% toluidine blue, dehydrated, and mounted with Permount. This technique is illustrated in Fig. 2f.

7. Genetics in the study of embryonic cell death

The study of cell death in embryos has been implicitly genetic for many years, starting with Saunders' and Hinchliffe's observations that interdigital cell death was reduced or absent in web-footed animals and ranging through associations of harelip with lack of cell death along the midline and the identification of morphological genetic defects such as hammertoe as defects in the patterning of cell death. Nevertheless, genetics has not played a major role in the study of cell death in the embryo. The most important studies were crucial to our understanding of the mechanisms of apoptosis, in that the cell death mutants identified in *Caenorhabditis* measured embryonic or developmental cell deaths, leading to the now well-known sequence of bcl-2-like molecules normally inhibiting cell death, the activation of caspase-like molecules destroying the cells, and the existence of "ready for phagocytosis" signals (phosphatidylserine and other molecules) on the surface of apoptotic cells and their counterpart receptors on the phagocytes. A similar series of genes, the reaper-hid-grim group, was identified from their effects on embryonic cell deaths in *Drosophila*, though this group unlike the cell death (ced) genes in *Caenorhabditis*, does not appear to be universal. Beyond these studies, incidental papers have detected cell death in pre-implantation mammalian embryos, and an interesting study suggested that there existed in amphibian embryos an inhibitor of apoptosis that disappeared at the maternal-zygotic transition (Hensey and Gautier, 1997). Similar observations in zebrafish have been reinterpreted to suggest that the capacity to undergo apoptosis is acquired at the maternal-zygotic transition, thereby providing access to the critical determinants of the apoptosis machinery. It is likely that the genetics of cell death will provide much information concerning the control of patterns of cell death (as in hammertoe mice), where the machinery itself is not affected, as well as of the machinery itself, which may well involve embryonic lethals.

8. Development and differentiation of the immune system

Positive and negative selection of thymocytes in embryonic and early postnatal mammals leads to a vast repertory of immunocompetent cells coupled with self-tolerance. The achievement of this grand design derives from a complex series of interactions including steroids, T-cell activations, and the relative expression of Fas and FasL (Osborne, 1998). The fact that lpr/lpr mice (which express little or no functional Fas) and gld/gld mice (which express little or no functional FasL) develop lupus-like syndromes indicates that this ligand–receptor interaction is important

in negative selection in the thymus. These tissues are generally suitable for studies by flow cytometry as well as immunohistochemistry, and have revealed appropriate up-regulation of bcl-2 in regions of the thymus in which cells survive and down-regulation in regions of cell death and have, like many other studies of thymocytes, contributed considerably to our understanding of the mechanisms of cell death.

9. Conclusions

Cell death is a prominent issue in development, and historically has been the source of ideas such as that of programmed cell death, the existence of cell death genes, a cell death pathway, and the role of caspases. The study of cell death in embryos could produce much more, including an understanding of the subtle mechanisms controlling patterning of cell death (and thereby the cell-to-cell and tissue-to-tissue interactions that are important) and the molecules defining the acquisition of the ability to undergo apoptosis, to name two obvious examples. Limitations to this research include the small size and regional specificity of embryos, obviating interpretation by analysis of the whole embryo, but these issues cannot be addressed in tissue culture. The techniques that are most functional for this type of research are primarily microscopic, though other techniques are possible following microsurgery. Genetic analysis using knockout or overexpression systems has not been as revealing as had been hoped, in part because of the ability of embryos to make use of redundant pathways. Transiently controlled alteration of expression is likely to produce more meaningful results. It is to be hoped that future researchers will exploit the unique possibilities in embryos to lead us to more profound understanding of both embryonic development and of the mechanisms of cell death.

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