Morphological Features of Cell Death

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Cell death is discriminated into two main forms: apoptosis and necrosis. In contrast to necrosis, apoptosis is a regulated, energy-dependent form of cell death leading to phagocytosis of cellular remnants by neighboring cells. Characteristic morphological features of these two forms of cell death will be discussed and correlated to underlying molecular mechanisms.

Cell death occurs frequently in complex, multicellular organisms to maintain tissue homeostasis. For example, cells die during embryonic development. Also, the killing of infected cells by cytolytic effector cells of the immune system is an example of cell death in tissue maintenance. It is now generally accepted that cell death is an important phenomenon, reflected by the appearance of numerous publications on the subject every year.

For more than 150 years, morphological features played the leading role in the description of cell death (2). However, during the past three decades cell death has been characterized on the molecular level, which markedly increased our understanding of the morphology.

In the following review, we will discuss the different morphological features of cells undergoing apoptotic and necrotic cell death and, briefly, correlate the underlying molecular mechanisms. Other forms of cell death will also be mentioned if appropriate.

Apoptosis

Cells undergoing apoptosis activated numerous proteins in a temporally as well as spatially tightly regulated sequence. Initiation is induced by various stimuli, including the binding of ligands to cell surface receptors of the tumor necrosis factor family, damage of DNA integrity by various stress factors (i.e., drugs), radiation, or major changes of the homeostasis of cells. A main theme in transduction of many of these signals further downstream is the oligomerization and interaction of proteins with death effector domains (20). These proteins with conserved structural modules like death and death effector domains have a number of different functions in the cell, including connecting membrane-bound receptors to cytosolic effector caspases. Proteins containing death domains like FAS receptor (CD95/APO-1) can bind other death domain-containing adaptor proteins like Fas-associated death domain (FADD), which then initiate the proteolytic activation of proteins like caspase-8 and -10. The activation of the caspase cascade is the most central step in apoptosis. This finding was initiated by the discovery in Caenorhabditis elegans that the Ced-3 gene encodes a protein related to mammalian caspase-1 (interleukin-1β-converting enzyme) (8). Caspases can initiate apoptotic signals (caspases-8 and -9) and execute the apoptotic program (caspases-3, -6, -7). Simultaneously with the activation of effector caspases, a mitochondrial pathway leading to apoptosis is activated. Numerous proteins (e.g., proteins belonging to the Bcl-2 family) translocate to mitochondria or are released [e.g., cytochrome c, apoptosis-inducing factor (AIF)] into the cytoplasm from mitochondria during induction of different pathways of cell death (3). The activation of the mitochondrial pathway is partially regulated by proteins containing death effector domains by forming a protein complex composed of apoptotic protease activating factor-1 (Apaf-1), cytochrome c, and caspase-9. Subsequent to activation of the apoptotic cascade, several vital proteins are cleaved. This cleavage allows the correlation of morphological events with underlying molecular mechanisms. Morphological alterations of apoptotic cell death that concern both the nucleus and the cytoplasm are remarkably similar across cell types and species (6, 11, 18). Usually several hours are required from the initiation of cell death to the final cellular fragmentation, although the time schedule is dependent on the cell type, the stimulus, and the apoptotic pathway. Apoptotic cell death occurs in normal as well as in pathologically altered tissues. It has been recognized as a characteristic type of cell death for over 100 years, and only the name changed from karyorrhexis or pyknosis to apoptosis in 1972, a term proposed by Kerr et al. (9).

Nucleus. Morphological hallmarks of apoptosis in the nucleus are chromatin condensation and nuclear fragmentation (Figs. 1 and 2). The condensation starts peripherally along the nuclear membrane, forming a crescent or ringlike structure. During later stages of apoptosis the nucleus further condenses, and finally it breaks up inside a cell with an intact cell membrane, a feature described as karyorrhexis (13). Condensation and fragmentation of the nucleus can be seen with electron microscopy (Fig. 1) but also with light microscopy (Figs. 2 and 3). Techniques to use DNA-binding stains like 4’,6-diamidino-2-phenylindole, Hoechst, and others makes the formation of apoptotic bodies more evident. In addition, the staining with DNA-binding markers can be performed in situ to characterize the apoptotic nuclei. DNA strand breaks can also be detected by DNA fragmentation assays (Fig. 3) and simplify the detection of apoptosis in situ (10). The condensation and fragmentation of the nucleus can be visualized by using the incorporation of labeled dUTP (TUNEL) method (Figs. 2 and 3). Techniques to use DNA-binding stains like 4’,6-diamidino-2-phenylindole, Hoechst, and others makes the formation of apoptotic bodies more evident. The TUNEL method uses specific enzymes to label the DNA and forms a labeled nick end labeling (TUNEL) method (Figs. 2 and 3) (10).

These morphological features can be initiated by the cleavage of various proteins by caspases. Lamin and nuclear mitotic apparatus proteins are able to maintain the structural integrity of the nucleus. Both can be cleaved by activated caspases-3...
and -6 (16). A major hallmark of apoptosis in the nucleus is the internucleosomal fragmentation of double-stranded DNA into fragments of 180- to 200-bp length. Responsible for this fragmentation of DNA are a number of caspase substrates that are involved in DNA repair and replication. Examples are DNA fragmentation factor (DFF) 40 and caspase-activated DNase (CAD). Both proteins are constitutively present as heterodimers with inhibitor proteins DFF45 and inhibitor of CAD (ICAD) (12). On activation, DFF40 and CAD are able to fragment DNA together with a large number of other endonucleases that are suggested to be involved in apoptosis (16). Other proteins that are cleaved during apoptosis resulting in inactivation are poly(ADP-ribose) polymerase and DNA-dependent protein kinase, both proteins involved in DNA repair mechanisms.

**Cell membrane and cytosol.** Early during the initiation of apoptosis, cells lose contact with neighboring cells. Membranes and organelles including mitochondria are well preserved during early apoptotic cell death (Fig. 1B). It was shown in embryonic epithelia but also in cultured Madin-Darby canine kidney cells growing in monolayers that dying cells generate signals activating actin- and myosin-dependent mechanisms in neighboring cells (17). This mechanism extrudes dying cells from the epithelia and monolayer. Subsequently, microvilli are lost and the cells start to show protrusions of the plasma membrane commonly referred to as blebs. The cells shrink, and finally the blebs separate, forming apoptotic bodies densely packed with cellular organelles and nuclear fragments that are engulfed by phagocytosis of surrounding cells (7). Morphologically these ingested apoptotic bodies can be observed for some time, but eventually they will degrade and no trace will be left that tells the tale of the lost cells in tissues. Apoptosis occurs without associated inflammation due to the containment of the cellular constituents by an intact membrane and the subsequent engulfment of apoptotic bodies. However, if the remnants of apoptotic cells are not phagocytosed they will undergo degradation that resembles necrosis and is called secondary necrosis.

The shrinkage of cells, blebbing, and forming of apoptotic bodies can be observed by using light and electron microscopy (Figs. 1 and 2). One of the earliest signs of apoptosis is the externalization of phosphatidylserine, providing an “eat-me” signal for phagocytosing cells (5). This can be employed in vitro as marker for the detection of apoptosis because annexin V binds to phosphatidylserine with a high affinity (Fig. 3C). Annexin V is a 35-kDa Ca\(^{2+}\)-binding protein that is available conjugated to fluorochromes for use in fluorescence microscopy and coupled to other markers suitable for light and electron microscopy. Another combined morphological-enzymatic assay for detection of apoptotic cells is the turnover of membrane-permeable, fluorescently quenched substrates for caspases. PhiPhiLux is one of these substrates that get cleaved by caspase-3, resulting in a bright fluorescent product that can be detected in unfixed cells by light microscopy.

A number of structural proteins are processed on initiation
of the apoptotic cascades. One of the most prominent of these proteins is actin (14). Actin forms microfilaments and regulates the cell shape in the cortical cytoskeleton. Examples of other proteins that are cleaved by caspases during apoptosis are spectrin, fodrin, β-catenin, gelsolin, growth arrest-specific 2, and p21-activated kinase 2. These proteins are involved in the maintenance, organization, and attachment of the cytoskeleton (e.g., gelsolin, Gas2, PAK2) and play a role in cell-to-cell junctions (e.g., spectrin, fodrin, β-catenin). The role of cleaved gelsolin and Gas2 is illustrated in overexpression experiments that result in changes in cell shape resembling apoptosis. Even blebbing can be linked to specific proteins. Death-associated protein kinase (DAP kinase) as well as DAP kinase-related kinase (DRP kinase) were recently found to be involved in membrane blebbing. Both kinases are independent of caspase activity, but it is known that activated DRP kinase activates caspase-dependent events. Dominant-negative mutants of DAP and DRP kinase showed reduced blebbing and, interestingly, reduced autophagy.

**Mitochondria.** Alterations in cellular stress responses and bioenergetic state play an important role in the initiation of apoptosis. Both events are continuously monitored by mitochondria that integrate multiple proapoptotic signals into common apoptotic degradation cascades (3). The mitochondrial membrane permeabilization has a central role during this process. Inducing factors are rather heterogeneous depending on the death stimulus. Proapoptotic members of the Bcl-2 family appear to be central during the initiation of the mitochondrial membrane permeabilization, whereas anti-apoptotic members of the Bcl-2 family inhibit this process. A number of proteins are released, including cytochrome c, AIF, Smac/DIABLO, and others when mitochondrial membranes are permeabilized. A complex called the apoptosome is formed by interaction of cytochrome c with Apaf-1, which results in Apaf-1 oligomerization and binding as well as activation of caspase-9. Smac/DIABLO facilitates the activation of caspases by binding to a family of proteins called inhibitor of apoptosis proteins. AIF, in contrast to the caspase-activating proteins, translocates to the nucleus and induces chromatin condensation and DNA fragmentation. Despite the importance of the mitochondria, their light- and electron-microscopic appearance is largely unaffected until late in the process of apoptosis, when they swell like other cell organelles.

**Necrosis**

Initially, the term necrosis was used to describe irreversible tissue damage, which apparently occurs after the involved cells have already died (13). Currently, the term necrosis is

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**FIGURE 2.** Morphological appearance of DNA fragmentation [terminal deoxynucleotide transferase nick end labeling (TUNEL staining)] in actinomycin D-treated apoptotic K562 cells (10). Fluorescence images (A–F) and corresponding interference contrast images (A’–F’). A and A’: nuclear boundaries are outlined by an intensely labeled rim, whereas the interior shows a diffuse homogeneous signal. Neither the nucleolar region (arrowhead) nor the cytoplasm are labeled. B and B’: similar to A and A’, but the nucleus is rounded up with peripheral crescents directly beyond the nuclear margins. Fluorescent bodies pinch off in the direction of the cytoplasm. C and C’: early fragmentation of the nucleus into separate bodies, with diffuse labeling of the interior and peripheral crescents. D and D’: fragmentation of the nucleus into many small, intensely fluorescent homogeneous bodies with diffuse labeling of the cytoplasm. E and E’: fragmentation of nucleus into many small bodies. F and F’: condensation without fragmentation of the nucleus. The cytoplasm shows a diffuse fluorescence. Bar = 9 μm.
used for a type of cell death that is not programmed but rather accidental. In contrast to apoptosis, necrosis is a passive form of cell death without the intricate regulatory mechanisms that are characteristic of apoptosis. The causes of necrotic cell death such as heat stress or toxic agents can in many cases also induce apoptotic cell death (13, 19). Often, the intensity of the insult but also the energy level of a cell decides the outcome as either apoptosis or necrosis. It could be shown that low ATP concentration or impaired ATP generation pushes the morphology of cell death toward necrosis (11, 15).

The morphology of cells dying by necrosis is fairly diverse. The cell membrane becomes permeable early during the death process (Fig. 1D). Organelles may dilate, and ribosomes may dissociate from the endoplasmic reticulum. The nucleus disintegrates late, and in some cases chromatin condensation occurs. However, pyknotic and fragmented nuclei are not a common feature in necrotic cell death. Due to early leakage of cellular contents, a massive inflammation in the tissue is triggered. Depending on the morphology and the involvement of lysosomes, two different types of necrotic cell death have been distinguished: autophagic and nonlysosomal disintegration (1). The autophagic cell death is characterized by numerous vacuoles in the cytoplasm filled with cellular remnants. The nonlysosomal cell death shows that a pronounced dilation of organelles and empty spaces are formed and the degeneration proceeds without any detectable involvement of lysosomes.

Detection of cells dying a necrotic type of cell death employs the fact that, in contrast to apoptosis, the cell membrane becomes permeable very early. Small charged molecules that normally do not traverse the cell membrane will then enter the cell. Propidium iodide, mono- or dimeric cyanine nucleic acid dyes (e.g., YOPRO-1) several others are often used for membrane permeability detection. These dyes have a high affinity to DNA and RNA, and the binding results in an increase of their fluorescence, which makes them suitable for live cell imaging. They can also be combined with membrane-permeable Hoechst stains to correlate the nuclear morphology. N-hydroxysuccinimidyl biotin (NHS-biotin) is another dye that is impermeable to viable cells with an intact membrane. NHS-biotin binds covalently to proteins and can be used if cells are to be fixed for analyses. The biotin moiety can be detected with appropriately labeled streptavidin, and the whole procedure is compatible with TUNEL staining (Fig. 3), making this method ideal for the combined detection of membrane damage and DNA fragmentation (10, 21). It is important to note that cells in late stages of apoptosis are also membrane permeable for all of these dyes (Fig. 3A) due to secondary necrosis.

**Intermediate forms of cell death**

In general, the mode of death and its morphological manifestation is dependent on the cell type, energy metabolism and level, signaling pathway, stimulus, and environment. Thus there exist various intermediate forms of cell death displaying both apoptotic and necrotic morphological characteristics, but the underlying activation of pathways are rather poorly understood. Cells may end in an apoptosis-like death without activation of caspases, reflecting an alternative pathway whereby lysosomal proteases play a crucial role. In these cases condensation of chromatin is low and fragmentation of nuclei usually does not occur. Cell death may also be initiated by a standard apoptosis program, which is then inhibited at the late stages of caspase activation and is finished by necrosis. This aponecrotic phenotype is characterized by the coexistence of fragmented nuclei and degenerated organelles (4). Currently, and even more so in the future when more cell types are investigated in vitro, but also in vivo, it becomes more and more apparent that intermediate forms of apoptosis and necrosis occur and that a stereotyped outcome either as apoptosis or necrosis cannot always be expected.

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