

APOPTOSIS: BIOLOGICAL AND CLINICAL ASPECTS

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Apoptosis is a form of cell death alternative to necrosis. Recently, the way cells die has focused the interest of many investigators, and apoptosis is being increasingly recognized as a common and fundamental event in many biological processes. This review summarizes the main biological aspects related to apoptosis, its involvement in physiological and pathological situations, and current research trends on this phenomenon.

Definition of apoptosis

Apoptosis, or programmed cell death, can be considered as the suicide of a cell that is going to die since this cell participates in its own destruction by activating a series of processes (a program) which ultimately lead to its complete elimination. Whereas necrosis is characterized by cell swelling, rupture of the plasma membrane and leakage of cellular contents into the extracellular environment,¹ apoptosis involves cell shrinkage,² nuclear condensation, chromatin clumping³ and final breakdown of the cell into the so-called apoptotic bodies, nuclear fragments bordered by a thin layer of cytoplasm that are removed *in vivo* by phagocytes.^{4,5}

From metabolic and biochemical points of view a number of aspects distinguish apoptosis from necrosis and account for the differences in morphologic and pathophysiological features between these two forms of cell death. The metabolic activity of necrotic cells is greatly damaged, energy production is impaired and osmotic pressures across the plasma membrane can no longer be regulated.^{6,7} As a result, there is a net movement of water into the cells with consequent swelling and rupture of the plasma membrane. Apoptotic cells, on the contrary,

preserve efficient metabolic activity, energy production and membrane integrity until late in the process of cell death. Thus, cellular content is released to the external environment only after it has been packaged within apoptotic bodies.⁸ Preservation of membrane integrity is evidenced by the ability of apoptotic cells to exclude vital dyes, sometimes even after they have already been fragmented into apoptotic bodies.⁹ It has been suggested that activation of transglutaminase activity leading to protein cross-linking strengthens the cell membrane and helps to prevent its rupture.¹⁰ That this phenomenon is an active process is also suggested by the observation that in several experimental models it requires active RNA and protein synthesis on the part of the dying cells.^{11,12}

Apoptosis usually starts with the appearance of cytoplasmic vesicles, probably derived from dilatation of the endoplasmic reticulum and cisternae (Figure 1). Dilated cisternae fuse with the plasma membrane and are expelled from cells, giving a bubbling aspect to the cell surface. These events lead to loss of water, cell volume reduction, increased cell density. Nuclei are also involved in the process; a loss of volume leads to nuclear condensation¹³ and is associated with alterations in chromatin structure and, finally, DNA breakdown.¹⁴

Biochemically, the pattern of DNA degradation is probably one of the best characterized aspects of the entire process. DNA degradation in apoptotic cells occurs through activation of specific calcium and magnesium-dependent endonuclease(s)¹⁵ that cut DNA between nucleosomes, producing oligonucleosomal DNA fragments.¹⁶ An endogenous deoxyribonuclease involved in DNA fragmentation during apoptosis has recently been isolated from rat thymo-

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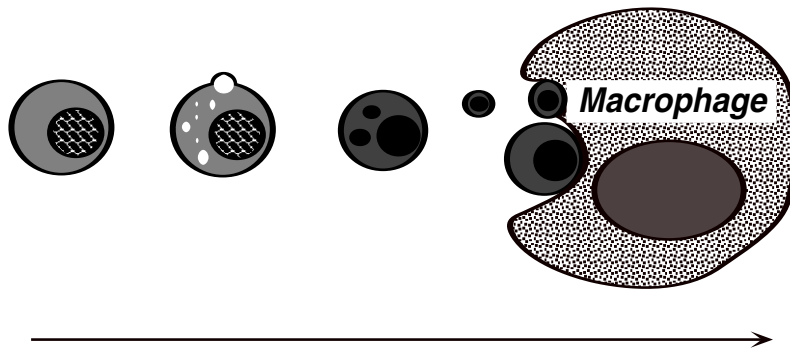


Figure 1. Schematic representation of the morphological aspects of apoptosis.

cytes and lymph nodes.¹⁷ This enzyme has been characterized in detail and identified with rat DNase I. The role of calcium in apoptosis, particularly in the activation of endogenous endonuclease(s), was initially suggested by McConkey et al.¹⁸ and Berke.¹⁹ More recently, it has been confirmed in other experimental models.²⁰ It has also been shown that during glucocorticoid-mediated apoptosis of lymphocytes there is early induction of the gene coding for the calcium-binding protein calmodulin.²¹ Calmodulin has complex functions in the modulation of several cellular activities, and its involvement in apoptosis is suggested by the observation that calmodulin inhibitors prevent apoptosis.¹⁸

Elimination of apoptotic cells or what remains of them, the apoptotic bodies, occurs through phagocytosis, and macrophages are the main effector cells. The mechanism of apoptotic cell phagocytosis is also an interesting field of investigation. It was initially demonstrated that phagocytosis followed recognition of dying cells by the macrophage vitronectin receptor.²² Recognition, however, is not direct but probably mediated by thrombospondin, a multifunctional glycoprotein involved in cell-cell interactions.²³ Other mechanisms of recognition also play a role in phagocytic removal of apoptotic cell remnants, including interaction of macrophage membrane lectins with side chain sugars on apoptotic cells following loss of sialic acid from these carbohydrates,²⁴ or the binding of phosphatidylserine on apoptotic cells by a phosphatidylserine receptor on the surface of phagocytes.²⁵

The differences between necrosis and apoptosis have significant consequences *in vivo*. In fact, the release of cell contents into the environment that occurs during necrosis leads to activation of chemotaxis, inflammation, diges-

tion of the extracellular matrix by lysosomal enzymes and, eventually, tissue repair by fibrosis.^{26,27} During apoptosis, on the contrary, cell debris packaged within apoptotic bodies is phagocytosed by macrophages and does not promote inflammation or tissue damage. For this reason apoptosis has been considered an altruistic form of cell death. This may be essential in situations where cell death is not due to aging and/or damage of any kind but is necessary for the maintenance of tissue homeostasis, such as in embryogenesis and morphogenesis,^{28,29} in tissue remodeling, in the clonal deletion of self-reactive immune system lymphocytes,^{30,31} and in the homeostatic regulation of cell number in tissues with high cellular turnover, such as the liver,³² digestive tract and hemopoietic system.^{33,34} From this point of view apoptosis can be seen as the physiologic mode that cells have for dying.

Demonstration of apoptosis

Apoptosis was originally defined according to the morphological aspects which distinguished it from necrosis, i.e. cell shrinkage, nuclear condensation and chromatin clumping as opposed to cell swelling. Subsequently, ultrastructural analysis also revealed mitochondrial swelling in cells undergoing necrosis, whereas mitochondria in apoptotic cells were both structurally and functionally normal until late in the process. Morphological and ultrastructural analysis are still fundamental approaches for evaluating apoptosis, but other methods have since been introduced, the best characterized being DNA structure analysis and cytofluorimetric examination of dying cells.

As previously stated, during apoptosis specific endonuclease(s) are activated which cut DNA

between nucleosomes, the primary structural units of chromatin. A single nucleosome contains 160 to 200 pairs of bases winding around a protein core of histones.³⁵ Activation of endonuclease(s) results in DNA degradation into oligonucleosomal fragments. Consequently, agarose gel electrophoresis of DNA obtained from apoptotic cells shows a ladder of fragments corresponding to multiples of 160-200 base pairs. Although a strict association between nucleosomal DNA fragmentation and apoptosis has been questioned recently,⁶ it is generally accepted that some form of DNA damage is linked with apoptosis, and may even be necessary for the process to occur.³⁶ The presence of fragmented DNA in apoptotic cells can also be shown through differential precipitation of the high and low molecular weight DNA fractions.¹⁵

Flow cytometry (FCM) techniques have provided interesting new opportunities for investigating programmed cell death. The specific metabolic, biochemical and molecular features which characterize apoptotic cells can all be detected by single and/or multiparametric analysis.³⁷⁻⁴⁰ The advantage common to all FCM-based techniques is the possibility to have an easy, rapid and accurate quantitation of apoptosis in both viable and fixed single cells.⁴¹

Apoptotic cells can be identified by their diminished stainability with several DNA-specific fluorochromes (i.e. propidium iodide, DAPI, Hoechst dyes) due to degradation and subsequent leakage of DNA from the cell. Apoptotic cells appear as an apparently hypodiploid population, which has been named the *sub G1* or *A0 region* (Figure 2).^{42,43} Initially it was not completely clear whether this finding was due to reduced DNA content or to altered conformation of chromatin now less accessible to staining. It was recently demonstrated that the reduced DNA stainability of apoptotic cells is a consequence of partial DNA loss following activation of an endogenous endonuclease. Evidence for this has been found in the similarity between the chromatin structure of fragmented apoptotic cell nuclei and that of non-apoptotic cells. Again, loss of DNA can be documented by leaving apoptotic cells in PBS for sometime after fixation and staining: reduction in DNA stainability is time dependent, and low molecular weight DNA can be demonstrated in the supernatant.³⁷

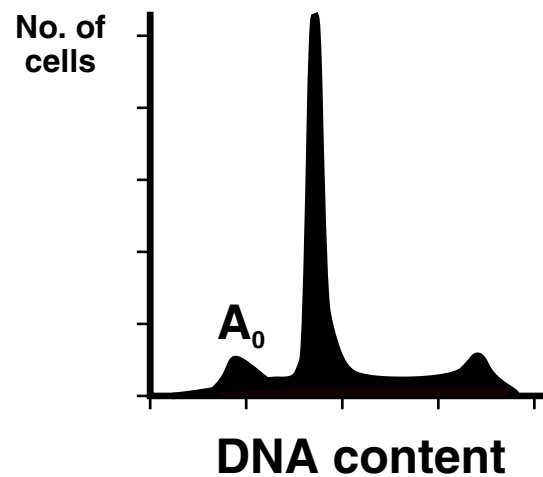


Figure 2. Schematic representation of flow cytometric DNA analysis of apoptosis.

Flow cytometric DNA analysis also allows definition of the relationship between the induction of apoptosis by different agents and their cell cycle phase specificity.⁴⁴⁻⁴⁶

Simultaneous flow cytometric measurement of DNA and total protein content revealed a decrease in both these constituents and made it possible to investigate a correlation between these processes.⁴⁴

Changes in the morphology of cells undergoing apoptosis affect their light scattering properties.⁴⁷ Flow cytometric analysis of forward scatter versus side scatter distinguishes apoptotic cells from normal ones⁴⁸⁻⁵⁰ and allows determination of the immunophenotype of cells undergoing apoptosis.⁵¹

The integrity of the plasma membrane of cells undergoing apoptosis is preserved in the early stages of the phenomenon and certain dyes such as propidium iodide (PI) do not enter.⁵² On the other hand, following exposure to dyes such as Hoechst 33342, apoptotic cells appear brighter than controls. Simultaneous staining with these two dyes provides a means of discriminating apoptotic from both living and necrotic cells.⁹ Hoechst 33342 diffuses through cell membranes so that this technique can also be associated with the phenotyping of apoptotic cells for surface antigens.

Apoptotic cell DNA shows markedly increased sensitivity to denaturation; thus, coloration

with the metachromatic dye acridine orange (which stains double-stranded and denatured DNA differently) allows easy and sensitive FCM detection of apoptotic cells.³⁷

A new assay for non radioactive *in situ* nick translation has recently been reported and employed to detect DNA strand breaks in apoptotic cells.⁵³ Combining this assay with PI-stained DNA, the authors were able to use FCM to reveal the cell cycle phase specificity of DNA breaks. This method has been successfully applied in the analysis of leukemic cell response to chemotherapy.⁵⁴

Considering the progress made in the identification and characterization of oncogenes involved in the control of apoptosis, simultaneous FCM analysis of DNA content and of some oncogene products and/or cell cycle-related proteins (i.e. bcl-2, p53, c-myc, cyclin, statin) should provide an important tool for assessing at the single-cell level the role these proteins have in the induction/prevention of apoptosis and their relationship with the cell cycle.^{46, 55}

Role of apoptosis in physiology and pathology

Apoptosis probably represents the physiological way cells have of dying. It plays an essential role in the regulation of tissue homeostasis since it can be modulated and is able to contribute to the finely tuned balance between cell death and proliferation. In addition, it is now clear that abnormalities in the regulation of apoptosis often contribute to disease.

Among the best-studied models of induction or inhibition of apoptosis in the normal regulation of biological systems are the apoptosis of

self-reactive lymphocytes during immune system development^{30,31,56-58} and the modulation of apoptosis by growth factors and cytokines.^{12,13,60-64} In this respect the observation that growth factors prevent apoptosis of factor dependent cell lines suggests that these factors are agents which promote the survival of target cells in addition to stimulating their proliferation.^{12,60} Signal transduction pathways activated by growth factors are being identified in more detail, and this research will probably lead to identification of second messenger systems involved in the prevention of apoptosis. On the other hand, transforming growth factor β and tumor necrosis factor (TNF) are cytokines that induce apoptosis of different cell kinds and are possibly involved in the negative control of cell growth.^{20,63-65} However, similarly to its pleiotropic effects on cell growth, which range from stimulation to inhibition, TNF's action on apoptosis also depends on its target cells; it protects monocytes and granulocytes from apoptosis,^{34,66} while inducing the phenomenon in several leukemic and adenocarcinoma cell lines and in fibroblasts.⁶⁵ NK cells and cytotoxic lymphocytes have also been shown to induce the apoptosis of their target cells.⁶⁷⁻⁶⁹

Dysregulation of apoptosis can be involved in the pathogenesis of disease states (Table 1). In this regard the most interesting example is probably represented by the follicular B-cell lymphomas associated with the t(14;18)(q32;q21) translocation. The proto-oncogene bcl-2 normally located on the long arm of human chromosome 18 is translocated to chromosome 14, near the immunoglobulin (Ig) heavy chain locus.⁷⁰⁻⁷² The expression of bcl-2 is

Table 1. Disease states associated with dysregulation of apoptosis.

<i>Disease</i>	<i>abnormality and gene(s) involved</i>
Follicular B-cell lymphoma associated with the t(14;18)(q32;q21) translocation	Aberrant expression of bcl-2 under the influence of the Ig gene promoter: inhibition of apoptosis
Blast crisis of chronic myelogenous leukemia	Deletion or joint mutations of p53: inhibition of apoptosis
Severe β -thalassemia	Excessive apoptosis of erythroid cells induced by α -globin chains
AIDS	Excessive apoptosis of CD4 ⁺ cells induced by HIV
Alzheimer disease	Apoptosis of neurons induced by β -amyloid peptide

increased severalfold under the influence of the Ig gene promoter and the gene acquires transforming activity. Although the biochemical function of the bcl-2 gene product is still unknown, it has been shown that its aberrant expression protects cells from apoptosis, leading to their accumulation and tumor development.⁷³ Investigation of this topic provided additional information on the cooperation between oncogenes in the multistep pathogenesis of tumors. In fact, while the constitutive expression of the c-myc proto-oncogene produces apoptosis of several kinds of cells,^{74,75} when lymphocytes are protected from apoptosis by bcl-2 the subsequent activation of c-myc does not induce apoptosis⁷⁶ but rather confers a more malignant phenotype on cells.⁷⁷ Conversely, the product of onco-suppressor gene p53 induces apoptosis of cell lines,⁶² but mutated forms of this gene, which are often found in tumors such as the blastic crisis of chronic myelogenous leukemia, are unable to induce apoptosis and in this way are thought to contribute to neoplasia. These observations indicate that tumors develop not only from abnormal cell proliferation and inhibition of differentiation, but also from reduced cell death due to inhibition of apoptosis. This hypothesis has recently been strengthened by the finding that the bcr/abl fusion gene, derived from the translocation that gives rise to the Philadelphia chromosome in chronic myelogenous leukemia, protects growth factor-dependent cell lines from apoptosis when they are cultivated in the absence of appropriate growth factors.⁷⁸

Enhanced apoptosis during the maturation of hematopoietic precursors in bone marrow has been suggested as a pathogenetic mechanism in some cases of myelodysplasia characterized by ineffective hematopoiesis.⁷⁹ Ineffective erythropoiesis is also a feature of severe β -thalassemia (Cooley's anemia). Analysis of bone marrow erythroblast DNA from patients with Cooley's anemia undergoing bone marrow transplantation showed a pattern of DNA degradation into oligonucleosomal-size fragments typical of apoptosis.⁸⁰ This pattern was less evident in the bone marrow donors, all carriers of the β -thalassemia trait, suggesting that enhanced apoptosis may be responsible for ineffective erythropoiesis in severe β -thalassemia.⁸⁰

Investigation of cell growth regulation in hormone-dependent tumors also shed some light

on the mechanism of action of some hormones. Androgens, estrogens and ACTH have been shown to protect hormone-dependent tumor cells from apoptosis,⁸¹ and it is likely that the corresponding normal tissues, for which these hormones represent trophic agents, are similarly protected and induced to grow.

Viral infections are another field in which induction or inhibition of apoptosis can play a pathogenetic role. The Epstein-Barr virus (EBV), for example, has been shown to inhibit apoptosis of B lymphocytes. The virus is thought to infect B cells, which normally have a limited life span. Following infection these cells enter a long-lived B-cell pool. EBV genes contributing to the inhibition of apoptosis in B lymphocytes have been identified and shown to be part of the family encoding for EBV latent proteins.⁸² It is noteworthy that one of these genes, BHRF1, shares sequence homology with bcl-2,⁷² possibly involving the latter in the pathogenesis of EBV-associated tumors. Another possibility is that EBV latent genes protect B-cells from apoptosis through induction of bcl-2 expression.⁸³ Conversely, induction of apoptosis might be involved in the selective destruction of CD4⁺ cells during HIV infection.⁸⁴ When activated in vitro by the T cell receptor for the antigen, CD4⁺ lymphocytes from HIV-infected subjects fail to proliferate and die through induction of an active process that is prevented by protein synthesis inhibitors and has the biochemical and ultrastructural features of apoptosis.⁸⁵

Recently, Loo et al.⁸⁶ observed that neuronal degeneration induced by β -amyloid peptide occurred by apoptosis, suggesting a role for this process in the loss of neurons associated with Alzheimer's disease.

A less-studied but equally interesting aspect of apoptosis in disease is the possibility that impaired phagocytosis of apoptotic bodies by macrophages may lead to the release of cytoplasmic and nuclear material by dying cells into the extracellular environment, thus exposing normally masked self-antigens to the immune system. This would contribute to inflammation and polyclonal lymphocyte activation with generation of self-reactive clones and development of autoimmune disease.⁸⁷ In systemic lupus erythematosus patients the release of nucleosomal DNA might contribute to the production of anti-DNA antibodies.

Present research trends on apoptosis

Since it is an active process, apoptosis can be modulated, and its modulation could provide new tools in the treatment of malignant diseases. It has already been shown that the inhibitors of topoisomerase II, including agents already used in cancer and leukemia therapy such as the epipodophyllotoxins VP16 and VM26, induce apoptosis in thymocytes.^{88,89} The same is also true of other agents active in therapy,⁹⁰ of Ara-C⁹¹ and of the purine nucleoside analogues 2-chloro-2'-deoxyadenosine and 9- β -D-arabinosyl-2-fluoroadenine,⁹² which are promising drugs in the therapy of lymphocytic malignancies. The mechanism of action of the epipodophyllotoxins has been especially well characterized.^{88,89,93}

Experimental research into the mechanism of action of growth factors and their signal transduction pathways is in progress. Protein kinases have been shown to be widely involved in signal transduction from growth factor receptors, and several inhibitors of protein kinases such as okadaic acid (an inhibitor of serine and threonine kinases), and tyrphostins (inhibitors of tyrosine kinases) induce cell cycle arrest and apoptosis.^{46,94} Herbimycin, another tyrosine kinase inhibitor, prolongs survival of mice inoculated with v-abl transformed leukemic cells.⁹⁵ These data suggest that in the future kinase inhibitors might represent a new family of chemotherapeutic drugs.

Development of new therapeutic strategies involving modulation of apoptosis is stimulating basic research into the molecular and biochemical mechanisms of apoptosis. Since new RNA and protein synthesis is required for apoptosis to occur in several systems, attempts are being made to identify the genes whose expression is necessary to activate the mechanisms leading to programmed cell death. Owens et al.⁹⁶ identified two messenger RNAs, RP-2 and RP-8, whose expression is associated with the apoptosis of immature thymocytes, and assigned them to a putative family of *death genes* specifically activated during the early phases of programmed cell death. Identification of other members of this family of *death genes*, elucidation of the mechanisms controlling their expression, and definition of their function will provide much deeper insight into the control of cell growth.

Another line of evidence suggests the exist-

tence of apoptosis-related antigens/receptors on cell membranes. Antibodies against the Fas/APO-1 antigen which is expressed on the surface of some T and B lymphocytes, can induce apoptotic forms of cell death.^{97,98} Antibodies to the CD22 antigen, expressed on mature B lymphocytes, are similarly cytotoxic and induce the DNA fragmentation pattern usually observed during apoptosis (Perfetti V., personal observations). Hopefully, the knowledge accumulating on the mechanisms controlling cell death will lead to the development of new drugs and new protocols for treating human disease.⁹⁹

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