Current concepts of apoptosis

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Apoptosis is a carefully regulated process involved in developmental and immunological events. The alteration of apoptotic pathways is important in the establishment and progression of neoplasia. Apoptosis allows for the orderly removal of excess cells but, in contrast to necrosis, it is not an inflammatory process. Many of the molecular components and effectors of apoptosis have been described. In this review the authors briefly discuss the current understanding of apoptosis in the context of the two prevailing hypotheses, the "conflicting signal" and "dual signal" theories.

Key Words * cell death * apoptosis * cell proliferation * regulatory gene

Among the paradoxes of multicellular existence is the organism's ability to produce sufficient cell populations to meet ongoing structural and functional requirements while concurrently protecting against potentially lethal neoplastic proliferation. The efficiency of this process is evident by the very small chance of a given cell division producing a rogue lineage. When we place the one-in-three odds of an individual developing cancer over a lifetime in the context of $10^{16}$ cell divisions, the observed rate of neoplasia is lower than expected. In the absence of mutagens, spontaneous mutations occur at a rate of $10^{-6}$ per gene per cell cycle, a value maintained by the fidelity of DNA replication and repair. Therefore, the cumulative number of mutations a given gene is likely to undergo is $10^{10}$.[5,37] Based solely on these facts, the homeostatic processes that safeguard against neoplasia appear to be quite efficient. Cells with an apparent selective advantage are somehow compelled to activate self-destructive pathways. Over the past decade appreciation has developed for the role that apoptosis may play in controlling unchecked cell proliferation. Parallel to this interest are advancements in the fields of cell signal transduction and cell cycle control. In this article we will briefly review the current understanding of apoptosis and explore the potential therapeutic dividends of controlling this process.

The carefully regulated processes of apoptosis are distinct from those of necrosis, which involve cellular swelling and lysis.[17,41,55] Furthermore, necrosis is accompanied by the release of noxious cellular content leading to a significant inflammatory response. A number of characteristic microscopic, subcellular, and molecular "footprints" of apoptosis have been described in previous studies. Nuclear chromatin condensation is among the earliest changes (Fig. 1), followed by DNA cleavage into 30- to 500-kb fragments. Subsequent digestion by Ca++/Mg++-dependent endonucleases yields 180 bp segments, which are resolvable by gel electrophoresis (Fig. 2) These characteristic fragments, also known as oligonucleosomes, are chromosomal DNA remnants that are wrapped around histone proteins,
which protect these stretches from activated endonucleases.[55] At the cellular level, as disclosed by time-lapse videomicography, multiple changes are visible within the cytosol and plasma membrane.[17] Activation of endogenous proteases[39] results in cell shrinkage and rounding, accompanied by the formation of membrane-bound blebs (Fig. 1). These blebs possess specific signaling molecules that encourage phagocytosis of cellular debris by neighboring cells and macrophages.[14,19] In contrast to necrosis, the apoptotic event is not associated with a significant inflammatory reaction and allows for the orderly removal of excess cells. Typically, apoptosis lasts 5 to 30 minutes, making the in vivo study of its processes technically challenging.

![Photomicrograph showing control U87 glioma cells grown in 10% fetal bovine serum. Right: Photomicrograph showing typical morphological changes, nuclear condensation, membrane dissolution, and blebbing in U87 cells grown in 10% fetal bovine serum with 1 µmol K252a, a protein phosphatase inhibitor.](image)

Apoptosis is a common pathway for myriad chemical and physical stimuli. Furthermore, it is likely that individual cell types have both convergent and divergent apoptotic pathways. Variations of these pathways are likely to be expressed in a tissue-specific manner, although some participants are conserved across cell type and among different species. Alterations in different portions of these pathways have been shown to be present in most cancers. In fact, it can be inferred from the accumulated data that apoptotic responses may need to be suppressed for neoplasia to progress.[17,41,55] Research into the loss of proliferative control induced by positive growth regulators has led to a growing appreciation of the significance of loss of negative controls, specifically the suppression of intrinsic apoptotic responses in the establishment and progression of neoplastic disorders.
Fig. 2. Left: Agarose gel displaying a molecular weight marker. Right: Gel showing oligonucleosomal laddering after U87 cell DNA was extracted following treatment with cisplatin for 48 hours. The 180-bp segments were resolved by electrophoresis on 1.2% agarose.

Many physical and chemical stimuli have been shown to induce apoptosis.[19,51] Among those that inflict physical damage are heat shock, gamma and ultraviolet radiation, viral infection, and oxidative stress. The physiological effectors of apoptosis enumerated in previous reports include the tumor necrosis factor/Fas ligands, transforming growth factor-β, neurotransmitters, glucocorticoids, retinoids, and growth factor withdrawal. Additional stimuli include chemotherapeutic agents, cisplatin, doxorubicin, bleomycin, cytosine, arabinoside, nitrosamines, methotrexate, and vincristine. Agents that affect signal transduction pathways, in particular kinases, phosphatases, and their inhibitors, have also been shown to induce apoptosis in appropriate cell lines and conditions.[8,23,35,53]

Two dominant theories concerning the regulation of apoptosis have been advanced. The "conflicting signal" theory states that such pathways are activated at times when an individual cell is receiving proliferative (mitogenic) and inhibitory signals simultaneously. A given cell exposed to growth inhibiting conditions such as serum-free media, with concomitant activation of regulatory genes, such as c-myc, will undergo apoptosis. More recently another hypothesis has gained support: the "dual signal" theory.[16,17,41] This model holds that signals for proliferation and apoptosis are integrated; cell cycling programs and apoptotic programs are activated concomitantly in the absence of specific regulatory signals. Thus, the cell is compelled toward apoptosis as a default condition, because without the presence of these specific regulatory factors the cycling cell is condemned. According to the dual signal hypothesis two factors are required for successful proliferation: one to activate mitogenesis and a complementary one to suppress apoptosis.[16,17]

Many of the key factors involved in apoptosis have been cloned and carefully characterized. Among the best understood are p53, a transcriptional regulator and tumor suppressor; c-myc, identified by virtue of its ability to co-transform; Bcl-2, the initial member of a growing family of regulatory genes shown to have antiapoptotic functions; and the interleukin-1β-converting enzyme (ICE) family of proteases that are increasingly implicated in apoptosis.
Cell cycle arrest, and under certain conditions apoptosis, are known to be triggered by \textit{p53}. It is likely that damage to genomic DNA, caused by physical or chemotherapeutic agents for example, triggers \textit{p53} activity.[33] Although these discrete \textit{p53}-dependent pathways have not been elucidated, evidence suggestive of its role in cell cycle arrest includes posttranslational accumulation and stabilization of \textit{p53} that is proportional to the amount of DNA damage.[33] The DNA binding and transcriptional regulatory properties of this molecule are likely early steps in the effector arm of this pathway. The \textit{p53} gene is thought to cause cell cycle arrest until genomic damage is repaired; if the damage is irreparable, apoptosis ensues. Fifty percent of cancer cell lines tested display mutations in the \textit{p53} gene, with alterations clustered around the DNA binding domain.[44] The strong selective advantage for cells with these mutations highlights the role of apoptotic suppression in the development of cancer.

The oncogene \textit{c-myc}, initially identified by virtue of its ability to cotransform, is dysregulated in most neoplasia.[46] Recent studies have shown that \textit{c-myc} can act as a potent inducer of apoptosis when it is expressed in conditions of cell cycle arrest, such as serum starvation or after withdrawal of specific growth factors. Fibroblasts that have been transformed to express \textit{c-myc} cycle continuously in the absence of mitogens.[18] However, the number of cells does not necessarily increase because a commensurately large number undergo apoptosis.[18,24] Similar mechanisms have been observed in hematopoietic cells.[26] The regions involved in apoptosis have been mapped to sites also responsible for autoregulation, DNA binding, and transcriptional activation.[18] Accumulating evidence suggests that the transcriptional activator mechanism of \textit{c-myc} participates in apoptotic responses, but few genes that \textit{c-myc} targets directly have been identified.[24,26] The conflicting signal theory holds that the function of \textit{c-myc} is a pathological one, whereas in the dual signal hypothesis \textit{c-myc} has an inherent physiological apoptotic function that is modulated by specific survival factors. Ectopic production of \textit{c-myc} in quiescent (G\textsubscript{0}) cells is sufficient to drive them into the cell cycle.[13,40,49,54] It is probable that \textit{c-myc} acts on intermediate pathways that control entry and exit from phases of the cell cycle.[16] Specifically, \textit{c-myc} has been found to cooperate with Ras in generating cyclin-dependent kinase activity, thus allowing the cell to enter the S phase of the cell cycle.[32]

The \textit{Bcl-2} gene was initially identified as a result of its location at the site of translocation between chromosomes 14 and 18, which are present in most follicular lymphomas. It was initially considered to be an oncogene; however, it was found to have no ability to promote cell cycling or division. Uniquely, it was discovered to prevent the initiation of apoptosis.[51] Mutations of \textit{Bcl-2} highlight the potentially important role for apoptotic suppression in tumorigenesis. Numerous in vitro and in vivo models have shown an antiapoptotic role for \textit{Bcl-2}. A striking enhancement of tumor formation in transgenic mice expressing \textit{c-myc} and \textit{Bcl-2} has been shown.[1] The \textit{Bcl-2} gene has been shown to inhibit \textit{c-myc}-induced apoptosis without affecting \textit{c-myc} mitogenic function, although the direct mechanism has yet to be elucidated.[2,20] It has been shown that \textit{Bcl-2} belongs to a group of genes that are homologous to Ced-9,[10,21] which is involved in the suppression of programmed cell death during the development of \textit{Caenorhabditis elegans}. This large family of genes can be divided into those that inhibit apoptosis and those that antagonize this protective effect. Evidence has mounted indicating that dimerization of these proteins is necessary for antiapoptotic function.[56]

A large number of epithelial and hematopoietic tumors express \textit{Bcl-2/Bcl-X}, and \textit{Bcl-2} expression has been correlated with a poor prognosis in some tumors.[12,38] In vitro studies have shown that \textit{Bcl-2/Bax} gene products are capable of inhibiting apoptosis induced by chemotherapy and ionizing radiation.[6,7,12] No clear molecular function has yet been ascribed to this family of genes, but it can be
inferred from recent data that cells expressing Bcl-2 are relatively resistant to proteolysis by members of the ICE family. It has also recently been discovered that Bcl-xL and Ced-9, its nematodal homologue, interact with and inhibit the function of Ced-4,[9] and Ced-4 can simultaneously interact with the caspases. Thus, the Bcl family of protective factors is linked with the proapoptotic proteases (ICE/caspases), suggesting a central role for Ced-4 in the cell death pathway.[9] A human homolog of Ced-4 has not yet been identified.

Much of the machinery for apoptosis is present on a constitutive basis. Cells treated with RNA or protein synthesis inhibitors can generate apoptotic responses.[4] Furthermore, pure nuclear extracts can produce DNA laddering,[29] and, with appropriate stimuli, cytosolic preparations can produce typically apoptotic appearing membranes.[48,52] Following protein synthesis blockade some cells undergo apoptosis and others do not. This difference may be explained by the relative amounts, activity, and stability of protective compared with proapoptotic protein that are present in the cytosol as well as their phosphorylation status.

Recent attention has focused on the possible role of proteases as important mediators of apoptotic responses.[39] Evidence to support this line of reasoning comes from a variety of physiological, biochemical, and genetic investigations. Proteolysis has been observed early in apoptosis,[25,28,29,42,48,52] and select protease inhibitors have been demonstrated to inhibit apoptosis.[3,43] Individual caspases have unique inhibitors: CPP32, a caspase known to cleave polyADP-ribose polymerase (PARP) is inhibited by DEVD-aldehyde. Interleukin-1ß-converting enzyme is inhibited by YVAD-aldehyde; its substrates include pro-interleukin-1ß and the ICE proenzyme.[39] Additionally, some viral proteins have been demonstrated to inhibit apoptosis by blocking protein cleavage.[43] The CrmA, or cytokine response modifier, gene, which is a component of the cowpox genome, has also been demonstrated to inhibit ICE, likely by modifying the response of host cells to viral infection.[43] Finally, recent knock-out experiments have demonstrated that proteases play an indispensable physiological role in apoptosis.[27]

Interleukin-1ß-converting enzyme is the initial member of a growing family of cysteine proteases[34,36] that share homology to Ced-3, a protease involved in the developmentally programmed apoptosis of C. elegans.[50] Numerous members of this family are emerging, each with differing specificities, inhibitor profiles, and expression.[39] Several similarities have been observed among the cysteine proteases: they are functionally similar to tetramers and share highly conserved sequences of their catalytic sites. These enzymes are also believed to autoactivate, thus activating other ICE homologues via a complex regulatory pathway.[39] Additional proteases involved in apoptosis include those released by cytotoxic T lymphocytes (Granzyme B) into target cells,[22] serine proteases,[28] calcium-dependent calpains,[47] and ubiquitin-mediated proteases.[11]

Many putative targets for these proteases have been suggested. It is certain that a wide range of cellular proteins are modified to produce the characteristic apoptotic morphological appearance. Although additional modifications may occur, most investigators suggest a critical role for proteases in the initiation and propagation of apoptosis. Most intracellular proteins, however, appear to be unaffected by proteases during apoptosis.[25,42] Thus far, unequivocally identified targets include PARP, lamins, and the 70-kD peptide of the U1 RNP particle.

Lamins are intranuclear intermediate filament proteins found between the nuclear membrane and chromatin.[45] The organizational changes of the nucleus during mitosis are distinct from those that
accompany apoptosis: mitotic disassembly of lamins is marked by their depolymerization, whereas in apoptosis lamins are cleaved by proteolysis.\[31,45\] Lamin cleavage appears to be a relatively late response, occurring approximately 20 minutes following appropriate stimuli; by comparison, the degradation of PARP is apparent after 3 minutes.\[30,31\] Putative targets include endonucleases, which may become active once they are cleaved. This hypothesis fits well with the observation that proteolysis precedes the production of oligonucleosomal fragments. An alternative theory proposes that proteases may digest DNA-associated proteins, thus making them more susceptible to degradation.\[15\] Histone proteolysis may also play a role in the fragmentation of genomic DNA, but it has been suggested that this occurrence follows PARP cleavage.\[30,42\]

As the mechanisms and machinery of apoptosis are delineated, the development of therapeutic strategies will follow. Most simplistically, cytoreduction may be achieved by exposing tumor cells to agents known to induce apoptosis, among which are traditional chemotherapeutic agents with dose-limiting toxicities. Agents that can produce specific and more direct stimulation of apoptosis are desirable candidates for future generations of chemotherapy. Alternatively, resistance to various therapies could be reduced by inhibiting antiapoptotic pathways, because these mechanisms have been shown to impact prognosis and response to treatment. Finally, vectors for gene therapy may come to include components or effectors of apoptotic pathways.

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