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Review

Markers of Apoptosis: Methods for Elucidating the Mechanism of Apoptotic Cell Death from the Nervous System

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ABSTRACT

Apoptosis is a highly conserved energy-requiring program for non-inflammatory cell death that is important in both normal physiology and disease. Numerous techniques have been used to study apoptosis. In the nervous system, apoptosis is necessary for normal development, but it also occurs in many acute and chronic pathologic conditions. This review places commonly used markers of apoptosis and their detection in the context of what is now known about the process of apoptosis. We review the potential role of apoptosis in nervous system and neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis). We then describe important morphological, immunocytochemical, and molecular genetic markers for apoptosis, including proteases, signal transduction molecules, and mitochondrial proteins. The possibility of manipulating apoptosis therapeutically in conditions of too many or too few cells is under active investigation.

INTRODUCTION

Advances in genetics, molecular biology, and immunology have allowed more detailed study of the cellular mechanisms of apoptotic death, and biochemical markers unique to apoptosis have been sought among the molecular changes that enact and accompany it. This research has demonstrated intermediate states that show characteristics of both apoptotic and non-apoptotic cell death (25,49) and mediators common to both apoptosis and necrosis (55). For example, cells protected from glutamate-induced necrotic death by glutamate inhibitors may, under further stress, die by apoptosis (49). Although apoptosis is thought to occur by the activation of cysteine proteases that cleave behind aspartate residues, called caspases, there is evidence for caspase-independent apoptosis in mouse embryogenesis (33). Finally, the presence of caspases locally in dendrites and their possible role in synaptic plasticity as well as cell death (41) suggest more complex roles for these proteases involving signal transduction. Thus, there is a continuum of pathways to cell death rather than an invariant "all or nothing".

Considering the clinical relevance of apoptosis, it is important to determine whether it causes or occurs in response to the disease in question. The advances and refinement of techniques used to detect apoptosis constitute important milestones in the design of therapeutic

tics. It is thus important to ask whether increasing or decreasing apoptosis leads to demonstrable benefit. There is some evidence that this may be so, at least in *Drosophila*: *ninaE-rh27* rhodopsin mutants expressing the anti-apoptotic caspase-inhibiting baculovirus gene p35 retained significantly better visual function as measured by a behavioral assay of oculomotor walking response than their light-reared *ninaE-rh27* counterparts without the apoptosis-inhibiting gene (13). Yet, inhibiting apoptosis may simply cause a cell to die by necrosis (28), since inflammation results. Another potentially important limitation on manipulating apoptosis is that exceeding the capacity of clearance mechanisms to remove apoptotic bodies may induce necrosis (26). After briefly describing apoptosis in the nervous system, we will review morphological, immunochemical, and genetic markers for detecting apoptosis.

INDUCERS OF APOPTOSIS

Programmed cell death is induced by many stimuli, including stress, hypoxia, radiation, trauma, and glutamate excitotoxicity. Apoptosis occurs as part of normal development and homeostasis but has also been demonstrated in pathologic conditions of the nervous system such as Alzheimer's disease, Parkinson's disease, stroke, brain trauma, and amyotrophic lateral sclerosis

(ALS). Apoptosis has been demonstrated in many organ systems, but there are significant differences in the kinds of signals that result in programmed cell death from tissue to tissue. In the developing and mature nervous system, the absence of a signal often initiates the apoptotic death cascade. Developing neurons in particular are poised to destroy themselves; the absence of growth factors at a tyrosine kinase receptor of the plasma membrane enables the dephosphorylated pro-apoptotic BAD protein to bind to Bcl-2, permitting the release of cytochrome c from mitochondria and the activation of caspase 3, a member of the family of cysteine proteases that affect the death program (16). In contrast, cells of the immune system also employ a ligand-mediated signaling system involving the Fas death receptor (also known as CD95 and a member of the tumor necrosis factor receptor family) to activate caspases to the same self-destructive end. Numerous inducers of apoptosis in the central nervous system (CNS) have been identified. The following methods for inducing apoptosis have potential therapeutic relevance.

Stress may take the form of changing extracellular ion concentrations and the absence of a normal extracellular serum milieu, with resultant production of reactive oxygen species. In such circumstances, cerebellar granule neurons undergo apoptotic death and may be rescued by brain-derived neurotrophic factor and other trophic factors (53). In cultured cortical neurons, hydroxyl radicals generate internucleosomal cleavage of DNA, but not other apoptotic morphologic features (31). Cyanide-induced hypoxia can cause apoptosis in differentiated PC12 cell cultures, as shown by oligonucleosomal fragmentation of DNA and electron microscopy; cell death was partially blocked by antioxidants ascorbate and catalase (43). Chronic oxidative stress also increases susceptibility to apoptotic cell death (3; see below on Cu/Zn superoxide dismutase). Heat (22) and pressure (1) have also been associated with apoptosis.

Hypoxia may be transient or prolonged, global or focal, and affects apoptotic cell death in both embryonic and mature neurons. It is also important in tumor surveillance. In the em-

bryo, relative hypoxia is required for both apoptosis and normal morphology (10). The newborn piglet brain shows interesting regional differences during transient global ischemia: cerebellar Purkinje cells do not die by apoptosis, while cerebellar granule cells often do. Less mature inner neurons of the hippocampal dentate region died by apoptosis, while more mature adjacent cells died by necrosis (71). In many tumors, regional hypoxia upregulates p53, which promotes apoptotic tumor cell death. In hepatocytes, hypoxia leads to apoptosis via the inhibition of gluconeogenesis. In stroke, both oxygen and glucose are decreased.

Glutamate excitotoxicity and excess calcium entry are important in necrotic cell death following ischemic cell injury (52). Ischemic models in mice with transient glucose and oxygen deprivation produce necrosis that can be partially prevented by glutamate antagonists. If this protective effect is overwhelmed by prolonged insult, cell death characteristic of apoptosis results and can be inhibited by the protein synthesis inhibitor cyclohexamide. There is evidence for apoptotic cell death in cells of the hippocampus following global ischemia and in the penumbra surrounding necrotic regions of focal ischemic injury (37). There is also evidence that inhibiting apoptosis in stroke can reduce infarct volume (21). For a review of apoptosis in stroke, see Reference 55.

Glutamate excitotoxicity is particularly relevant in the nervous system since many neurons express both NMDA and AMPA glutamate receptors, and their interplay may modulate apoptotic signals and cell death pathway decisions. Glutamate stimulation at these receptors leads to depolarization and the influx of calcium. Low levels of glutamate depolarization actually protect cultured cerebellar granule cells from apoptotic death through non-depolarizing stimuli (69), yet over-stimulation can result in both apoptotic and necrotic death. Glutamate concentrations change both acutely, as in stroke, and chronically, which may play a role in neurodegenerative disorders.

Experimentally altering extracellular and synaptic glutamate concentration induces DNA changes in vitro, and chronic change in cerebellar cell cul-

tures induces reversible DNA nicking and ultimately apoptosis (19). Though much evidence points toward apoptotic death following glutamate stimulation, there is some evidence that glutamate may cause toxic death by a non-apoptotic mechanism. In one report (38), the alkaloid protein kinase inhibitor staurosporine and glutamate toxicity patterns were compared. Apoptotic nuclear morphology and DNA fragmentation was seen with the staurosporine but not glutamate in cortical neuron cultures.

A recent report (27) in cortical and hippocampal cultures indicates that caspase-dependent degradation of the AMPA subtype of glutamate receptors occurs early during cell stress and may favor apoptotic rather than necrotic cell death. Combined with evidence in the adult rat striatum of combined necrotic morphology with the DNA-laddering characteristic of apoptosis (49), this further supports the notion of a continuum of cell death modes. Significantly, merely activating caspases through an excitotoxic stimulus does not necessarily commit neurons to apoptosis. There appears to be a time window in which the program may be reversed (18).

Radiation also induces apoptotic cell death in neurons. In cultured mouse hippocampal cells, ionizing radiation increased levels of tumor-suppressor and pro-apoptotic gene p53 product levels, which apparently triggered apoptosis. Caspase activity increased as well, but caspase inhibition did not prevent apoptotic cell death (34). Immature neurons are more likely to undergo radiation-induced apoptosis than mature neurons, but when treated with high extracellular potassium to induce depolarization, these immature neurons were resistant to ionizing radiation-induced apoptosis (61).

In cases of head trauma, increased levels of bax and, in 30% of cases, p53 were found in tissue samples, while bcl2 was not expressed (46). Cerebrospinal fluid (CSF) from severe head trauma patients was found to inhibit neutrophil apoptosis in cells collected from healthy patients undergoing orthopedic surgery. Antibody-mediated neutralization of the increased levels of granulocyte-colony-stimulating factor found in trauma patients reduced the anti-apoptotic effect. (23).

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Neurodegenerative Diseases and Apoptosis

In neurodegenerative disease such as Alzheimer's, genetic predisposition may favor apoptosis. Familial early-onset Alzheimer's disease is associated with alterations in the protein presenilin, which interacts with and may affect the processing of regulatory apoptotic proteins including Bcl-XL (68). P53 and C95 proteins are also elevated in Alzheimer's disease (15). To what degree these relationships, and apoptosis itself, is fundamental in Alzheimer's or other neurodegenerative diseases is unclear, since a more fundamental pathologic process may cause cell injury that leads to apoptosis. One possibility is that the DNA fragmentation seen in Alzheimer's disease results from prolonged metabolic disturbance and that the predominant mode of cell death in this disease is necrotic (58). Our recent results using a number of sensitive methods for detection of apoptosis show that cortical cells overexpressing amyloid precursor protein (APP) undergo apoptosis (6). In another familial neurodegenerative disease, ALS, mutations in the Cu/Zn superoxide dismutase are associated with increased apoptotic cell death. Cultured mouse neurons overexpressing transgenic Cu/Zn SOD showed increased susceptibility to kainic acid mediated excitotoxic apoptosis that may be due to chronic oxidative stress (3).

Apoptosis in Parkinson's disease has also been described (59), but its role and extent have been questioned (5). Dopamine and other catecholamines may contribute to programmed cell death directly and through a N-methyl D aspartate (NMDA) receptor-mediated mechanism (72). The latter mechanism may play a role in other neurodegenerative diseases as well (66). Methods used to detect apoptosis in Parkinson's disease brains after autopsy showed apoptotic DNA fragmentation and caspase 3 activity only in microglia (32), perhaps reflecting a slow rate of cell death with subsequent ingestion of apoptotic bodies. Alternatively, apoptosis may not play a prominent role in this disorder. Moreover, in a mouse model of Parkinson's disease, the use of caspase inhibitors caused a transition from apoptotic to necrotic death (30).

Table 1. Nuclear, Cytoplasmic and Membrane Changes After Exposure to Apoptosis-Inducing Agents

| Alteration in Apoptotic Parameters | Time After Treatment |
|--|----------------------|
| Increase in caspase 3 activity | 30 min |
| PARP Cleavage | 1 h |
| Chromatin Condensation | 3–5 h |
| DNA Fragmentation | 5 h |
| Decrease in Cellular Metabolism | 8–12 h |
| Membrane Permeability Compromised | 8–12 h |
| Lamin β Cleavage | 8–12 h |
| A summary time course of DNA damage and apoptosis after exposure to staurosporine (0.1 μ M) or etoposide (10 μ M). | |

MARKERS OF APOPTOSIS

Recent advances in the development of methods for the detection of apoptosis have facilitated our understanding of the signaling pathways involved in this process. These markers include an increase in caspase 3 activity, poly(ADP-ribose) polymerase (PARP) cleavage, chromatin condensation, DNA fragmentation, decreased cellular metabolism, compromised membrane permeability, and cleavage of nuclear envelope proteins (lamins). These are summarized for cultured neuroblastoma cells in Table 1 by time after exposure to the apoptosis-inducing agents staurosporine or etoposide (7). In addition, the release of cytochrome c from mitochondria and the change in mitochondrial membrane potential are thought to occur relatively early in apoptosis (see below). Morphological, immunochemical, and both pro- and anti-apoptotic genetic marker assays are outlined below. It is generally accepted that both visualization of morphological changes and at least one other technique to detect DNA changes should be used in combination when asserting the presence of apoptosis.

Morphological Markers of Apoptosis

Cell and nuclear shrinkage with chromatin condensation and margination followed by karyorrhexis were visualized in the early 1970s using electron microscopy (35). The cell then disintegrates into membrane-bound bodies ingested by nearby cells. These apoptotic

bodies contain nuclear fragments and intact organelles and may undergo secondary necrosis if not cleared promptly (reviewed in Reference 26). An important membrane change in apoptosis involves the flipping of anionic phospholipids and phosphatidyl serine groups to the outer surface of the membrane for recognition by neighboring cells. In vitro detection of externalized phosphatidyl serine can be achieved through interaction with the anticoagulant annexin V. Annexin V conjugated to FITC binds to the exposed phosphatidyl serine, thus allowing the detection of apoptosis by fluorescent microscopy or flow cytometry.

Chromatin condensation can be detected by dyes that bind to chromatin. One method uses bisbenzamide, which binds to normal and condensed chromatin; resulting patterns in the nuclei of intact cells can be visualized by fluorescence microscopy. Cortical cultures contain both neurons and glia. Neuronal nuclei can be readily visualized by labeling neurofilaments with a neurofilament antibody and neuronal nuclear chromatin with bisbenzidine (Figure 1A). Exposing cortical neurons to glutamate results in chromatin condensation and shrunken nuclei (Figure 1B). The number of apoptotic nuclei after exposure to an apoptotic stimulus can be quantified by counting total nuclei and expressing apoptotic nuclei as a percent of total nuclei counted (6).

DNA Breaks

Single-stranded breaks can be detected by using the Klenow fragment of

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

endogenous DNA polymerase to catalyze the addition of biotin-labeled (and unlabeled) deoxynucleotides to exposed 3OH ends of DNA fragments. Streptavidin-HRP conjugate is added and reacts with biotinylated nucleotides. The product can be visualized in intact cells under bright-field microscopy. The technique also labels dsDNA breaks, but under certain stimulus conditions, single-stranded breaks may be seen without double-stranded breaks in apoptotic cells (19). Klenow fragmentation detection kits are available from various companies including Oncogene Research and Trevigen.

Double-stranded breaks are commonly visualized with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling

(TUNEL) techniques. Here, peroxidase is used to detect digoxigenin-nucleotide residues added to the 3OH ends of DNA strand breaks by exogenous TdT. Reaction with diaminobenzadine (DAB) generates a brown product that can be visualized with light microscopy. Kits are available for this technique as described above. TUNEL labels necrotic and apoptotic DNA, and there is increasing caution in equating TUNEL-positive nuclei with apoptosis (39). As with chromatin labeling and Klenow fragment techniques, TUNEL can give quantitative measures of apoptosis based on cell counting.

Figure 2 shows a comparison of these three techniques. Cerebellar cultures were chronically exposed to L- α -amino-adipate (LAA), an inhibitor of

the glutamate uptake transporter. No changes in nuclear morphology were seen when the cells were stained with bisbenzamide (stains chromatin) or Apoptag (a kit for TUNEL labeling that labels mostly dsDNA breaks). Those cells processed for in situ nick translation using the Klenow fragment of DNA polymerase I (shows ssDNA breaks) show changes as detected by the brown reaction product (Figure 2) (19). Thus, the Klenow fragment method is more sensitive for detecting DNA breaks than the other two methods in this system.

DNA Laddering

Detecting characteristic DNA fragmentation is a common method that provides evidence for apoptosis. DNA damage is not limited to apoptotic cells, and an important function in healthy cells is repairing damage that occurs through a variety of processes. As related to apoptotic DNA damage per se, ssDNA breaks may precede double-stranded breaks. Ultimately the DNA is cleaved between histones giving DNA fragments of about 180 bp. DNA laddering, in which purified DNA extracts are run electrophoretically on an agarose gel, shows characteristic fragments generated by apoptotic-specific endonuclease. Figure 3 shows a typical DNA laddering on gel electrophoresis (8). It is important to note that not all cells that undergo apoptosis show DNA fragmentation that can be visualized on DNA laddering.

Common Immunochemical Markers of Apoptosis

ELISAs for histones can quantitatively detect DNA fragmentation in ruptured cells. In this technique, an anti-histone antibody labeled with biotin simultaneously binds to the histone associated with cleaved DNA and to streptavidin-coated plates. A second antibody, anti-DNA peroxidase-conjugated-mouse-monoclonal antibody reacts with the DNA component of nucleosomes. Nucleosomes associated with degraded DNA are then analyzed photometrically (Figure 4) (6–8).

PARP is an endogenous nuclear enzyme cleaved by caspases. It is activated by binding to DNA nicks and breaks. It

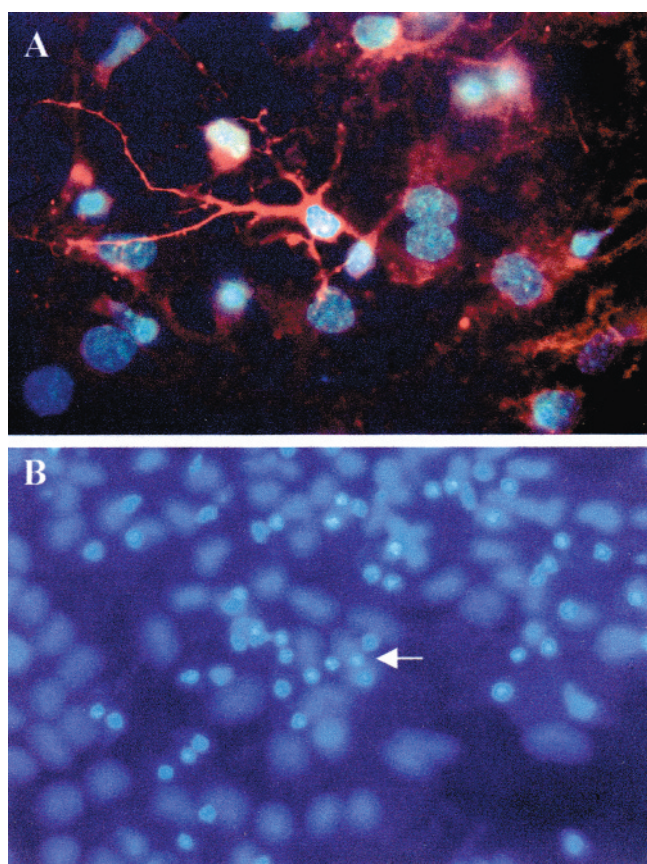


Figure 1. Cortical cells nuclei labeled with bisbenzimide. (A) Control cells were double-labeled with an antibody to a neurofilament protein and with a secondary antibody conjugated to Texas Red® (red). The nuclei of the same cells were stained with bisbenzimide (blue), and the cells were viewed under a fluorescent microscope. By interchanging the fluorescent filters, the nuclei of neurons can be readily identified. The normal nuclei are round and regular in shape. (B) Cortical cells treated with glutamate (100 μ M) for 12 h. Note the apoptotic nuclei (arrow) showing condensation of chromatin.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

functions in DNA repair and cell death by catalyzing poly(ADP-ribosyl)ation of nuclear proteins, including itself and histone H1. The PARP inhibitor 3-aminobenzamide prevents both this function and internucleosomal DNA cleavage in cells undergoing apoptosis induced by DNA-damaging agents (70). PARP cleavage by caspases in apoptosis probably reflects a decoupling of DNA damage from DNA repair. Whether this happens before DNA nicks and breaks in response to a feedforward signal or after DNA damage as a direct response to that damage is unknown.

In neuroblastoma apoptosis, PARP cleavage and caspase 3 activity were more sensitive for DNA damage and may occur earlier than nick translation (Table 1 and Figure 5A). PARP cleavage catalyzes ADP ribose polymer linkage to PARP itself and possibly other proteins. The process uses NAD⁺ as a substrate and releases nicotinamide, lowering NAD⁺ and ATP concentrations in the cell. PARP proteins can be measured by protein isolation followed by Western blotting and immunolabeling with PARP antibodies. Antibodies that recognize the intact and cleaved 85-kDa portion of PARP are available commercially (e.g., BioMol, Sigma Cell Biochem, and Santa Cruz Chemicon). The role of PARP in apoptosis is reviewed in Reference 29.

Later in apoptosis, the lamin proteins surrounding the nuclear membrane are cleaved. Proteolytic breakdown may be dependent on the apoptotic stimulus used, occurring relatively earlier in heat stress-induced apoptosis versus radiation (22). Caspase 3 has been shown to cleave and activate protein kinase C in leukemia cells, while caspase 6 cleaves lamin (11). This protein and its cleavage products can be analyzed with techniques analogous to those described for PARP. Figure 5B illustrates increase in a 43-kDa lamin cleavage product beginning about 8 h after apoptosis is induced in human neuroblastoma cells.

Genetic Markers of Apoptosis

The complete set of apoptosis effector and regulatory molecules and their roles in different cell lines at different developmental stages are unknown.

Other variables include the stimulus used to induce apoptosis, whether the experimental system is *in vivo* or *in vitro*, and what experimental organism is used in transgenic studies. Despite multiple sources of complexity, the beginnings of an integrated description of apoptosis exist. An enormous number of experimental techniques have been applied to studying apoptotic cellular processes; particularly important are transgenic organisms that lack, overexpress, or have targeted mutations that

impair protein product function. Caspase knockout mice are reviewed in Reference 42. Key components in emerging apoptotic mechanisms include signaling, mitochondria, the bcl-2 protein family, effector proteases, and physiologic control of apoptosis via tumor suppressor mechanisms. After a cell recognizes a death signal, apoptosis proceeds through the proteolytic activity of a family of cysteine proteases, the caspases, that cleave after aspartate. There appear to be two broad pathways to caspase ac-

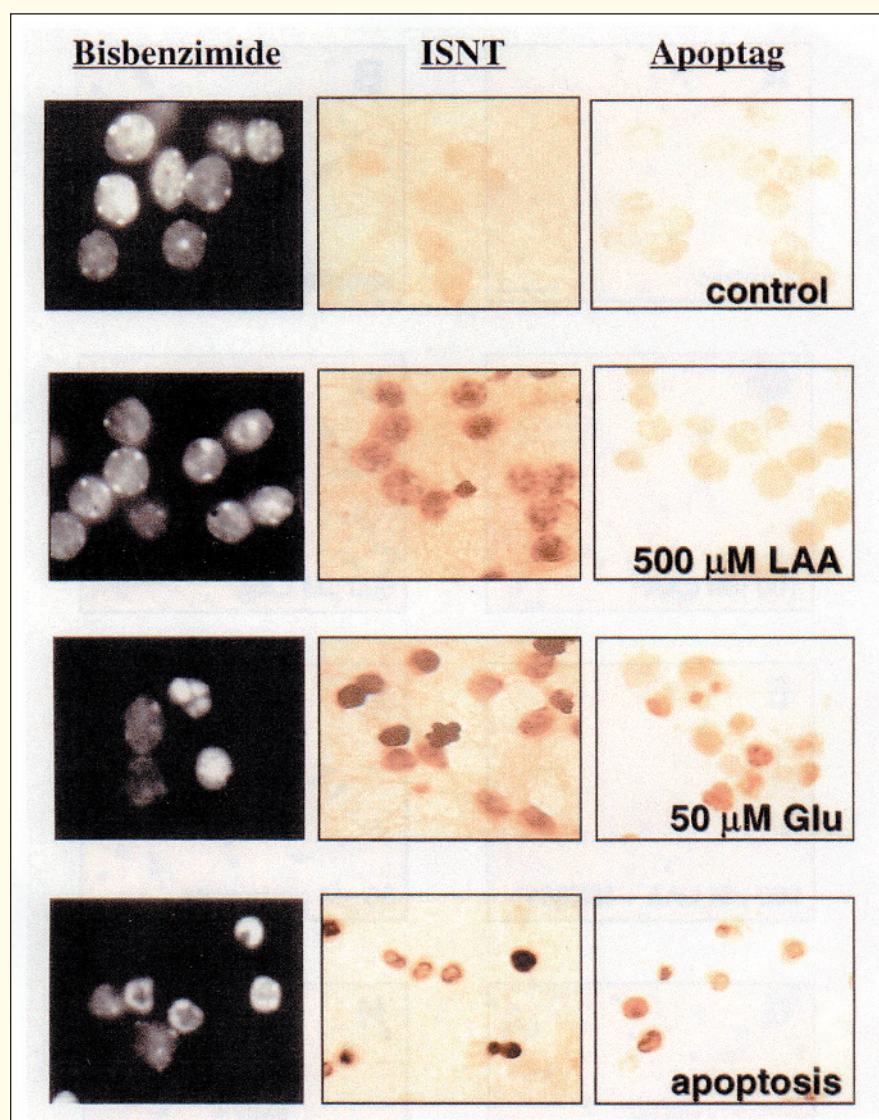


Figure 2. Comparison of nuclear morphology changes using three different methods before and after exposure to apoptosis-inducing agents such as LAA glutamate or low K⁺ (5 mM) concentration. Cells stained with bisbenzamide were observed under the fluorescent microscope, and those processed for ISNT or Apoptag were observed under a light microscope. Cells treated with LAA showed no changes in nuclear morphology after staining with bisbenzamide or Apoptag. However, DNA damage was observed in LAA-treated cells with the ISNT detection method. The results suggest that the ISNT technique is more sensitive in detection of DNA damage than the other two methods.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

tivation: signal transduction and mitochondrial release of cytochrome c, though they are not mutually exclusive.

Signal Transduction

Signal transduction involves receptors of the tumor necrosis factor (TNF) family, which includes the C95/Fas and TNFR1 receptors. Antibodies for the detection of these receptors are available. At the Fas receptor, ligands cause receptor clustering and the formation of an intracellular complex with a protein that includes a Fas-associated death domain (FADD). This domain is specialized to interact with and activate caspase 8. Domains on other proteins that interact with caspases are collectively known as caspase recruitment domains (CARDs). Caspase 8 activates caspases 3, 6, and 7 and with them attacks cell substrates, leading to the orderly changes of programmed cell death. Caspase 8 also cleaves the bcl-2 family protein bid (see below), which is involved in the release of cytochrome c from mitochondria.

The TNFR1 receptor is activated by TNF produced mostly by immune cells responding to infection. As with the CD95 ligand, TNF binding produces receptor clustering, and an adapter protein, TNFR associated death domain (TRADD), is present. In contrast to more direct CD95-associated activation of a caspase, TRADD stimulates the activation of nuclear transcription factors $\text{NF-}\kappa\text{B}$ and JNK, which is involved with the synthesis of inhibitor of apoptosis proteins (IAPs). Here, apoptosis is more likely when protein synthesis is inhibited, and inhibition of $\text{NF-}\kappa\text{B}$ makes programmed cell death more likely (2). Caspase 8 appears to be involved in this receptor-mediated pathway. Figure 6 diagrams the major signaling pathways.

Probes for Mitochondria

The release of cytochrome c and changes in the mitochondrial membrane potential are major markers of apoptosis. There is much debate over which of these events occur first or whether they occur simultaneously. Nevertheless, both the release of cytochrome c and the changes in the mi-

tochondrial membrane potential are early apoptotic events detectable by the techniques described below.

Mitochondrial Release of Cytochrome C

Cytochrome c is a heme-containing component of the electron transport chain in mitochondrial membranes. It is released from mitochondria in some forms of apoptosis, particularly those dependent on the interaction of pro- and anti-apoptotic members of the bcl2 family. The mechanism of cytochrome c release is unknown but probably involves interactions of pro-apoptotic bcl-2 family proteins (see below) with structures like the adenine nucleotide transporter (ANT) and the voltage-dependent ion channel (VDAC) in the mitochondrial membrane (24). Release is significant, for it allows activation of caspase 9 with subsequent activity of caspases 3, 6, and 7. Signal transduction through Fas or TNF receptors with activation of caspase 8 also stimulates cytochrome c release via the cleavage products of bcl-2 family member bid. Immunofluorescent microscopy can detect released cytochrome c within cells. ELISA and Western blot analysis can quantitate cytochrome c released into the media of cultured cells or isolated from homogenized cells.

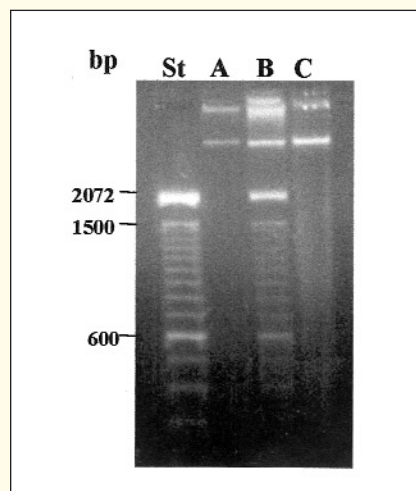


Figure 3. DNA ladder in glioblastoma cells treated with staurosporine. St denotes the migration distance and size (bp) of DNA standards. (A) Control cells. (B) Internucleosomal DNA ladder produced after staurosporine treatment. (C) Glioblastoma treated with etoposide do not show DNA fragmentation.

Mitochondrion-Selective Markers

Recently, voltage sensitive dyes have been used to study the mitochondrial membrane potential in whole cells (4). The uptake of most mitochondrial-selective dyes is dependent on the mitochondrial membrane potential. A number of cell-membrane-permeable mitochondrial-selective dyes are available (Molecular Probes, Eugene, OR, USA). Some of these MitoTracker™ probes such as MitoTracker Orange and MitoTracker Red accumulate in active mitochondria and withstand fixation. These dyes are suitable for double-label studies with antibodies. The dual-emission potential-sensitive probes such as JC-1 and JC-9 are non-fixable mitochondrion-selective probes useful for the detection of the disruption of the mitochondrial membrane. At low membrane potential, cells exposed to JC-1 fluoresce green, and at higher concentration (above 0.1 μM) they fluoresce red. The ratio of red to green JC-1 fluorescence is dependent only on the membrane potential and not other factors such as mitochondrial size, shape, and density that may influence other fluorescent signals. Thus, the dual-emission dyes can be used to investigate the mitochondrial potentials in live cells by ratiometric techniques (50).

Bcl-2 Gene Family: Pro- and Anti-Apoptotic

The bcl-2 gene family is important in both preventing and permitting apoptosis and is intimately involved in cytochrome c release from mitochondria. Overexpression of anti-apoptotic bcl-2 marker prevents apoptosis in the neurons of transgenic mice during experimental ischemia (40). Bcl-2 probably has multiple sites of influence in preventing apoptosis. The balance between pro- and anti-apoptotic bcl-2 family members helps determine whether programmed cell death occurs. The story becomes more complicated when different cell lines at different developmental stages with different death stimuli are considered. For example, there is recent evidence that in developing neurons, neither bax nor caspase 3 is required for apoptosis (20). Bcl-2 family proteins have been studied by immuno-

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

chemical and standard protein quantification techniques. Transgenic mice overexpressing bcl-2 family members or lacking one or more bcl-2 family genes have also been important in elucidating the signaling pathways.

Anti-apoptotic bcl-2 family members are associated with the outer mitochondrial membrane and are thought to contribute to mitochondrial integrity by affecting ATP trafficking, membrane potential, and the channel or pore through which cytochrome c is released (42). In HeLa and cerebellar neurons treated with staurosporine or serum deprivation, pro-apoptotic bcl-2, translocated from cytosol to mitochondria, induced a conformational change in bax, and allowed cytochrome c release into the cytosol (17). Bax was associated weakly with the outer mitochondrial membrane before interacting with bcl-2 but strongly associated after the interaction. Induced conformational change may permit bax dimers to form and insert into the mitochondrial membrane.

Anti-apoptotic properties of bcl-2 and bcl-xl derive in part from interfering with the bcl-2 interaction, though they apparently bind to a different site on bax than bcl-2 does. A transgenic mouse study found that bax-deficient sympathetic neurons were protected from nerve growth factor (NGF)-induced apoptosis but showed significant metabolic changes and atrophy (14). An emerging view is that the BH3-only members of the bcl-2 family connect Fas-mediated and other death signals to the mitochondria by altering the conformation of other mitochondrially associated bcl-2 family members such as bad (17) and bak (65).

Proteolytic Effectors: Caspases and Calpains

Effector proteases carry out the death program by enzymatic actions on substrates like PARP (decoupling DNA damage from repair), lamins (changing the structure of the nucleus), and the inhibitor of caspase-activated deoxynuclease (ICAD), resulting in DNA fragmentation. The first caspase was called interleukin 1 β -converting enzyme (ICE) since it activated IL-1 β . By analogy with a protease active in nematode apoptosis—ced 3 in *Caenorhabditis elegans*—the existence and pathways of

mammalian caspases began to be studied. One review of mammalian caspases (42) defines two phylogenetic groups: those analogous to ced3, including caspases 3, 6, 7, 8, 9, and 10, and those analogous to caspase 1, including caspases 4, 5, and 13. The ced3 group has proven roles in apoptosis.

The activation of caspase 9 by mitochondrial damage, release of cytochrome c, and interactions with Apaf1 was described above, along with receptor-mediated activation of caspases 8 and 10. In general, “upstream” caspases 8 and 9 activate the “downstream” caspases 3, 6, and 7 with multiple targets throughout the cell. Caspase substrates are reviewed in Reference 9. There are three main substrate target groups: large aromatic group amino acids, aliphatic or hydrophobic group amino acids, and a large group targeting aspartate (42). Important nuclear targets include ICAD and PARP, which are cleaved by caspases 3 and 7. Caspase 6 cleaves lamins. The cleavage products are readily recognized on immunoblots using enzyme-specific antibodies. Available kits measure caspase activity in cell extracts (e.g., Promega and Calbiochem-Novabiochem). Kits are also available for studies of caspase activation and subsequent downstream events in whole cells using flow cytometry.

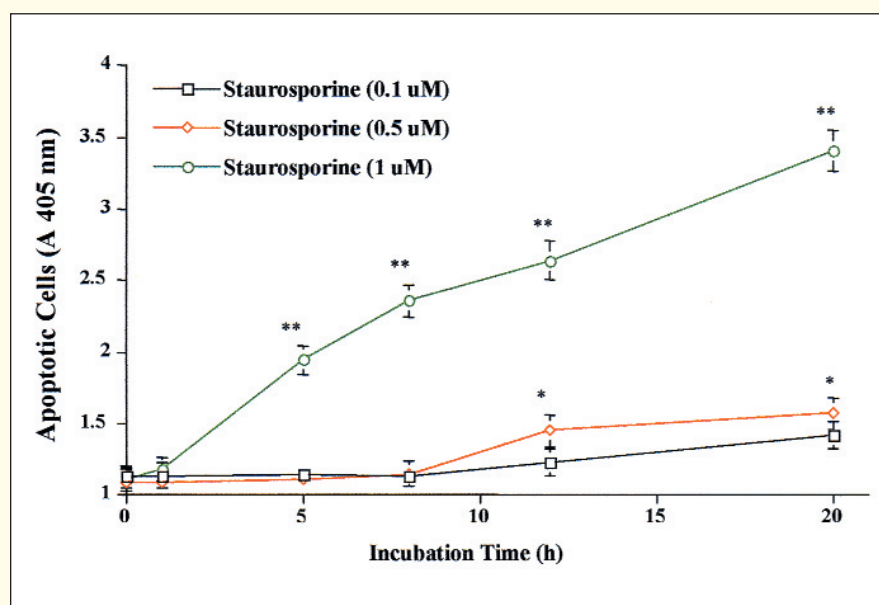


Figure 4. DNA fragmentation enzyme immunoassay. The cell death ELISA is a spectrophotometric assay that allows the detection of internucleosomal degradation of genomic DNA that occurs during apoptosis. Cells treated with staurosporine show a significant increase in DNA fragmentation 5 h after treatment.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Table 2. Comparison of Commonly Used Methods in Studies of Apoptosis

| Assays | Sensitivity ^a | Time Detection ^b | Method of Detection | Useful for |
|---|--------------------------|-----------------------------|--|--|
| Bisbenzamide | ++ | Intermediate | Fluorescence microscope | Detection of chromatin condensation in viable and non-viable cells (Hoechst 33342) |
| DNA Ladder | ++ | Intermediate | Gel electrophoresis | Detection of fragmentation of genomic DNA |
| Cell Death Detection ELISA Plus | ++ | Intermediate | ELISA | Quantification of histone complexed DNA fragments |
| PARP Cleavage | +++ | Early | Western blots | Detection of the cleaved form of PARP, a target for some caspases |
| TUNEL-TdT Labeling | ++ | Intermediate | Fluorescent or light microscopy, flow cytometry | Detection of dsDNA breaks, independent of a template |
| In situ nick translation using Klenow fragments | +++ | Intermediate | Fluorescent or light microscopy | Detects one strand of dsDNA nicks. Template dependent |
| Lamin B cleavage | ++ | Late | Western blots, light or fluorescent microscopy | Degradation of Lamin B. breakdown of nuclear envelope |
| Caspase Activity | +++ | Very early | ELISA | In vitro detection and quantification of caspase activity |
| Caspase Cleavage | +++ | Early | Western blots, immunocytochemistry | Detection of proteolytic cleavage products |
| Cytochrome c and AIF release | +++ | Early-Intermediate | Western blots | Detection of disruption of mitochondrial permeability. Cytochrome c release promotes caspase activation |
| Annexin V | ++ | Early-Intermediate | Flow cytometry, fluorescent microscopy | Detection of apoptotic cells with cytoplasmic membrane alterations (phosphatidylserine translocation) Differentiation of apoptotic from necrotic cells by simultaneous staining with propidium iodide |
| Apoptosis-related proteins: (a) FAS (CD95/Apo-1) | ++ | Intermediate | Immunocytochemistry, flow cytometry or Western blots | Detection of cell surface receptor genes that could mediate apoptotic cell death |
| (b) Bcl-2 protooncogene | ++ | Early | Immunocytochemistry or Western blots | Detection of the apoptosis suppressing Bcl-2 protein |
| (c) P53 tumor suppressor | ++ | Intermediate | Immunocytochemistry | Detection of p53 protein. Induces apoptosis in potentially malignant cells |

^aThe sensitivity of a technique is arbitrarily divided into three categories: +++, very sensitive; ++, moderately sensitive; and +, low sensitivity.

^bTime of detection is divided into three categories and is based on the initial time when apoptosis was induced.

etry or fluorescent microscopy. Targets and relative specificity of the caspases are still being worked out. Caspases can also be activated by a killer T-cell serine protease, granzyme B (47).

Caspase Inhibitors

TNF receptor activation can result in apoptosis, yet also involves the synthesis of IAPs via transcription factor activity. These proteins likely work in part by inhibiting caspases (60). Inhibitors of the IAPs themselves such as Grim and

Reaper have been described in *Drosophila*, and a recent report (62) indicates that the vertebrate protein diablo/smac can remove IAP inhibition and make transfected human cells sensitive to UV.

Other anti-apoptotic proteins are produced by viruses and include the false-substrate baculovirus protein p35. This protein has been shown to specifically inhibit caspases 3, 6, 7, 8, and 10 (73) and to inhibit apoptosis in transgenic mice expressing the protein (63). A cell-permeable false-substrate caspase inhibitor, benzyloxycarbonyl V-A-

D(OMe)-fluoromethylketone (zVAD-fmk), also has broad caspase-inhibiting properties and has been shown to ameliorate ischemic injury in brain tissue (12). Other inhibitors targeted to specific caspases before their activation may prove useful in delaying or preventing cell death.

In addition to the caspase family of apoptotic proteases, the protease calpain has also been implicated in apoptosis (57). Drug-induced apoptosis via caspase 9 implicates calpain in the cleavage of bax (67). In rat spinal cord injury, pro-

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

tection was afforded from apoptotic cell death by calpain inhibition (51). Calpain may be activated before caspases in apoptosis induced by radiation (64). In a mutant mouse model, calpain protein was increased in brain and spinal cord in the setting of apoptotic cell death, as measured by in situ hybridization and Western blot methods (36).

The transcription-regulating p53 gene product is up-regulated from negligible baseline nuclear amounts in the face of DNA damage. DNA damage can be readily detected with the techniques described above. The accumulation of wild-type p53 following DNA damage leads to either DNA repair following cell cycle arrest or apoptosis. When DNA damage includes a mutation in the p53 gene itself, DNA repair and apoptotic responses are defective, and cancer may result. p53 mutations are present in 40%–45% of human cancers (56).

The apoptotic function may result from p53-mediated up-regulation of bax and down-regulation of bcl-2 (45); a p53-negative response element has been identified in the bcl-2 gene (44). Colorectal cells lacking functional p53 undergo apoptosis after wild-type p53 is added with a viral vector (48), and a similar gene transfer resulted in apoptosis in post-mitotic neurons (54).

OVERVIEW OF COMMONLY USED TECHNIQUES IN STUDIES OF APOPTOSIS

Numerous methods have been utilized to detect apoptosis. Some of these techniques are summarized in Table 2. This list is by no means inclusive but gives an overview of common methods used to study apoptosis based on the desired experimental endpoint. It is important to emphasize the need for concordant (positive) results from several techniques before asserting the presence of apoptosis in a given system. A useful framework for organizing these methods comprises four categories: DNA changes, membrane changes, enzyme activity, and apoptosis-regulating genes. Within each category, there may be multiple assays and detection methods. Methods based on the characteristic DNA changes of apoptosis include DNA dyes, DNA ladders on gel electrophore-

sis and the in situ nick translation (ISNT) and TUNEL. TUNEL labels blunt ends of dsDNA breaks independent of a template. On the other hand, ISNT, which uses Klenow (DNA polymerase I), catalyzes the template-dependent addition of nucleotides when one strand of a dsDNA molecule is nicked. This technique detects DNA damage earlier than TUNEL (19) and is useful in detecting not only apoptosis but also the random fragmentation of DNA by multiple endonucleases. TUNEL and ISNT are both based on DNA strand breaks and label both apoptotic and necrotic cells.

DNA dyes such as propidium iodide (non-intercalating) and Hoescht 33342 (intercalating) reveal apoptotic nuclear morphology and can be visualized directly under light, fluorescent, or confocal laser microscopy. These can be combined with annexin V techniques (see below) to distinguish necrotic from apoptotic cells. Alternatively, DNA ladders indicate that nucleosomal cleavage has taken place but do not show what proportion of cells in the sample exhibits this pattern. Furthermore, not all cells undergoing apoptosis show a DNA ladder. Thus, the importance of using more than one independent assay

cannot be overemphasized.

Membrane changes in both the whole cell and mitochondrial membranes occur during apoptosis. Phosphatidylserine relocates from the inside to the outside of the cell membrane early in the process. Annexin V, a calcium-dependent binding protein specific for phosphatidylserine, binds to the surface of the apoptotic cells. This labeling is detected with flow cytometry or fluorescent microscopy. Double labeling of cells with annexin V and propidium iodide can readily distinguish apoptotic from necrotic cells. In the mitochondria, the change in the membrane potentials associated with the release of cytochrome c serves as an important marker of apoptosis. This change in potential can be assessed with voltage-sensitive dyes. The release of cytochrome c can be assessed with Western blots.

Enzymes, particularly the caspases, affect the apoptotic program as outlined above. Detection of the enzymes themselves, as a form of protein, or the results of their activity on substrates forms a third category of apoptosis detection. The products of PARP (early) and lamin B (late) cleavage can be assessed with Western blots or immunocytochemical

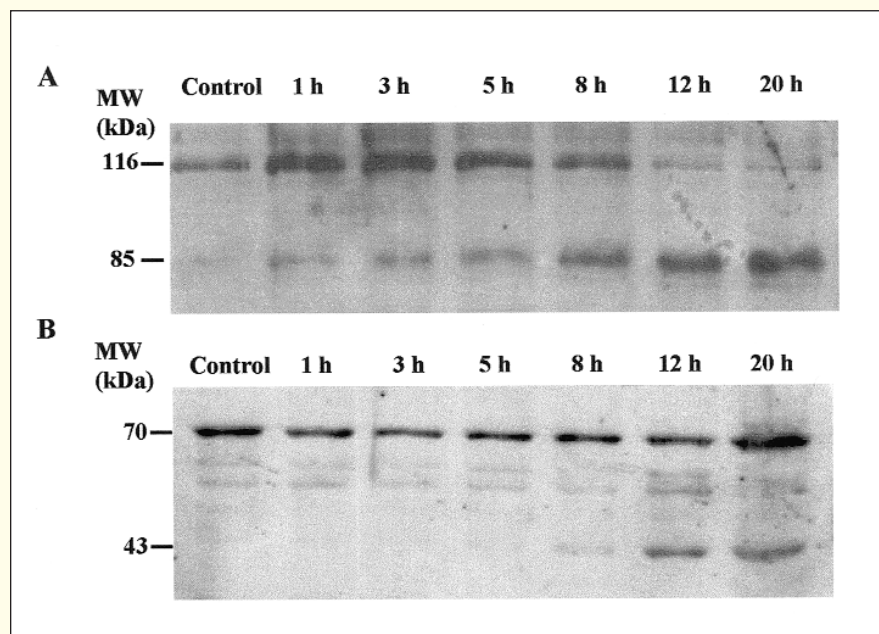


Figure 5. Western blots showing cleavage of PARP and lamin B after exposure of neuroblastoma cells to staurosporine. (A) Cleavage of PARP into an 85-kDa product is seen 1 h after treatment. (B) Cleavage of lamin into a 43-kDa band is noticeable 8 h after treatment and is most pronounced 12 h after exposure to staurosporine. This suggests that the dissolution of the nuclear membrane occurs after PARP becomes inactive.

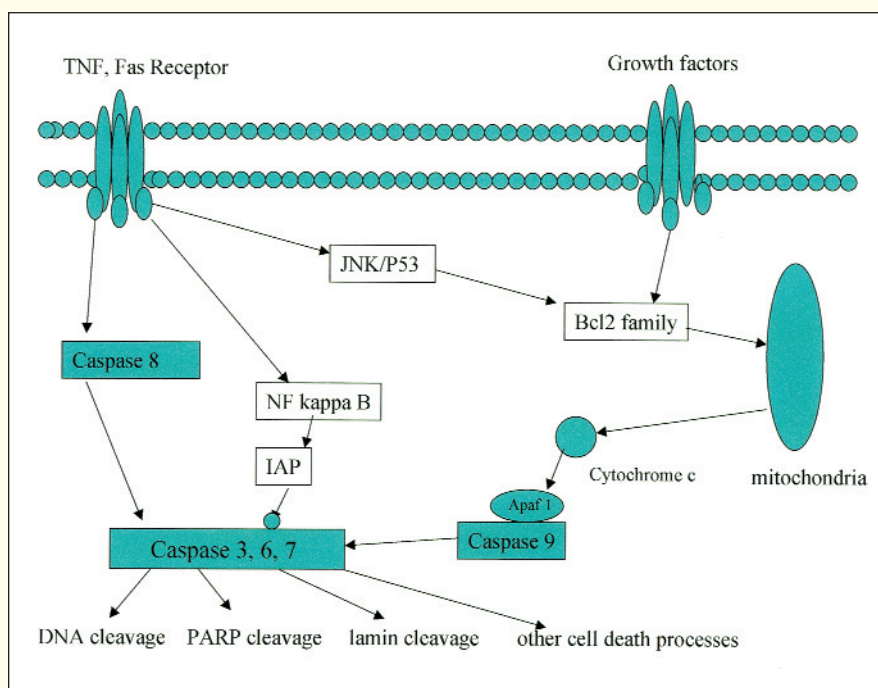


Figure 6. A signal that activates the TNF, Fas receptor, or the withdrawal of growth factors initiates the apoptotic cascade. Both of these events activate caspases. The receptor-mediated pathway activates caspase 8, followed by downstream caspases. This results in the cleavage of many substrates or the activation of transcription factors such as NF κ B and the release of pro-apoptotic factors such as apoptosis-inducing factor, cytochrome c, and apoptosis protease activating factor (Apaf-1) into the cytosol. There is also the IAP family of proteins that prevent cell death by binding to and inhibiting active caspases. Caspases can also be activated via the mitochondrial pathway. Here the Bcl-2 family associates with the mitochondria and releases cytochrome c and other proteins that activate caspase 9, followed by the downstream caspases. Both pathways lead to the activation of caspase 3, which is considered the "central executioner of apoptosis".

methods. Quantitative, in vitro caspase activity can also be assessed as a very early marker of apoptosis using ELISA.

The fourth category of apoptosis detection focuses on signaling proteins such as FAS/CD95, the bcl2 protooncogene, and the p53 tumor suppressor gene product. These can be detected using immunocytochemistry, Western blotting, or flow cytometry.

Thus, there are many methods for studying apoptosis at different stages in the process by visualizing or detecting the signals that initiate and regulate it, the enzymes that carry the program out, and the resulting products. Appropriately chosen methods should allow sound conclusions to be drawn regarding the presence of apoptosis in a given system.

CONCLUSION

Apoptosis is a highly conserved biological program that has attracted phe-

nomenal research interest since the mid-1980s. Its appeal rests on intrinsic interest and the dual potential for uniting theories of pathology and inspiring new therapies in virtually every organ system. First described using the electron microscopic techniques of the 1970s, morphologic criteria are still important for asserting apoptotic cell death. These and newer methodologies are of crucial importance in enhancing our understanding of apoptotic pathways. Emerging details about these pathways have deepened and extended our appreciation for the role of apoptosis in normal development, including massive CNS neuronal pruning in early life, homeostatic functions such as tumor surveillance, neurodegenerative diseases, and brain injury.

Recent research has revealed much about the mechanisms of apoptotic death and how it occurs. An overview is shown in Figure 6. Stimuli arising in the nucleus (DNA damage and p53 in-

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

duction), the mitochondria (with release of cytochrome c and activation of caspase 9 through the apoptosome), or the cell surface (via members of the TNF receptor family, including Fas and TNFR1) can ultimately activate caspases and give rise to apoptotic cell death.

Much remains unknown about the mechanisms of apoptosis within cells and the relationship between death pathways that overlap. Moreover, signals and effectors of cell death pathways may have multiple roles with intriguing connections to other cell functions such as cellular plasticity. There are also important questions about the benefits of modifying apoptotic programs in clinical situations. The functionality of cells rescued from apoptosis initiated by physiologic stimulus is a fundamental question. Effective delivery of apoptosis-inhibiting or promoting agents (e.g., through viral vectors or targeted parenteral infusion) will also be challenging, particularly in the blood-brain-barrier-protected CNS.

Given the existence and importance of apoptosis, one of the most fundamental questions for those researching its mechanisms is, "is it present?" From there, one asks whether it is primary or secondary to a separate underlying pathology and whether or not modifying apoptotic programs will be demonstrably beneficial. The common methods reviewed above for detecting apoptosis address the first question. The methods for inducing apoptosis can help to unravel the other questions. As more is learned, new techniques with increasing specificity can be anticipated.

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