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Review

Apoptosis: A New Pharmacodynamic Endpoint

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INTRODUCTION

Apoptosis or programmed cell death was first reported in 1972 by Kerr et al (1), and has been the focus of intense research interest since the recognition of its active involvement in development and disease etiology. The number of research articles that contain apoptosis and/or programmed cell death as key words have grown from 145 during 1980–90 to 8,493 during 1990–97. Most of the published works are by molecular biologists, biochemists, and molecular pharmacologists, and have focused on the mechanisms and regulation of apoptosis, the role of apoptosis in cancer etiology, and the induction of apoptosis by external stimuli including drugs. Relatively little is known about the kinetics and pharmacodynamics of drug-induced apoptosis. The purpose of this review is to provide pharmaceutical scientists an overview of the current knowledge of the apoptotic process, with an emphasis on drug-induced apoptosis. This review is organized in three sections, (a) mechanisms and regulation, (b) induction by drugs, and (c) apoptosis as a pharmacodynamic endpoint.

MECHANISMS AND REGULATION

Apoptosis, a controlled physiological process of fundamental importance to all multicellular organisms, occurs in a morphologically and biochemically distinct manner which ultimately leads to cell suicide. Apoptosis plays a central role in embryogenesis and normal adult tissue homeostasis by regulating the balance between cell death and cell proliferation. It is also important for eliminating cells with nonrepairable genotoxic injury. Deregulation of apoptosis is involved in the etiology of diseases including degenerative diseases of the central nervous system, autoimmune diseases, viral infection, and cancer (2–4).

The apoptotic process involves a sequence of events including cell shrinkage, increased cytoplasmic density, chromatic condensation and segregation into sharply circumscribed masses, and the formation of membrane-bound smooth surface apoptotic bodies (5,6). Apoptosis is often accompanied by the activation of endogenous Ca^{2+}- and Mg^{2+}-dependent endonucleases. These enzymes cleave DNA at internucleosomal sites, where DNA molecules are complexed with histone proteins, to produce fragments in multiples of approximately 185 bp (7). Apoptotic cells are phagocytosed from the midst of living tissue by neighboring cells or macrophages without eliciting an inflammatory reaction.

In spite of the rapid gain of knowledge in recent years, the biochemical mechanisms of apoptosis are far from being fully understood. Multiple genes and their protein products are involved in the induction and execution of apoptosis. The purpose of this section is to provide an overview of the most well recognized regulators of apoptosis and is not intended to give an exhaustive review of the literature at large. Readers are referred to a number of excellent reviews on the molecular and biochemical regulation of apoptosis (8–12).

Apoptosis is linked to cell cycle progression. In vivo, physiological apoptosis can be detected in self-renewing tissues, such as intestinal crypts, epithelium of the adrenal cortex, differentiating spermatogonia, and germinal centers. Apoptosis becomes particularly evident in tissues after periods of rapid proliferation, such as mammary tissue following weaning, in the endometrium at estrus, during ovarian follicular atresia, and in malignant tumors (13). It has been suggested that apoptotic cells utilize the same proto-oncogene products and regulators of the cell cycle in a unique manner to induce a tightly controlled cell death (14). Several typical events of early cell cycle traverse are associated with apoptosis, e.g. upregulation of proto-oncogenes such as c-myc, ras, c-fos, c-jun, cdc-2, and phosphorylation of the protein product of the tumor suppressor retinoblas-

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Genes are denoted in lower case: bcl-2, bax, bcl-x, bcl-x_L, bcl-x_S, bak, bad, p53, c-myc, gadd45, ras, c-fos, c-jun, cdc2, rb, cdk2, cdk4, ced-3, ced-4, mcl-1, p2. Protein products of genes are capitalized except p53 and p21: Bcl-2, Bax, Bcl-X, Bcl-X_L, Bcl-X_S, Bak, Bad, Bik, Ced-3, Ced-4, GADD45, Ras, Raf-1, BAG-1, BCR/ABL, Blf-1/A1, Mcl-1, Nr13, R-Ras, C-H-Ras.

ABBREVIATIONS: Fas, FAS/APO1/CD95; FasL, Las ligand; p21, $p21^{cip1/waf1}$; PCNA, proliferating cell nuclear antigen; rb and Rb, retinoblastoma gene and protein; ICE, interlukin-1β converting enzyme; YAMA, YAMA/CPP32/apopain.caspase 3; FLICE, FLICE/MACH/caspase 8; AIF, apoptosis inducing factor; DFF, DNA fragmentation factor; CAPs, cytotoxicity-dependent APO-1 associated proteins; TNF, tumor necrosis factor; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; AIF, apoptosis inducing factor; TUNEL, terminal deoxunucleotidyl transferase mediated dUTP nick end labeling; ISEL, in situ end labeling.

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toma gene (15). Some of the molecules that induce apoptosis are also involved in the regulation of proliferation and differentiation. For example, the nuclear transcription factor c-myc which is classically associated with the promotion of cell growth has also been demonstrated to be a central mediator of apoptosis (16). Ceramide, a hydrophilic component of sphingolipids (especially sphingomyelin) which induces differentiation, growth suppression and cell cycle progression, also induces apoptosis (17).

There are distinct cellular thresholds, or set points, for apoptosis induction and subsequent signaling. As a result, different cell types vary in their susceptibility to activate the apoptotic pathway. There are two general categories of apoptosis, i.e., primed apoptosis and unprimed apoptosis (18). Primed apoptosis is found in most cell types of normal or transformed hematopoietic lineages. In primed apoptosis, all of the effector molecules are expressed in the cell and the apoptosis program can be executed directly after it is initiated without the requirement of active gene transcription. In unprimed apoptosis, active gene transcription is required and the process occurs more slowly than primed apoptosis, cells successfully progress through one round of the cell cycle, but die in the subsequent cycle.

Apoptosis is controlled by multiple genes that have been evolutionarily conserved from the nematode *Caenorhabditis elegans* to mammals. These genes encode ligands and their receptors, and a number of signaling molecules which are linked to second messengers that bridge membrane events to transcription factors and gene expression. The resulting gene products act to either stimulate or block apoptosis. This paradigm holds true for both physiological and exogenous induction of apoptosis.

Stages of Apoptosis

Apoptotic signaling can be categorized in four stages (Figure 1). The earliest stage, induction, describes how a cell interprets environmental or intrinsic cues to provoke the apoptotic response. Different insults, including drugs and irradiation that cause damage to DNA, drugs that cause damage to microtubules, ligands, binding to cell surface receptors, cytotoxic T cells, and growth factor withdrawal, can induce apoptosis. During the second or detection stage, the apoptotic signal is detected and transduced to downstream effectors. Activation of the signaling molecules at this stage varies according to the type of stimuli. The third stage describes the effector arm of the apoptotic pathway, which involves a family of cysteine proteases (now called caspases) and endonucleases, as well as other positive and negative regulators of apoptosis. There is a preponderance of recent evidence indicating that the many signal transduction pathways converge at a common endpoint at the effector stage to elicit the apoptotic response (3,6,11, 15,16,19,20). The final and least understood stage of apoptosis, corpse disposal, is the stage where the apoptotic cells are phagocytized and digested by neighboring cells or macrophages.

As will be evident from the following discussion, there are multiple apoptotic pathways that are activated under different circumstances and in different cell types. A well studied apoptosis pathway is the Fas/FasL pathway. The molecular events and signaling pathway for the Fas/FasL-mediated apoptosis is discussed to illustrate the multi-stage apoptosis process. Other critical genes/proteins involved in different apoptosis pathways are also discussed.

FAS/FasL-Mediated Apoptosis

Figure 2 shows the Fas/FasL-mediated apoptosis pathway. Fas/APO-1/CD95 (Fas) is a 48 kDa surface receptor protein. Fas belongs to the nerve growth factor/tumor necrosis factor (TNF)-receptor superfamily (21). The Fas ligand (FasL) also shows significant homology to TNF, a multi-functional cytosine that causes necrosis of transplanted tumors in mice and induces apoptosis under selected conditions (21–23). Fas was first identified on the surface of lymphoid cells, and subsequently in a

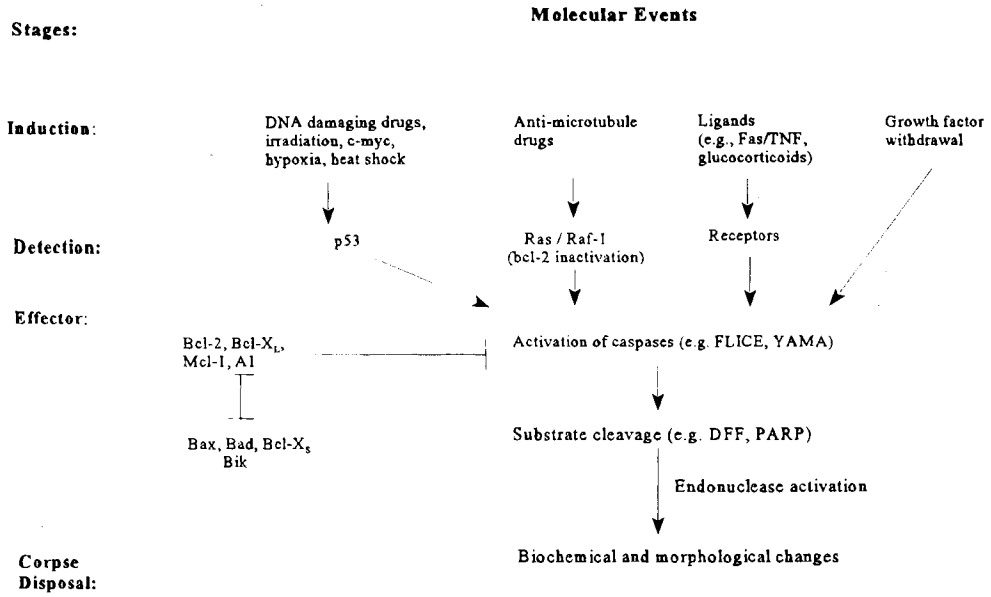

Fig. 1. A model of molecular events and pathways of apoptosis. Arrows do not necessary indicate direct interactions.

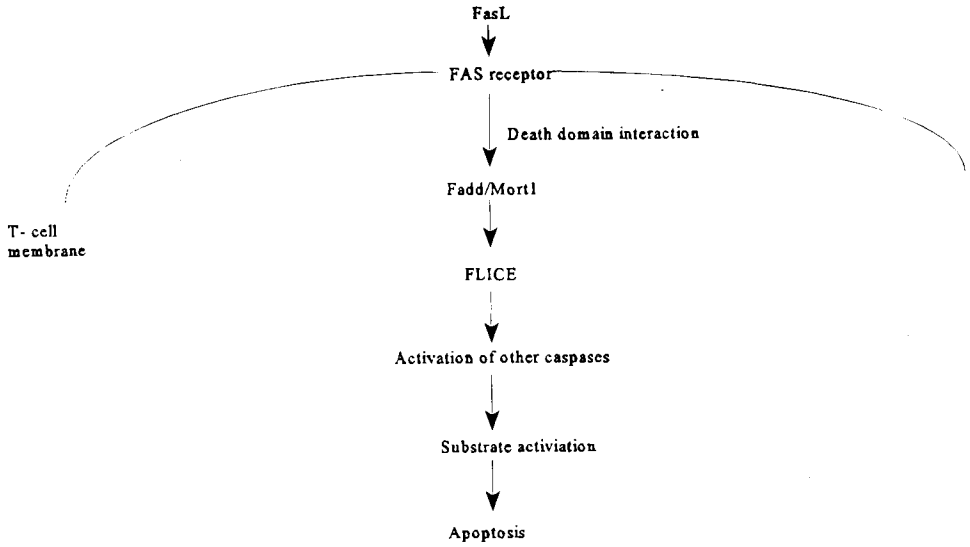

Fig. 2. Example of apoptosis pathway: Induction of apoptosis in T-lymphocytes by Fas/FasL.

variety of tissues including thymus, activated T and B lymphocytes, heart, lung, ovary, breast, and liver as well as in tumor cells from various origins (21,24). Activation of Fas by FasL or coupling of anti-Fas antibody to Fas induces a rapid apoptotic response in a variety of cells (21). Fas-induced apoptosis plays an important role in regulating the immune system and maintaining normal levels of T lymphocytes (24,26). For example, injection of anti-Fas antibody causes rapid regression of murine xenografts of Fas-expressing human lymphoid cell lines, accompanied by enhanced apoptosis of the grafted cells (2); Fas knockout mice display massive lymphadenopathy and splenomegaly, and deletion of peripheral T cells (27).

Fas and TNF-α receptor share a cytoplasmic motif termed the death domain. This death domain is also found in cellular proteins, named cytotoxicity-dependent APO-1 associated proteins (CAPs). Some examples of CAPs are FADD/MORT-1, RIP, TRADD, TRAIL/Apo-2L (related to FasL/TNF), RAIDD, CAR1, and CRADD (28–38). Some CAPs, termed adaptors, serve to relay the signaling from Fas/FasL to downstream caspases. For example, one signal transduction pathway for Fas or TNF is via association with CAPs such as FADD and RAIDD through the death domain and formation of a complex, e.g. Fas-associated Death-Inducing Signaling Complex (39–42), and recruitment of caspases such as FLICE/MACH/caspase-8. The subsequent heterotetramerization of FLICE results in self-activation of proteolytic activity and triggering of downstream caspases which acts on substrates, such as nuclear lamins, poly (ADP-ribose) polymerase, and the recently discovered DNA fragmentation factor, to induce morphological and biochemical changes in cells (43).

p53

p53, a tetrameric, sequence-specific DNA binding phosphoprotein, is the product of a tumor suppressor gene whose mutation represents a genetic lesion found in 50–55% of all cancers (44). The vast majority of p53 mutations in tumors are clustered within the central portion of the protein, which is critical for its sequence-specific DNA-binding function (45). p53 plays a crucial role in apoptosis. p53 functions at the detection stage of the apoptotic pathway. The consensus is that p53 assists in making the decision between cell growth arrest and apoptosis, depending on the insults and/or cell types.

For growth arrest, p53 is upregulated in response to selected forms of DNA damage (especially double stranded DNA breaks), and activates one of its downstream genes, $p21^{cip/waf1}$ (p21) (46). The p21 protein inhibits kinase activity in various cyclin/cyclin-dependent kinase complexes (e.g., cyclin D1/CDK4, cyclin E/CDK2, cyclin A/CDK2, cyclin A/Cdc2), which are key components in cell cycle progression (47–49). For example, a quaternary complex between p21 protein, CDK4, cyclin D1, and proliferating cell nuclear antigen (PCNA) functions to recognize the damage and arrest the cell at the G1 checkpoint until DNA repair is completed, or to direct the cell towards the apoptotic pathway if the damage is extensive and/or if repair is unsuccessful (50–53). This mechanism protects the genome from accumulating excess mutations (54,55).

p53 mediates apoptosis induced by chemotherapeutics, adenovirus E1A expression and oncogene c-myc expression, as well as a more general stress response to suboptimal grow conditions such as hypoxia, heat and starvation (56–58). The pathway by which p53-mediated apoptosis occurs depends on cell types and experimental conditions, and can be by direct and/or indirect induction (59). The direct induction of apoptosis by p53 employs direct protein signaling without activation of gene transcription. For example, p53-mediated apoptosis induced by DNA damaging agents is not affected by treatment with agents that inhibit either RNA synthesis or protein synthesis (60). Similarly, transfection with a p53 cDNA fragment, which is devoid of the regions necessary for transcriptional activation or DNA binding, induces apoptosis in ovarian carcinoma cells (61). However, transfection of the same p53 cDNA into lung carcinoma cells fails to induce apoptosis (62), indicating the cell type specificity of the direct p53-mediated apoptosis pathway. The targets of the direct protein signaling by p53 are not known. The indirect p53-mediated apoptosis pathway involves activation of gene transcription. p53 functions as a transcription factor which either activates of represses the transcription of apoptosis-related genes (45). For example, p53 is

a negative regulator of bcl-2, an apoptosis suppressor gene, and a positive regulator of bax, an apoptosis inducer gene (63–65). Loss of p53 leads to increases in Bcl-2 and decreases in Bax levels, resulting in resistance to apoptosis. p53 also regulates the expression of a gene encoding the insulin-like growth factor-binding protein-3 (IGF-BP3) (66). IGF-BP3 inhibits the IGF mitotic signaling pathway by blocking IGF binding to IGF receptor, and subsequently potentiates apoptosis or lower mitogenic potential of the cell.

Bcl-2 Family

Bcl-2 family proteins play a key role in the effector arm of the apoptotic pathway. These proteins probably act upstream to the family of cysteine proteases, now renamed caspases (42,67). Bcl-2 (named after the B cell lymphoma from which its gene was cloned), was the first protein discovered in a multigene family of apoptosis regulators. Inappropriate gain and over-expression of Bcl-2 is often observed in hematological as well as other non-hematopoietic malignancies including carcinomas of the lung, colon, and prostate (68–72). The bcl-2 gene and its family members belong to a category of highly conserved oncogenes which induce cell survival instead of cell proliferation. Protein products of these genes either positively or negatively control apoptosis. Bcl-2 inhibits apoptosis in response to a variety of stimuli, including chemotherapeutic agents, glucocorticoids, irradiation, viral infection, growth factor withdrawal, and Fas/FasL signal, indicating that it acts close to the final irreversible steps of apoptosis on which different apoptotic pathways converge (3,73–75).

To date, there are at least eight mammalian homologs of Bcl-2 which can generally be separated into two categories based on their modulation of apoptosis. The anti-apoptotic proteins which function like Bcl-2 are Bcl-X_L, Mcl-1, Blf-1/A1, Nr13, and the pro-apoptotic proteins which antagonize Bcl-2 function are Bax, Bcl-X_S, Bad, Bak, and Bik (76,77). There are occasional exceptions to this generalization. For example, pro-apoptotic proteins such as Bak and Bax have also been shown to inhibit apoptosis under specific situations (78,79).

The Bcl-2 related proteins have unique interactions to affect apoptosis. The different proteins in this gene family bind to each other or themselves, thereby forming heterodimers or homodimers. The BH1 and BH2 domains on the C-terminus of the Bcl-2 related proteins are important for dimerization, and are required for interactions between Bax and Bcl-2, Bcl-2 and Bcl-X_s, and Bad and Bcl-X_L (80–81). A third conserved domain BH3 identified in Bak and Bax is required for the interaction of Bax with Bcl-2, and of Bak with Bcl-X_L (82). These protein-protein interactions create a biological rheostat which modulates the activity of these proteins and accordingly decides the fate of cells. For example, bax-bax homodimers are permissive for apoptosis, whereas Bax-Bcl-2 heterodimers block apoptosis, suggesting that the Bax:Bcl-2 ratio determines the apoptotic potential of the cell (78,83,84). Similarly, an overtone of the Bcl-X_L:Bcl-X_s ratio supports cell survival.

There is intense research interest to delineate the mechanism of action of Bcl-2. Many mechanisms have been proposed. The subcellular locations of Bcl-2, primarily in the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum (85,86), have given rise to two theories. One theory relates to calcium homeostasis. Calcium homeostasis is linked to activation of the effector enzymes including endonucleases and caspases (87). Apoptosis is linked to increased intracellular calcium concentration, a slow loss of sequestered Ca^{2+} in endoplasmic reticulum and a rise of Ca^{2+} in mitochondria (87–89). Over-expression of Bcl-2 affects the sequestration of Ca^{2+} and subsequently inhibits Ca^{2+} release from the mitochondria of cells exposed to uncouplers of oxidative phosphorylation, thereby blocking entry of Ca^{2+} into nuclei (87–89). Another theory relates to reactive oxygen species. Because the sites of Bcl-2 location are the main source of production of reactive oxygen species and because effects of oxygen free radicals are similar to hallmark properties of apoptosis, it has been proposed that Bcl-2 blocks the oxidative cell deaths by decreasing the generation of reactive oxygen species or suppressing oxygen radical-induced lipid peroxidation, or that Bcl-2 functions as a pro-oxidant to influence the level of reactive oxygen intermediates that induce endogenous cellular antioxidants (90,91). This hypothesis is supported by the ability of Bcl-2 to block H_2O_2 induced cell death in a dose dependent manner (92,93). The two findings that oppose this hypothesis are the ability of Bcl-2 to inhibit cell death at nearly anaerobic conditions, and that mitochondria are not necessary for either induction of apoptosis or action of Bcl-2 (94,95). The latter findings suggest that oxidative damage may be a downstream event of apoptosis and not the only means to mediate apoptotic signaling.

Bcl-2 may act by other mechanisms. It may act via its control of the mitochrondria-to-cytosol translocation of cytochrome C, which directly activates caspases (96–98). The bcl-2-related genes may exert their functions by interfering with various signal transduction pathways. Protein kinase C (PKC) is identified as both a positive and negative regulator of apoptosis depending on the cell type-specific responses to triggering agents. Similar to Bcl-2, PKC inhibits calcium depletion from endoplasmic reticulum. Activation and phosphorylation of Bcl-2 by PKC has been described as a mechanism of inhibition of apoptosis by hematopoietic growth factors (99).

In addition to the Bcl-2 homologs, several other proteins may also bind to and interact with Bcl-2. These include R-Ras, a member of the Ras family of low-molecular weight GTPases, Raf-1, a serine/threonine-specific protein kinase, and BAG-1, Bcl-2-associated AthanoGene-1. These proteins bind to Bcl-2 and function in cooperation with Bcl-2 in protecting cells from apoptosis (100). The adenoviral protein E1B is functionally equivalent to Bcl-2 in the inhibition of the apoptosis pathway induced by another adenoviral protein E1A (57). E1B also interacts with and inactivates Bax (101).

Bcl-2 family proteins are regulated by phosphorylation. An example of phosphorylation resulting in Bcl-2 overtone is the phosphorylation of Bad mediated by the phosphorylation of another kinase, Raf-1. Bcl-2, by localizing Raf-1 to the mitochondrial membrane, causes Raf-1 phosphorylation (102). The phosphorylated Raf-1 kinase in turn phosphorylates the pro-apoptotic protein Bad, leading to the failure of Bad to heterodimerize with Bcl-2-like anti-apoptotic proteins, resulting in Bcl-2-driven survival (103). An example of phosphorylation resulting in apoptosis is the inactivation of Bcl-2 via phosphorylation, which has been shown to correlate with apoptosis induced by anti-microtubule agents (discussed later).

Caspases

The nematode *C. elegans* has been used extensively to study the genetic regulation of apoptosis. In *C. elegans,* the

ced-3 and ced-4 genes play a central role in the initiation of cell death (104). The ced-3 gene show significant homology to the mammalian protease interleukin-1β converting enzyme (ICE/caspase-1) (105,106). Over-expression of ICE or transfection of ced-3 in cultured mammalian cells induces apoptosis (107), whereas inhibition of ICE by CrmA (a cowpox virus) renders neuronal cells resistant to apoptosis induced by growth factor withdrawal (108). However, ICE knockout mice surprisingly show normal development with only minor defects in Fas-induced apoptosis, and ICE-deficient thymocytes taken from ICE knockout mice are resistant to Fas-induced apoptosis but remain susceptible to apoptosis induced by radiation, glucocorticoid or ageing (109–111). Collectively, these findings indicate that ICE plays a role in apoptosis but is probably not the most important death effector, and indicate the involvement of other caspases.

To date, at least 10 other caspases homologous to Ced-3 and ICE have been identified. These include Nedd2/Ich-1/caspase-2, YAMA/CPP32/apopain/caspase-3, TX/Ich-2/ICE_{rel}II/capase-4, TY/ICE_{rel}III/capase-5, Mch2/caspase-6, ICE-LAP-3/Mch-3/CMH1/caspase-7, FLICE/MACH/caspase-8, ICE-LAP-6/caspase-9, Mch-4/FLICE 2/caspase-10, and Ich3/caspase-11 (112–128). Of these caspases, YAMA is the most intensively studied; multiple observations have indicated that this caspase is both necessary and sufficient to induce apoptosis.

All of the known caspases fall within a unique group of cysteine proteases that are initially translated as inactive zymogens that must be cleaved at aspartate residues and assembled into heterotetramers in order to become active. One of the two major functions of caspases is self-activation and activation of other caspases by cleaving at aspartate residues. For example, ICE is activated by itself or Ich2 and activates pro-YAMA and pro-Nedd2 (108), and Nedd2 is processed by granzyme B, a protease involved in cytotoxic T cell-mediated death. Studies using cell-free systems have shown a hierarchical cascade of caspase activation with FLICE as the most proximally activated caspase, and that FLICE may be sufficient to trigger the proteolytic activation of other downstream caspases (129,130). The hierarchical cascade activation model is supported by the finding that specific inhibition of ICE proteases prevents activation of YAMA proteases, whereas the reverse does not occur (131).

The second function of the caspases is to cleave nuclear and cytoplasmic substrates during apoptosis. These substrates include topoisomerases, protein kinase Cβ1, histone H1, sterol-regulatory element binding proteins, nuclear lamins, adenomatous polyposis coli gene, poly (ADP-ribose) polymerase (PARP), and the recently discovered DNA fragmentation factor(DFF). DFF is a cytosolic, heterodimeric protein located downstream to YAMA, and has been shown to induce nuclear DNA fragmentation. Of these proteins, PARP is cleaved by YAMA, ICE, NEDD2, Mch2 and Ich2, DFF by YAMA, and nuclear lamins by an ICE-like protease LamP (116,132,133). The finding of normal development of PARP knockout mice suggests that PARP cleavage is probably not critical for apoptosis (134). Whether the cleavage of DFF by YAMA plays an indispensable role in apoptosis remains to be shown.

Interactions Between Bcl-2 Related Proteins and Caspases

As discussed above, proteins in the Bcl-2 and caspase families are involved in the effector stage in apoptosis. There is evidence to suggest that interaction among these proteins regulates apoptosis and that Bcl-2 family proteins regulate caspases. With respect to the question on molecular ordering, i.e. which proteins act upstream and downstream to others, a recent study in Jurkat neoplastic T cells shows that Bcl-2 and Bc-X_L function upstream to the mammalian caspases YAMA and ICE-LAP3 (42). Three intermediates between Bcl-2 family proteins and caspase have been suggested. These possible intermediates are cytochrome C (96–98), the *C. elegans* Ced-4 (135), and apoptosis inducing factor (AIF) (136). Translocation of cytochrome C from mitochondria to cytosol and the coincidental YAMA activation are inhibited by over-expression of Bcl-2, suggesting that Bcl-2 regulates YAMA activation by controlling cytochrome C translocation. Ced-4 is thought to be an intermediate because apoptosis induced by Ced-4 over-expression in mammalian cells is inhibited by over-expression of Bcl-X_L and by caspase inhibitors, indicating that caspase activation by Ced-4 is controlled Bcl-2 family proteins. Although no mammalian homolog of Ced-4 has yet been identified, there is evidence consistent with the presence of such an analog (135). Bcl-2, through the opening of permeability transition pores, regulates AIF, which is a 50 kDa mitochondria-derived factor that induces apoptosis in isolated nuclei (137). AIF is a caspase-activator, thereby implicating AIF as an intermediate between Bcl-2 and caspases (136). The three intermediaries may act independent from each other, act in parallel or sequentially to induce apoptosis (138).

In addition to caspase-dependent apoptosis, the recent finding of a caspase-independent apoptosis upon over-expression of Bax in Jurkat T cells indicates that there are additional mechanisms by which Bcl-2 family proteins regulate apoptosis that do not involve direct or indirect regulation of caspases (139).

DRUG-INDUCED APOPTOSIS

Apoptotic death of tumor cells can be induced in vitro and in vivo by radiation and different classes of drugs. Anticancer drugs that induce apoptosis include doxorubicin, vincristine, vinblastine, 5-fluorouracil, methotrexate, cytosine arabinoside and its derivatives, mitoxantrone, camptothecin, teniposide, etoposide, paclitaxel (taxol), cisplatin, amsacrine, cyclophosphamide, glucocorticoid, and retinoid acid (18). Generally, the mode of cell death, i.e., apoptosis or necrosis, evoked by a drug or irradiation, is dependent on drug concentration or radiation dose. Necrosis occurs usually in response to a very high drug concentration and high radiation dose, whereas apoptosis is induced as a result of lower but clinically relevant doses (18,140,141). It is believed that apoptosis is the predominant mode of death of cells treated with antitumor drugs (142). Depending on the drug, the apoptosis-inducing injury may be the result of stresses such as damages to DNA, RNA, or microtubules.

Different cell types vary greatly in their susceptibility to apoptosis. For example, neurons and epithelial cells are intrinsically more resistant to chemotherapy-induced apoptosis compared to germs cells and hematopoietic cells (143). It is believed that normal and transformed hematopoietic cells undergo primed apoptosis where activation of gene transcription is not needed (142). In general, induction of apoptosis in epithelial cells requires activation of gene transcription and is suppressed by inhibitors of mRNA or protein synthesis (144). Most solid

tumors are derived from epithelial cells and are inherently resistant to drug-induced apoptosis.

Cell Cycle and Drug-Induced Apoptosis

As discussed previously, apoptosis is closely related to cell proliferation status. Over-expression and activation of genes and proteins related to increased proliferation and cell cycle progression lowers the threshold for apoptosis (145). For example, hyper activation of cdc2 is observed in apoptosis induced by Taxol (146). The expression of c-myc, cdk2, PCNA and cyclin A increases when the AGF T cells undergo apoptosis (147). Staurosporine, a known activator of CDKs, and is an acute activator of apoptosis in human cells (13).

While apoptosis can occur during any phase of the cell cycle (148), sensitivity of proliferating cells to various death stimuli is usually cell cycle phase specific. For example, human promyelocytic HL60 leukemia cells are preferentially affected in G1 phase by 5-azacytidine, nitrogen mustard, and hyperthermia. Ionizing radiation and H7 (a serine/threonine kinase inhibitor) induce apoptosis in HL60 cells preferentially in G2/M phase, whereas cells progressing through S phase are susceptible to apoptosis induced by camptothecin, teniposide, m-AMSA, mitoxantrone, hydroxyurea, cytosine arabinoside, and H7 (149). S phase arrest also potentiates apoptosis induced by agents with a wide spectrum of pharmacological activities, including staurosporine, 6-dimethylaminopurine, okadaic acid, caffeine, and gamma-radiation (13). IMR90 fibroblasts and Hela cells in G0/G1 transition and during mitosis are much more sensitive to Taxol-induced apoptosis than cells in other positions of the cell cycle (150). Our laboratory has also shown that the maximum apoptotic effect of Taxol in human bladder, breast, head and neck, ovarian, and prostate tumors is positively correlated with the proliferative status of the tumor, i.e. a higher apoptosis for rapidly proliferating tumors, and that apoptosis occurs after completion of DNA synthesis (151–155). Arrest of cell cycle progression by cycloheximide or thymidine renders cells resistant to apoptosis induced by Taxol (146,156). Another example is the apoptosis of proliferating T lymphocytes, induced by antibody ligation of their antigen receptor, is inhibited when cells are blocked in G1 by mimosine, deferoxamine, or dibutyryl CAMP, but is enhanced when cells are arrested at the G1/S interphase by aphidicolin or in early S phase by thymidine (157).

In some cases apoptosis occurs with little or no cell cycle specificity (158). For example, no significant cell cycle specificity was observed in the case of the DNA topoisomerase II inhibitor fostriecin, the presumed tyrosine kinase inhibitor genistein, the protein synthesis inhibitor cycloheximide, or DNA cross linking agent cisplatin.

P53 and Drug-Induced Apoptosis

Many anticancer drugs, ionizing radiation and UV light directly induce DNA damage. Other agents indirectly induce DNA damage, by blocking DNA replication, interfering with DNA topology, or blocking the segregation of chromosome. Apoptosis induced by these various DNA damaging agents can be divided into two distinct types, depending on the presence or absence of the G1 checkpoint which is regulated by p53.

The first type of apoptosis is observed in cells that have functional p53 and a functioning G1 checkpoint. Exposure to DNA damaging agents increases the p53 level, which in turn activates the transcription of p21 and thereby arrests cells at the G1 checkpoint. The duration of cell cycle arrest at the G1 checkpoint is proportional to the extent of DNA damage and the rate of DNA repair. DNA repair may be activated through p53-stimulated expression of gadd45, or p53-stimulated p21/CDK4/cyclin D1/PCNA complex. The normal cell cycle resume when DNA repair has been completed. Alternatively, when DNA damage is extensive or if repair is unsuccessful, the high level of functional p53 will direct the cell towards the apoptotic pathway, presumably through the transcriptional inactivation of bcl-2 gene and activation of bax gene or direct protein signaling as discussed earlier.

The second type of apoptosis in response to various DNA-damaging agents occurs in cells with malfunctioning G1 checkpoint. This is generally the case when p53 is mutated. As a consequence of the loss of functional p53, DNA damage does not arrest cells at the G1 checkpoint. Instead, cells can enter S phase, although their progression through S phase may be suppressed as a function of drug concentration (159). Prolonged drug-induced suppression of cell cycle progression, termed defective progression, leads to growth imbalance, which in turn results in secondary changes including activation of new genes and subsequently apoptosis (142).

Bcl-2 and Tubulin-Binding Agents

Bcl-2 proteins are regulated by phosphorylation. Depending on the site of phosphorylation, Bcl-2 function is either enhanced or abrogated. Abrogation of Bcl-2 function by the microtrubule-active agent Taxol was first shown in leukemic cells and later in prostate cancer cell lines (160,161). Taxol and other drugs which bind to and damage microtubule integrity induce Bcl-2 phosphorylation. The process is not fully understood, but is mediated by a Ras/Raf-1 pathway (162,163). Upon microtubule disturbances, Ras activates Raf-1 kinase which subsequently phosphorylates and inactivates Bcl-2, thereby triggering apoptosis. Bcl-2 is considered as guardian of microtubule integrity (164); the Bcl-2 phosphorylation cascade guards against microtubule damage as p53 guards against DNA damage.

Chemoresistance to Apoptosis

Prior to the discovery of apoptosis as a common drug-induced response, events that are believed to be central to drug resistance were divided into three categories: (a) alteration of effective drug concentration (e.g., influx of efflux modification, increased detoxification, decreased activation, over-expression of scavengers such as glutathione), (b) modification of the molecular targets (e.g. increased concentration), (c) repair of drug-induced damage (e.g. over-expression of DNA repair enzymes). It is now believed that another major mode of drug resistance may be insensitivity to apoptosis induction, which often shows a multidrug resistance pattern.

The role of p53 in chemoresistance is controversial. The loss of functional p53 inhibits the induction of apoptosis by anticancer drugs and radiation, and correlates with poor response to chemotherapy (165,166). For example, cells lacking p53 expression or transfected with mutated p53 gene exhibit increased resistance to apoptosis induced by anticancer agents

and radiation, and thymocytes and small intestine of p53 knockout mice are less sensitive to radiation-induced apoptosis as compared to normal controls (54,166–172). However, two recent reports have shown that alterations in the p53 gene may actual increase sensitivity to DNA damaging agents in bladder carcinoma (173,174).

It should be noted that for drugs that do not induce DNA damage, the presence of functional p53 may not be necessary for apoptosis. For example, null mutation of p53 only reduces thymocyte susceptibility to apoptosis induced by DNA damage but not by other triggers such as glucocorticoids or activation via ligands to cell surface receptor CD3/TCR (54).

Over-expression of anti-apoptotic genes can render cancer cells resistant to drug effects. Bcl-2 and Bcl-X_L have the capacity to block apoptosis induction by a wide spectrum of chemotherapeutic drugs as well as gamma-radiation under in vivo and in vitro conditions (175–181). Transfection of the bcl-2 gene correlates with a 5 to 10,000 fold resistance to cytotoxicity of various drugs; the extent of resistance depends on the drug and cell line (100). In humans, bcl-2 over-expression has been correlated with poor response to chemotherapy in lymphomas, acute myelogenous leukemia, and some types of solid tumors (71,182,183). It is known that Bcl-2 does not prevent entry of drugs into cells, does not alter the extent of drug-induced DNA damage, does not alter the rate of DNA repair, and does not affect the nucleotide pool or rate of cell cycling. In fact, anticancer drugs can still induce cell cycle arrest when bcl-2 is over-expressed, but cells fail to die. It has been proposed that bcl-2 can convert the action of anticancer drugs from cytotoxic to cytostatic (100).

Other oncogenes and tumor suppressor genes which participate in the apoptotic mechanism may also impinge on cell kill by anticancer agents. Expression of C-H-Ras in rat rhabdomyosarcoma cells promotes cell survival after treatment with doxorubicin (184). BCR-ABL, an oncogene which functions as a transcription factor, protects cells from apoptosis induced by a variety of agents (185).

In summary, most drugs work at the first stage of apoptosis program by disrupting a central macromolecular network, leading to apoptosis via multiple apoptotic pathways. Further understanding of the signal transduction pathways involved in apoptosis may help with the development of new drugs targeting appropriate molecular lesions.

APOPTOSIS AS A PHARMACODYNAMIC ENDPOINT

The following discussion focuses on using apoptosis as a pharmacodynamic endpoint of anticancer drug action, but is also applicable to other studies that involve apoptosis measurement. In cancer pharmacodynamic studies, drug-induced cytotxicity is often measured as changes in cell number due to drug treatment. For reason of expediency, cell number is often measured by monitoring surrogate markers of cellular components. The two commonly used methods are the tetrazolium dye assay which measures the ATP-mediated reduction of the dye and the sulforhodamine B assay which measures the total protein content. Measurements of drug-induced changes in cell number do not distinguish cytostatic and cytotoxic (cell kill) effects. On the other hand, measurement of apoptosis specifically identifies cell kill.

Apoptosis can be measured by a number of methods that fall into three general categories, (a) measurement of products of the hallmark event of apoptosis, e.g. fragmented DNA or PARP cleavage, (b) detection of apoptotic cells by morphological changes, and (c) measurement of enzymes involved in DNA cleavage. For studies involving monolayer or suspension cell cultures, agarose gel electrophoresis can be used to provide a semi-quantitative measurement of the extent of apoptosis, whereas flow cytometry and ELISA measure the level of DNA-histone complex resulting from fragmented nucleosomes released from nucleus in a more quantitative manner. For studies involving solid tissues that cannot be readily dissociated into single cell suspension or where the maintenance of 3-dimensional structures is desired (e.g. to determine if apoptosis is dependent on the cell type or micro environment), other semi-quantitative methods using immunohistochemical techniques to detect DNA fragments or morphological evaluation to identify apoptotic cells can be used. Measurements of enzymes involved with the decision and execution phases of apoptosis, i.e. caspases and endonucleases, are also used. As discussed below, each of these methods have advantages and limitations.

DNA Electrophoresis

Internucleosomal DNA fragmentation is considered the most characteristic feature of apoptotic cell death. Endogenous endonucleases which are activated during apoptosis cleave DNA first into large fragments of 50–300 kb that are subsequently cleaved to smaller multiple fragments of ~185 bp. Detection of the 500-300 kb fragments requires the use of pulse field electrophoresis where alternating electric currents are applied to facilitate the resolution of relatively large DNA fragments. This method is not commonly used because of the requirement of specialized equipment. The more commonly used method is detection of the smaller fragments of multiples of ~185 bp using agarose gel electrophoresis, where apoptosis corresponds to a characteristic DNA laddering pattern. Figure 3 shows the DNA fragmentation pattern for paclitaxel-induced apoptosis in human tumors. The agarose gel electrophoresis method has several limitations. First, it measures only end-stage apoptosis cells and not early-stage apoptotic cells. Second, DNA laddering may not have the sensitivity to detect a low incidence of apoptosis as is frequently observed in solid tumors. Third, agarose gel electrophoresis uses DNA extracted from whole cell population, hence it cannot evaluate apoptosis in individual cells. A third DNA electophoretic method, i.e. the comet assay or single gel assay, provides a qualitative measures of DNA strand breaks in individual apoptotic cells. In the comet assay, cells are lysed after being embedded in agarose gel, and DNA fragments migrating in an electric field are visualized (186). It should be noted that although uncommon, apoptosis can occur in some cells without DNA laddering (187,188), in which case measurement of DNA fragmentation would not be useful for monitoring apoptosis.

Morphological Identification

Apoptosis was originally distinguished from necrosis on the basis of its ultrastructure (1). Electron microscopy still provides the most reliable method for distinguishing the two processes. In many cases, however, they can be identified using

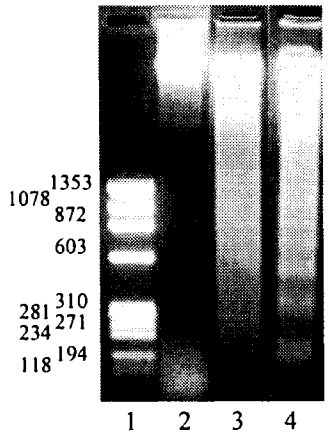

Fig. 3. Apoptosis and necrosis identified by agarose Gel electrophoresis. Lane 1: ϕX174/Hae III DNA size markers. Lane 2: untreated control. Lane 3: Doxorubicin-induced necrosis in human bladder cells, showing DNA smearing (i.e. no discrete bands). Lane 4: Taxol-induced apoptosis in a human head and neck tumor, showing DNA laddering (i.e. discrete bands).

light microscopy. Figure 4A shows the morphologies of apoptotic cells. Apoptosis typically involves scattered individual cells in a tissue without local inflammatory reaction. Apoptotic cells are morphologically characterized by condensation of chromatic which is marginated against the nuclear envelop, condensation of cytoplasm, membrane blebbing, and apoptotic bodies (189). The advantage of morphological identification is that it is technically simple and quantitative. The disadvantages are that the evaluation is subjected to operator bias, and that it may be difficult to detect the early stages of apoptosis with little or no morphological changes. Furthermore, apoptotic cells may not be readily distinguished from other elements with condensed chromatic such as lymphocytes and cells undergoing mitosis (189).

Immunohistochemical Methods

Three methods have been described in recent years for detecting apoptosis in histological sections by in situ labeling of DNA fragments. The first method is the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method. The exposed 3′-OH ends of fragmented DNA incorporates dUTP labeled by digoxigenin or biotin. The labeled cells are detected by immunohistochemical methods (e.g. antibody to digoxigenin and streptavidin coupled to biotin) and measured using light microscopy (190). The second method is in situ end labeling (ISEL), which uses DNA polymerase I (or the active Klenow fragment of this enzyme) to label the 3′ ends of DNA fragments (191). ISEL only labels the 3′-recessed ends whereas TUNEL does not have this requirement favoring both protruding and blunt ends. Hence, the TUNEL method shows a higher sensitivity than ISEL. Figures 4B and 4C show drug-induced apoptotic and necrotic cells in human xenograft tumors detected by the TUNEL method. The third method is post-exonuclease III-bromodeoxyuridine labeling, which is less frequently used. In this method, exonuclease III digests single-strand DNA at break points and exposes the pre-incorporated bromodeoxyuridine in the complementary strand. In the absence of DNA breaks, exonuclease III digest only the two ends of the intact DNA molecule and the bromodeoxyuridine-labeled DNA can be detected only after DNA denaturation. Hence, detection of bromodeoxyuridine after exonuclease III treatment indicates multiple breaks in DNA (192).

In situ immunohistochemical staining methods can detect individual cells with DNA fragmentation occurring in early and late stages of apoptosis, and are therefore more sensitive than morphological methods. However, these immunohistochemical methods also label random DNA fragmentation during necrosis, and are not specific for detecting apoptosis. To overcome this limitation, in situ immunohistochemical analysis should be used in conjunction with morphological evaluation to distinguish labeled necrotic cells from labeled apoptotic cells.

Flow Cytometry

This technique uses fluorescent DNA dyes to label DNA and is often used to analyze the proportion of cells in G0/G1, S, and G2/M phase. Flow cytometry is used to detect apoptotic cells based on the morphological and structural changes in cells, as follows: (a) Apoptotic bodies containing DNA fragments appear as sub-G1 DNA or hypodiploid DNA content in a flow cytometry histogram. (b) Changes in cell size and granularity (or density) of apoptotic cells because of cell shrinkage or compaction give lower forward scatter values and higher side scatter values than nonapoptotic cells which have a greater consistency of nucleus-to-cytoplasm ratio. (c) Fragmented DNA in apoptotic cells can be end-labeled by TUNEL or ISEL and then detected quantitatively by flow cytometry. (d) Plasma membrane integrity, which is lost in necrotic but not in apoptotic cells, can be probed by exclusion of DNA dyes such as propidium iodide. Combination of DNA dyes, e.g. propidium iodide followed by Hoechst 33342, has been shown a good method to distinguish live, necrotic, early- and late-stage apoptotic cells (193). (e) Apoptotic cells lose membrane phospholipid asymmetry and expose phosphatidylserine on the outer leaflet of plasma membrane. The exposed phosphatidylserine is labeled with Annexin V. The labeled apoptotic cells are detected by flow cytometry as well as by histochemical methods. Figure 4D shows the early and late stages of apoptosis as detected by the ApoAlert Annexin V assay.

Flow cytometric analysis of apoptotic cells is relatively rapid and quantitative. However, because apoptotic changes may vary for different cell types, flow cytometric results should be verified with other methods to confirm that the selected methods indeed label apoptotic cells (142). A second limitation is that flow cytometry can only be used with dissociated cells and cannot be applied to systems where the 3-dimensional structure is maintained. For example, flow cytometry cannot be used to detect apoptotic cells in different regions of a solid tumor or tissue specimen (142).

Quantification of DNA Fragments

Cleavage of DNA to mono- and oligo-nucleosomes, which are tightly complexed with the core histones H2A, H2B, H3

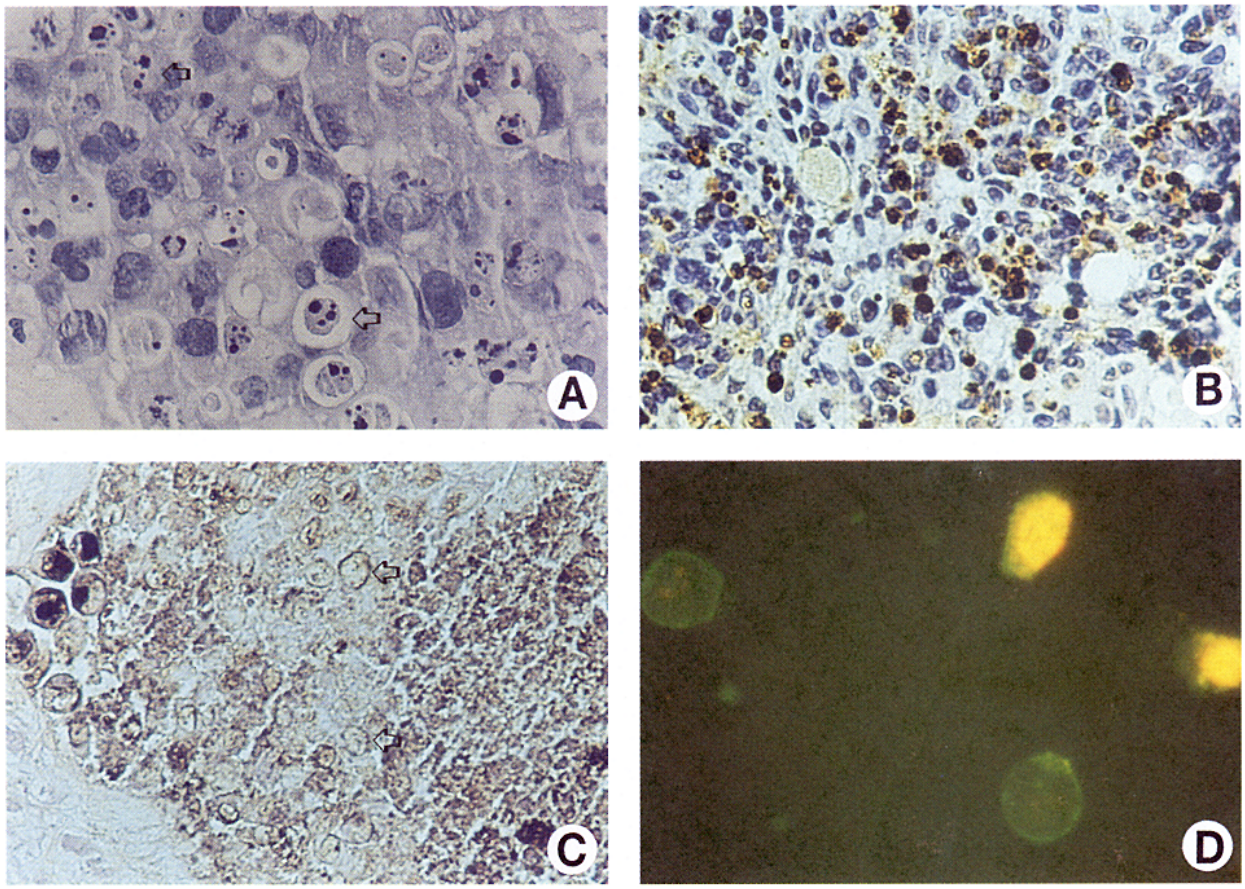

Fig. 4. (A) Apoptotic cells induced by 1 μM Taxol in a human bladder tumor, showing condensation of nuclear chromatic with loss of nuclear membrane, disappearance of nucleoli, formation of apoptotic bodies and cell shrinkage. (B) Apoptotic cells induced by 10 μM geldanamycin in a human prostate CWR22 xenograft tumor, detected by TUNEL (stained brown). Hematoxylin counterstained (blue). (C) Necrotic cells in a human prostate CWR91 xenograft tumor, after treatment with 5 mM suramin for 96 hr, showing TUNEL-stained ghost cells. (D) Stages of apoptosis in Taxol-treated human breast MCF7 cancer cells (100 nM for 12 hr). Early apoptotic cells were stained with Annexin V-FITC (green color on cell surface). As the plasma membrane became increasingly permeable during the later stages of apoptosis, propidium iodide penetrated cell membrane, resulting in the cytoplasmic staining of the later stage apoptotic cells by propidium iodide (yellow-red color).

and H4, occurs several hours prior to the disintegration of the plasma membrane. The nucleosomal DNA-histone complexes released into cytoplasm of apoptotic cells are quantified by an ELISA-based assay. In another method, i.e. filter-binding assay (194), cells pre-labeled with a radiolabeled DNA precursor are loaded and lysed on a protein-adsorbing filter. Small DNA fragments not bound to proteins such as histones are eluted and quantified by liquid scintillation.

Both the ELISA-based and filter-binding assays provide a quantitative measurement of fragmented DNA, and are rapid. However, these two methods may underestimate apoptosis, because these assays measure the end-stage apoptotic cells. The ELISA assay only measures apoptotic cells after mono- and oligonucleosomes are released into the cytoplasm, and the filter-binding assay measures only the DNA fragments not linked to proteins.

Analysis of Endonucleases and Caspases

Because endonucleases are responsible for the DNA fragmentation during apoptosis, detection of enzyme activity indicates apoptosis. In this method, DNA-free nuclear extracts of cells are incubated with other DNA sources, such as supercoiled or linearized plasmid DNA, radiolabeled or nonradiolabeled DNA immobilized on SDS-polyacrylamide gel matrix, HeLa cell nuclei or chicken red blood cell nuclei. Generation of DNA fragments in nucleosomal segments indicates enzyme activity. A difficulty with this method is the uncertainty whether the selected assay conditions are optimal for the endonuclease(s) responsible for executing the DNA fragmentation. It is not yet known if a single or multiple endonucleases are responsible for the different apoptotic pathways. Different endonucleases have different pH and metal ion requirements, e.g. DNase I and Nuc 18 are Ca^{2+}- and Mg^{2+}-dependent and active in neutral pH conditions whereas DNase II is independent of Ca^{2+} and Mg^{2+} and requires and acidic pH for activation. More importantly, the detection of endonuclease does not establish its involvement in apoptosis, because endonucleases can be artificially activated in vitro (195). Accordingly, endonuclease activity is only an indirect measurement of apoptosis.

The activation of caspases is indirectly measured by their cleavage of particular substrates including PARP, lamias and β-actin. For example, activation of YAMA, Mch2, and ICE-

LAP3 leads to PARP cleavage. The cleavage of PARP was initially discovered in etopside-induced apoptosis and is an early event in apoptosis (196,197). Proteolytic cleave of PARP, a 166 kDa DNA polymerase, into two fragments of approximately 25 kDa and 85 kDa, can be detected and resolved with polyacrylamide gel and immunoblot assay using PARP antibodies (114,116,123,198,199).

PERSPECTIVES

In summary, apoptosis is an important process in cancer chemotherapy but is also important in other diseases. This process involves multiple genes and protein products that are tightly regulated by molecules which are linked kinetically. For example, the formulation of homodimers and heterodimers of the Bcl-2 family proteins, which are negative and positive regulators of apoptosis, is controlled by the concentrations and kinetic interaction among these proteins. The majority of studies and literature reports on apoptosis primarily focus on the qualitative aspects of molecular events in the apoptotic pathway. The kinetics of interaction among the various proteins have received relatively little attention. Quantitation of the kinetics of the intersecting regulatory mechanisms and proteins is likely to improve our understanding of the relative importance of the different steps in the apoptotic pathway, and represents a research area that warrants attention from scientists with a quantitative orientation.

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