THE EFFECT OF DRUGS AND TOXINS ON THE PROCESS OF APOPTOSIS

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SUMMARY

In this review we examine the modifying effect of specific drugs on apoptosis. Apoptosis is a type of cell death prevalent during many physiological and pathological conditions, consisting of several steps, namely, initiating stimuli, transduction pathways, effector mechanisms, nuclear fragmentation, and phagocytosis. Pharmacological substances such as glucocorticoids can either induce or inhibit the process of apoptosis in various cells depending on the type of drug and its concentration. Understanding the mechanisms of interaction of drugs with cells undergoing apoptosis could encourage novel therapeutic approaches to human diseases in which apoptosis has a critical role.

KEY WORDS

apoptosis, cell death, chemotherapeutic drugs

1. CELL DEATH

The basis of all diseases is injury to the cell. If the injury is too great or extensive, this results in irreparable changes in structure and function, leading to the death of the cell. Cell death has fundamental importance in most pathological processes and it also plays an essential role in the regulation of normal tissue turnover by eliminating all debris formed from aged and dying cells. Ultrastructural abnormalities shown in cells dying in a variety of circumstances indicate two common patterns of morphological changes /1-3/. In the first, cell death is initiated through reactions to defined stimuli, followed by a sequence of intracellular changes. These morphological changes include marked swelling of mitochondria and the appearance of dense structures in their matrix followed by progressive dissolution of the entire cell. This type of cell death is named necrosis /4-7/. Necrosis refers to the progressive and complete degradation of cell structure that occurs after death. It represents irreversible damage to cellular membranes associated with various injurious stimuli, such as hypoxia, bacterial or viral infection, or corrosive chemicals, resulting in lysis /8/.

The second form of cell death, named apoptosis, is characterized by cell shrinkage, rapid condensation of the cytoplasm and nuclear chromatin, accompanied by blebbing of the plasma membrane. This subsequently leads to the fragmentation of the cells into a cluster of membrane-bound structures, apoptotic granular bodies, in which the integrity of various subcellular organelles is initially maintained. The apoptotic bodies are incorporated by phagocytes or neighboring cells, DNA breaks up at the internucleosomal spaces to oligome fragments. This type of cell death is present in physiological conditions.

Naturally occurring cell death, unrelated to any causative agent, is also found in almost all tissues. Various terms have been used to describe this natural death, such as physiological cell death or programmed cell death, to distinguish it from pathological death brought about by disease. In physiological circumstances and during development, different sequences of events occur involving prominent nuclear changes in response to hormonal stimuli and changes in other subcellular targets due to T cell or natural killer (NK) cell killing activities /9-11/.

These two distinct forms of cell death show major differences. Necrosis is a degenerative process that is associated with irreversible injury /12,13/. Apoptosis is connected with cellular self-destruction rather than degeneration /7,10,11/, and requires protein synthesis and fusion of subcellular components for its execution /14-16/. The phenomenon of apoptosis is also implicated in the physiological process of regulating organ size. Morphologically, apoptosis involves fragmentation of the nucleus, fusion of the nuclear chromatin and cytoplasm, resulting in membrane-encapsulated bodies. The presence of these bodies interferes with normal cell function and these granules are disposed of by neighboring cells without inflammation.

2. FEATURES OF APOPTOSIS

2.1 Occurrence

Apoptosis is involved in the programmed elimination of cells in physiological conditions. This is an irreversible mechanism for the elimination of excess or damaged cells. Apoptosis also occurs during embryonic and fetal development. In adult life apoptosis regulates the size of organs and tissues. In pathological conditions apoptosis is

responsible for the reduction of cells in different types of atrophy and in the regression of hyperplasia. It develops spontaneously in cancer cells and it is increased in both neoplasm and during normal cell proliferation triggered by a variety of agents applied in cancer chemotherapy. Apoptosis is enhanced by cell-mediated immune reactions and various toxins that also produce necrosis.

2.2 Morphology

Apoptosis manifests in single cells scattered in the affected organ in an asynchronous (apparently random) fashion and it is not associated with inflammation /2,3,10,11/. Electron microscopic studies show that at the earliest stage, nuclear chromatin is aggregated into dense masses attached to the nuclear membrane and cytoplasm becomes concentrated. These changes are followed by further condensation of the cytoplasm, and the nucleus breaks up into small fragments. The chromatin is segregated and some protuberations develop on the cell surface (blebbing). The pedunculated protuberances are separate and become bounded by plasmalemmal sealing membrane, producing apoptotic bodies. These dense masses have a different texture from chromatin and sometimes present in the lucent part of nuclei or in its fragments. The condensation of the cytoplasm is often associated with the formation of vacuoles. Nuclear fragmentation and cellular budding usually characterize cells with a high nucleus/cytoplasm ratio, such as thymocytes /11/. In the acinar cells of the salivary gland and pancreas, the rough endoplasmic reticulum is rearranged into whorls before the cell becomes fragmented /3/.

The apoptotic bodies are usually quickly phagocytosed by neighboring cells and degraded with phagolysosomes. In epithelial and tumor cells, similar processes are manifested and specialized mononuclear phagocytes also participate in the degradation /10,11/. In lining epithelia the apoptotic bodies are extruded from the surface /1,17,18/.

Light microscopic studies of apoptosis show diverse pictures. The shrinkage and budding of the cell is complete within a few minutes and discrete apoptotic bodies can be demonstrated at the end of the process /19,20/. The size of the apoptotic bodies varies considerably. They can be round or oval; some represent a single relatively large nuclear fragment surrounded by a thin cytoplasmic rim, others consist mostly of cytoplasm with a variable number of nuclear fragments.

2.3 Biochemistry

Early investigations of apoptosis revealed that it is an active process rather than degeneration of the cell /7/. It is connected with cytoplasmic and membrane surface changes, protein synthesis and internucleosome cleavage of DNA.

The process of condensation observed by ultrastructural examinations and associated with an increased density suggest that the surface convolution and the removal of the apoptotic bodies are associated with redistribution of cytoplasmic microfilaments /21,22/. The rapid uptake of apoptotic bodies by neighboring cells probably depends on changes in the carbohydrates on the surface of these bodies. It may be that the changes in carbohydrates represent the consequences of incorporation into the plasmalemma of membranes surrounding the cytoplasmic vacuoles that are formed during the development of apoptotic bodies. Discharge of the vacuole content has been described /5,6,15/. In the early stages of apoptosis lysosomes are intact and it is unlikely that lysosomal enzymes are involved in triggering off this type of cell death /7,23/.

Protein synthesis seems to be a requirement in the formation of the apoptotic bodies. Inhibitors of protein synthesis suppress the occurrence of apoptosis of thymocytes and chronic lymphocytic leukemia cells treated with glucocorticoids /7,23/. Protein synthesis inhibitors also reduce the formation of apoptotic bodies in T lymphocytes deprived of interleukin-2 /1/, in epithelial cells at the plane of fusion of the palliative processes in normal rat embryo /24/ and in various cells exposed to radiation or to cytotoxic drugs /14,25-27/. All of these results indicate that protein synthesis is a required process in the development of apoptosis, but it is uncertain what is the role of these proteins. The synthesis of several proteins is increased following the treatment of thymocytes with glucocorticoids /28/ but, in contrast, protein synthesis inhibitors do not block apoptosis induced by T lymphocytes /29/.

Among the biochemical events of apoptosis, double-strand cleavage of nuclear DNA at the regions between nucleosomes is reported for all cell types. This cleavage produces oligonucleosome fragments and it is catalyzed by the endonuclease enzyme /30-32/. The endonuclease activity and DNA breakdown is inhibited by zinc /33/. Some papers have reported that zinc deficiency enhances apoptosis in gut

crypts /34,35/. Itoh *et al.* have shown that DNA fragmentation does not occur during cell death of immature thymocytes /36/.

Several recent studies have shown that the activation of the interleukin-1-β-converting enzyme/Ced-3 family of proteases represents the end point in apoptotic cell death /37/. Other investigations have indicated that the loss of mitochondrial membrane potential is the critical step in cell death /38,39/. Many members of the Bcl-2 family of genes play major roles in the regulation of the programmed cell death in many systems /40/. This family, including Bcl-x_l, are potent inhibitors that modulate cell death through inhibition of activation of caspases, a family of cysteine proteases /37,41,42/. Bcl-x_l may thus facilitate protection against cell death /43/. Bcl-x_l can prevent apoptosis and maintain cell viability by averting the loss of mitochondrial membrane potential that occurs as a consequence of interleukin 1β-converting enzyme/Ced-3 protease activation /44/. The breakdown of Bcl-x_l during the execution phase of cell death converts it from a protective to a lethal protein /43/.

Apoptosis is involved in the death of hematopoietic progenitor cells after removal of the appropriate colony-stimulating factor. Pharmacological investigations indicated the role of protein kinase C in the suppression of apoptosis in interleukin-3 and granulocyte-macrophage-colony-stimulating factor dependent human myeloid cells /45,46/. Overexpression of some protein kinase C isoform in factor-dependent human TF-1 cells enhances cell survival in the absence of cytokine. This effect is associated with induction of Bcl-2 protein expression, an increase over the levels in empty vector transfections /47/.

3. ACTIVATION OF APOPTOSIS

Apoptosis develops in four different phases: (a) The presence of genes regulates the occurrence of programmed cell death. This prerequisite has been documented in developing organisms /48,49/, and in cell cultures /50/; (b) Various signals trigger the genetic program, or an unbalanced signaling system can prevent the action of repressors. Specific signaling molecules include calcium ions, glucocorticoid hormones and sphingomyelin. Initiation can also occur by imbalanced signaling such as lack of a growth factor /51/, or the signaling pathway can be inhibited by the action of a toxicant /52/; (c) The progression

of the condition leads to the expression of genes manifesting in structural alterations, such as cytoskeletal changes, cell shrinkage, nuclear pyknosis, chromatin changes and DNA fragmentation /53/; and finally, (d) Death and engulfment by phagocytosis of the whole cell or cell fragments terminates the apoptotic process /54/ (Fig. 1).

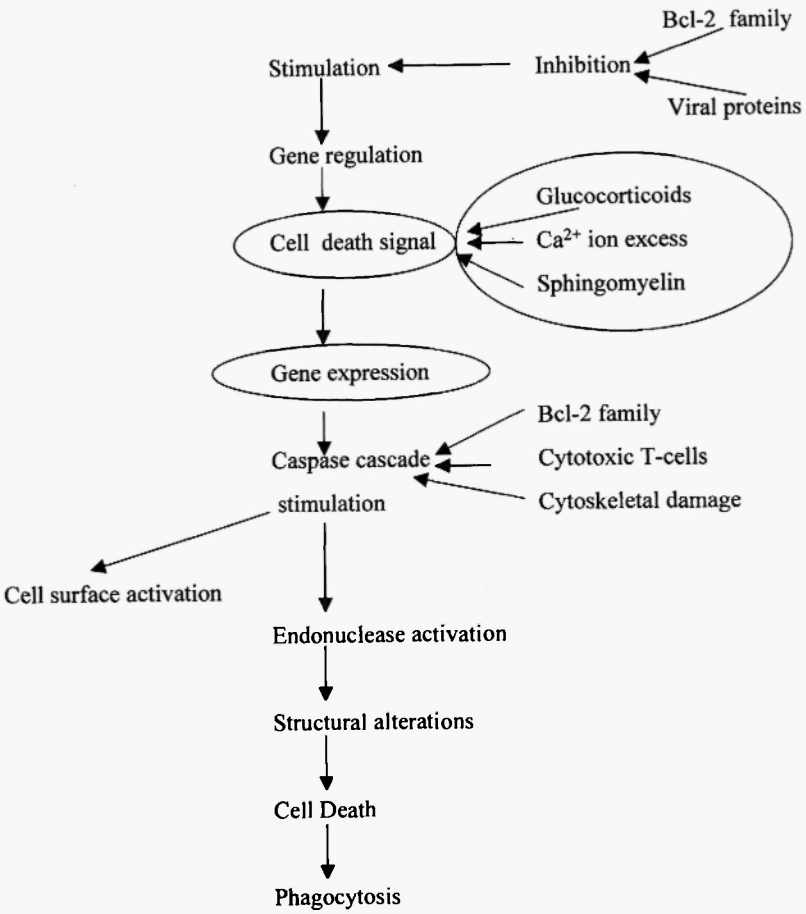

Fig. 1: Schematic illustration of various phases of apoptosis. Various stimuli such as DNA damage, radiation, thermal actions, steroids, cytokines and other growth factors, oxidants and other cytotoxic substances, anticancer chemicals, withdrawal of trophic hormones, autoimmune disease, viral infections, signaling agents and caspase cascade activation can lead to structural damage, death and elimination of cell debris by phagocytosis. Modified from Cameron and Feuer /8/.

Apoptotic signaling cascades are expressed in most if not all cells; they are usually present in inactive forms /11,55/. Apoptosis can be triggered by a variety of physiological and stress stimuli which initiate one or several distinct signaling pathways. The activation of the specific pathway is dependent on the cell type and on the subcellular organelles which are targets of each type of stress. The various signaling pathways converge into a common final effector mechanism that disintegrates the dying cell /56/. The activation mechanism includes the ICE/Ced-3 family of cysteine proteases that reorganize subcellular structures in an orderly fashion. The integrity of the plasma membrane is preserved and the disintegrated subcellular organelles are aggregated into apoptotic bodies (membrane-bound vesicles). Cellular fragments or dead cells are finally eliminated by neighboring cells or macrophages, by phagocytosis. The overall result of this process is that individual cells can be abolished without an inflammatory reaction producing tissue damage.

Intracellular Ca^{2+} signals activate apoptosis /57/. Calcium overload can trigger several lethal processes including disruption of the cytoskeletal organization, DNA damage, and mitochondrial dysfunction. When Ca^{2+} accumulates within the cytoplasm or other intracellular compartments, sudden increase of intracellular Ca^{2+} can quickly lead to cell necrosis. Disturbances of Ca^{2+} signaling can also induce apoptosis /58/. Removal of extracellular Ca^{2+} can prevent nuclear changes manifesting in apoptosis, such as apoptotic body formation and DNA degradation, demonstrating a requirement for Ca^{2+} in apoptosis /57/. Transfection of WEHI 7.2 thymoma cells with calbindin, a Ca^{2+}-binding protein, prevents apoptosis caused by calcium ionophore, cAMP or glucocorticoids /59/. Several *in vitro* models of apoptosis are connected with a loss of the regulation of intracellular Ca^{2+} level and activation of Ca^{2+} dependent endonuclease activity /60/. Ca^{2+}-mediated endonuclease activation is associated with the cytotoxicity of aributyltin and TCDD in thymocytes /52,60/. Ca^{2+} can induce endonuclease activity and initiate apoptosis in malignant cells and in cells infected with viruses /57/.

Several studies described the sphingomyelin signal transduction pathway as an essential part in the mediation of apoptosis related to environmental stresses and to several cell surface receptors /61,62/. The sphingomyelin pathway is ubiquitous. Most, probably all, mammalian cells are able to signal through the sphingomyelin system. The

functioning sphingomyelin pathway is connected with the formation of ceramide that acts as a secondary messenger by activating a variety of cell functions /63,64/. Distinct receptors signal via the sphingomyelin pathway following ligand binding. Ceramide mediates apoptosis and several cellular functions, including differentiation of promyelocytes, proliferation of fibroblasts, and the survival of T9 glioma cells. The involvement of the sphingomyelin signaling system in apoptosis is associated with stress activation of acid sphingomyelinase to produce ceramide, and ceramide as a secondary messenger initiates apoptosis. Several environmental stresses that induce apoptosis, such as ionizing radiation, heat shock, exposure to UV-C rays and oxidative stress, bring about rapid generation of ceramide through the activation of sphingomyelinase /62,65/. Understanding the role of pro- and antiapoptotic signaling involved in apoptosis mediated by ceramide, including their mode of action, may provide an opportunity to develop pharmacological means for intervention in the process of apoptosis /66/.

4. APOPTOSIS IN PHYSIOLOGICAL CONDITIONS

4.1 Apoptosis in embryonic and fetal development

Controlled cell death is part of normal development. Several morphological studies reported that apoptosis is involved in the programmed elimination of cells during the embryonic and fetal period, such as the deletion of the redundant epithelium at the plane of fusion of the palatine processes /67/, in the differentiation of the gut mucosa /68,69/ and the retina /70,71/ and in the removal of the interdigital webs /10/.

4.2 Cell turnover in adult tissues

Proliferating normal mammalian cells undergo spontaneous apoptosis, responsible for the continuous removal of the aged cells /7,72-76/. In slowly proliferating tissues, apoptosis balances necrosis over a time period /11/, and the oscillation between these two processes may be regulated by soluble factors produced locally /77/. In rapidly proliferating tissues, the deletion of the cell is associated with movement from the site of production and apoptosis. These types of

changes characterize the basal compartment of seminiferous tubules and gut crypts /72,76/.

During the normal terminal differentiation of cells, the double-strand cleavage of DNA shows great similarity to processes occurring in apoptosis. This is exemplified by differentiation in the lens of the eye /78/. Similarly, the residues of megakaryocytes remaining after platelet release in bone marrow greatly resemble the typical ultrastructural changes associated with apoptosis /79/. Apoptotic bodies are found in lymphoid germinal centers of follicle cells due to apoptosis /2/, and are formed from macrophages in spleen /80/.

4.3. Involution of adult tissues

The growth of various cell populations is controlled by hormones and growth factors. Reduction or excess addition of these substances triggers off a rapid decrease of cell number. In these circumstances, the fall of trophic hormone stimulation leading to cell deletion is connected with apoptosis. This occurs in the human premenstrual endometrium /81/, in the human breast towards the end of the menstrual cycle /82/, in the endometrium of the hamster at estrus /83/, in ewe endometrium following parturition /84/, in the theca interna of sheep ovarian follicles during atresia /85/, and in the adrenal cortex of the neonatal rat /86/.

5. APOPTOSIS IN PATHOLOGICAL CONDITIONS

5.1 Regression of hyperplasia

In several cases in the processes of regression of hyperplasia apoptosis is involved. This occurs after the removal of the proliferative stimulus producing hyperplasia in hepatic parenchymal cells by phenobarbital, lead nitrate or cyproterone acetate /87,88/, bile duct proliferation brought about by α-naphthyl isothiocyanate or ligation of the main bile duct /89/, or pancreatic hyperplasia induced by trypsin inhibitor /90/. In some cases hormone withdrawal is connected with the occurrence of apoptotic processes, such as hormone-induced hyperplasia of the adrenal cortex /11/. Apoptosis is reported in renal parenchyma atrophy in hydronephrosis and in hepatic atrophy brought about by mild ischemia /91/. Apoptosis occurs in tissue regression

such as involution of hair follicles /92/, and resorption of tissue around erupting teeth /93/. Pancreas atrophy and salivary gland duct obstruction are associated with enhanced loss of secretory cells by apoptosis /20,72,94/, and apoptotic changes in the vascular endothelial cells /72/. Apoptosis is involved in the normal regression of the corpus luteum /95/.

5.2 Pathological atrophy and apoptosis

This is frequently associated with increased levels or withdrawal of hormones, or with reduction of growth factor. Increased progesterone levels bring about apoptosis in cat oviduct lining /96/; increased glucocorticoids induce apoptosis in chronic lymphocytic leukemia cells /15/, in the cells of some lymphoid lines /97/, and in thymocytes /98/. Castration leading to pathological atrophy of the rat prostate or withdrawal of testosterone stimulation are connected with apoptosis of the epithelial cells /98-101/. Withdrawal of adrenocorticotropic hormone by excess prednisone administration significantly increases apoptosis in the adrenal cortex of rats /86/.

In T lymphocytes isolated from the blood of patients with infectious mononucleosis, the withdrawal of the T lymphocyte growth factor, interleukin-2, induces apoptosis /102,103/.

6. DRUGS AND APOPTOSIS

Many drugs have been found to induce apoptosis in experimental conditions or as side effects /104/. Some of these actions are direct by affecting the death pathway; some drugs interfere with biochemical mechanisms and these effects lead indirectly to apoptosis - for example, azide administration inhibits ATP synthesis, diphtheria toxin interferes with protein synthesis and subsequently apoptosis is induced. Since varying pharmacological agents provoke the same reaction, it may be that the effect of drugs is associated with a nonspecific stress response leading to the formation of apoptotic bodies (Table 1).

Chemotherapy drugs are a major example of pharmacological agents which serve as inducers of the process of apoptosis in a number of tissues and with a number of different cell types (Table 1). Most prominent of this class of inducers are cytosine arabinoside (ara-C), cisplatin, doxorubicin, etoposide, methotrexate, and taxol /81/.

TABLE 1

Drugs and toxins that serve as inducers of apoptosis

Chemotherapeutic drugs	**Toxins**
Adriamycin	Abrin
Bleomycin	Albitocin
Cisplatin	β-Amyloid peptide
Cytosine arabinoside (ara-C)	Aphidicolin
Doxorubicin	Azide
Etoposide	Colcemid
Methotrexate	Colchicine
Myleran	Copper salts
Taxol	1,1-Dichloroethylene
Vincristine	Ethanol
	Heliotrine
	Mercury salts
	Mycin
	Raw soya flour
	Ricin

Several mechanistic steps have been identified by studying the effects of ara-C on the process of apoptosis in various cell types. It has been demonstrated that mitogen activated protein kinase and protein kinase C are critical to the apoptotic effects of ara-C in HL-60 promyelocytic leukemic cells /105/. Nandy *et al.* found that leukemic cell apoptosis was further potentiated by drug synergism with ara-C and a new class of iso-indole derivatives /106/. The mechanism of this synergistic effect on apoptosis was reported to be related to inhibition of ribonucleotide reductase. In another study by Nakamura *et al.* using human non-lymphocytic leukemic cells, they showed the possible involvement of Fas and the Fas ligand system in the apoptotic effect of

ara-C therapy by using anti-Fas IgM monoclonal antibodies *in vitro* /107/. Cisplatin-induced apoptosis in human proximal tubular epithelial cells *in vitro* is associated with the activation of the Fas ligand system /108/.

Doxorubicin or adriamycin is a commonly used drug in the chemotherapy of breast cancer but its mechanism of action at the cellular level is not well understood /109/. It had been suggested that doxorubicin acts to suppress apoptosis of human breast cancer cells but *in vitro* studies revealed that drug resistance to apoptosis with doxorubicin correlated more with an increase in DNA synthesis.

Etoposide-induced apoptosis appears to be mediated by protein kinase C and caspases /I10/. In another *in vitro* study of etoposide-induced apoptosis, it was shown that a calcium binding protein of the endoplasmic reticulum had protective functions against calcium-induced apoptosis /111/. Etoposide, used mainly for the treatment of testicular cancer and small cell lung carcinomas, has been shown to be a p53 activating topoisomerase II inhibitor. The main side effect of etoposide therapy is bone marrow depression with leukopenia and thrombocytopenia. In an attempt to identify the genes responsible for etoposide-induced apoptosis in a variety of tumor cell lines, Wang *et al.* utilized DNA chip technology to simultaneously display changes of gene expression during etoposide-induced apoptosis using a number of cell lines, and at least 12 genes were characterized which were shown to be p53 responsive genes /112/.

The apoptosis of peripheral human T cells *in vitro* when exposed to methotrexate for eight hours was shown to be independent of CD95 when given at a dose range of 0.1-10 μM /113/. In contrast, the apoptotic cell death of human keratinocytes *in vitro* after exposure to 0.1 μM methotrexate was associated with the overexpression of p53 /114/.

Taxol shows cytotoxicity to tumor cell lines in the form of apoptosis at doses of 0.005-0.5 μM concentration and necrosis at drug concentrations of 5 to 50 μM *in vitro*. Taxol is shown to increase the polymerization of microtubules and stimulate formation of microtubule bundles which block entry into the S phase /115/. The inhibition of S phase entry and of cell proliferation led to the induction of necrosis in various breast cancer cell lines. In a similar study by Torres and Horwitz, low concentration exposures led to disruption of the normal microtubule cytoskeleton, and at higher concentrations

there was terminal mitotic arrest and cytotoxicity by means of the Raf-1-dependent pathway /116/. Apoptosis by taxol of various breast cancer cell lines was shown to be mediated by phosphorylation of Bcl-2 and by means of cytosolic accumulation of cytochrome *c* /117/. This process was shown by Ebrato *et al.* to be related also to p34 mediation and inhibited by Bcl-x_l overexpression /118/. Fan showed that taxol-induced apoptosis seemed to be dependent on a sustained block of mitosis by means of its effects on microtubules and cell cycle arrest /119/. Using a member of the taxoid family of chemotherapy drugs, mainly taxotere which is a semi-synthetic compound prepared from needles of the yew tree, Birchem *et al.* also showed that phosphorylation of the apoptosis blocker Bcl-2 seemed to be responsible for sensitizing the MCF-7 breast cancer cell lines to doses and concentrations as low as 5 nM taxotere leading to apoptotic cell death/120/.

7. GLUCOCORTICOID-INDUCED APOPTOSIS

King and Cidlowski showed that glucocorticoids induced G1 cell cycle arrest and apoptosis in transformed lymphoid cells /121/. Decreased expression of cell cycle components c-myc and cyclin D3 was essential for glucocorticoid-induced growth arrest and death in these dividing cells. Thompson showed further that the induction and suppression of the c-myc gene was quantitatively controlled by a number of intracellular variables, and inappropriate expression of c-myc or gross overexpression of the c-myc gene can lead to apoptosis. Cells may also be sensitized to a variety of apoptotic agents by the expression of c-myc /122/.

Schmidt *et al.* showed that glucocorticoid-induced human monocyte apoptosis was mediated through the introduction of interleukin-1β /123/. Ramdass *et al.* showed that the glucocorticoid dexamethasone inhibited cell growth *in vitro* of human leukemic T cells, leading to G1 arrest and also significant internucleosomal DNA fragmentation /124/. In another study of glucocorticosteroid-induced apoptosis of lymphocytes, by Distalhorst and Dubiak, it was found, with respect to the role of extracellular calcium, that peripheral T lymphocytes were not responsive whereas thymocytes were sensitive to calcium-mediated glucocorticoid-induced apoptosis /125/. Fan showed that glucocorticoids also had an inhibitory action on apoptotic cell death induced by taxol /119/. Similarly, studies by Huang and Cidlowski showed that

glucocorticoid treatment protected T lymphocytes from apoptosis *in vitro* when T cells were serum depleted /126/. Rogatsky *et al.* further showed that not only the repressing or inhibiting activity of glucocorticoids on apoptotic cell death but also the inducing activity of glucocorticoids on apoptosis is mediated through the glucocorticoid receptor /127/. Hofmann *et al.* found that the induction or inhibition activities of glucocorticoids (Table 2) varied depending on the type of natural or synthetic steroid hormone used /128/. For example, betamethazone, triamcinolone, dexamethasone and clobetasol were efficient inducers of gene expression of the glucocorticoid receptor and of apoptosis.

TABLE 2

Pharmacological agents that serve as inhibitors of apoptosis

Calpain inhibitors
Cysteine protease inhibitors
α-Hexochlorocyclohexane
Phenobarbital
PMA (phorbol ester)
Miscellaneous drugs

Inflammatory responses are mediated by polymorphonuclear leukocytes which persist in tissues during inflammatory processes and are eliminated by apoptosis and phagocytosis during the resolution of the inflammatory process. Liu *et al.* have studied a variety of anti-inflammatory glucocorticoids for their effect on this clearance mechanism /129/. They found that pretreatment of semi-mature 5-day human monocyte-derived macrophages for 24 hours with methyl prednisone, dexamethasone, or hydrocortisone potentiated the phagocytosis of apoptotic neutrophils. This effect was not seen with non-glucocorticoid steroids, aldosterone, estradiol or progesterone.

8. TOXIC CHEMICALS

Chronic copper administration is connected with increased hepatic apoptosis in sheep /130/. Acute lethal doses of copper or mercury to rainbow trout cause massive apoptosis in the gills /131/. Various hepatotoxins, such as 1,1-dichloroethylene, albitocin and heliotrine, given to experimental animals in high doses, produce zonal necrosis; administered in smaller doses, they enhance apoptosis in less severely affected hepatic parenchyma /6,7,23/. Colchicine causes apoptosis in gut crypt /132/, interphase lymphocytes /133/, and affects microtubules. Toxic plant proteins, mycin and also diphtheria toxin, inhibitors of protein synthesis, all induce apoptosis in mouse colonic crypts /134/. Apoptosis is also involved in the damage of the adrenal cortex of rats brought about by 9,10-dimethyl-1,2-benz[*a*]anthracene /135/. In acute mesodermal cell death, apoptotic changes produced by the teratogenic compound 7-hydroxymethyl-12-methylbenz[*a*]anthracene in the developing rat are probably the consequence of the site-specific induction of this condition in the embryo /136/. Shiga toxin from *Shigella dysenteriae* causes apoptosis in the absorptive epithelial cells of rabbit small intestine /137/.

Treatment of several cultured mammalian cells with cell cycle phase specific antiproliferative drugs commonly results in apoptosis /138/. The cytotoxic outcome of low concentrations of colcemid, an anti-mitotic drug, on HeLa 53 cells is the induction of multipolar spindles and multipolar divisions. Aphidicolin, an inhibitor of DNA synthesis, causes apoptosis which varies as a function of aphidicolin concentration. It occurs at later times after the cells have progressed further through the S phase /139/. These results indicate that the target of drug action in the cell cycle differs with colcemid and aphidicolin, which has secondary importance in the induction of cytotoxicity and apoptosis.

8.1 Ethanol

Dalhoff *et al.* revealed that neutrophils derived from ethanol-treated human volunteers showed increased apoptosis *in vitro* compared to control human neutrophils /140/. Ethanol-induced apoptosis of peritoneal macrophages harvested from ethanol-treated rats was shown by Singhal *et al.* to be mediated by TGFβ produced by the macrophages /141/. These investigators also showed that ethanol-

induced neutrophil apoptosis was associated with nitric oxide generated by the neutrophils /142/. When normal human primary hepatocytes and HepG2 cells were exposed to ethanol concentrations of 40-80 mM, the rate of apoptosis was dose dependent /143/.

9. CONCLUSIONS REGARDING APOPTOSIS *IN VIVO*

Apoptosis is a well established process that plays an important role in a variety of physiological and pathological conditions. Apoptosis represents a process of cell death that is manifested in all multicellular organisms. The phenomenon of apoptosis varies with cell type and the stimulus. The unique character of apoptotic cell death is that it is regulated developmentally; it is also called programmed cell death /144/. Cells dying during development undergo a unique and distinct set of structural changes which are similar or identical to changes occurring in cells dying in a wide variety of circumstances outside of development, such as normal cell turnover in several tissues and in tumors, killing by T-cells, atrophy induced by endocrine and other physiological stimuli, negative selection within the immune system, and cell turnover following exposure to some toxic compounds, chemotherapy, hypoxia or low doses of ionizing radiation. The process of cell death by apoptosis is clearly different from necrosis which is the consequence of extreme alterations of the cellular microenvironment.

The process of apoptosis can be divided into several steps: (a) the stimulus that initiates the cell death response; (b) the pathway by which the message is transferred to the cell; and (c) the effector mechanisms that carry out the death program /145/ (Fig. 1). The dying cell separates from its neighbors with a loss of specialized membrane structures and undergoes a period of distortion. Diverse stimuli may trigger the death response in the cell in different ways, but the pathways converge onto the same effector mechanisms with several identical key components, including a family of proteases called caspases. Following activation, these proteases are directly or indirectly responsible for the varying morphological or biochemical changes characteristic of apoptosis. Finally, the neighboring cell efficiently phagocytoses the apoptotic cells.

Apoptosis is a gene-regulated phenomenon, and great progress has been made in revealing the mechanisms involved in this type of cell

death. The occurrence of apoptotic cell death may provide a new insight into certain diseases. Further studies at the molecular level may lead to a clear view of the etiology and development of these diseases. A comprehensive understanding of the great variety of cellular processes that occur during apoptosis and further application of our knowledge concerning cell death can provide a solid basis for the development of novel therapeutic approaches and more effective ways of vaccination or gene therapy /104,145,146/. It may also open new avenues to the application of pharmacological substances in diseases associated with apoptotic cell death.

10. EXPERIMENTAL STUDIES OF DRUG-INDUCED APOPTOSIS

A variety of man-made and naturally occurring chemicals can induce apoptosis in a number of cell types /8,147/. We have been studying the process of the induction of apoptosis by selected drugs *in vitro* and *in vivo*. The chemotherapeutic drug methotrexate induces apoptosis in skin cells and liver cells *in vitro* and, in addition, apoptosis of hepatocytes was observed in liver biopsies of patients treated with methotrexate for psoriasis. In a series of further studies, we also examined the drugs acetaminophen and valproic acid for their apoptotic inducing effects on hepatocytes *in vitro* /147/.

10.1 Methotrexate-induced apoptosis

Methotrexate is an antimetabolite which binds to the enzyme dihydrofolate reductase. It acts by inhibiting the synthesis of purine and pyrimidine nucleotides and appears to exert its toxicity by means of DNA strand breakage in cells of the liver and skin /148/. The mechanism of methotrexate toxicity to hepatocytes has been studied by a number of groups /149-153/. It was suggested from these studies that one mechanism of apoptosis induction in hepatocytes is associated with CD95 receptor ligand interaction. Methotrexate is known to upregulate CD95 receptors. Methotrexate-induced apoptosis of hepatocytes was also shown to be mediated by caspases /151/. In our studies, we investigated the effect of this compound on normal neonatal primary skin cells, epidermal skin cells of A431 line, normal human primary hepatocytes, and human HepG2 cells. The presence of cytokines and the level of cytotoxicity in apoptosis were examined, as

well as cytoviability, by transmission electron microscopy and glutathione content. In addition, we attempted to quantify the differences in morphology found in electron micrographs from liver biopsies of patients with methotrexate toxicity. We also examined the effect of methotrexate in combination with ethanol. It was concluded that at low doses, methotrexate or ethanol will not cause cellular apoptosis; however, ethanol produces oxidative stress which can then promote methotrexate-induced apoptosis /147/.

Methotrexate added alone at a dose of 10 mM caused some hepatocytes to enlarge in parallel with mild steatosis with the appearance of lipid droplets. Similarly, the addition of 40 mM ethanol to hepatocytes for 24 hours *in vitro* showed few, if any, differences compared to control cells. We had previously found that a dose of 80 mM ethanol to similar cells for 24 hours induced a number of toxic effects, including changes in mitochondria and striated endoplasmic reticulum (SER), and accumulation of abundant lipid vesicles /154/. The addition to hepatocytes of this subtoxic dose of 40 mM ethanol with 10 mM methotrexate for 24 hours *in vitro* resulted in a number of toxic manifestations, including increases in numbers of lipid droplets, enlargement of the SER, and changes in mitochondria with a reduction in the number of mitochondrial cristae. Similar effects were further accentuated if an additional dose of the same combination of ethanol and methotrexate were added for another 24 hours. There was a threefold increase in the number of lipid vesicles, further ballooning of the SER and further alterations in mitochondria. In addition, many hepatocytes became apoptotic, as evidenced by dense aggregations of nuclear chromatin. Image analysis of hepatocytes exposed to the combination of ethanol and methotrexate showed that these cells were much larger at 6025 ± 345 microns compared to controls exposed only to plain medium which were 4425 ± 525 microns in size. In addition, electron microscopic morphometry showed the hepatocytes exposed to methotrexate plus ethanol had a threefold increase in the length of mitochondria, 2.5-fold increase in the diameter of lipid droplets and a twofold increase in the number of lipid droplets per cell compared to control untreated hepatocytes *in vitro* /147/.

Methotrexate has been a commonly used and effective drug in the treatment of psoriasis, a skin condition which involves the formation of scaly and itchy plaques on the skin. Heenen *et al.* /114/ found that keratinocytes from psoriatic plaques were resistant to apoptosis.

Psoriatic plaques have also been shown by Wrone-Smith *et al.* to overexpress Bcl-x_l, an apoptosis inhibiting protein /155/. Methotrexate may serve to reduce the hyperplasia characteristic of psoriatic skin by means of the induction of apoptosis in keratinocytes /114/. Snyder proposed that the mechanism of methotrexate toxicity involves the depletion of cellular deoxynucleoside triphosphate pools which affect the DNA excision repair process in cultured human fibroblasts /156/. This effect on DNA synthesis can lead to a deoxynucleotide pool imbalance and subsequently to apoptosis. Skin cells which were studied were obtained from two sources: one source was skin obtained of healthy neonates and the second was cultured skin cells of the epidermal cell line A431, obtained from the Wistar Institute, Philadelphia, PA. When keratinocytes of the A431 cell line were exposed to a combination of 40 mM ethanol and 10 mM methotrexate for two doses over 48 hours in culture, multiple apoptotic skin cells were evident, similar to what was seen with hepatocytes *in vitro* /147/.

10.2 Acetaminophen-induced apoptosis of hepatocytes and skin cells *in vitro*

Exposure to acetaminophen *in vitro* is cytotoxic to human hepatocytes, particularly when glutathione is depleted. Glutathione substrates are depleted in the process of detoxification of acetaminophen and can be replenished by sulfhydryl compounds from the diet or by cystine-containing drugs, such as *N*-acetylcysteine. Protection against acetaminophen hepatotoxicity, therefore, could be induced by agents such as *N*-acetylcysteine. Acetylcysteine acts in a similar manner to glutathione by preventing the binding of the toxic metabolite of acetaminophen to liver cell macromolecules. Apoptosis was observed in hepatocytes *in vivo* when high doses of acetaminophen were administered to ICR mice. DNA fragmentation began at 2 hours post treatment and extended to 24 hours. The morphological appearance of apoptosis, namely, nuclear condensations, began as early as 2-6 hours after exposure to acetaminophen. We have shown similar responses *in vitro* /147/.

10.3 Valproic acid-induced apoptosis of hepatocytes *in vitro*

Valproic acid is a drug frequently used in the treatment of epilepsy. This drug has excellent therapeutic effects in the treatment of several

forms of epilepsy but has been linked in rare cases to severe and fatal hepatotoxicity /157/. Anti-convulsants such as valproic acid typically present with idiosyncratic hepatotoxicity, but the risk of fatal hepatotoxicity has been rare, in one study reported as 1 in 50,000 /158/. This study also reported that 90% of patients with valproic acid-induced fatal hepatic failure were below the age of 20.

Various studies have elucidated possible mechanisms of hepatotoxicity /157-162/. One significant factor derived from these studies is the production of the toxic metabolite 4-en-valproate, which is the favored metabolite when the metabolism of valproic acid is shifted from the usual β-oxidation to ω-oxidation. Induction of cytochrome P450 activity favors the shift towards this type of metabolism of valproic acid. The reactive metabolites formed by this pathway then bind to macromolecules, deplete glutathione, and inhibit fatty acid metabolism, resulting in hepatic microvesiscular steatosis. Patients taking valproic acid had low levels of the cofactors carnitine, coenzyme A and acetyl-coenzyme A, which are necessary for the β-oxidation of fatty acids. Carnitine deficiency may predispose these patients to hepatoxicity due to the increasing serum fatty acid levels which then promote the shift of metabolism of valproic acid towards the pathway which generates reactive intermediates. Takeuchi *et al.* reported that co-administration of D,L-carnitine and albumin reduced valproic acid hepatotoxicity /159/. Studies by Fisher *et al.* showed that the toxicity of valproic acid and its metabolites had a range of toxicity in liver slices from adult or weanling rats that were similar to the toxicities in slices derived from human livers /163/. A study by Jurima-Romet *et al.* found that depleted levels of glutathione were critical for valproic acid toxicity to rat hepatocytes *in vitro,* and found a protective effect of antioxidants such as vitamins C and E /161/.

We have shown that valproic acid hepatotoxicity is enhanced *in vitro* by inducers of cytochrome P4502E1 /143/. Normal human hepatocytes *in vitro* treated with a combination of valproic acid and 40 mM ethanol for 24 hours show apoptosis. Cells treated with valproic acid alone, however, showed only microvesicular steatosis without apoptosis. In contrast, liver cells exposed only to 40 mM ethanol without valproic acid showed mild steatosis without apoptosis /147/.

10.4 Conclusions regarding drug-induced apoptosis *in vitro*

We have been able to show in a series of *in vitro* studies using skin cells and human liver cells that a variety of different drugs are able to induce apoptosis in hepatocytes and skin cells, including methotrexate, acetaminophen and valproic acid. The addition of these drugs to tissue culture environments presenting specific metabolic stresses to these cells, such as induction of specific cytochrome P450s or depletion of glutathione, has been shown to enhance the induction of apoptosis *in vitro* for skin cells and human liver cells. Intracellular ATP levels in human T-cell lines have been shown by Eguchi *et al.* /164/ to be critical in directing the process of cell death so that cells in ATP-depleted conditions undergoing apoptosis can be driven towards necrosis. Apoptosis of hepatocytes was also observed in liver biopsies of patients treated with methotrexate for psoriasis. In summary, it has been possible to undertake mechanistic studies of the induction of apoptosis of human skin cells and human liver cells *in vitro*.

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