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Probing the Molecular Program of Apoptosis by Cancer Chemopreventive Agents

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Abstract This paper provides a rational molecular basis for studies intended to clarify the interactions between cancer chemopreventive agents and apoptosis, one of the natural forms of cell death that overlaps molecular mechanisms with other forms such as programmed cell death and specialized forms of physiological cell death. Molecular details of the process show the existence of distinct molecular pathways leading to the activation of critical effector elements (apoptosis gene products) functioning under the control of a network of negative regulatory elements. Dysregulation of either apoptosis or anti-apoptosis genes has a significant role in multistage carcinogenesis. Inhibition of apoptosis is one of the underlying mechanisms of the action of tumor promoters. The network of apoptosis and anti-apoptosis gene products provides multiple targets for compounds with cancer chemopreventive potential. Many data in the literature show initiating, potentiating or inhibitory effects of such compounds on apoptosis. However, the molecular mechanism of these effects is largely unknown. We initiated a series of studies using mouse thymocytes which undergo apoptosis through distinct molecular mechanisms after T-cell receptor activation (TCR pathway), following the addition of glucocorticoids (DEX pathway) or DNA damaging agents (p53 pathway). All *trans*-and 9-*cis*-retinoic acid induced apoptosis, elicited through the DEX pathway, inhibited the TCR pathway, and did not affect p53- initiated apoptosis. *N*-acetylcysteine can inhibit all forms. Sodium salicylate enhanced spontaneous cell death, decreased p53-dependent apoptosis, and did not affect the DEX and TCR pathways. These preliminary results, which show differential effects of the studied compounds on distinct molecular pathways of apoptosis, warrant further investigations in the effort to utilize the molecular elements of apoptosis in proper cancer chemoprevention, and find biochemical targets for apoptosis-related surrogate endpoint biomarker assays of chemoprevention.

Key words: Apoptosis, carcinogenesis, cancer chemopreventive agents, NAC, retinoic acid, salicylate, selenium, thymocytes

Apoptosis, the most common naturally occurring form of cell death, has recently become a challenging issue in biomedical research. Both normal and malignant cell populations in living tissues are significantly affected by the actual rate of apoptosis, a process thought to complement mitosis in regulating cell numbers [1,2]. Compounds which modulate apoptosis, such as cancer chemopreventive agents, can therefore affect steady-state cell number; this effect may be beneficial, harmful, or both depending on the site of action. Before reviewing experimental data, we will consider some of the main points from recent apoptosis research.

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APOPTOSIS AND CARCINOGENESIS

Apoptosis: A Naturally Occurring Form of Cell Death Initiated through Distinct Molecular Pathways

Naturally occurring forms of cell death can be divided into at least three major groups (Fig. 1).

One is apoptosis, which itself covers several distinct molecular pathways. Although the three major forms differ in morphology as well as in some characteristic molecular features [3–6], they share some common basic elements. These include elimination of the nuclei by internucleosomal cleavage of DNA, efficient elimination of the dying cells without leakage of intracellular macromolecules and inflammation, involvement of various forms of intracellular proteases and transglutaminases, and controlling death by negative regulatory elements such as BLC-2 and related proteins. In this early stage of natural cell death research, it is hard to predict the extent of overlap among these processes in molecular terms. Nevertheless, if a compound influences one form of cell death by interacting with a biochemical element shared by several of the molecular pathways, tissue homeostasis will be modulated in a broad range of organs and in developmental and differentiation settings.

Some of the Molecular Elements of Apoptosis Have Been Recognized

Intense research efforts in recent years have revealed some of the cell death machinery [7–12] operating during apoptosis (Fig. 2). Either the appearance of apoptosis factors (TNF, FAS ligand, antigen, glucocorticoids, retinoic acid, *etc.*) or the disappearance of survival factors (various interleukins, erythropoietin, c-*kit* ligand, NGF, testosterone, *etc.*) may prime cells for death, leading to the expression of apoptosis genes through the action of various transcriptional factors and nuclear receptors with transactivation potential. Sometimes priming may occur in the absence of an apoptosis factor or without the disappearance of a survival factor. The triggering signal, which generates increased intracellular concentrations of Ca^{2+} or other second messengers such as ceramide, may be an internally programmed event or some nonspecific change in the extracellular mill-

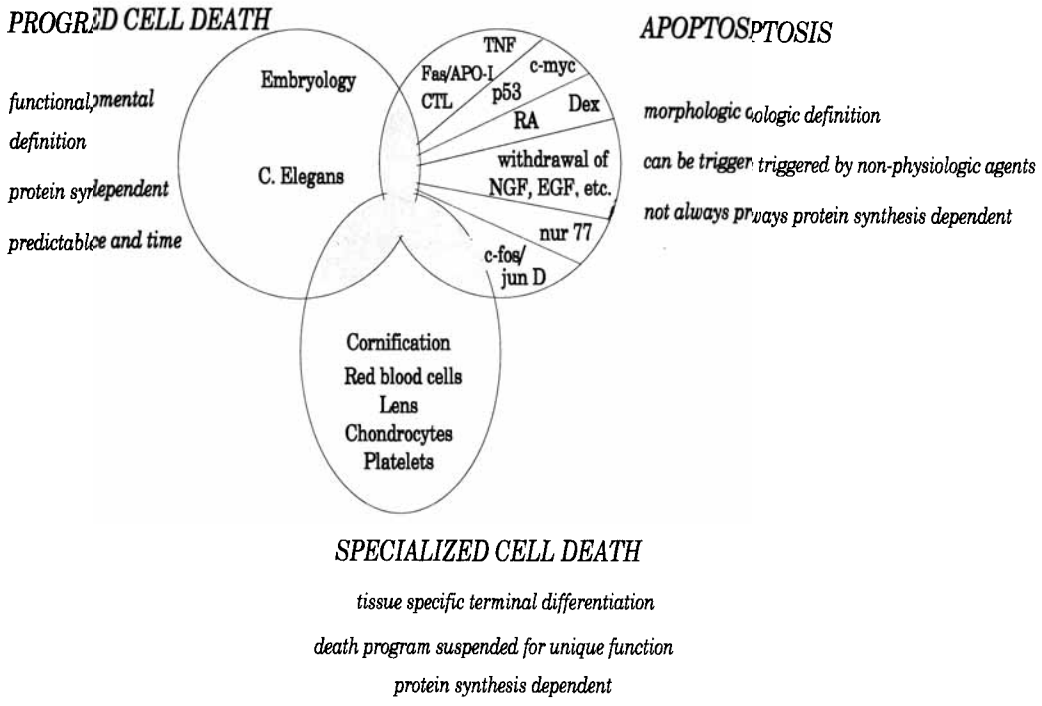

Fig. 1. *Schematic illustration of the interrelationship among various forms of naturally occurring cell death and apoptosis.*

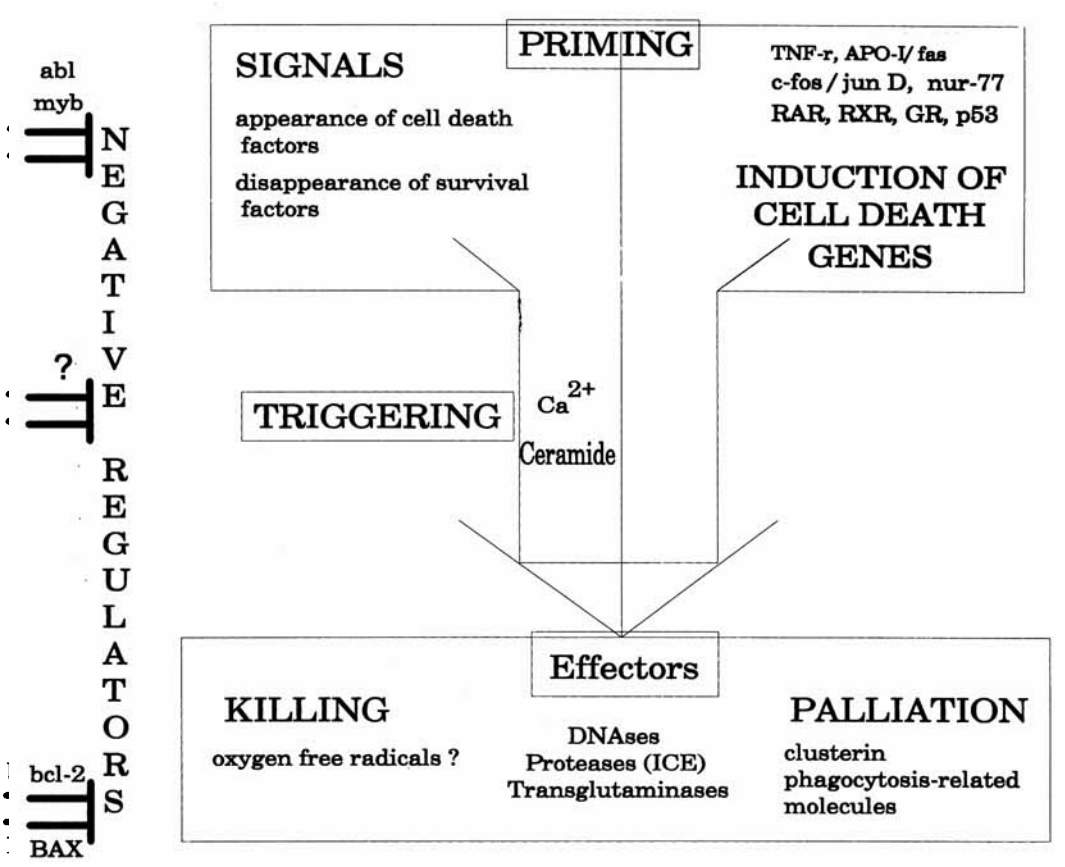

Fig. 2. Schematic illustration of the molecular elements of the apoptosis machinery.

ieu (irradiation, oxidative stress, or physical damage). The trigger usually activates the effector elements/enzymes which kill the cell and keep the death process under control, facilitating degradation of intracellular structures and macromolecules, crosslinking proteins, and inducing rapid phagocytosis. It is still not clear what actually kills cells during apoptosis. However, a negative regulatory network keeps the apoptosis machinery under control. One prominent member of this anti-apoptosis system is BCL-2, an integral membrane protein present in mitochondria, endoplasmic reticulum and the perinuclear membrane, which may act as an antioxidant or as a regulator of Ca^{2+} fluxes. There are several BCL-2 related proteins, some inhibiting apoptosis (BCL-x_L), others regulating BCL-2 (BCL-x_S, BAX). The most compelling evidence for both an apoptosis and anti-apoptosis molecular network comes from viral studies [13–14]. Many viruses contain proteins which prevent apoptosis for persistent infection (Table I); some of these proteins are homologous to BCL-2, others are not related to BCL-2 and act on distinct molecular targets in the biochemical pathway of apoptosis. The target of many such proteins is unknown. It is also predictable that these proteins have mammalian counterparts (anti-apoptosis proteins) controlling specific steps of the death process.

Malignant Transformation Involves Genetic Changes in the Apoptosis Machinery

In the largely genetically determined, multistep process of carcinogenesis, activation of proto-oncogenes and/or the inactivation of tumor suppressor genes are critical events. Recent evidence suggests that oncogenes and tumor suppressor genes can regulate the rate or suscepti-

TABLE I. Viral Proteins Interacting with the Apoptosis Machinery

Related to BCL-2	
Epstein-Barr Virus BHRF1	Oxygen free radical
African Swine Fever LMW5-HL	Oxygen free radicals
Not Related to BCL-2	
Adenovirus E1B 19kD	TNF receptor, FAS/APO1 receptor
*cpm*A (cowpox)	ICE cytosolic protease [32]
Simian virus large T antigen	p53 Transcription factor
Adenovirus E1B 55kD	p53
Epstein-Barr Virus BCRF-1 (IL-10)	?
activated T24 *ras*	?
v-*abl*	?
Baculovirus genes (p35 and *iap*)	?
So Far Unrevealed	
?	DNase
?	Transglutaminase

For citations see [13,14].

bility of cells to undergo apoptosis; the deregulation of apoptosis pathways is also of critical importance in multiple types of cancer development [13]. Some oncogenes (*bcl*-2, *myb*, *bcr-abl*) are anti-apoptosis genes [13,15,16]; their overexpression extends the life-span of cells and in the case of *bcl*-2, increases susceptibility to malignant transformation. Some of the antioncogenes have the potential to induce apoptosis; their loss may lead to generations of premalignant cells, particularly in case of somatic mutations of the p53 gene which is now considered the most common cancer-related genetic change. The p53 tumor suppressor protein in wild-type but not mutant form, is able to induce apoptosis in cells [17,18]. Wild type p53 protein appears critical to initiate growth arrest and apoptosis in response to DNA damage [19,20], and p53-null mice develop spontaneous tumors [21]. Future studies will likely find that activation of viability-enhancing anti-apoptosis genes (recognized or not as oncogenes) and inactivation of genes that normally mediate cell death (either known or not as anti-oncogenes) are critical events in carcinogenesis.

Tumor Promoters Inhibit Apoptosis

Though we still don't know what common denominators underlie the mechanism of tumor promotion, inhibition of apoptosis have been suggested as one such element. Apoptosis does not seem to occur randomly in tissues; it eliminates old, preneoplastic or damaged cells preferentially. Tumor promoters inhibit apoptosis in a reversible manner both *in vivo* and *in vitro* [22,23]. Obviously, reversing tumor promotion by inducing apoptosis is a major issue in both assessing health risks of tumor promoting compounds and interpreting the action of cancer chemopreventive agents.

MANY CANCER CHEMOPREVENTIVE AGENTS MODULATE APOPTOSIS

The molecular network of apoptosis and anti-apoptosis proteins provide a broad surface and large number of potential targets with which cancer chemopreventive agents can interact. Such interactions are presumed to be beneficial, facil-

itating the elimination of premalignant cells. However, some biochemical effects of chemopreventive compounds may do harm either blocking beneficial apoptosis or depleting cells critical for cancer immune defense mechanisms.

In several types of cells (particularly bone marrow-derived and neuronal types), apoptosis initiated by withdrawal of survival factors is accompanied by generation of oxygen free radicals, thought to play critical roles in the physiological death process. Glutathione and *N*-acetylcysteine, analogously to BCL-2 protein, prevent the generation of these reactive radicals and prevent apoptosis [24,25].

Tamoxifen given to mice in a spontaneous mammary carcinogenesis model resulted in growth arrest of the glands and epithelial cell apoptosis [26]. Toremifene causes growth inhibition of estrogen-sensitive breast cancer cells by inducing some cells to undergo apoptosis and inhibiting other cells from entering mitosis [27].

Selenodiglutathione, the initial metabolite of selenite, induces apoptosis in cultured cells; it also induces accumulation of p53 protein in cells that contain normal p53 [28]. Recent mammary carcinoma cell line studies show that induction of apoptosis by selenium compounds may partially account for their chemopreventive activity [29].

Proteases, particularly SH proteases (calpain and proteases related to interleukin-1β converting enzyme), seem to have a decisive role in some apoptosis processes. Consequently, protease inhibitors, selective for cysteine or serine proteases or calpain, have successfully blocked apoptosis of several cell types [30–32].

Exposure of cells to sodium butyrate causes apoptosis [33]. Such treatment of virus-transformed human lung fibroblasts results in induction of apoptosis. The therapeutic effects of sodium butyrate in ulcerative colitis and neoplastic growth may be related to the induction of apoptosis in colonic mucosa (Paul Birckbichler, personal communication).

The above results are sporadic, differ in various cell types, usually do not offer molecular explanations, and do not provide a clear understanding of the significance of apoptosis modulation in cancer chemoprevention. Systematic studies that dissect the effects of these compounds on well-described apoptosis pathways are needed to make a direct link between the effect on apoptosis and cancer chemopreventive action, as well as to define clear chemopreventive strategies based on the modulation of apoptosis.

THYMOCYTE APOPTOSIS: AN EXPERIMENTAL SYSTEM TO DEFINE APOPTOSIS MOLECULAR PATHWAYS

T-cells differentiate into mature T-lymphocytes within the thymus. During this process they proliferate, randomly generate their T-cell receptors (TCRs) and, in the CD^4CD^8 double-positive stage of differentiation, become selected based on the capability of the TCR to interact with antigens presented in association with a major histocompability complex (MHC) molecule on the surface of an antigen-presenting cell (APC). Cells which express potentially autoreactive TCRs after interacting with the APC, undergo apoptosis (negative selection). This selection process can be mimicked *in vitro* by simultaneous addition of phorbol dibutyrate and a calcium ionophore [34]. Cells which express functionally acceptable TCRs can recognize and interact with self MHCs after interacting with the APC, become positively selected, escape the cell death pathway, and differentiate into mature, single-positive thymocytes. However, most T-cells express functionally unacceptable TCRs, cannot interact with the APC, and will enter the apoptotic program. The rate of apoptosis accelerates when cells are exposed to high levels of glucocorticoids [35], or effects which induce DNA breakage (ionizing radiation, topoisomerase II inhibition) [36]. The apoptotic programs induced in these cases are dependent on *de novo* gene expression; they are indistinguishable by morphologic criteria, involve activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease and require induction of tissue type transglutaminase (unpublished observations). However, these signals work through different signal transduction pathways: TCR stimulation induces changes in second messenger systems, glucocorticoids bind to cytoplasmic steroid receptors that translocate to the nucleus, while topoisomerase II inhibition by etoposide causes direct DNA damage. Each of these pathways appears to induce distinct sets of genes. For example, the transcripts RP-2 and RP-8 are expressed in thymocyes following treatment with glucocorticoids [37]. Similarly, the immediate-early gene *nur77* is

induced in response to TCR signals, but not by glucocorticoids or ionizing radiation [38]. Antisense inhibition of *nur77* expression prevents apoptosis in TCR stimulated cells, but not if the death was induced by other stimuli. DNA damage, on the other hand, leads to p53 induction. Thymocytes lacking p53 are resistant to the lethal effects of ionizing radiation or etoposide, but not to other treatments [19,20].

In our experiments apoptosis was induced via three different signalling pathways—dexamethasone, 1 μM (DEX); A 23187, 0.5 μM + PdBu, 5 g/ml (TCR); or etoposide, 50 μM (p53)—in primary cultures of mouse thymocytes. Apoptotic response was measured [described in 39] in the presence and absence of the investigated compounds.

DIFFERENTIAL EFFECTS OF SOME CANCER CHEMOPREVENTIVE AGENTS ON VARIOUS BIOCHEMICAL PATHWAYS OF THYMOCYTE APOPTOSIS

Retinoids

Vitamin A exerts most of its effects on cellular processes after being converted to all-*trans*- and 9-*cis*-retinoic acid within the cells. Both are physiological ligands for the retinoic acid receptors (RARs, RXRs) belonging to the steroid/thyroid/retinoid receptor family. These receptors are ligand-inducible transcription factors which bind to specific hormone response elements (RAR-E, RXR-E) on DNA and transactivate specific target genes. all-*trans*-Retinoic acid and 9-*cis*-retinoic acid are equipotent in activating RAR, while 9-*cis*-retinoic acid activation of RXR is 50-fold greater than that of all-*trans*-retinoic acid [40]. In the presence of physiological concentrations of retinoids, only 9-*cis*-retinoic acid is expected to activate RXR. Retinoic acid receptors can function as either RAR/RXR heterodimers or RXR/RXR homodimers in the presence of retinoic acids [41]. RXR, however, can also form heterodimers with various members of the steroid/thyroid/retinoid receptor family (thyroid receptor, vitamin D_3 receptor, COUP-TF). The presence of RXR in most of the heterodimers enhances cooperative binding of these receptors to DNA, and requires only the presence of cognate vitamin D_3 receptor, thyroid receptor, and RAR ligands [42]. These complex interactions explain the pleiotropic effects of retinoids in practically all cells [42].

To determine whether retinoic acid can induce DNA fragmentation, freshly isolated thymocytes were exposed to increasing concentrations of retinoic acids, viability and the amount of fragmented DNA were determined after 6 hr. Part of the thymocytes entered the apoptotic program spontaneously due to removal of the protective thymic environment, resulting in about 25% DNA fragmentation (Fig. 3A). Both all-*trans*- and 9-*cis*-retinoic acid induced a further DNA fragmentation; 9-*cis*-retinoic acid was more effective. However, the induction seemed to be dependent on the initial rate of cell death. Those cultures where initial DNA fragmentation was higher had better responses to the retinoic acids (data not shown). Both compounds, as shown in Figure 3B, also enhanced the effect of the glucocorticoid dexamethasone. After 6 hr of incubation in saturating concentrations of dexamethasone, cultures showed 61 ± 3% DNA fragmentation, further increasing to 83 ± 4% DNA fragmentation in the presence of retinoids. Again, 9-*cis*-retinoic acid and all-*trans* retinoic acid were effective at 0.01-0.3 μM and 0.1-10 μM concentrations, respectively. The observed maximum DNA fragmentation represents the cell death of the total glucocorticoid-sensitive thymocyte population. Retinoids, on the other hand, failed to affect the etoposide-induced DNA fragmentation (Fig. 3C). To determine whether retinoids influence the TCR-mediated cell death, thymocytes were treated with the combination of the calcium ionophore, A 23187, and phorbol dibutyrate. The treatment alone resulted in a 25% increase in DNA fragmentation after 6 hr of culture (Fig. 3D). Both retinoids prevented activation-induced cell death, and again the 9-*cis*-retinoic acid showed a more pronounced effect.

These results, obtained as a continuation of previous works [43–45], demonstrate that retinoids are able to interact differentially with the three cell death pathways of mouse thymocytes, while they enhance the effects of glucocorticoids, exhibit no effect on the p53 signalling pathway, and inhibit the TCR-mediated apoptosis. These different interactions provide further evidence for the existence of the three independent signaling pathways of cell death in mouse thymocytes. In our experiments, retinoids seemed to induce DNA fragmentation in isolated mouse thymo-

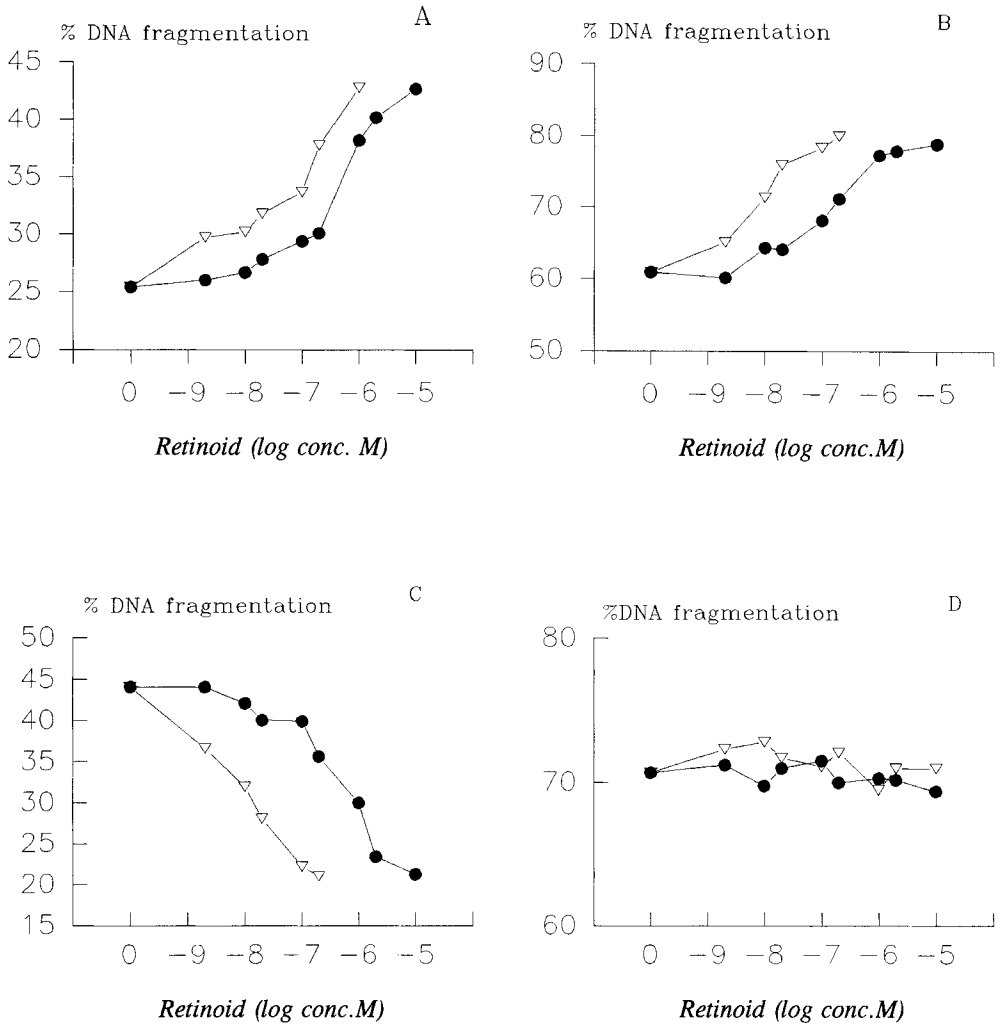

Fig. 3. Effect of all-*trans*- (•) and 9-*cis*-retinoic acids (▽) on various forms of thymocyte apoptosis. A: spontaneous; B: DEX-pathway; C: TCR-pathway; D: p53-pathway.

cytes. However, isolated thymocytes might already have been affected by exposure to endogenous glucocorticosteroid production in the animal [46]. The differences in endogenous glucocorticoid levels may be reflected in different "spontaneous" cell death rates of cultured thymocytes originating from different animals. Since retinoids enhance the apoptosis-inducing effect of glucocorticoids, the observed effect of retinoids on spontaneous apoptosis may be related to enhancement of the endogenous glucocorticoid effect. RARs and RXRs have overlapping but distinct functions responsible for the activity of retinoic acids. In our experiments, 9-*cis*-retinoic acid was more potent than all-*trans*-retinoic acid in influencing cell death pathways. This finding strongly suggests involvement of the RXR receptor in mediating retinoic acid activity in the apoptosis pathway. However, more detailed studies using specific RAR and RXR receptor analogs are required to prove or exclude the involvement of specific RARs or RXRs in the observed effects of retinoids.

N-Acetylcysteine

N-Acetylcysteine (NAC) is an effective antioxidant capable of direct reduction of *reductive*

TABLE II. Effect of 2 hr Pretreatment with *N*-acetylcysteine on the DNA Fragmentation and the Loss of Cell Viability of Cultured Mouse Thymocytes Induced by Various Signaling Pathways

Treatment	*N*-Acetylcysteine (mM)	Amount of DNA Fragmented (%)	Viability (%)
Control	0	34.0 ± 1.2	68 ± 4
	10	34.3 ± 3.1	69 ± 3
	25	27.4 ± 2.5	72 ± 3
	50	20.1 ± 1.3	80 ± 2
	75[a]	14.0 ± 3.1	41 ± 5
	100[a]	3.2 ± 0.7	10 ± 2
Dexamethasone (1 μM)	0	55.4 ± 3.4	46 ± 4
	10	56.0 ± 2.5	48 ± 2
	20	38.2 ± 1.8	61 ± 3
	30	30.9 ± 2.2	65 ± 3
	40	25.1 ± 2.7	70 ± 4
	50	18.8 ± 0.6	76 ± 2
A 23187 (0.5 μM) + PdBu (5 ng/ml)	0	421. ± 2.0	69 ± 3
	10	40.8 ± 1.8	71 ± 2
	20	34.4 ± 2.4	78 ± 4
	30	28.6 ± 1.7	80 ± 2
	40	22.1 ± 0.3	84 ± 4
	50	18.9 ± 1.9	83 ± 3
Etoposide (50 μM)	0	71.3 ± 4.1	26 ± 4
	10	70.4 ± 3.9	28 ± 2
	20	70.9 ± 2.8	29 ± 3
	30	70.9 ± 3.1	28 ± 2
	40	65.4 ± 2.3	34 ± 3
	50	59.4 ± 3.1	36 ± 3

[a] Toxic concentrations of *N*-acetylcysteine.

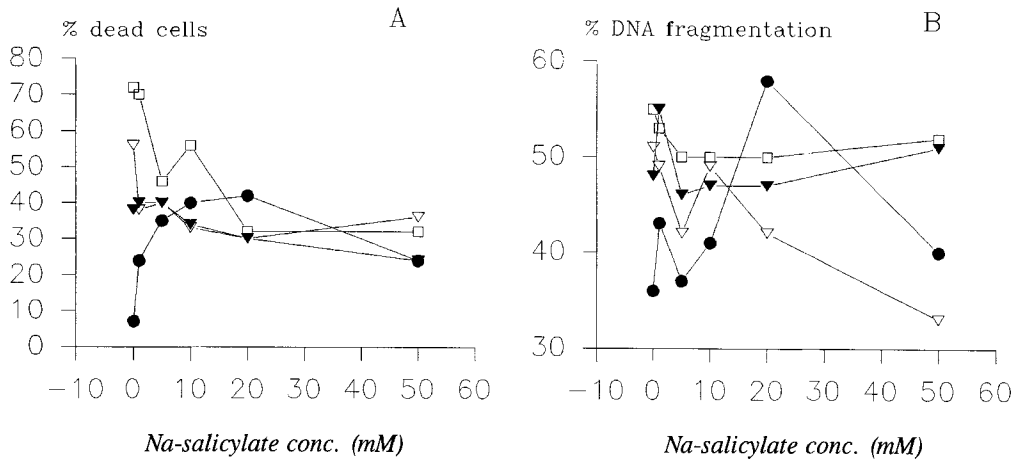

Fig. 4. Effect of sodium salicylate on various forms (• spontaneous; □ DEX-pathway; ▼ TCR-pathway; ▽ p53-pathway) of thymocyte apoptosis. Sodium salicylate was added 2 h before various stimuli.

oxygen species and by deacetylation, it forms cysteine and increases glutathione production. Glutathione is an important antioxidant within the cells, where it serves as a substrate in the reduction of peroxide catalyzed by the selenoenzyme glutathione peroxidase [47]. In addition, glutathione itself can be a scavenger for the hydroxyl radical. Treatment of mouse thymocytes with various concentrations of NAC increased thymocyte viability and decreased the rate of spontaneous DNA fragmentation (Table II). The most pronounced effect was found at 50 mM concentrations of NAC; higher concentrations had a toxic effect and strongly decreased viability. Pretreatment for 2 hr before the addition of compounds enabled the same concentration of NAC to partially inhibit cell death induced via the three different signalling pathways and also inhibited, though less efficiently, the amount of fragmented DNA.

There is increasing evidence that production of reactive oxygen species may participate in the signalling or the effector part of the apoptotic pathway. BCL-2, previously shown to inhibit each of these three forms of thymocyte death [48], was suggested to regulate an antioxidant pathway [24] and did inhibit transactivation of genes by NF-κB, a transcription factor which is unregulated if hydrogen peroxide levels increase within the cell (Patrick Baeuerle, personal communication). Our data confirm observations showing that pretreatment of thymocytes with an antioxidant compound partially prevents all three forms of thymocyte death.

Sodium Salicylate

Recent experimental data suggest that salicylic acid influences prostaglandin-independent signalling processes; some of these effects are linked to the inhibition of NF-κB by high doses of sodium salicylate [49]. According to our preliminary experiments, various pathways of apoptosis are differentially affected by sodium salycilate; it did not influence the DEX and TCR pathways, but high doses increased the rate of spontaneous apoptosis (particularly at 20 mM concentration) and inhibited p53-induced DNA fragmentation (Fig. 4). Salicylic acid seems to have some nonspecific effect as well, affecting the rate of DNA fragmentation (Fig. 4B) and cell viability (Fig. 4A) differently, especially in case of DEX-induced death.

SUMMARY

Could we modulate apoptosis using cancer chemoprevention compounds? Multistage carcinogenesis involves genetic changes in the apoptosis machinery; these changes may be effectively counterbalanced by compounds which initiate or potentiate apoptosis. Clearly, different cell types vary profoundly in their susceptibility to apoptosis induction. In addition, there are distinct

molecular pathways leading to apoptosis even in one cell type; the biochemical machinery of these pathways may be differentially influenced by various compounds, lowering or increasing the cellular thresholds of apoptosis induction. Since some cancer chemopreventive agents inhibit apoptosis, the benefit of their use should be re-evaluated with respect to the significance of apoptosis in eliminating precancerous cells or in cancer chemotherapy. Systematic molecular studies of the interaction between cancer chemotherapeutic agents and the elements of the various apoptosis pathways, especially the one which involves p53, are needed to design rational chemoprevention trials and perhaps new compounds for such trials.

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