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Regulation of Apoptosis by Steroid Hormones

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Steroid hormones play major roles in regulation of growth, development, homeostasis, and cell death. Together with other hormones and growth factors, steroids regulate both the function and cellular composition of organs throughout the body. In this article we will discuss the mechanisms of steroid hormone regulated apoptosis. Emphasis will be placed on the effect of glucocorticoids on lymphoid cells.

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INTRODUCTION

The development and function of higher organisms requires a balance between many homeostatic mechanisms. Of pivotal importance is achieving equilibrium between cellular proliferation and death. Such physiologic cell death occurs by an active, energy requiring process known as apoptosis (programmed cell death). Apoptosis is the mechanism by which organization and structure is resolved during development and is involved in scar resolution, in the involution of endocrine-dependent tissue after withdrawal of hormones, and in immune cell selection in adults [1, 2]. In addition, apoptosis appears to be involved in many pathologic processes including carcinogenesis [3]. The induction of apoptosis by addition or withdrawal of steroid hormones from various tissues and cell lines has provided a convenient model for the study of the regulation of this process [2].

MORPHOLOGIC AND BIOCHEMICAL CHARACTERISTICS OF APOPTOSIS

Apoptosis is "programmed" since its induction and progression requires the temporal occurrence of a set of biochemical and morphological changes. Membrane fusion early in apoptosis causes fluid loss and therefore cell shrinkage [4]. Chromatin condenses in apoptotic cells and is deposited on the inner surface of the nuclear membrane [2]. Macromolecular synthesis decreases and usually (a few exceptions exist) cellular DNA is cleaved at internucleosomal sites, resulting in fragmentation of DNA into multiples of approx. 180 bp [2, 5–11]. This DNA is eventually released from cells in membrane bound vesicles which are eliminated *in vivo* by phagocytic cells [2]. Since intracellular contents are never released into interstitial fluid, inflammation does not readily occur. The presence of membrane-bound vesicles in cells is a definitive sign of apoptosis, and is easily visualized by staining of the cells with hematoxylin/eosin. Figure 1 shows normal murine S49-neo thymoma cells (control) and those treated with dexamethasone for 18 h (dex) which have been stained in this manner. The control cells possess a normal lymphoid morphology consisting of a dense nucleus surrounded by scant cytoplasm. In contrast, most of the dexamethasone-treated cells have an apoptotic morphology consisting of multiple vesicles containing nuclear material enclosed in intact cytosol. Lanes from an agarose gel in which DNA from control and dexamethasone-treated S49-neo cells has been electrophoresed are shown beside the photomicrographs of the cells. DNA from normal S49-neo cells is intact and remains near the top of the lane, while that from apoptotic S49-neo cells forms a clearly visible "ladder" of DNA which represents fragments which are multiples of approx. 180 bp.

Exceptions in which morphological apoptosis occurs in the apparent absence of internucleosomal DNA cleavage have led to some confusion regarding whether all programmed cell death really represents a manifestation of the same process [6–12]. In several of these cases, DNA was only cleaved into larger fragments (300 and/or 50 kb) which probably correspond to the

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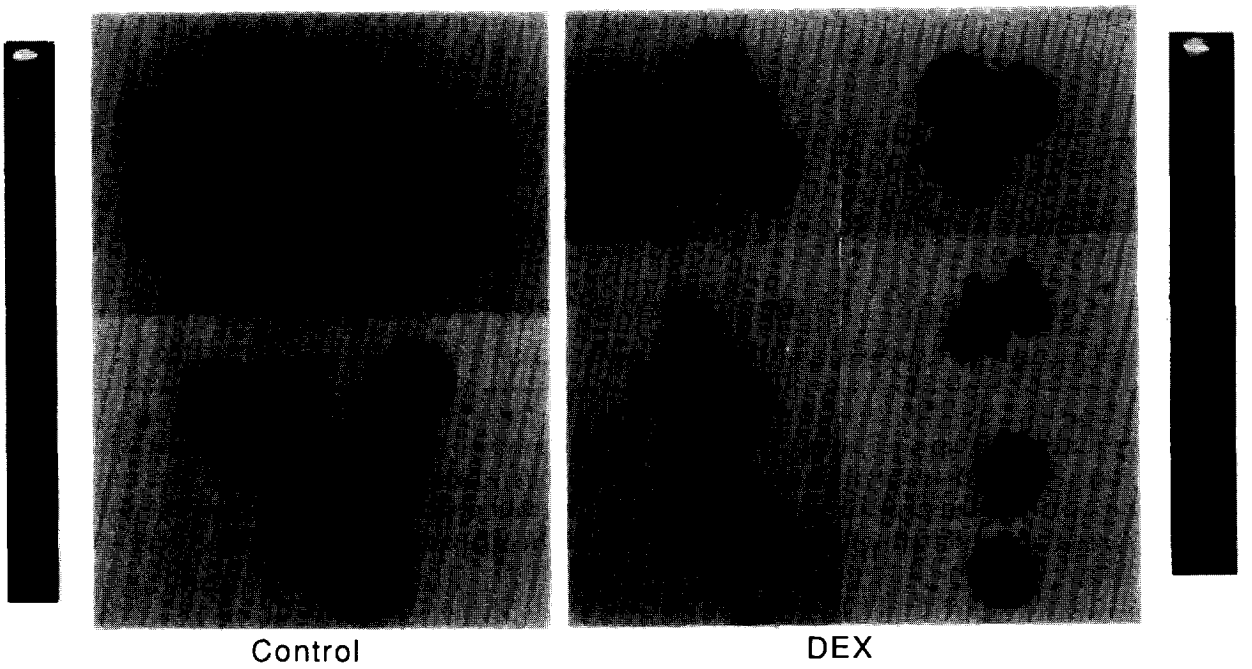

Fig. 1. Morphology and DNA degradation of S49-neo cells. A murine thymoma cell line (S49) was stably transfected with a recombinant amphotropic retrovirus carrying a G418 antibiotic resistance gene alone. Cells were maintained at 37°C in a 7% CO_2 incubator in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 4 mM glutamine, 100 U/ml penicillin, and 75 U/ml streptomycin sulfate. Cells were resuspended at 5×10^5/ml in the same media but with 5% FCS, and either had nothing added (labeled Control) or had 1×10^{-7} M dexamethasone added for 18 h (labeled DEX). After incubation, a small aliquot of cells was centrifuged, the media aspirated, and cells were fixed in 3:1 ethanol:acetic acid, applied to slides, and stained with hematoxylin and eoxin. Photomicrographs of these cells show normal cells (Control) and apoptotic cells (DEX). DNA was isolated from the remaining cells, and 15 μg electrophoresed through a 1.8% agarose gel. The agarose gel was stained with ethidium bromide, and photographs taken. The gel lane containing DNA from normal cells (Control) contains intact chromosomal DNA, while the lane containing DNA isolated from apoptotic cells shows the characteristic "ladder" of DNA seen during apoptosis.

hexameric rosettes and loops of higher order chromatin structure [7, 10, 13]. In other instances, cleavage of DNA into these large fragments appears to precede internucleosomal DNA cleavage, suggesting that these large fragments could be precursors for internucleosomal DNA fragmentation [14, 15]. Although this is an attractive model, it has not been substantiated. DNA fragmentation in these experiments was detected by agarose gel electrophoresis of DNA followed by staining with ethidium bromide. More molecules of ethidium bromide will intercalate into large fragments of DNA than into small fragments, thus, large fragments will be detected in lesser amounts. Internucleosomal DNA cleavage and DNA fragmentation into larger pieces may therefore be occurring simultaneously, but the internucleosomal DNA fragments may be below the detection limits of this system. This suggests that apoptosis in many diverse systems shares certain features, but apparent differences in DNA cleavage patterns seen during apoptosis may eventually lead to a need for the re-examination of the definition of programmed cell death, and perhaps the development of a new nomenclature system.

It is unclear at what point in the apoptotic process cellular changes become irreversible. DNA cleavage occurs hours before a decrease in cellular viability is observed and may represent the step beyond which cell death is inevitable [16]. Glucocorticoids are widely known to rapidly inhibit glucose uptake and protein and RNA synthesis in various tissues and cell lines [17]. Although one might suspect that such early changes could represent irreversible steps in apoptosis, this does not appear to be the case since many of these early effects of steroid treatment have been shown to occur in cells that do not become apoptotic [18].

STEROIDS AND APOPTOSIS

The induction of apoptosis by addition or withdrawal of steroid hormones from responsive tissues has been well documented. For example, the ablation of androgens by castration causes regression of the prostate via apoptosis of ventral prostate epithelial cells, and the withdrawal of progestins causes apoptotic regression of lactating breast tissue [19, 20]. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis, progesterone suppresses apoptosis in uterine epithelial cells, and the thymus gland shows marked regression in response to administration of glucocorticoids [2, 21, 22]. Thus, it is not surprising that ablation

or addition of steroid hormones has been successfully used for treatment of a variety of human tumors arising from such tissues [23, 24].

When steroid hormones bind to their cognate receptor the complexes translocate to the nucleus where they may activate or repress expression of genes which contain the appropriate hormone response elements (HREs) [25]. These complexes may also affect mRNA stability and translational efficiency [25]. In the thymus of adrenalectomized rats, the glucocorticoid antagonist RU 486 prevents dexamethasone-induced apoptosis [16]. While these observations demonstrate that the induction of apoptosis in this gland by glucocorticoids is receptor mediated, it does not distinguish whether this effect is due to activation or repression of transcription, or to some other effect.

Requirement for transcription

The requirement for transcription for the induction of apoptosis in various tissues has been studied extensively. Apoptosis is suppressed by inhibitors of protein synthesis in irradiated thymocytes and in thymocytes treated with chemotherapeutic agents or glucocorticoid [2, 26, 27]. These data are consistent with a need for the expression of particular genes for induction of apoptosis. In contrast, inhibitors of macromolecular synthesis induce apoptosis in several cell types including HL-60 cells and S49 murine lymphoma cells [2]. Such studies imply that the machinery required for apoptosis to occur may actually be constitutively present in a repressed form in certain cells, and that derepression of the apoptotic machinery by inhibition of translation of an inhibitory molecule might be occurring. Thus, it appears that regulation of apoptosis varies among different cell types and that transcription dependent steps are part of the signalling cascade in certain cells.

In an attempt to clarify whether repression or activation of gene transcription is required for induction of apoptosis by glucocorticoids, several investigators have examined the requirement for various regions of the glucocorticoid receptor (GR) for induction of programmed cell death in lymphoid cells. One study using GR deletion mutants demonstrated that the carboxy-terminus and the vast majority of the amino-terminus of the GR are dispensable for induction of apoptosis by dexamethasone in human leukemic lymphoblasts [28]. None of the deletion mutants had the entire amino-terminus deleted (although the entire tau 1 transcriptional activation region was deleted). Nonetheless, these data strongly suggest that only the DNA binding domain of the GR is required to induce apoptosis in this cell type, and therefore that repression rather than induction of transcription could be the mode by which dexamethasone acts to induce apoptosis in these cells. In S49 murine thymoma cells transfected with wild-type GR (wt-GR) and a mutant GR in which most of the amino-terminus is deleted, wt-GR complements the lysis defect of the mutant GR, implicating the amino-terminus as being required for induction of apoptosis in these cells [29]. It is interesting that there are some cells which contain normal glucocorticoid receptor which cannot be induced to undergo programmed cell death by treatment with dexamethasone [30] (and unpublished data of authors). All of these results must reflect differences in the cell types in which these experiments were performed. Some cells may only require the presence of regulatory proteins to suppress the activity of constitutively present enzymes which cause apoptosis, while others may require synthesis of proteins for the induction of apoptosis. Cells are able to respond to environmental changes signalled by hormones or other signal transducing molecules more quickly if the majority of the components of the signal transduction pathways are already present, rather than waiting for protein synthesis. Thus, it would be advantageous for a cell which needs to rapidly undergo apoptosis to have the members of this pathway constitutively present. Portions of signal transduction pathways culminating in apoptosis may be intact in all cells, but the speed and manner in which the remaining components are synthesized and activated may differ.

Many groups have been trying to definitively identify genes in specific cell types whose induction or repression are associated with apoptosis after the addition or withdrawal of steroids, and to determine the functions of the products of these genes. The thymus and cell lines derived from this organ have provided a very useful model to study steroid-mediated apoptosis since they are exquisitely sensitive to induction of apoptosis by treatment with glucocorticoids [2]. An 18 kDa protein which possesses endonuclease activity is activated in the thymuses of adrenalectomized rats after glucocorticoid treatment [31]. This was assayed by the activation of nuclease activity with Ca^{2+} and Mg^{2+} after renaturation of the proteins in a polyacrylamide gel containing ^{32}P-labeled DNA, followed by autoradiography [32]. Nuclease activity was visible as a clear space devoid of ^{32}P-DNA on an otherwise dark autoradiogram. The 18 kDa nuclease (designated NUC 18) is a Ca^{2+}/Mg^{2+}-dependent nuclease which can also be detected in the S49 rat thymoma cell line [31]. The purification and subsequent protein sequencing of NUC 18 revealed that it has high homology with rat cyclophilins, and recent data has demonstrated that purified rat cyclophilins A, B, and C all possess endonuclease activity [33, 34]. However, neither NUC 18 nor the rat cyclophilins have been shown to possess internucleosomal DNA cleavage activity. Which nucleases are responsible for what type(s) of DNA cleavage during apoptosis is not clear at this time. It is possible that the cyclophilins and homologous nucleases are responsible for the introduction of DNA breaks which correspond to the appearance of large (>50 kb) DNA fragments prior to the occurrence of internucleosomal DNA cleavage in apoptotic cells. The nuclease(s) responsible for the internucleosomal DNA cleavage has

yet to be definitively identified, but several candidates exist. These include a variety of Ca^{2+}/Mg^{2+}-dependent nucleases, and DNases I and II [31, 32, 35–41].

Calmodulin

The arrest of macromolecular synthesis in whole rat thymocytes will inhibit induction of apoptosis by glucocorticoids, but internucleosomal DNA fragmentation can be induced in isolated thymocyte nuclei by incubation with Ca^{2+} and Mg^{2+} [2]. These data suggest that internucleosomal DNA cleavage activity (which is regulated at some level by Ca^{2+} and Mg^{2+}) is constitutively present in an inactive form in rat thymocytes, but that synthesis of at least one component of the regulatory pathway for apoptosis is required for induction of programmed cell death in intact lymphoid cells. This component is glucocorticoid-regulated in rat thymocytes, and could be calmodulin, which may be required for nuclear transport of calcium [42, 43]. Calmodulin regulates the Ca^{2+}-dependent protein serine/threonine phosphatase calcineurin (also known as protein phosphatase 2B) [44]. Interestingly, the immunosuppressive drug cyclosporin A forms a complex with cyclophilin, which then binds to and inhibits the activity of calcineurin [44]. Cyclosporin A also increases the nuclease activity of the cyclophilins [34]. Therefore, glucocorticoid induction of calmodulin may affect calcineurin activity, which in turn may profoundly affect nucleases involved in programmed cell death by altering their phosphorylation status.

Kinases and phosphatases

Phosphorylation of substrates regulated by proteins other than calcineurin may also be involved in induction of apoptosis. Dexamethasone treatment of MCF-7 tumors in mice decreases the activity of casein kinase II [45]. Casein kinase II may be involved in energy, protein, and RNA metabolism, as well as in the regulation of gene expression. These findings suggest that decreased phosphorylation of casein kinase II substrates is involved in the inhibitory effect that glucocorticoids generally have on breast tumor cell lines. In other cases, however, estrogen and testosterone increase casein kinase II activity [45]. The activation of a tyrosine-specific protein kinase in a human B-cell precursor is associated with onset of apoptosis induced by radiation, and induction of apoptosis by cAMP is via the activation of protein kinase A [46, 47]. The induction of calmodulin by treatment of cells with dexamethasone may activate a known calmodulin/Ca^{2+}-dependent protein kinase, which may affect regulation of apoptosis by phosphorylation of its as yet unidentified substrates [48]. Finally, treatment of a rat mammary tumor cell line with glucocorticoids or serum induces the serine/threonine specific protein kinase *sgk*, however, it is currently unknown if this kinase plays a role in apoptosis [49].

The dephosphorylation of certain substrates may also be important for the induction of apoptosis. Many studies have demonstrated that glucocorticoids induce alkaline and acid phosphatases [50, 51]. Several proteins were dephosphorylated during apoptosis in a Burkitt's lymphoma cell line and several leukemia cell lines [52]. Okadaic acid (an inhibitor of protein phosphatases I and 2A) inhibited dephosphorylation of these proteins, and inhibited apoptosis. The inhibition of kinases could also be responsible for the decreased phosphorylation of proteins observed in some apoptotic cells. Calmodulin inhibits the activity of casein kinase 2, and programmed cell death is induced in mouse thymocytes incubated with tyrosine kinase inhibitors [46, 53]. Thus, alterations in the phosphorylation of key substrates may be required for the induction of programmed cell death (at least in certain cell types). The regulation of homeostatic pathways depends on a careful balance of phosphorylation/dephosphorylation. It is therefore logical that the regulation of apoptosis may rely on a careful balance of these. One model suggests that the nuclease responsible for internucleosomal DNA cleavage during apoptosis exists in an inactive form associated with an inhibitor in certain cells [54, 55]. If this is the case, perhaps the association of the inhibitor is regulated by the phosphorylation status of the inhibitor or of the nuclease itself, which could be regulated by steroids. Future studies will certainly clarify what role steroid hormones play in achieving such a balance.

Other genes regulated by steroids with possible roles in regulation of apoptosis

Several other genes regulated by steroids may be involved in the induction of apoptosis. Unfortunately, in no case has a clear-cut relationship between the function of any of these genes and apoptosis been demonstrated, suggesting that the requirement for expression of many of these genes may be cell-type specific. It is exceedingly difficult to ascribe a function during apoptosis to any particular gene, however, a compendium of these genes follows to give an overview of those which have been implicated in programmed cell death.

Testosterone-repressed prostate message-2 (TRPM-2; also known as SGP-2, clusterin, and several other names) is expressed during androgen ablation-induced regression of the prostate, during apoptosis induced in the mammary gland after weaning, and in glucocorticoid-treated thymocytes, as well as in several examples of non-steroid induced apoptosis including renal atrophy after ureter obstruction, and in regression of a tumor induced by chemotherapeutic agents [20]. TRPM-2 might appear to be a good candidate for a protein required for apoptosis, however, it is not expressed in all cells undergoing programmed cell death, and is expressed at high levels in some cells not undergoing apoptosis [20]. Thus, the requirement for expression of TRPM-2 for apoptosis may be tissue/cell

type specific. The only function that has thus far been ascribed to TRPM-2 is the inhibition of complement-mediated lysis [20]. It may therefore act in certain systems *in vivo* to suppress inflammation by complement activation, but may not be required for the induction of programmed cell death.

The activities of tissue type and urokinase type plasminogen activators increases during programmed cell death in the regressing prostate and mammary gland, causing increased processing of plasminogen to plasmin (a serine protease) which in turn activates metalloproteases [20]. Plasmin and the metalloproteases are both involved in the degradation of basement membrane. The plasminogen activators are regulated by inhibitors I and II (PAI-1 and PAI-2). Glucocorticoids inhibit the induction of apoptosis in both of these glands, and decrease steady state levels of mRNA encoding the urokinase type plasminogen activator and increase expression of the PAI-1 gene, thereby presumably inhibiting activation of plasmin and metalloproteases and subsequent destruction of basement membrane. Cathepsin D is another protease whose activity increases during regression of the prostate, and may be involved in the protein degradation observed during programmed cell death [20]. Like TRPM-2, the activity of these proteases may be a secondary consequence of induction of apoptosis in certain tissues, and therefore may not be required for actual induction of apoptosis.

The increased expression of tissue transglutaminase is observed during prostatic and mammary gland involution, during apoptosis induced by glucocorticoid treatment of thymocytes, and during apoptosis induced by EGF-treatment of neonatal rat liver [20]. This enzyme cross-links proteins and may be responsible for the formation of the insoluble protein matrix detected in membrane bound vesicles in apoptotic cells, and is probably required to preserve the integrity of apoptotic bodies during programmed cell death.

Expression of poly (ADP-ribose) polymerase has been observed in regressing prostate and is known to be stimulated by DNA strand breakage [20]. This enzyme is bound to chromatin and catalyzes poly(ADP-ribosyl)ation of structural chromosomal proteins and nuclear enzymes. Several of these enzymes are involved in DNA repair, and it is possible that this polymerase is induced in a futile effort to repair the strand breakage observed during apoptosis. ADP-ribosylation is believed to inhibit a Ca^{2+}/Mg^{2+}-dependent endonuclease in certain cells, but it is unclear if this nuclease is involved in apoptosis [56]. Peak expression of poly (ADP-ribose) polymerase in regressing prostate occurs days after initiation of programmed cell death, so its role in apoptosis is unclear [20]. Several additional genes have been identified whose expression is induced by glucocorticoid treatment of murine thymocytes. These include the mouse VL30 retrovirus-like element, the murine homolog of chondroitin sulfate proteoglycan core protein, mitochondrial phosphate carrier protein, GP-70, and the autoantigens for Lupus and Graves diseases [57]. The increased expression of several genes whose functions are unknown have also been demonstrated during glucocorticoid-induced apoptosis of murine thymocytes, including one which shares similarity with G-protein-coupled receptors, another which shares similarity with a human placental-specific protein, another which encodes a protein containing a probable membrane spanning α-helix, and one which encodes a Zn^{2+}-finger sequence suggestive of a transcription factor [58–60].

Other investigators have reported that c-*myc*, c-*fos*, and c-*jun* expression increase during apoptosis in rat thymocytes, and the DNA binding activities of the transcription factors AP-1 and NF-κB increase (while those of Oct-1 and CREB decrease) [61, 62]. Glucocorticoid treatment of murine lymphosarcoma cells (P1798) causes decreased transcription of c-*myc*, thymidine kinase, thymidylate synthase, and ornithine decarboxylase [63, 64]. CEM human leukemia cells show rapid suppression of c-*myc* expression after glucocorticoid treatment, and in S49 murine lymphoma cells, c-*myc*, c-*myb*, and c-Ki-*ras* are all down-regulated [65, 66]. This may reflect a decrease in proliferation induced by glucocorticoid treatment rather than being specific for apoptosis. In human thymocytes and leukemic cell lines treated with glucocorticoids, increased synthesis of glutamine synthetase, the interleukin-1 receptor, insulin receptor, and β-galactoside binding protein has been observed [57, 67]. Eleven apoptosis-induced genes were identified by subtractive hybridization of cDNA from normal versus glucocorticoid-treated T-cells, but their identities have not been determined [58]. Another study identified an RNase which is activated after initiation of condensation in the prostate and liver [20]. The Yb1 subunit of glutathione-S-transferase, RVP.1, RSG-2 and RSG-8, glutathione-S-transferase (GST), matrix carboxyglutamic acid, and γ-actin are expressed in the pre-condensation phase of apoptosis in the prostate and mammary gland [19, 20]. GST expression is also increased in serum-starved NIH 3T3 cells which are not undergoing apoptosis, suggesting that expression of this gene is not specific for apoptosis [19]. The function of any of these gene products during apoptosis remains unknown.

Clearly, the need for activation or repression of expression of certain genes for induction of apoptosis varies among types of cells (and probably depends on the inducer of apoptosis). Many of these genes may act in signal transduction pathways which are required for induction of apoptosis in certain cell types but not in others. In all cell types, these pathways culminate in a set of morphological and biochemical changes leading to cell death. Much further investigation is required to identify common pathways for induction of apoptosis.

Suppression of glucocorticoid-induced apoptosis

Over the past several years, interest in the ability of certain cells to repress apoptosis by the expression of specific proteins has increased. Bcl-2 is such a protein, and was originally identified by the location of its gene at the translocation breakpoint between chromosomes 14 and 18 in many human follicular B cell lymphomas [68]. Bcl-2 is an integral membrane protein which is found in greatest abundance in the inner mitochondrial membrane [69]. Expression of the *bcl*-2 gene has been demonstrated to protect a variety of murine lymphoid cell lines from apoptosis induced by glucocorticoids and several other compounds, but does not reduce the expression of glucocorticoid receptor, nor does it affect the ability of glucocorticoid to suppress proliferation in such cells [18, 70]. Bcl-2 does not afford protection under all conditions which are able to induce apoptosis, as demonstrated in a study in which *bcl*-2 expression in several cell lines was insufficient to protect against apoptosis induced by interleukin deprivation [71]. Several investigators have suggested that this protein could be involved in nuclear transport, since it seems to be associated with nuclear pores in some cases, or that it might act in an antioxidant pathway, but definitive data demonstrating how Bcl-2 inhibits programmed cell death does not exist [72, 73].

Bcl-2 almost completely blocks apoptosis induced by dexamethasone in murine S49 thymoma cells without altering the ability of this steroid to inhibit proliferation [18]. Figure 2 demonstrates that treatment of S49-neo cells and the same cells in which Bcl-2 is constitutively expressed with increasing concentrations of dexamethasone decreases cellular proliferation equivalently in both cell types. This treatment does not, however, decrease the viability of the cells expressing Bcl-2, while that of the S49-neo cells decreases precipitously. These data demonstrate that glucocorticoids are able to affect those metabolic pathways involved in cell growth in S49 cells without affecting those involved in induction of apoptosis (implying that these pathways are different), that apoptosis can be separated from growth suppression in this cell type, and that Bcl-2 does not act by altering the growth potential of cells. For the first time, the antiproliferative effects of glucocorticoids have been separated from their apoptotic effects.

Several genes which share homology with *bcl*-2 have now been identified. These include *bcl*-x and *bax* [74, 75]. The Bax protein can heterodimerize with Bcl-2, and thereby modulate the ability of Bcl-2 to suppress apoptosis [75]. Although definitive data explaining how Bcl-2 inhibits apoptosis remains elusive, it is clear that its function is regulated by interaction with other proteins.

In conclusion, the regulation of cellular proliferation and death by steroid hormones is complex and exceedingly important. The correct implementation of the apoptotic pathway requires a balance between cellular signals for proliferation and death. Few genes expressed during apoptosis in different tissues are the same (other than some common nucleases), thus, several different pathways culminating in apoptosis must exist in various cells. The complexity of the genes which can suppress apoptosis complicates analysis of these pathways. Steroids regulate these processes in many normal tissues, and continue to afford an excellent model for studies of apoptosis.

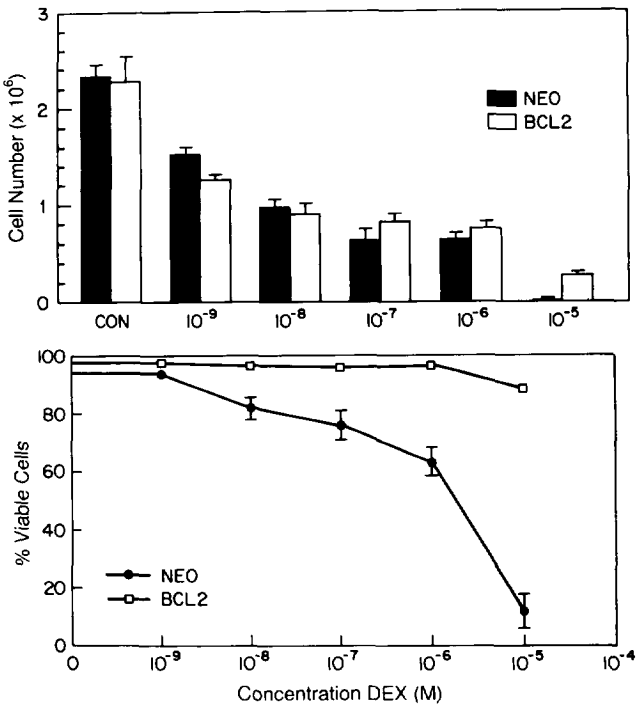

Fig. 2. The effect of dexamethasone on cell number and viability of S49-neo cells and S49-Bcl-2 cells. Murine S49 thymoma cells were stably transfected with a recombinant amphotropic retrovirus carrying a G418 antibiotic resistance gene alone (S49-neo) or in combination with a *bcl*-2-cDNA. These cells were grown and prepared for experiments as described in Fig. 1, and then treated with increasing concentrations of dexamethasone for 24 h, and counted for cell number and viability. Top panel: total viable cells/ml vs DEX concentration. Bottom panel: % cell viability vs DEX concentration. [From L.-A. M. Caron-Leslie *et al.*, *FASEB J.* 8 (1994) p. 639. With permission].

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