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An elevated *bax/bcl-2* ratio corresponds with the onset of prostate epithelial cell apoptosis

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Abstract

The prostate gland in adult male rats is highly dependent on androgenic steroids. Castration initiates the regression of this tissue through a process involving the loss of the vast majority of cells by means of apoptosis. We studied this well characterized *in vivo* model of apoptosis to evaluate how the expression of two particular gene products, *bcl-2* and *bax*, known to be important for the regulation of apoptosis were affected by castration. An RNase protection assay designed to quantify the levels of *bax* mRNA showed that this transcript was transiently elevated after castration, reaching a peak in expression at 3 days and declining thereafter. In contrast, *bcl-2* mRNA expression was continuously elevated over a period of up to 7 days after castration. The distinct changes in the expression of the mRNAs encoding these two genes were confirmed by an *in situ* hybridization analysis of regressing rat ventral prostate tissues. The elevation in mRNAs were apparently restricted to the secretory epithelial cells of the gland, the cellular compartment of the tissue most affected by castration. Finally, SDS–PAGE/Western blot analysis of *bax* and *bcl-2* protein expression in the regressing rat prostate gland with *bax* and *bcl-2*-specific antibodies showed that the changes in the *bax* and *bcl-2* protein levels were similar and consistent to that found for the mRNAs. In summary, the expression of both *bax* and *bcl-2* gene products are uniquely modulated during castration-induced regression of the rat ventral prostate gland. The changes we observed identify a transient but marked increase in the *bax/bcl-2* expression ratio of the tissue that peaks on the second and third days after castration, coinciding with the peak periods of prostate cell apoptosis. These data support previous studies done on *in vitro* systems wherein it was shown that the *bax/bcl-2* ratio determines the apoptotic potential of a cell.

Keywords: prostate; castration; apoptosis; *Bax*; *Bcl-2*

Abbreviations: RPA, RNase Protection Assay; DEP, diethylpyrocarbonate; PBS, phosphate buffered saline; TBS, Tris-buffered saline

Introduction

Bcl-2 and *bax* are two discrete members of a gene family involved in the regulation of cellular apoptosis. Though these two genes share partial nucleotide sequence homology and their encoded polypeptides have critically conserved amino acid sequences in two functional domains, their individual products appear to have opposing functions with regards to their effects on the apoptotic sensitivity of a cell.[1] Extensive experimentation involving the manipulation of *bcl-2* and *bax* expression in cultured cells has revealed that the *bcl-2* protein can be functionally characterized as an apoptosis-suppressing factor[2,3] whereas the *bax* protein is more functionally characterized as an apoptosis-promoting factor.[4] Based on the results of this type of experimentation and the apparent antagonistic action these proteins exert in apoptosis control, they have described as a cellular 'rheostat' of apoptosis sensitivity in the sense that the intracellular ratio of *bax*/*bcl-2* protein can profoundly influence the ability of a cell to respond to an apoptotic signal.[3,4] According to this concept, a cell with a high *bax*/*bcl-2* ratio will be more sensitive to a given apoptotic stimuli when compared to a similar cell type with a comparatively low *bax*/*bcl-2* ratio.

To evaluate this concept *in vivo*, we studied *bcl-2* and *bax* expression in a well-described model of *in vivo* apoptosis, the regressing rat ventral prostate gland. In this model, extensive prostate epithelial cell apoptosis is predictably induced by androgenic steroid depletion therapy (usually simple castration) of a mature male rat.[5–7] Most of the cells of the mature rat prostate gland are dependent on androgenic steroids for viability and up to 85% of the cells of the gland will be lost by apoptosis during the 2 weeks following castration.[8] The daily apoptosis rates in regressing rat prostate tissues have been previously quantified by directly counting the apoptotic bodies and by measuring the loss of DNA from this tissue.[9,10] These studies consistently show that prostate epithelial cell loss by apoptosis begins approximately 24 h after castration, and peaks by the third day after castration, declining thereafter. The relative synchrony in the onset of apoptosis in this tissue has previously

enabled the characterization of some molecular events that appear to accompany apoptotic signaling in the rat prostate gland, including transient induction of proto-oncogenes (c-*fos*, c-*myc*), heat shock response molecules (hsp-70), sulfated glycoprotein-2 and tumor suppressor protein, p53.[11–13] Here, we describe our experience utilizing several different molecular techniques to determine whether the expressions of the *bax* and *bcl-2* gene products are modulated during the onset of apoptosis in the regressing rat ventral prostate gland. Our analysis of *bax* and *bcl-2* expression show that these gene products are differentially regulated during castration-induced regression of the rat ventral prostate gland in a manner that supports the concept that an increase in the cellular ratio of *bax*/*bcl-2* protein accompanies the onset of apoptosis in this tissue *in vivo*.

Results

Expression of *bcl-2* and *bax* mRNA in the regressing rat ventral prostate gland

Adult male rats (n=3) were surgically castrated and were sacrificed to obtain the regressing ventral prostate tissues. The tissues were frozen then processed to extract total RNAs. The RNAs from each time point were analyzed by means of an RNase Protection Assay (RPA) to evaluate the time-dependent expression of mRNAs encoding *bax* or *bcl-2*. The radiolabeled antisense RNA probes utilized in this assay will protect a 353 nucleotide fragment from *bax* mRNA or a 452 nucleotide fragment of *bcl-2* mRNA from RNase degradation following hybridization. Autoradiography of the acrylamide gel utilized for the RPA analysis showed that *bax* mRNA was transiently upregulated after castration (Figure 1a), reaching a peak of expression at 3 days after castration and declining thereafter to day 6. LIkewise, autoradiography of the acrylamide gel used to examine protected *bcl-2* transcript fragments showed that *bcl-2* mRNA expression was also significantly upregulated after castration. However, in contrast to the transient upregulation of *bax* mRNA, *bcl-2* mRNA was induced in a more continuous manner throughout the 7 days following castration (Figure 1b). The individual bands corresponding to the 353 bp fragment protected by the *bax* probe and the 452 bp fragment protected by the *bcl-2* probe were excised from the gel and the radioactivity in each band was counted in a scintillation spectrophotometer. The radioactive counts of the individual bands allowed us to quantitatively determine the relative mRNA levels for *bax* and *bcl-2* after castration (Figure 1). This evaluation revealed that, *bax* transcripts increased after castration to a level approximately 13-fold higher than in control, (unoperated) rats by the third day after castration, and subsequently decline back to near control levels by the sixth day after castration. In contrast, *bcl-2* mRNA levels accumulate continuously after castration, reaching a level of approximately 13-fold higher than control levels at 6 days after castration. Comparing the relative *bax* mRNA expression level to the *bcl-2* mRNA expression level over the 6 day period after castration (expressed as a relative *bax*/*bcl-2* ratio) we found that the *bax*/*bcl-2* ratio dramatically but transiently increases to a maximum on day 2 after castration and declines thereafter (Figure 2).

In situ hybridization confirms upregulation of *bax* and *bcl-2* mRNA in regressing rat ventral prostate tissue

To confirm changes in *bax* and *bcl-2* mRNA expression and to localize the cells in which the differential regulation of these genes was occurring, we performed an *in situ* hybridization analysis of regressing rat ventral prostate tissues for *bax* and *bcl-2* mRNA expression. Ventral prostate glands were obtained from control, unoperated rats or from rats at various daily intervals after castration. Tissue sections were hybridized to digoxigenin-labeled sense or antisense riboprobes for *bax* or *bcl-2* mRNA. After washing hybridization of digoxigenin-labeled probe to the tissue was evaluated with an anti-digoxigenin antibody and an alkaline phosphatase-based detection system. Tissue sections that were hybridized under equivalent conditions to the sense riboprobes (for *bax* or *bcl-2*) did not show any significant alkaline phosphatase staining in this assay (not shown). In contrast, in sections in which the *bax* antisense riboprobe was utilized in the

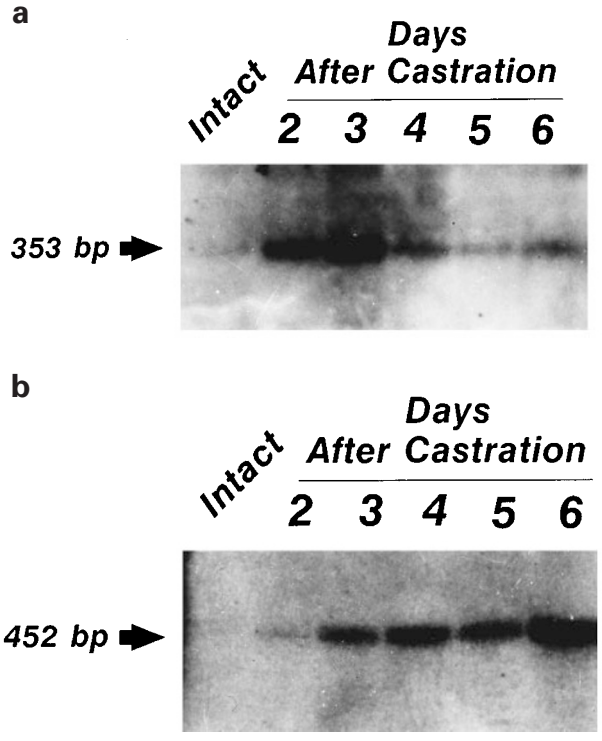

Figure 1 RNase protection assays identify upregulation of *bax* and *bcl-2* mRNAs in the regressing rat ventral prostate glands. Total RNA was extracted from ventral prostate glands from control (uncastrated rats) and from rats at sequential daily intervals after castration (days 2–6). These RNAs were tested in an RPA to evaluate the relative expression of *bax* or *bcl-2* mRNA at different times after castration. (**a**) Autoradiography of acrylamide gel utilized for *bax* RPA shows a transient increase of the 353 base protected *bax* mRNA fragment peaking on day 3 and declining thereafter. (**b**) Autoradiography of acrylamide gel utilized for *bcl-2* RPA shows a continuous increase of the 452 base protected fragment of *bcl-2* mRNA over a 6-day period after castration

hybridization reaction, we could identify a very low but continuous level of hybridization to the epithelial cell compartment of the control prostate gland. The hybridization was much more intense on the sections obtained from a 3-day castrated rat. Likewise, the *bcl-2* antisense riboprobe was found to hybridize to the prostatic epithelial cell compartment in the control rat prostate gland in a pattern identifying a similar low level expression of *bcl-2* mRNA to clusters of cells throughout the epithelium (Figure 3b). Some epithelial cells did not show significant hybridization. This pattern was markedly different when compared to a 10-day castrated rat prostate where hybridization was very intense in all the epithelial cells of the regressed prostate gland.

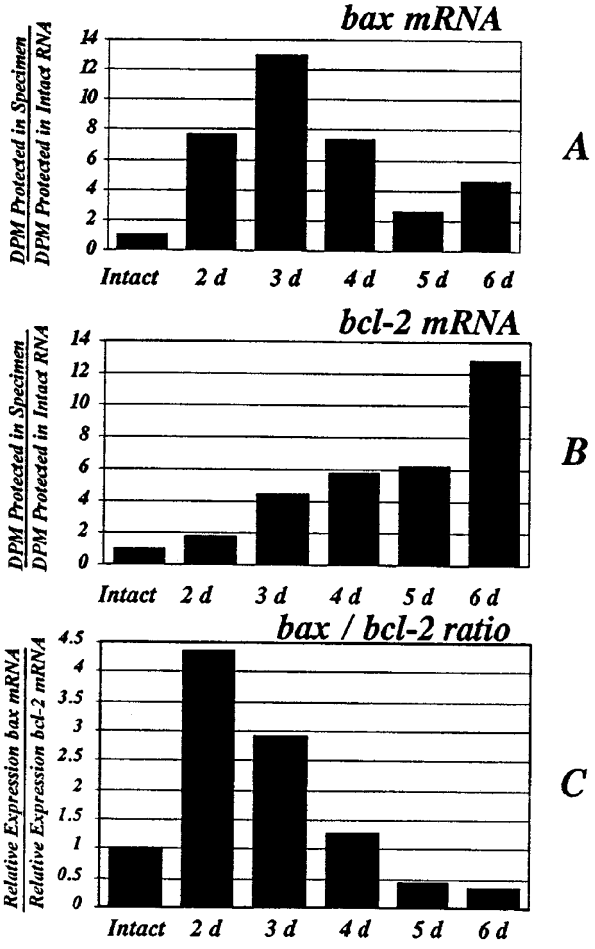

Figure 2 Graphical description of the expression of *bax* and *bcl-2* mRNA during castration-induced regression of the rat ventral prostate gland. (**A**) Bands corresponding to protected *bax* mRNA fragments in the RPA assay were individually cut from the acrylamide gel and counted by scintillation spectrophotometry. Each time point shows a relative comparison of *bax* riboprobe DPMs protected at any given time after castration to the *bax* riboprobe DPMs protected in the control (intact rat) prostate RNA. Bax expression increases approximately 13-fold on the second day after castration and decreases thereafter. (**B**) Bands corresponding to protected *bcl-2* mRNA fragments in the RPA assay were individually cut from the acrylamide gel and counted by scintillation spectrophotometry. Each time point shows a relative comparison of *bcl-2* riboprobe DPMs protected at any given time after castration to the *bcl-2* riboprobe DPMs protected in control (intact rat) prostate RNA. (**C**) Relative *bax*/*bcl-2* mRNA expression ratio during regression of the rat ventral prostate gland. Bars identify change in expression of *bax versus* change in expression of *bcl-2* in ventral prostate RNAs at each time point after castration. The relative *bax*/*bcl-2* mRNA ratio reaches a maximum (4 : 43) at 2 days after castration and declines thereafter

Western blot analysis of *bax* and *bcl-2* protein expression in regressing rat ventral prostate tissue

Lysates were prepared from frozen rat ventral prostate tissues obtained at sequential daily intervals after castration and aliquots of these lysates containing equivalent amounts of protein were co-electrophoresed on an SDS polyacrylamide gel. The proteins in the gel were transferred to a nitrocellulose filter by electroblotting techniques and the Western blot was probed with an antibody against *bax* or *bcl-2*. A chemiluminescent detection system was utilized to identify binding of the anti-*bax* or anti-*bcl-2* antibody. Figure 4, top, shows Western blot detection of the bax protein product in lysates of prostates obtained from control (intact) rats or rats at 3 or 7 days post-castration. A strong band of reactivity at 21 kd, the observed size of the *bax* gene product, was present in the control extract and was highly amplified in the extract obtained from the 3-day castrated rat. Similar to our observations on bax mRNA expression, expression of the 21 kd *bax* protein in a lysate from a 7-day castrated rat was much lower than in the 3-day castrated rat, and even lower than control levels. In contrast to *bax* protein expression, the 25 kd *bcl-2* protein appears to be continuously enriched over a 7 day period after castration in protein lysates from regressing rat prostates (Figure 4, bottom), once again, consistent with the expression of the *bcl-2* mRNA as was determined by RPA.

Discussion

In this study we utilized a variety of molecular analytical techniques to evaluate whether the expression of the rat *bax* or *bcl-2* gene were significantly modulated during regression of the rat ventral prostate gland after castration. RPA analyses showed that *bax* mRNA expression is rapidly, but transiently elevated in the tissue, reaching a peak expression on the third day after castration and declining thereafter. In contrast, *bcl-2* mRNA expression appears to be continuously induced during the 7 day period of observation after castration, a situation that has been previously observed by others.[14] These mRNA expression patterns were confirmed by an *in situ* hybridization analysis using digoxigenin-labeled riboprobes to detect *bax* or *bcl-2* mRNA in the ventral prostate gland. Moreover, our *in situ* evaluation of *bax* and *bcl-2* mRNA expression allowed us to localize the changes to the secretory epithelial cell compartment of the ventral prostate gland, the cells that are known to be the most affected by castration. The expression of *bcl-2* mRNA throughout the epithelial layer of the regressed rat ventral prostate gland (ten days after castration) differs remarkably from the control (intact) tissue both with regards to its distribution (in striking contrast to sporadic clumps of *bcl-2* hybridizing epithelial cells in the control tissue) and with regards to the significant intensity of the hybridization signal (in contrast to the low level signal in control prostate epithelial cells). The primary observation that *bcl-2* expression is changed from individual or scattered clumps of epithelial

cells throughout the intact prostate gland to a continuous distribution throughout the regressed prostate suggests that *bcl-2* expressing epithelial cells originally present in the rat prostate might be selected for by castration. This concept is highly supported by the known expression of *bcl-2* in prostate basal cells[19] (scattered throughout the epithelium of the normal rat prostate) and by the accumulation of basal epithelial cells (subsequent to the loss of the luminal epithelial cells) in the fully regressed rat prostate gland. The drastically increased intensity of the *in situ* hybridization signal for *bcl-2* in the regressed prostate epithelium, however, also suggests that some form of *bcl-2* expression upregulation is involved in the transition from the normal to the regressed rat prostate epithelium following castration. Finally, we have shown here by Western blot analytical procedures that the changes in *bax* and *bcl-2* mRNA expressions ongoing in the regressing rat ventral prostate gland are reflected by similar changes in the protein products of these genes.

There are three specific findings from this study that deserve further comment: (1) our results indicate that the *bax* and the *bcl-2* genes are differentially regulated during regression of the rat prostate gland; (2) the ratio of *bax*/

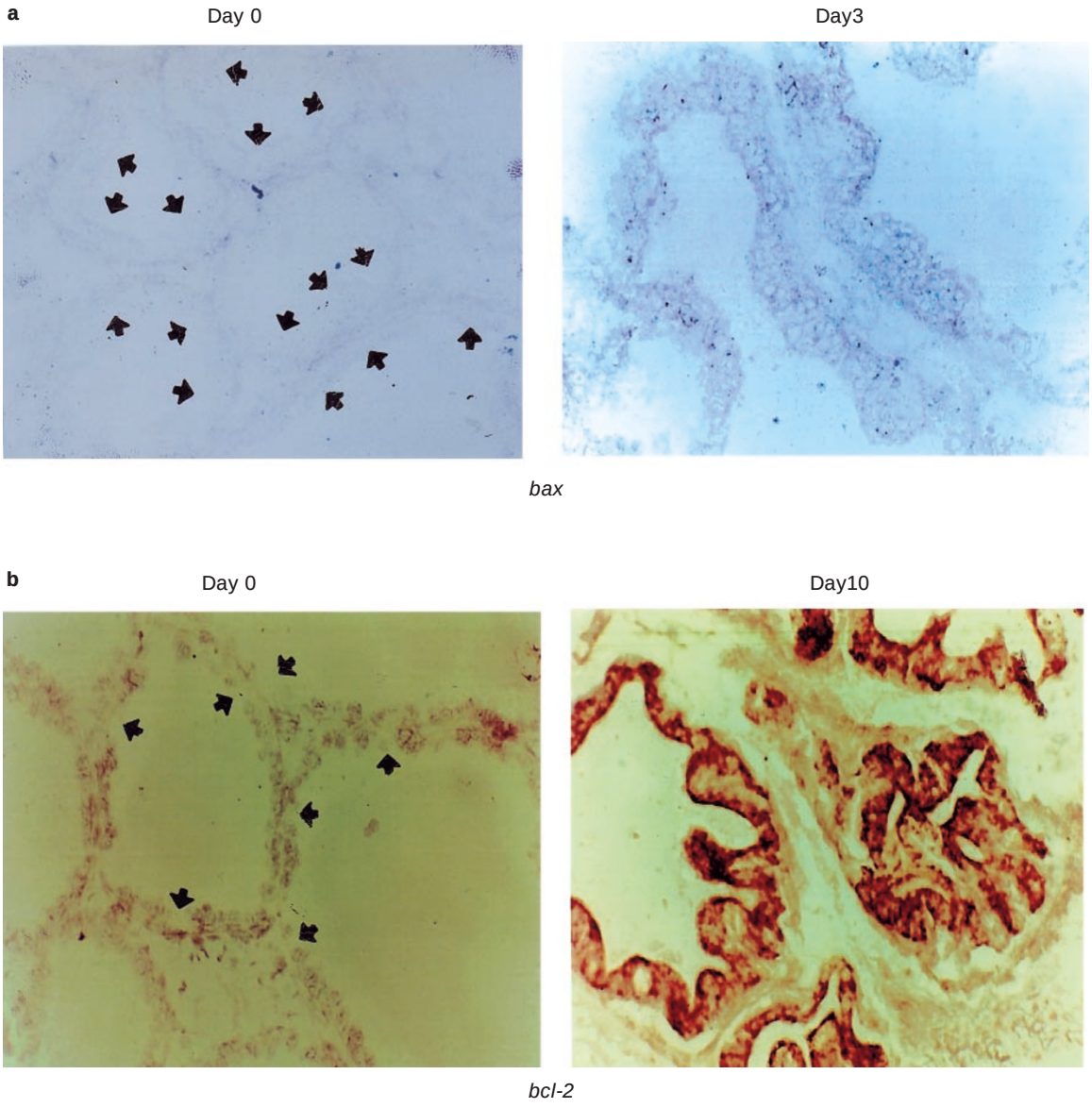

Figure 3 *In situ* hybridization analysis for the expression of *bax* and *bcl-2* mRNAs in control and regressing rat ventral prostate glands. (**a**) Hybridization of an antisense riboprobe for rat *bax* identifies low level expression in the epithelial cell compartment of the intact rat ventral prostate (left side). Arrows identify sites of epithelial cell layers. The intensity of the hybridization signal increases significantly in ventral prostate tissue obtained from a 3 day castrated rat (right side). (**b**) Hybridization of an antisense riboprobe for rat *bcl-2* identifies low level expression in clusters of epithelial cells in the intact rat ventral prostate (left side). Arrows identify sites on the epithelium where epithelial cells are present but do not stain for *bcl-2* mRNA. The intensity of the hybridization signal increases significantly and occurs over all residual epithelial cells in ventral prostate tissue obtained from 10 day castrated rats (right side)

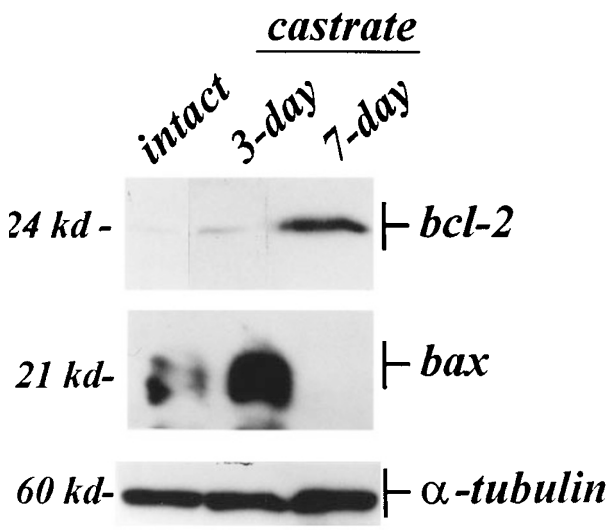

Figure 4 Western blot analysis for the expression of *bax* and *bcl-2* protein in protein lysates made from regressing rat ventral prostate tissues. Top panel: Immunochemical detection of the 25 kd *bcl-2* protein in extracts from rat ventral prostate glands of control (intact) rats, or at 3 or 7 days after castration. *Bcl-2* protein continuously increases over this 7 day period. Middle panel: Immunochemical detection of the 21 kd *bax* protein in extracts of control (intact) rat ventral prostate or at 3 or 7 days after castration. *Bax* protein is drastically increased at 3 days after castration but becomes undetectable in the 7 day specimen. Bottom panel: same blot reprobed for the presence of the 60 kd α-tubulin protein shows even distribution of this protein throughout all extracts

bcl-2 gene products in the ventral prostate gland becomes the highest at the peak onset of apoptosis in this tissue; and (3) the prostate epithelial cells that survive castration maintain very high *bcl-2* levels when compared with the population of prostate epithelial cells that were present in the tissue prior to castration.

With regards to the first observation, the fact that simple androgen depletion of the rat drastically alters the expression of *bax* and *bcl-2* within the prostate gland, suggests that there is a mechanism within the susceptible cell population of the tissue by which androgenic steroid stimulation can ultimately affect the expression of these two gene products. It is of interest that a prior study of a human malignant prostatic epithelial cell line, LNCaP, that maintains the androgen regulatory response system showed that removel of androgens from the medium of these cells grown *in vitro* will chronically upregulate *bcl-2* expression, similar to what we observed here for rat prostate epithelial cells *in vivo*.[15] No similar analysis has been performed to date for *bax* expression.

Oltvai and his colleagues[4] initially proposed the paradigm of *bax* and *bcl-2* interaction in the regulation of cellular apoptotic sensitivity. Their hypothesis was derived from the results of experimental studies wherein *bax* and *bcl-2* expression was modulated by genetic manipulation of cultured cells[16] and by immunoprecipitation studies wherein it was shown that *bax* and *bcl-2* can bind to each other to form heteroduplexes.[4,17] The remarkable observation of a considerably increased *bax*/*bcl-2* ratio associated with the peak period of apoptosis in the regressing rat ventral prostate gland highly supports the contention originally put forth by these researchers and suggests that *bax* and *bcl-2* gene products are involved in the androgen regulation of apoptosis of the rat prostate epithelial cell.

Finally, there is a great interest in defining the gene products that control the apoptotic response of the prostate epithelial cell to androgen deprivation because of the enormous health problem posed by prostate cancer in Western society. There is a fairly simple treatment for this disease, castration, that works because, just like the normal cells of the prostate gland, most prostate cancer cells are also dependent on androgenic steroids and will undergo apoptosis in their absence. Inevitably, however, this therapy is only transient and palliative because some fraction of prostate cancer cells are able to resist apoptotic initiation in an androgen deprived environment and it is these hormone resistant cells that go on to cause the extensive morbidity and mortality associated with prostate cancer. Genetic manipulations of prostate cancer cell lines have shown that increased *bcl-2* expression can induce a hormone independent phenotype in these cells.[18] Several surveys of human prostate tissues have already identified a unique pattern of *bcl-2* expression that correlates with an increase associated with the progression of prostate cancer to the hormone independent state.[14,19–21] The experiments presented in this study continue to support the involvement of *bcl-2* expression in the generation of hormone resistance of prostate cancer by showing that the epithelial cells of the normal rat prostate gland that express *bcl-2* might be selected for survival post-castration as well as by showing that those epithelial cells that survive castration also upregulate and maintain a high *bcl-2* expression.

Materials and Methods

Laboratory animals and tissues

Male Sprague Dawley rats (325–350 g) were obtained from Camm, Inc. (Camden, NJ, USA) and were surgically castrated under anesthesia as previously described.[11] At sequential daily intervals after castration, groups of rats were euthanized by sodium pentobarbitol overdose and the ventral prostate glands were recovered and frozen in liquid nitrogen for RNA/protein extraction or for cryosectioning. These tissues were stored at −80°C until processing.

Construction of riboprobe expression vectors for rat *bax* and *bcl-2*

cDNA fragments for rat *bax* or *bcl-2* were amplified from reverse-transcribed RNA extracted from a rat ventral prostate gland utilizing oligonucleotide primers designed with the assistance of a primer-analysis software program (Oligo) applied to the sequence of human *bcl-2* and human *bax* cDNAs present in genbank.[4,22] The 353 bp *bax* cDNA fragment and 452 bp *bcl-2* cDNA fragment were directly cloned into the TA cloning vector (Invitrogen, Inc., San Diego, CA, USA) and the inserts were sequenced by standard dideoxynucleotide methods to confirm *bax* and *bcl-2* identity of the cDNAs as well as to determine orientation of the cDNA insert with regards to the T7 and SP6 promotors flanking the insert. The *bax* cDNA expression vector was termed *rbax1* and the *bcl2* cDNA expression vector is termed *rbc1*.

RNA extraction and the RNase protection assay

Total RNA was extracted from pulverized frozen prostate tissues (three per time point) utilizing the RNAzole B reagent (Tel Test, Inc., Friendswood, TX, USA) as previously described.[13] Lyophilized pellets of RNA were resuspended in diethylpyrocarbonate- (DEP)-treated water and RNA concentration was determined by spectrophotometry at 260 nM.

Labeled antisense riboprobes were synthesized, *in vitro*, from linearized expression plasmids, *rbax1* or *rbcl1*, utilizing T7 or SP6 RNA polymerase in the presence of ^{32}P-UTP, as previously described.[13] The RNA probes synthesized in these reactions were electrophoresed on acrylamide gels and the full length labeled antisense riboprobes were excised from the gel and were eluted into a TE buffer. Aliquots containing 2×10^5 c.p.m. of antisense riboprobe were added to 20 μg of test RNA extracted from control or castrated rat ventral prostate glands and the mixtures were co-precipitated with ethanol at -20°C. RNA pellets were resuspended in a small amount of hybridization buffer, heated to 65°C for 5 min then incubated overnight at 42°C. Reactions were then digested with RNase A/T, as previously described[13] and the digests were applied to 8% acrylamide sequencing gels for electrophoresis. Control reactions included probe alone, with or without RNase digestion. The resulting gel was exposed to X-ray film to produce an autoradiograph. Developed films were subsequently utilized as a template to excise the individual labeled bands from the gel and each band was placed in a vial containing scintillation fluid (ScintiVerse, Fischer Scientific) and the radioactivity associated with individual bands was counted by scintillation spectrophotometry (Beckman LSC 2800, Beckman Instruments, Inc.).

In situ hybridization analysis

Digoxigenin-labeled riboprobes for *bax* and *bcl-2* were synthesized from *rbax1* and *rbcl1* plasmids using T7 or SP6 transcription reaction buffers in the presence of digoxigenin-UTP (dig, Boehringer Mannheim, Indianapolis, IN, USA). Probes were quantified by a dot-blot analysis. Ventral prostates obtained from control rats (uncastrated), and from rats at 3 and 10 days after castration were frozen over liquid nitrogen and sectioned with a microtome in a cryostat. The sections were fixed with 4% paraformaldehyde and were then washed three times in DEP-treated water. Sections were treated with 10 μg/ml Proteinase K and were then washed in 0.2% glycerol in PBS. Sections were prehybridized in a 20$\times$ Denhardt solution containing 250 μg/ml of yeast tRNA for 4 h and the prehybridization solution was replaced with a hybridization solution containing 50% formamide and 10 ng/ml sense or antisense riboprobe for *bax* or *bcl-2*. After overnight incubation in a humidified chamber at 42°C, sections were washed in a successive series of solutions containing decreasing amounts of SSC (from 2$\times$ SSC to 0.1$\times$ SSC) at 50°C.

The hybridization of the dig-labeled probe was detected by immunohistochemical analysis of sections with an anti-digoxigenin antibody (Boehringer Mannheim, Inc., Indianapolis, IN, USA) diluted 1 : 250 in blocking reagent. After 1 h of incubation, slides were washed and then incubated with substrate (37.5 μg/ml nitroblue tetrazolium salt and 17.5 μg/ml 5-bromo-5 chloro-3-indolyl-phosphate) for 1 h at pH 9.0. Slides were washed in water and mounted under crystal mount for microscopy.

Preparation of prostate protein lysates and Western blot analysis of *bax*, *bcl-2* proteins

Frozen tissues from control or castrated rat prostates were pulverized and suspended in RIPA lysis buffer containing protease inhibitors prior to homogenization with a Polytron tissue homogenizer. The homogenate was centrifuged at 10 000$\times g$ for 15 min. The supernatant was analyzed for protein concentration by the Bio-Rad protein assay and aliquots containing 100 μg of protein were suspended in SDS–PAGE sample buffer. Specimens were boiled then co-electrophoresed in 12% SDS-polyacrylamide gels and the proteins in the gels were transferred to nitrocellulose filters by an electrotransfer device to produce Western blots. The blots were blocked in a solution containing 10% nonfat milk in Tris-buffered saline (pH 7.6) (TBS), washed repeatedly in TBS then incubated for 2 h with a rabbit polyclonal anti-*bcl-2* antibody or with a rabbit polyclonal antibody against *bax* (Santa Cruz Biochemicals, Inc., Santa Cruz, CA, USA). Binding of the primary antibodies to the Western blots was detected by secondary sheep anti-rabbit IgG conjugated to horseradish peroxidase in an ECL Western blot Detection System (Amersham, Inc., Arlington Heights, IL, USA). The blot was exposed to Kodak XAR-5 X-ray film to identify bands.

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