Overexpression of bcl-2 Protects Prostate Cancer Cells from Apoptosis in Vitro and Confers Resistance to Androgen Depletion in Vivo

Anthony J. Raffo, Harris Perlman, Min-Wei Chen, Mark L. Day, Jack S. Streitman, and Ralph Buttyan

ABSTRACT

Normal (nonneoplastic) human prostatic secretory epithelial cells do not express the bcl-2 protein. However, a recent immunohistochemical survey of neoplastic human prostate tissues showed that a fraction of primary untreated prostate adenocarcinomas cells expressed this apoptosis-suppressing oncoprotein at significant levels (Colombel et al., Am. J. Pathol., 143: 390–400, 1993). Additionally, a number of hormone-refractory prostatic adenocarcinomas obtained from hormonally-treated patients (subsequent to surgical or drug castration therapy) were examined and were found to be uniform in their elevated expression of bcl-2 oncprotein. The results of this preliminary survey imply that bcl-2 expression distinguishes a subgroup of primary human prostate cancers and that the expression of this protein might be a factor enabling prostate cancer cells to survive in an androgen-deprived environment. The current study was undertaken to determine the degree to which overexpression of bcl-2 can protect human prostate cancer cells from apoptotic stimuli in vitro and in vivo. Human prostate cancer cells (LNCaP) were transfected with a neomycin-selectable eucaryotic expression vector containing cDNA encoding human bcl-2. Transfected clonal variants that express bcl-2 protein (LNCaP/bcl-2) were unaltered with regard to their basal growth rate in 10% serum-containing medium, or with regard to their expression of the differentiated human prostate cell gene products prostate-specific antigen or androgen receptor protein. The bcl-2-transfected clones were altered, however, with regard to their growth rate in charcoal-stripped serum lacking dihydrotestosterone. Additionally, in contrast to the parental or control-transfected cell lines, LNCaP/bcl-2 cells were highly resistant to a variety of apoptotic stimuli in vitro including serum starvation and 10 mM phorbol ester (phorbol 12-myristate 13-acetate) supplementation of the medium. Lastly, the overexpression of bcl-2 by these prostate cancer cells altered their tumorigenic potential in a nude mouse assay. s.c. injections of 106 LNCaP/bcl-2 cells into male nude mice resulted in earlier and larger tumor formation compared to an equivalent injection of parental or control-transfected LNCaP cells. When these variant cell lines were injected into castrated male nude mice, only the LNCaP/bcl-2-transfected cells gave rise to tumors. Moreover, LNCaP/bcl-2 tumors grown in intact male nude mice were refractory to the growth-inhibiting effects of castration demonstrated by parental LNCaP cells. Data obtained in this study demonstrate that the bcl-2 oncprotein can protect prostate cancer cells from apoptotic stimuli in vitro and suggest that such protection correlates with the ability to form hormone-refractory prostate tumors in vivo.

INTRODUCTION

Therapy for the advanced form of prostate cancer generally involves either surgical gonadectomy to remove the major source of androgens or drug treatments that suppress androgen production and action. These treatments are at least palliatively effective because they can initiate apoptosis of prostate cancer cells. Unfortunately, some fraction of the prostate cancer cells inevitably survives these therapies and continues to grow. It is clear that these cells have a means of avoiding or inhibiting the cellular mechanism that activates the cell death pathway so intrinsic to normal prostate epithelial cells after androgen withdrawal. Therefore, our ability to comprehend the mechanism of this apoptotic inhibition and overcome it has significant ramifications for the development of new and more effective therapies to successfully treat this malignancy.

On the basis of the rapidly increasing knowledge of the genetic components involved in the apoptotic pathway, we recently screened a large number of human prostatic tissues to characterize whether any of these tissues expressed the bcl-2 oncprotein, a potent and broad-spectrum suppressor of apoptosis (1, 2). The results of this immuno-histochemical survey showed that no normal cells of the adult human prostatic secretory epithelium expressed this protein. In contrast, a subset of primary prostatic adenocarcinomas obtained from untreated prostate cancer patients positively immunostained for bcl-2, as did all of the hormone-refractory prostatic adenocarcinomas obtained from hormone-treated patients (3). This intriguing pattern establishes the potential involvement of bcl-2 in the developmental pathway of prostate cancer leading to hormone resistance. The results that we obtained by experimental manipulation of bcl-2 expression in prostate cancer cells, presented here, further support this hypothesis.

When exogenously expressed in cultured cells and in transgenic mouse systems, bcl-2 has been shown to protect against apoptotic stimuli as diverse as chemotherapeutic agents and growth factor deprivation (1, 2, 4–6). This protection extends to certain forms of hormone-activated apoptosis, most prominently, glucocorticoid-induced apoptosis of thymocytes (7). To date, the question of whether bcl-2 expression can protect endocrine-dependent cells from hormone withdrawal has not yet been established. To test this for prostate cells, we genetically manipulated a commonly used prostate cancer cell line, LNCaP, so that these cells would overexpress the human bcl-2 protein. This cell line is a popular model for the study of prostate cancer (8, 9) because it retains some of the most prominent differentiated features of the human prostate cell, including the production of the prostate secretory protein, PSA3 (10), the prostate-specific membrane antigen (11), and the AR (12). Moreover, LNCaP cells have proven to be growth responsive to androgenic steroids in vitro (13). Additionally, these cells show an androgen-dependent phenotype in vivo with regard to their ability to form tumors in male but not castrated male nude mice (8, 9).

In this series of experiments, we examined whether exogenously expressed bcl-2 protein can alter the expression of the differentiated prostate gene products or the basal growth rate of LNCaP cells. Furthermore, we tested whether enhanced bcl-2 expression could alter the LNCaP cell growth response to DHT or the apoptotic response of these cells to serum withdrawal or phorbol ester supplementation in vitro. Finally, the results of a study of LNCaP tumor formation in intact and castrated male nude mice strongly support the hypothesis.

1 The abbreviations used are: PSA, prostate-specific antigen; FBS, fetal bovine serum; TPA, phorbol 12-myristate 13-acetate; AR, androgen receptor; DHT, dihydrotestosterone.

Received 4/4/95; accepted 8/2/95.

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1 This work was supported by United States Public Health Service Grants NIH-CA58089 and NIH-CA47848, as well as grants from the CaP Cure Foundation and the Koch Foundation. M.W. C. is a research fellow of the American Foundation for Urological Disease.

2 To whom requests for reprints should be addressed, at Department of Urology, Columbia University, College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.
that enhanced bcl-2 expression can make prostate cancer cells resistant to androgen deprivation in vivo.

MATERIALS AND METHODS

Cell Culture and Derivation of Transformed Cell Lines. The human prostate cancer cell line LNCaP was originally derived from a patient with metastatic prostate cancer to the pelvic lymph nodes (8, 9). These cells were received from Dr. Warren Heston (Memorial Sloan-Kettering Cancer Center, New York, NY). LNCaP cells are propagated as monolayer culture in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cultures were routinely given fresh medium 3 times a week, and confluent cultures were dispersed, approximately weekly, with RPMI 1640 containing 0.05% trypsin and 0.02% EDTA for subsequent passage.

For transfection, 5 × 10^6 LNCaP cells were seeded into each well of a 6-well tissue culture plate and transfected 48 h later with the neomycin-selectable pSVFF/bcl-2 plasmid (Ref. 14; obtained from Stanley Korsmeyer, Washington University School of Medicine, St. Louis, MO) or with a control, neomycin-resistant expression vector (pBK-CMV, Stratagene Cloning Systems, Inc., La Jolla, CA). Aliquots containing 30 μg of plasmid and 5 μg of lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD) in 2 ml of serum-free OPTI-MEM (Life Technologies, Inc.) were preincubated for 15 min and were then added to a culture well. The transfection media was replaced 6 h later with RPMI-10% FBS medium, and 24 h later, this was replaced with RPMI-10% FBS containing 600 μg/ml G418 sulfate (Genetecin; Life Technologies, Inc.). Individual colonies were selected from these plates after approximately 3–4 weeks of routine maintenance in neomycin selection medium using a cloning ring strategy. Colonies were dispersed with trypsin/EDTA and were reseeded into fresh wells of a 6-well plate.

For experiments requiring counts of viable cell numbers, 10^6 cells were seeded into 25-cm² tissue culture flasks and were allowed to grow for the specified time at which trypan/EDTA was used to disperse the cells for counting. Cells were pelleted and resuspended in a small amount of medium containing 0.04% trypan blue. Cell number was estimated from the count of trypan blue-excluding cells observed microscopically in a hemocytometer. Triplicate cultures were quantitated for each time point. A modified medium was used to test androgen effects on cell growth rate (phenol red-free RPMI with 10% charcoal-stripped serum). DHT (Sigma Chemical Co., St. Louis, MO) was supplemented to this medium when indicated at a concentration of 0.5 nM. Modified media were also used to induce apoptosis in LNCaP cells and transfected cell variants including serum-free RPMI or RPMI-10% FBS containing 10 nm trypsin (TPA; Sigma Chemical Co.).

RNA Extraction and Northern Blot Assay for PSA and bcl-2 mRNA. Comparative measurement of PSA and bcl-2 mRNA in parental and transformed cell lines was performed by Northern blot analysis of RNAs extracted from individual cell lines. Total RNA was isolated from monolayer cell pellets (containing 10⁶ cells) using the RNAzole B Reagent (Ref. 15; Tel-Test, Inc., Indianapolis, IN). RNase (5 μg/ml) was added to the lysate and was incubated at 37°C for 30 min. Proteinase K was added to 50 μg/ml, and incubation continued at 50°C for 1 h. The extract was then applied to an affinity column following the manufacturer’s recommendations. DNA was eluted from the column and was precipitated with an equal volume of isopropanol. The precipitate was collected by centrifugation at 12,000 × g for 15 min, washed in 70% ethanol, air dried, and then redissolved in TE buffer [10 mM Tris-HCl (pH 8.0)-0.1% SDS-0.5% sodium deoxycholate]. DNA was quantitated by spectrophotometry at 260 nm.

Aliquots of DNA containing 10 μg were electrophoresed through a 1.8% agarose gel in TEA buffer, and the gel was stained with ethidium bromide. Fluorescent DNA bands were visualized through an UV transilluminator and photographed. A ladder pattern representing fragments of DNA in multiples of 180–200 bp provided evidence for apoptosis.

Measurement of PSA Protein Production by Individual Cell Lines. PSA protein production was determined from cell culture medium (RPMI 1640–10% FBS) that was exposed to confluent monolayer cultures for only 2 h. The PSA in the medium was measured by the TANDEM-PSA automated immunoassay (Hybritech, Inc., San Diego, CA). This test quantitates PSA levels as ng/ml. The number of cells in each culture was determined as described above. PSA production for individual cell lines was then expressed as molecules of PSA/cell/h.

Measurement of AR and bcl-2 Protein Production in Individual Cell Lines. AR and bcl-2 protein levels were evaluated by Western blot analysis of protein extracts made from the various cell lines. Cell pellets containing 10^6 cells were lysed in 50 μl of ice cold RIPA buffer [50 mM Tris-HCl (pH 8.0)-150 mM NaCl-0.1% SDS-1% NP40-0.5% sodium deoxycholate] on ice for 30 min. Insoluble debris was removed by centrifugation at 10,000 × g for 10 min. Protein concentrations were determined in these extracts using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell extracts containing 50 μg of protein were made 100 mM in 2-mercaptoethanol and were briefly heated (in boiling water), then coelectrophoresed on a 12% Laemmli SDS-polyacrylamide gel at 200 V for 45 min. Electrophoretic transfer to a nitrocellulose filter was for 60 min at 200 V in 25 mM Tris-HCl (pH 8.0)-0.192 mM glycine-20% methanol. The filter was blocked in a solution of 10% nonfat milk powder at 4°C overnight and then incubated for 2 h with a mouse monoclonal anti-bcl-2 antibody (Dako bcl-2, 124; Dako Corp., Carpinteria, CA) diluted 1:2000 in TBS-T buffer [20 mM Tris-HCl (pH 8.0)-0.136 M NaCl-3% BSA-0.5% Tween 20]. After a series of washes in TBS-T buffer, the filter was incubated with the secondary antibody (sheep antimouse IgG-horseradish peroxidase complex) supplied by Amersham, Inc. in the enhanced chemiluminescence Western blotting analysis system (Amersham Life Sciences, Arlington Heights, IL). Chemiluminescent detection of antibody binding was accomplished using the reagents provided in this system after exposure of the blot to Kodak XAR-5 X-ray film. For immunodetection of the human AR protein, 10^6 cells were solubilized in 50 mM Tris (pH 8.0)-0.5% SDS-100 mM 2-mercaptoethanol buffer and heated, and aliquots containing 100 μg of protein were electrophoresed on an 8% SDS-polyacrylamide gel. Monoclonal mouse anti-AR (clone G122–434; Pharmingen, Inc., San Diego, CA), at a concentration of 2 μg/ml, was utilized for Western blot detection of AR.

Apoptois DNA Fragmentation Assay. The occurrence of apoptosis in LNCaP cells was confirmed by the detection of apoptosis-specific internucleosomal DNA degradation in these cells. This was shown by means of an electrophoretic analysis of DNA extracted from monolayer cultures grown either in serum-free medium or in phorbol ester-supplemented medium. Parental and transfected LNCaP variants were plated at 5 × 10^6 cells/75-cm² culture flask. Forty-eight h later, the growth medium was replaced with test medium, and the cells were incubated for an additional 12 h. At this time, cells were harvested by scraping into the medium and were pelleted at 1500 × g for 10 min. The cell pellets were lysed in a lysis buffer provided with the A.S.A.P. High Molecular Weight DNA Isolation kit (Boehringer Mannheim Inc., Indianapolis, IN). RNase (5 μg/ml) was added to the lysate and was incubated at 37°C for 30 min. Proteinase K was added to 50 μg/ml, and incubation continued at 50°C for 1 h. The extract was then applied to an affinity column following the manufacturer’s recommendations. DNA was eluted from the column and was precipitated with an equal volume of isopropanol. The precipitate was collected by centrifugation at 12,000 × g for 15 min, washed in 70% ethanol, air dried, and then redissolved in TE buffer [10 mM Tris-HCl (pH 8.0)-1 mM EDTA]. DNA was quantitated by spectrophotometry at 260 nm.

Aliquots of DNA containing 10 μg were electrophoresed through a 1.8% agarose gel in TEA buffer, and the gel was stained with ethidium bromide. Fluorescent DNA bands were visualized through an UV transilluminator and photographed. A ladder pattern representing fragments of DNA in multiples of 180–200 bp provided evidence for apoptosis.

Tumorigenicity of LNCaP Cell Variants in a Nude Mouse Assay. Male athymic (nude) mice were obtained from Harlan Bioproducts for Science, Inc. (Indianapolis, IN) and were housed aseptically in the Athymic Animal Facility of the Columbia University Health Center.

Parental LNCaP cells, control-transfected LNCaP cells (LNCaP/neo²-Line 3), and LNCaP/bcl-2-transfected cells (Line 3 and Line 5) were trypsinized, washed, counted, and adjusted to a concentration of 4 × 10⁶ cells/ml in RPMI containing 10% FBS. This was mixed with an equal volume of Matrigel basement membrane matrix (Collaborative Biomedical Products/Becton Dickinson, Bedford, MA), and 0.5 ml aliquots (10^6 cells) were injected into the s.c. flank of the mice. Tumor development was followed in individual animals by daily sequential caliper measurements of length, width, and depth. Tumor volume was calculated by multiplying these 3 numbers and then multiplying by 0.5236 (18).

Statistical Analysis. All numerical data are expressed as the average of the values obtained, and ± SE was calculated. The significance between the values obtained in different experimental treatments was determined by conducting a paired Student’s t test, and a probability that the means are significantly different is reached at the P = 0.05 level. Both the SE and the paired t test were
ANDROGEN RESISTANCE IN PROSTATE CANCER MEDIATED BY bcl-2

RESULTS

Derivation and Characterization of bcl-2-transformed LNCaP Cells. Monolayers of parental LNCaP cells were transfected with either a neomycin-selectable eucaryotic expression plasmid containing cDNA for human bcl-2 (pSFFV/bcl-2) or a control neomycin-selectable expression plasmid containing no cDNA (pBK-CMV). Medium containing the antibiotic G418 was used to select for transfected cells, and isolated colonies that formed were cloned and expanded. Individual clones (LNCaP/bcl-2 or LNCaP/neo) were then characterized for the expression of bcl-2 mRNA and protein. Western blot analysis done on protein extracts obtained from several different bcl-2 transfected lines showed greatly enhanced expression of bcl-2 protein in these cells when compared to the parental LNCaP line or to a control-transfected LNCaP/neo line (Fig. 1). The results for bcl-2 protein expression were reiterated by Northern blot analysis for bcl-2 mRNA expression that demonstrated significant elevation of this mRNA in bcl-2-transformed cell lines (not shown here).

In contrast to the results for bcl-2, there was little difference between parental cells and various clonal isolates in the expression of the human prostate epithelial cell protein PSA at either the mRNA or protein levels. This is shown both by the results of a Northern blot assay for PSA mRNA (Fig. 2) and also by a measurement of PSA secretion into the cell medium (Table 1). Although there was some variability between various cell clones in PSA message and secretion rates, these changes were not significant and did not correlate with any other cell characteristics. Likewise, as evaluated by a Western blot assay for human AR, all clonal isolates express this protein (detected at Mr, 100,000) at similar levels to the parental LNCaP cells (Fig. 3). In this Western blot the LNCaP/Bcl-2 (B3) and the LNCaP/Neo' clones showed an increase in AR protein levels compared to the parental line; however, this difference is not significant. Another LNCaP/Bcl-2 clone (B6) showed levels similar to the parental line (Fig. 3), and this line mimicked the B3 line in regard to androgen insensitivity in vitro and in vivo (data not shown). Therefore, it appears that the overexpression of the oncprotein bcl-2 in LNCaP cells does not significantly affect their expression of a prostate-cell differentiation gene product (PSA), nor the expression of the AR protein that regulates the ability of these cells to respond to androgenic stimulation.

Table 1 PSA protein expression in LNCaP cell lines

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<thead>
<tr>
<th>Cell Line</th>
<th>Molecules of PSA protein/cell/h (X 10⁶)</th>
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<tr>
<td>LNCaP</td>
<td>5.20 ± 0.23</td>
</tr>
<tr>
<td>LNCaP/Neo</td>
<td>1.73 ± 0.04</td>
</tr>
<tr>
<td>LNCaP/Bcl-2 (clone 3)</td>
<td>7.17 ± 0.26</td>
</tr>
<tr>
<td>LNCaP/Bcl-2 (clone 5)</td>
<td>5.47 ± 0.01</td>
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Overexpression of bcl-2 protein did not appear to affect the relative growth rate of LNCaP cells in a culture medium containing 10% FBS. Fig. 4 graphically displays a 3-day growth pattern of parental LNCaP cells, control-transfected LNCaP/neo cells, or LNCaP/bcl-2 (line 3) cells were electrophoresed and transferred to a nitrocellulose filter. The Northern blot was probed with a 32P-labeled PSA cDNA. The autoradiograph of the hybridized Northern blot identifies the 1.7-kb mRNA transcript for human PSA present in similar amounts in each of the cell lines.

Fig. 2. Northern blot analysis of RNAs extracted from LNCaP cell lines to evaluate the expression of human PSA mRNA. Equal aliquots of RNA extracted from parental LNCaP cells, LNCaP/Neo, or LNCaP/bcl-2 (clone 3) cells were electrophoresed and transferred to a nitrocellulose filter. The Northern blot was probed with a 32P-labeled PSA cDNA. The autoradiograph of the hybridized Northern blot identifies the 1.7-kb mRNA transcript for human PSA present in similar amounts in each of the cell lines.

Fig. 1. Western blot analysis of protein lysates derived from LNCaP cell lines to evaluate the expression of the Mr 24,000 bcl-2 protein. Equal aliquots of protein extracted from parental LNCaP cells, LNCaP/Neo (clone 3), or two clones of LNCaP/bcl-2 (lines 3 and 5) were electrophoresed, and the proteins were transferred to a nitrocellulose filter. The Western blot was probed with a mouse monoclonal anti-human bcl-2 antibody. Binding of the antibody on the Western blot was detected using a chemiluminescent detection system. The film above shows the Mr 25,000 band of human bcl-2 protein. Mr, molecular weight in thousands.

Fig. 4 graphically displays a 3-day growth pattern of parental LNCaP cells, control-transfected LNCaP/neo cells, and LNCaP/bcl-2 cells.
There was no significant difference of growth rate between any of the cell lines in this complete medium. However, when the LNCaP clones were grown in a medium containing 10% charcoal-stripped serum (depleted of endogenous steroids), we were able to demonstrate a significantly enhanced difference in the growth rate of LNCaP/bcl-2 cells when compared to the parental LNCaP or LNCaP/neoR (Fig. 5). More interesting, this apparent difference in the growth rate between transformed and control cells was abolished when 0.5 nm DHT was supplemented to the charcoal-stripped serum-containing medium (Fig. 5). These results indicate that bcl-2-transformed LNCaP cells grow at an accelerated rate in the absence of DHT (and other steroids) and suggest that the expression of bcl-2 protein might influence the cellular signaling pathway through which androgenic steroids stimulate prostate cell growth.

Expression of bcl-2 Protects LNCaP Cells against Apoptotic Stimuli in Vitro. LNCaP cells will undergo apoptosis in vitro when stimulated by culture conditions as diverse as phorbol ester supplementation (19), tumor necrosis factor supplementation (20), and as we have found in our experiments presented here, serum deprivation and the presence of monoclonal anti-fas antibody. We tested whether exogenous bcl-2 expression might protect LNCaP cells against these apoptotic stimuli by the use of two assays: (a) a cell viability assay to determine cell survival in the presence of these stimuli; and (b) a DNA fragmentation assay that will identify the 180-bp apoptotic DNA “ladder” fragmentation pattern that is caused by preferential nuclease digestion within the internucleosomal region of nuclear DNA.

The response of LNCaP cells and various sublines of the transformed derivatives to serum starvation was also investigated. As shown by a cell viability assay at 24 h after serum starvation, parental and control-transfected LNCaP cells are killed by this treatment, whereas the bcl-2 transformed derivative is highly resistant (Table 2). The invulnerability of bcl-2-expressing LNCaP cells to the serum starvation is further evidenced by the lack of an apoptotic DNA ladder pattern in electrophoresed DNA extracted from serum-starved bcl-2-transformed lines (Fig. 6) in contrast to the evident apoptotic DNA ladder generated in parental LNCaP or LNCaP/neoR cells. In a similar manner, the bcl-2-transformed LNCaP cells are much more resistant to phorbol ester treatment (10 nm TPA), a condition in which the vast majority of LNCaP and neomycin control-transfected cells are killed (Table 2). Examination of the electrophoretic DNA pattern from cells treated with TPA again demonstrates the lack of the apoptotic ladder pattern in the DNA extracted from bcl-2-transformed LNCaP lines (Fig. 7). Finally, DNA fragmentation studies demonstrated that the
Table 2  Effect of serum withdrawal and phorbol ester incubation on the viability of the LNCaP cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Serum withdrawal</th>
<th>Phorbol ester (10 nm)</th>
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<tbody>
<tr>
<td>LNCaP</td>
<td>14.1 ± 1.7</td>
<td>19.3 ± 3.2</td>
</tr>
<tr>
<td>LNCaP/Neo</td>
<td>14.8 ± 2.6</td>
<td>20.7 ± 1.2</td>
</tr>
<tr>
<td>LNCaP/Bcl-2</td>
<td>110.1 ± 16.1</td>
<td>50.5 ± 2.9*</td>
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a Percentage of viable cells after 24 h, compared to pretreatment levels.
b This value is significantly different than that obtained for the parental LNCaP (or LNCaP/Neo controls) after the corresponding treatment (P < 0.03).

Fig. 6. LNCaP/bcl-2 cells are resistant to internucleosomal DNA fragmentation when grown in a serum-free medium. LNCaP cells and transfected derivatives (LNCaP/neo, LNCaP/bcl-2) were grown in medium containing 10% FBS; then the medium was removed and replaced with growth medium containing 10 nm phorbol ester (TPA). After 12 h in this TPA-supplemented medium, cells were collected, and DNA was extracted. Equal aliquots of DNA extracted from the LNCaP cells, LNCaP/neo cells, or LNCaP/bcl-2 cells (lines 3 and 5) were electrophoresed on an agarose gel. After staining with ethidium bromide, the DNAs from the TPA-exposed parental and control LNCaP show evidence of an internucleosomal DNA ladder characteristic of apoptosis as described previously (19). In contrast, the LNCaP/bcl-2 lines do not show evidence of apoptotic DNA fragmentation. M.W., molecular weight.

Fig. 7. LNCaP/bcl-2 cells are resistant to internucleosomal DNA fragmentation when grown in medium supplemented with 10 nm TPA. LNCaP cells and transfected derivatives (LNCaP/neo, LNCaP/bcl-2) were grown in growth medium containing 10% FBS; then the medium was removed and replaced with growth medium containing 10 nm phorbol ester (TPA). After 12 h in this TPA-supplemented medium, cells were collected, and DNA was extracted. Equal aliquots of DNA extracted from the LNCaP cells, LNCaP/neo cells, or LNCaP/bcl-2 cells (lines 3 and 5) were electrophoresed on an agarose gel. After staining with ethidium bromide, the DNAs from the phorbol ester-exposed parental and control LNCaP show evidence of an internucleosomal DNA ladder characteristic of apoptosis as described previously (19). In contrast, the LNCaP/bcl-2 lines do not show evidence of apoptotic DNA fragmentation. M.W., molecular weight.

no serum

bcl-2-transfected LNCaP cells were also highly resistant to monoclonal fas antibody-induced apoptosis (not shown here) in striking contrast to parental LNCaP cells. In summary, these experiments indicate that clonal derivatives of bcl-2-transfected LNCaP cells that were exogenously expressing high amounts of bcl-2 protein were extremely resistant to in vitro stimuli that can initiate apoptosis of the parental LNCaP cells or neomycin-expressing control-transfected LNCaP cells.

Expression of bcl-2 Enables LNCaP Cells to Form Tumors in Castrated Male Nude Mice. In the original description of the LNCaP cell line, it was found that these cells, when injected s.c. into nude...
mice, could form tumors in male but not castrated male mice (8, 9). More recent reports on the passage of this cell line indicate that they are only weakly tumorigenic by themselves, but that this tumorigenicity can be significantly increased by the inclusion of an extracellular matrix substance, Matrigel, in the inoculate (21–23). Our initial experience implanting these variants of the LNCaP cell line into nude mice support the difficulty in obtaining tumors without the inclusion of Matrigel. When 10⁶ cells were injected into the s.c. flank of male nude mice, 0 of 10 mice given injections of LNCaP cells formed palpable tumors within 30 days. Likewise, 0 of 10 mice given injections of LNCaP/Neo⁸ cells showed signs of tumor formation. In contrast, 3 of 10 of the mice given injections of LNCaP/bcl-2 cells formed palpable tumors. This experience already indicated that the bcl-2-transformed LNCaP cells are more tumorigenic than the parental or control-transfected cell lines.

As was reported previously (22, 23), the inclusion of Matrigel substance in the nude mouse inoculum greatly increased the tumorigenicity of the parental LNCaP cells, as well as all of the variant transformed LNCaP lines. Table 3 details the results of experiments in which 10⁶ parental LNCaP cells or variant transformed LNCaP cells were mixed with an equal volume of Matrigel before their s.c. implantation in the flanks of male nude mice. In this experiment, all of the cell line variants formed tumors at the site of injection. Histological analysis of these tumors showed that they were invariably adenocarcinomas that expressed high amounts of human PSA, as assessed by immunostaining for that protein. Of interest, the bcl-2-transformed cell line LNCaP bcl-2/3 formed significantly larger tumors when compared to the parental cells or to control-transfected (LNCaP/neo⁸) cells (P < 0.02).

When these same cell lines were inoculated s.c. with Matrigel substance into male nude mice that had been castrated 1 week previously, however, there was a remarkable difference in the tumorigenicity of the bcl-2-transfected variants when compared to the parental or control-transfected cells (Table 3). At 34 days after inoculation, 6 of 6 LNCaP/bcl-2-inoculated mice had large tumors, whereas 0 of the 6 mice inoculated with parental LNCaP and only 1 of 7 control (LNCaP/neo⁸)-inoculated mice developed a small tumor (when compared to the LNCaP/bcl-2 tumors). Clearly, the bcl-2-expressing transfected LNCaP cells have a significant advantage with regard to in vivo tumor formation in an androgen-deprived environment.

Further indication of a hormone-resistant phenotype of bcl-2-transfected LNCaP cells is shown by an experiment in which intact male nude mice were inoculated with 10⁶ cells (parental LNCaP or bcl-2 transfected LNCaP, in Matrigel). The tumors were allowed to develop to an approximate size of 500 mm³ (22 days for LNCaP/bcl-2 tumors, 36 days for LNCaP tumors), at which time the tumor-bearing mice were castrated. Tumor size in the individual mice was then monitored continuously from the date of castration. Fig. 8 compares the relative growth curve of the LNCaP/bcl-2-transfected cell tumors to the parental LNCaP cells. From this figure, it is evident that the LNCaP/bcl-2 cells have a distinct and significant growth advantage subsequent to androgen deprivation of the host. As with the previous experiment, these data demonstrate that LNCaP cells exogenously expressing high levels of bcl-2 oncoprotein are significantly more resistant to androgen withdrawal in vivo.

**DISCUSSION**

The oncoprotein encoded by the bcl-2 gene is well accepted for its involvement in the development of human follicular B-cell lymphomas (1, 2, 24). Likewise, this gene is suspected of participating in the development of other types of human malignancies (rare lymphomas, breast, and prostate cancer; Refs. 3, 25–27). Because of the unique and intriguing pattern of expression of bcl-2 protein in human prostate tissues and in various developmental stages of prostate cancer, we and others have suggested that the bcl-2 oncoprotein plays a role in the development and progression of human prostate cancer, particularly to the hormone-resistant state (3, 27).

The data obtained in this study, wherein we manipulated bcl-2 expression in a prostate cancer cell line and showed that the elevated expression of this oncoprotein causes resistance to apoptotic stimuli in vitro, support numerous other studies of cultured cells in which elevated expression of bcl-2 has proved to be highly protective against a wide variety of apoptotic stimuli (1, 2, 4–7). More important, we have shown here that elevated expression of bcl-2 oncoprotein enables

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**Table 3**

**Athymic mice tumor volumes after injection of LNCaP cell lines (10⁶ cells with Matrigel)**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type of animals injected</th>
<th>Percent of animals in which tumors had formed (n)</th>
<th>Average tumor volume in mm³ ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>Males</td>
<td>92 (11/12)</td>
<td>193.9 ± 43.7</td>
</tr>
<tr>
<td>LNCaP/Neo</td>
<td>Males</td>
<td>100 (7/7)</td>
<td>113.3 ± 12.3</td>
</tr>
<tr>
<td>LNCaP/bcl-2</td>
<td>Males</td>
<td>100 (12/12)</td>
<td>649.2 ± 120.0</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Castrated males</td>
<td>0 (0/10)</td>
<td>n.a.</td>
</tr>
<tr>
<td>LNCaP/Neo</td>
<td>Castrated males</td>
<td>13 (1/7)</td>
<td>58.1</td>
</tr>
<tr>
<td>LNCaP/bcl-2</td>
<td>Castrated males</td>
<td>100 (6/6)</td>
<td>180.1 ± 25.0</td>
</tr>
</tbody>
</table>

⁸Tumor volumes were measured 36 days postinjection for the normal males and 34 days postinjection for the castrated males.

This value is significantly different than the corresponding LNCaP or LNCaP/Neo tumor volumes at P < 0.02.
the normally androgen-sensitive LNCaP prostate cancer cells to rapidly form tumors in an androgen-depleted host (the castrated nude mouse). Likewise, our data demonstrate that bcl-2 expression enables continued growth of LNCaP-derived tumors, even after castration of the tumor-bearing host. The work presented here thus strongly supports the hypothesis that this gene product is a factor in the development of hormone resistance in human prostate cancers.

This hypothesis is highly pertinent to the development of more effective treatments for prostate cancer. Androgen deprivation by surgical or drug-induced methods, the current therapies for metastatic prostate cancer, provide simple, nontoxic procedures to suppress prostate tumor growth. They are, however, only palliatively useful therapies; ultimately hormone-resistant prostate tumor cells will continue to grow and metastasize, leading to the predominantly morbid and mortal outcome of the advanced form of prostate cancer. Because the preferred target of a successful therapy against advanced prostate cancer must be the hormone-resistant cell, identification of a specific genetic cause for this phenotype would focus efforts on the means to correct the defect in the hormone-resistant tumor cells, thereby restoring hormone dependency to the malignant prostate cells.

The classical view of the genetic basis for hormone resistance of prostate cancer generally invoked a lack of, or mutations in, the AR protein (28). Certainly frank mutations in the AR have been identified in prostate cancer cells, including a well characterized mutation in the AR of LNCaP cells, the prostate cancer cell line that was utilized in these experiments (12). Thus, although early immunohistochemical studies demonstrated abundant ARs in hormone-resistant prostate cancer cells and a corresponding low frequency of mutations in AR (29), more recent studies using extremely sensitive techniques have identified AR mutations and reduced AR expression in up to 35% of the cancer specimens examined (30, 31). If ongoing analysis continues to support the latter estimate, AR defects might account for a significant proportion of hormone resistance in prostate cancer. In any case, this proportion still represents only a minority of hormone-resistant tumors.

Another genetic factor that might influence the hormonal dependence of prostate cancer is a defective p53 tumor suppressor protein. A number of recent studies have identified the p53 protein as a molecular switch that regulates the cellular decision to proliferate, enter quiescence, or undergo apoptosis (32). Studies of genetically manipulated mice in which the p53 gene has been deleted showed that the normal prostate epithelial cells will still undergo apoptosis in these animals after castration (33). However, the loss of prostatic epithelial cells occurs at a significantly more delayed and reduced rate than in castrated homozygous p53 wild-type mice (34). Although some studies find a low percentage of p53 mutations (in contrast to other human tumors; Ref. 35), other studies identify a much higher frequency of occurrence (36–38), particularly in association with hormone-resistant human prostate cancer specimens.

As a genetic target to suppress hormone resistance in prostate cancer, the bcl-2 protein might be more preferred from the standpoint that the goal would be to eliminate it from cells rather than to replace it, as would be required for correcting AR or p53 defects. Antisense oligonucleotide strategies have already been proposed for bcl-2 elimination therapy (39), and it remains to be determined how such types of therapies might be made so that they are as effective in vivo as they are in vitro. Given the strategic importance of this protein in the apoptotic cell death pathway, there is growing information on its putative mechanism of action. One intriguing hypothesis concerns the importance of the interaction of bcl-2 protein with a homologous protein referred to as bax (40). According to this model, bcl-2 protein blocks apoptosis by binding to and suppressing the action of bax, a positive regulator of cell death. Therefore, alternate therapeutic strategies for hormone-resistant prostate cancer might be derived from drugs or peptides that prove capable of blocking bcl-2/bax interaction.

In follicular B-cell lymphomas, bcl-2 expression is increased because the bcl-2 gene (normally on chromosome 18) is translocated adjacent to the heavy chain immunoglobulin gene enhancer (on chromosome 14; Ref. 24). In prostate cancer, chromosomal anomalies involving chromosome 18 have been observed (41) but are not common. Neither is there any reason to suspect the occurrence of regular gene translocation events in the formation of prostate tissues as are necessary for the formation of the mature immunoglobulin genes during differentiation of B-cells. Most likely, bcl-2 overexpression in prostate cancers would then be a function of the inappropriate regulation of the normal gene structure. Given the recent demonstration that p53-null mice have enhanced bcl-2 protein expression in their prostate tissue (42), it is therefore possible that p53 mutations enable bcl-2 dysregulation in aggressive prostate cancers. With a plethora of activity focused on describing and characterizing the bcl-2 gene and bcl-2 homologous gene family, we should expect significant revelations concerning the means by which these genes are regulated. The study presented here, along with our results from a previous report, suggest that continued research on this intriguing gene product, its regulation, and its mechanism of action will form the basis for a better understanding of prostate cancer.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Susan Dong.

REFERENCES

Overexpression of bcl-2 Protects Prostate Cancer Cells from Apoptosis in Vitro and Confers Resistance to Androgen Depletion in Vivo

Anthony J. Raffo, Harris Perlman, Min-Wei Chen, et al.