

Knock-down of Bcl-2 by antisense oligodeoxynucleotides induces radiosensitization and inhibition of angiogenesis in human PC-3 prostate tumor xenografts

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Abstract

Expression of the proto-oncogene Bcl-2 is associated with tumor progression. Bcl-2's broad expression in tumors, coupled with its role in resistance to chemotherapy and radiation therapy–induced apoptosis, makes it a rational target for anticancer therapy. Antisense Bcl-2 oligodeoxynucleotide (ODN) reagents have been shown to be effective in reducing Bcl-2 expression in a number of systems. We investigated whether treating human prostate cancer cells with antisense Bcl-2 ODN (G3139, oblimersen sodium, Genasense) before irradiation would render them more susceptible to radiation effects. Two prostate cancer cell lines expressing Bcl-2 at different levels (PC-3-Bcl-2 and PC-3-Neo) were subjected to antisense Bcl-2 ODN, reverse control (CTL), or mock treatment. Antisense Bcl-2 ODN alone produced no cytotoxic effects and was associated with G₁ cell cycle arrest. The combination of antisense Bcl-2 ODN with irradiation sensitized both cell lines to the killing effects of radiation. Both PC-3-Bcl-2 and PC-3-Neo xenografts in mice treated with the combination of antisense Bcl-2 ODN and irradiation were more than three times smaller by volume compared with xenografts in mice treated with reverse CTL alone, antisense Bcl-2 ODN alone, irradiation alone, or reverse CTL plus radiotherapy ($P = 0.0001$). Specifically, PC-3-Bcl-2 xenograft tumors treated with antisense Bcl-2 ODN and irradiation had increased rates of apoptosis and decreased rates of angiogenesis and proliferation. PC-3-Neo xenograft tumors had decreased

proliferation only. This is the first study which shows that therapy directed at Bcl-2 affects tumor vasculature. Together, these findings warrant further study of this novel combination of Bcl-2 reduction and radiation therapy, as well as Bcl-2 reduction and angiogenic therapy. [Mol Cancer Ther 2007;6(1):101–11]

Introduction

Depending on the initial serum prostate-specific antigen level, Gleason score on biopsy, or clinical stage, only 33% to 56% of patients undergoing external beam radiotherapy (XRT) for localized prostate cancer were disease-free 5 years after initial radiation therapy (1–6). Tumor response to radiation is dependent on the total dose of radiation that can be safely delivered. Prostate tumors are notoriously resistant to total doses of 75 to 80 Gy. Higher doses are not generally administered because of the increased risk of toxicity and the lack of clinical data supporting improved local tumor control. Because improved local tumor control is a therapeutic goal for most cancers, various strategies for sensitizing tumors to radiation have been tested over the last 20 years (7–12). All of these strategies have involved the systemic administration of drugs or other agents that have specific toxicities of their own, which almost always limits pharmacologic doses to levels below what is needed to sensitize tumors. The only successful sensitizing strategy thus far has been hormonal deprivation in combination with radiation therapy, which has been shown to improve cause-specific survival in men with advanced prostate cancer (13). Despite ongoing clinical trials, no sensitizing strategies are currently available for widespread use.

An important determinant of response to radiation therapy in prostate cancer seems to be the oncogene Bcl-2. Bcl-2 has been implicated consistently in postradiotherapy tumor recurrence (14–17). In a case-control study, our group showed that overexpression of Bcl-2 is associated with the development of radiation-resistant prostate tumors (18). Other researchers have noted that many patients who failed radiation therapy had increased reactivity of Bcl-2 and decreased immunohistochemical reactivity of Bax (a proapoptotic molecule in the Bcl-2 family) in prostate biopsies obtained from patients before radiation therapy (19).

This information suggests a basis for the development of new strategies for radiation sensitization that are based on overcoming Bcl-2 overexpression. Our group previously reported on the use of a nonreplicating adenovirus vector to express the *PTEN* gene in prostate cancer cells that overexpressed Bcl-2. The overexpression of PTEN was associated with the down-regulation of Bcl-2, and ultimately, with the

Received 6/26/06; revised 10/3/06; accepted 11/27/06.

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doi:10.1158/1535-7163.MCT-06-0367

sensitization of cells (20) and xenograft tumors (21) to irradiation. Realizing the limitation of a virus-based approach to gene therapy, we sought to manipulate Bcl-2 expression through other avenues, specifically antisense therapy.

Antisense oligodeoxynucleotides (ODN) are ssDNA sequences that are complementary to RNA regions of a target gene. Through hybridization to single-stranded, target RNA transcripts, antisense ODNs can prevent a specific target gene from synthesizing protein. Phosphorothioate modification of ODNs stabilizes them against cellular nuclease digestion by substituting one of the nonbridging phosphoryl oxygens of DNA with sulfur (22). The potential clinical utility of antisense Bcl-2 ODNs was shown by Tolcher et al. (23, 24) in two clinical trials that combined antisense Bcl-2 ODN with docetaxel or mitoxantrone in patients with hormone-refractory prostate cancer.

The combination of Bcl-2 knockdown and irradiation represents a novel approach to the treatment of human prostate tumors that had been previously inadequately explored. In the present study, we investigated the sensitization of PC-3-Bcl-2 cells to irradiation by treatment with antisense Bcl-2 ODN *in vitro* and *in vivo*. We show that antisense Bcl-2 ODN sensitized PC-3-Bcl-2 to irradiation in clonogenic assays, and that antisense Bcl-2-treated and irradiated xenograft tumors were markedly smaller, had higher rates of apoptosis and decreased proliferation than control tumors. Furthermore, Bcl-2 expression was associated with increased tumorigenicity and angiogenesis. When therapy was directed at Bcl-2 expression in tumors with high Bcl-2 expression, we noted a significant reduction in vascular endothelial growth factor (VEGF) production in these tumors, which ultimately led to the inhibition of angiogenesis.

Materials and Methods

Cell Lines and Culture

Human prostate cancer cell lines PC-3-Bcl-2 (characterized by marked Bcl-2 overexpression, PTEN deletion, and p53 mutation) and PC-3-Neo (characterized by low Bcl-2 expression, PTEN deletion, and p53 mutation) were a generous gift from Dr. Timothy McDonnell (The University of Texas M.D. Anderson Cancer Center, Houston, TX). These cells were maintained in DMEM (Mediatech, Inc., Herndon, VA) with 4.5 g/L of glucose, 4 mmol/L of L-glutamine, 10% fetal bovine serum, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 500 µg/mL of G418. All cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Reagents

The following antisense Bcl-2 ODNs were a kind gift from Genta Incorporated (Berkeley Heights, NJ): antisense Bcl-2 ODN G3139 (sequence, 5'-TCTCCCAGCGTGCCG-CAT-3') and reverse-polarity control (CTL) ODN G3622 (sequence, 5'-TACCGCGTGCGACCC-TCT-3'). These ODNs are 18-polymer phosphorothioates that target the

translation initiation site of Bcl-2. All phosphorothioate oligonucleotides targeting Bcl-2 were purified by high-pressure liquid chromatography and dissolved in sterile saline before use. Sterile saline served as a vehicle (mock).

In vitro Cytotoxicity Assay

Both prostate cancer cell lines (PC-3-Bcl-2 and PC-3-Neo) were seeded in 96-well plates at a density of 2.5×10^3 cells per well and treated with antisense Bcl-2 ODN, reverse CTL ODN, or mock. The ODNs were administered at concentrations ranging from 0 to 10,000 nmol/L. After 1 to 3 days, 100 µL of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO) solution was added to appropriate plates and allowed to incubate at 37°C for 2.5 h. Each reaction was stopped with lysis buffer [200 mg/mL SDS, 50% *N,N*-dimethylformamide (pH 4)] at room temperature for 1 h, and the absorbance was read on a microplate autoreader (Bio-Tek Instruments, Winooski, VT) at 560 nmol/L. Absorbance values were normalized to the values obtained for the mock-treated cells to determine survival percentage. Each assay was done in triplicate, and the means were calculated from these three assays. Cellular viability was confirmed by means of the trypan blue exclusion test.

Western Blot Analysis

For protein extraction, cells were lysed, or tumor samples were minced and incubated in lysis buffer [250 mmol/L Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol] and protein inhibitor cocktail (Sigma-Aldrich). A standard protein assay was done using the DC Protein Assay kit (Bio-Rad, Hercules, CA). Western blot analysis was completed as described previously (25). Immunoblotting was done by first incubating the proteins with primary antibodies against Bcl-2 and γ -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) and then with horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Protein antibody complexes were detected by means of chemiluminescence (Amersham, Arlington Heights, IL).

Cell Cycle Analysis

Cells were seeded at 5×10^5 cells in 10-cm tissue culture dishes and incubated overnight. Cells were then incubated with 500 nmol/L of antisense Bcl-2 ODN, a reverse CTL ODN, or mock only for 48 h, followed by irradiation to a total dose of 0 or 2 Gy. Twenty-four hours later, cells were trypsinized, washed with 1× PBS and incubated in 70% ethanol at 4°C overnight, and then immediately treated with 0.02% RNase A and 0.002% propidium iodide prior to analysis. Cell cycle distribution was determined by analysis of at least 10,000 gated cells in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Each cell cycle analysis was done in triplicate.

Clonogenic Survival

For clonogenic survival assays, a technique previously used by our laboratory was employed (20). In brief, 5×10^5 cells were plated into sterile T 25 flasks and allowed to attach overnight. The next day, cells were treated with 500 nmol/L of antisense Bcl-2 ODN, a reverse CTL ODN, or mock only. Forty-eight hours later, flasks were irradiated with Gamma 40 (0.7 Gy/min) to a total of dose of 0, 2, 4, or

6 Gy. Immediately after irradiation, cells were trypsinized, serially diluted, replated onto 10 cm dishes, and incubated for 14 days. Next, colonies were stained with 0.2% crystal violet and counted. The surviving fraction was calculated relative to the control, nonirradiated cells. Each experiment was done in triplicate, and the mean surviving fraction for each set of three experiments was calculated.

Animals

Male athymic BALB/c nude mice were obtained from Harlan (Indianapolis, IN). The mice were maintained under specific pathogen-free conditions and used at 6 to 8 weeks of age. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, the Department of Health and Human Services, and NIH. Animal experiments were done according to the guidelines of the Institutional Animal Care and Use Committee of the University of Florida (Gainesville, FL).

S.c. Implantation of Tumor Cells

Cultured PC-3-Bcl-2 and PC-3-Neo cells (60–70% confluent) were prepared for injection (26). Utilizing a 27-gauge needle, tumor cells (5×10^6) in 1 mg/mL of Matrigel (Becton Dickinson Labware, Bedford, MA) were inoculated s.c. in the flank of each mouse. The formation of a bulla indicated satisfactory injection. Tumors were measured thereafter twice weekly and their volumes calculated according to the following formula: length (L) \times width (W) \times height (H) \times 0.5236. When a mouse's tumor reached a volume of $\sim 65 \text{ mm}^3$, the mouse was assigned randomly to a treatment arm.

In vivo Therapy of Human PC-3 Growing in the Subcutis of Athymic Nude Mice

Each treatment group contained 16 mice. Treatment commenced when tumors reached a volume of $\sim 65 \text{ mm}^3$. Mice were treated with 5 mg/kg/d antisense Bcl-2 ODN, reverse CTL ODN, or mock via i.p. injection daily for 14 days and then twice a week for the remainder of the study. On day 8, half of the mice had only their tumors irradiated to a total dose of 4 Gy with Gamma 40 (0.7 Gy/min) under pentobarbital anesthesia. No signs of toxicity were noted throughout the study. Two mice in each group were euthanized by CO₂ inhalation and necropsied on days 10, 17, and 24. Their tumors were resected on these days, and a portion of each tumor was (a) placed in 10% formalin for 24 h and then embedded in paraffin, (b) immediately embedded in optimum cutting temperature compound (Miles Laboratories, Elkhart, IN) and snap-frozen in liquid nitrogen, or (c) placed in cryotubes and snap-frozen in liquid nitrogen. The remaining mice (10 per group) were sacrificed and their tumors were resected on day 56.

Immunohistochemical Analysis

Frozen sections (8–10 $\mu\text{mol/L}$ thick) were mounted on Superfrost Plus slides (Fisher Scientific, Hampton, NH) and fixed in cold acetone for 5 min. Paraffin-embedded tissue sections were obtained by placing harvested tumors in 10% formalin and subsequently embedding them in paraffin after 24 h. Paraffin sections (3–5 $\mu\text{mol/L}$) were placed on

Superfrost Plus slides (Fisher Scientific), deparaffinized in xylene, and rehydrated in graded ethanol. The slides were rinsed twice with PBS, and endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 15 min. The tissues were washed thrice with PBS and blocked for 2 h at room temperature with 5% normal horse serum and 1% normal goat serum in PBS. Frozen tissue samples were incubated overnight at 4°C with a 1:100 dilution of a monoclonal rat anti-CD31 antibody (PharMingen, San Diego, CA). Paraffin-embedded tissue samples were incubated for 18 h at 4°C with a 1:200 dilution of a monoclonal mouse anti-Bcl-2 (Santa Cruz Biotechnology), a 1:750 dilution of rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology), or a 1:50 dilution of monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Carpinteria, CA). The following day, samples were washed four times with PBS, treated with the appropriate dilution of a biotinylated anti-rabbit IgG, anti-mouse IgG, or anti-rat IgG and incubated at room temperature for 1 h. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted with Permount.

Quantification of Immunostaining Intensity

The intensity of Bcl-2 and VEGF immunostaining was measured in three areas of each sample by an image analyzer equipped with MCID Elite software (Imaging Research, Inc., Ontario, Canada). The immunostaining intensity of these three areas were quantified and averaged to yield a mean (27). The results were presented relative to the value for the control group, which was set at 100.

Quantification of Microvessel Density

Microvessel density (MVD) was determined by light microscopy after immunostaining of sections with anti-CD31 antibodies using the procedure of Weidner et al. (28). Mean MVD was expressed as the average of the five areas of highest intensity identified within a single $\times 200$ field.

Terminal Deoxynucleotidyl Transferase – Mediated Nick End Labeling Assay

For terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assays (R&D Systems, Minneapolis, MN), frozen tissue sections (10 $\mu\text{mol/L}$ thick) were treated with 1:500 proteinase K solution (20 $\mu\text{g/mL}$) for 30 min, and endogenous peroxidase was blocked by using 3% hydrogen peroxide for 5 min. The samples were incubated for 1 h at 37°C with terminal deoxynucleotidyl transferase buffer and for 1 h at 37°C with anti-bromodeoxyuridine. After counterstaining with peroxidase-conjugated streptavidin, the slides were incubated with diaminobenzidine, counterstained with methyl green, and then mounted.

Quantification of Cell Proliferation and Apoptosis

Cell proliferation and apoptosis were determined by light microscopy after anti-PCNA immunostaining and TUNEL assay of sections. The density of proliferative cells and apoptotic cells was expressed as the average of the five areas of highest intensity identified within a single $\times 200$ field.

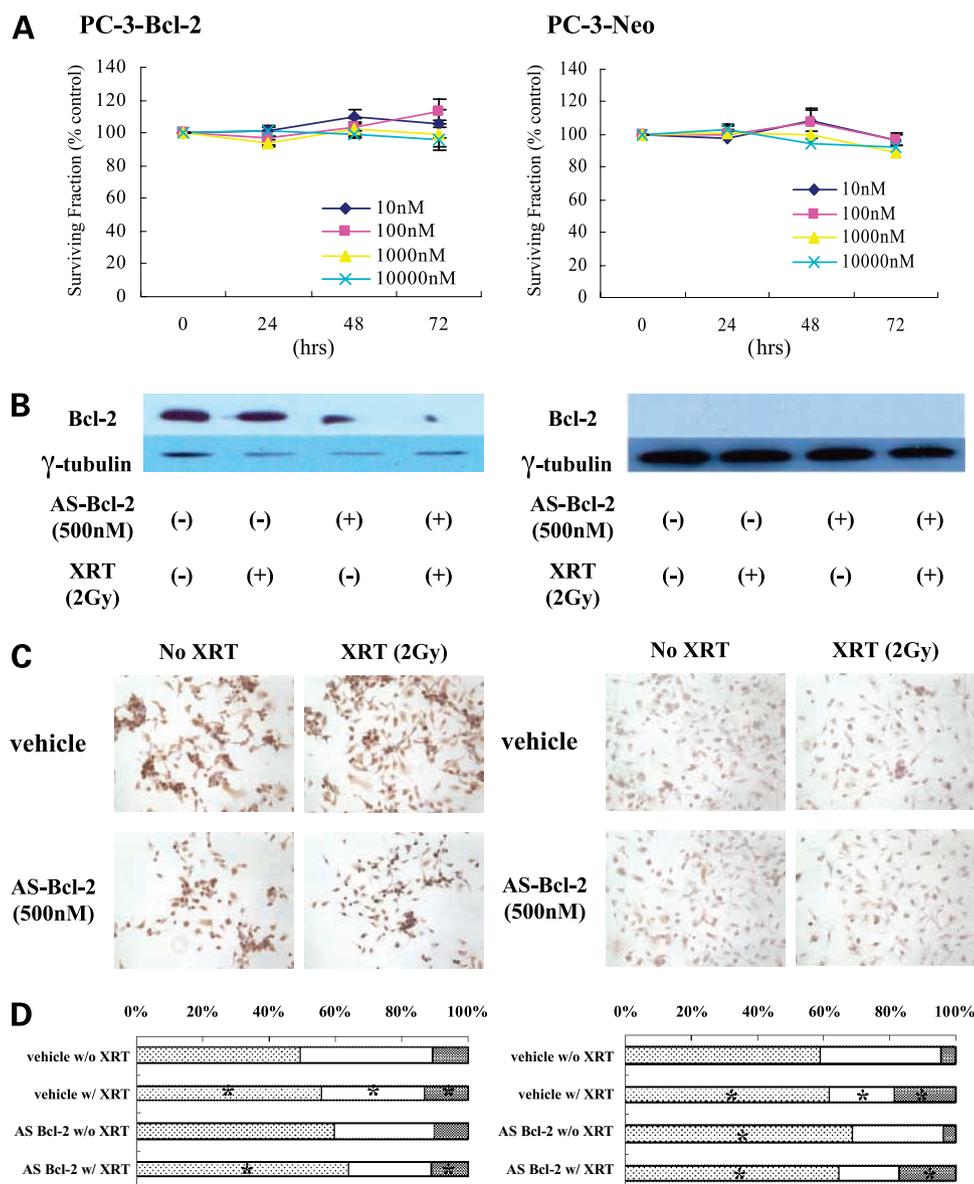


Figure 1. **A**, cell proliferation analysis in PC-3-Bcl-2 and PC-3-Neo prostate cancer cell lines. Both cell lines were treated with AS *Bcl-2* ODN at concentrations ranging from 0 to 10,000 nmol/L and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. X-axis, hours; Y-axis, percentage of surviving fraction compared with mock. Neither cell line showed cytotoxicity due to AS *Bcl-2* ODN, CTL, or mock treatment. Each assay was done in triplicate, and the mean for all three experiments were calculated. The trypan blue exclusion test confirmed no increase in cell death AS *Bcl-2* ODN, CTL, or mock-treated cells. **B**, Western blot analysis of *Bcl-2* expression in PC-3-Bcl-2 and PC-3-Neo prostate cancer cell lines. PC-3-Bcl-2 cells clearly overexpressed *Bcl-2*. The overexpression was down-regulated with the addition of AS *Bcl-2* ODN (500 nmol/L). PC-3-Neo cells expression of *Bcl-2* was near negligible. γ -Tubulin was used as the loading control. **C**, immunohistochemical analysis of *Bcl-2* expression in PC-3-Bcl-2 and PC-3-Neo prostate cancer cell lines. AS *Bcl-2* ODN (500 nmol/L) decreased the immunoreactivity of *Bcl-2* in PC-3-Bcl-2 prostate cancer cells. **D**, flow cytometric analysis of PC-3-Bcl-2 and PC-3-Neo cells stained for DNA content. Cells were treated with 500 nmol/L of AS *Bcl-2* ODN, CTL, or mock. Experiments were done in triplicate. *Speckled columns*, G₁ phase; *white columns*, S phase; *gray columns*, G₂-M phase. Exposure of PC-3-Bcl-2 and PC-3-Neo cells to AS *Bcl-2* ODN was associated with G₁ cell cycle arrest whereas radiation was associated with a G₂-M block in both cell lines. The combination of AS *Bcl-2* ODN and irradiation potentiated the G₁ arrest caused by AS *Bcl-2* ODN in both PC-3-Bcl-2 cells and PC-3-Neo cells. *, significant change in the phase of cell cycle compared with mock, where $P < 0.05$.

Immunofluorescent Double Staining of Apoptotic Endothelial Cells

For immunohistochemical analysis, frozen tissue sections (10 μ mol/L thick) were fixed in cold acetone. The samples were washed thrice with PBS and incubated for

20 min at room temperature with protein-blocking solution containing PBS (pH 7.5), 5% normal horse serum, and 1% normal goat serum. The samples were incubated for 18 h at 4°C with a 1:400 dilution of rat monoclonal anti-CD31 antibody (PharMingen). The samples were

rinsed four times with PBS and incubated for 1 h at room temperature with a 1:200 dilution of secondary goat anti-rat IgG conjugated to Texas red (Vector Laboratories, Burlingame, CA). The sections were washed twice with PBS. TUNEL assay was done using a commercially available kit according to the manufacturer's protocol (Promega Corp., Madison, WI). The tissue sections were fixed in 4% formaldehyde at room temperature for 15 min and then permeabilized by incubation with 0.5% Triton X-100 in PBS for 5 min at room temperature. Next, the slides were rinsed twice with PBS for 5 min and incubated with equilibration buffer for 10 min. The equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme were added, and the tissue sections were incubated in a humid atmosphere at 37°C for 1 h in the dark. So that cell nuclei could be identified, the slides were incubated with 100 ng/mL of 4',6-diamidino-2-phenylindole stain for 10 min at room temperature. The slides were examined under a microscope, and images were captured using a digital camera. In this procedure, the endothelial cells of CD31-positive microvessels were indicated by localized red fluorescence, and the nuclei of apoptotic cells were indicated by the localized green fluorescence resulting from the incorporation of fluorescein-12-dUTP at the 3'-OH ends of fragmented DNA in the TUNEL assay. All cell nuclei were indicated by blue fluorescence. The proportion of apoptotic endothelial cells was expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in five random 0.011 mm² fields at ×400.

Immunofluorescent Double Staining of Bcl-2 and Endothelial Cells

The procedure for immunofluorescent double staining of Bcl-2 and endothelial cells was similar to the double staining of apoptotic endothelial cells described above. In brief, the samples were incubated for 18 h at 4°C with a 1:400 dilution of rat monoclonal anti-CD31 antibody (PharMingen) and a 1:200 dilution of mouse monoclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology). Samples were rinsed four times with PBS and incubated for 60 min at room temperature with a 1:200 dilution of secondary goat anti-rat IgG conjugated to Texas red (Vector Laboratories) and goat anti-mouse IgG conjugated to FITC (Sigma-Aldrich). The sections were washed twice with PBS. To identify all cell nuclei, slides were incubated with 100 ng/mL of a 4',6-diamidino-2-phenylindole stain for 10 min at room temperature. Slides were examined under a microscope, and images were captured using a digital camera. The endothelial cells of CD31-positive microvessels were indicated by localized red fluorescence; Bcl-2-expressing cells were identified by localized green fluorescence. All cell nuclei are indicated by blue fluorescence.

Statistical Analysis

Statistical differences in the number of vessels, proliferative cells, apoptotic cells and in the intensity of Bcl-2, VEGF, and CD31 staining within the three prostate tumors were analyzed by the Mann-Whitney test. Tumor incidence and tumor size (in at least 10 mice) were analyzed

by the χ^2 test. Data points from all mouse experiments were expressed as average tumor volumes \pm SE based on at least 10 determinations. $P < 0.05$ was considered significant.

Results

Antisense Bcl-2 ODN Treatment Does Not Induce Cytotoxic Effects on Prostate Cancer Cell Proliferation

To determine the effects of antisense Bcl-2 ODN on cell proliferation and viability, PC-3-Bcl-2 and PC-3-Neo prostate cancer cell lines were treated for 72 h with antisense Bcl-2 ODNs (at concentrations ranging from 0 to 10,000 nmol/L) reverse CTL ODN, or mock. Even at high doses, antisense Bcl-2 ODN had no cytotoxic effects on either cell line (Fig. 1A). Antisense Bcl-2 ODN down-regulated Bcl-2 expression in a dose-dependent fashion. However, after treatment with a 500 nmol/L dose, no increase in efficacy was noted (Fig. 1B and C). This dose was used in all of our *in vitro* experiments and it was the same dose previously used by Gleave et al. in their *in vitro* studies (22).

Antisense Bcl-2 ODN Treatment Affects Cell Cycle Distribution

Cell cycle analysis was done in both PC-3-Bcl-2 and PC-3-Neo cell lines. Exposure to 500 nmol/L of antisense Bcl-2 ODN was associated with G₁ cell cycle arrest, whereas exposure to radiation was associated with G₂-M block in both cell lines. The combination of antisense Bcl-2 ODN and irradiation potentiated the G₁ arrest seen after antisense Bcl-2 ODN treatment in both PC-3-Bcl-2 and PC-3-Neo cells (Fig. 1D).

Exposure to Antisense Bcl-2 ODN Sensitizes Prostate Cancer Cells to Radiation

In six controlled experiments, the clonogenic responses of PC-3-Bcl-2 and PC-3-Neo cells to treatment with antisense

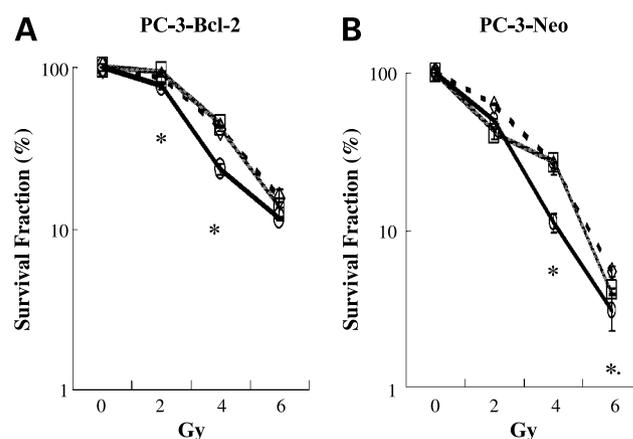


Figure 2. Clonogenic surviving fraction of 500 nmol/L of AS Bcl-2 ODN (solid black line), 500 nmol/L of reverse CTL ODN (solid gray line), or mock (dotted line). PC-3-Bcl-2 (A) and PC-3-Neo (B). Each experiment was done in triplicate, and the mean surviving fraction for all three experiments was calculated. Points, mean; bars, ± 1 SE (*, $P < 0.05$). After controlling for plating efficiencies, PC-3-Bcl-2 cells proved to be more radioresistant than PC-3-Neo cells. Treatment with AS Bcl-2 ODN (500 nmol/L) rendered both cell lines susceptible to the cytotoxic effects of irradiation.

Bcl-2 ODN (500 nmol/L) followed by irradiation were characterized. Both cell lines were relatively resistant to irradiation alone; however, after controlling for plating efficiencies, PC-3-Bcl-2 proved to be more resistant to irradiation. In experiments in which cells were irradiated to a total dose of 2 Gy without prior antisense Bcl-2 ODN treatment, 85.0% of PC-3-Bcl-2 cells and 62.5% of PC-3-Neo cells survived (Fig. 2). In experiments in which cells were treated with antisense Bcl-2 ODN and then irradiated to 2 Gy, only 50.8% of PC-3-Bcl-2 cells and 45.0% of PC-3-Neo cells survived. Thus, the addition of antisense Bcl-2 ODN caused a shift to the left in the slope of the clonogenic survival curves between 2 and 4 Gy for both PC-3-Bcl-2 and PC-3-Neo prostate cancer cells ($P = 0.020$ and 0.045 , respectively), a sign that antisense Bcl-2 ODN sensitized both cell lines to irradiation (29).

Inhibition of the Growth of Established Human Prostate Xenografts Overexpressing Bcl-2

Combination therapy with antisense Bcl-2 ODN and irradiation was significantly more effective at reducing

tumor size than single modality treatment with either antisense Bcl-2 ODN or irradiation alone (Fig. 3). Median PC-3-Bcl-2 tumor volumes were reduced from 1,052.1 mm³ in untreated controls to 719.3 mm³ in mice treated with antisense Bcl-2 ODN alone, 750.1 mm³ in mice treated with irradiation (4 Gy) alone, and 258.1 mm³ in mice that received the combined therapy ($P = 0.001$ for the combined therapy versus other treatments). This inhibition was detected in PC-3-Bcl-2 tumors as early as 10 days after implantation and persisted throughout the study. Similarly, PC-3-Neo tumors treated with antisense Bcl-2 ODN were sensitized to the effects of irradiation ($P = 0.005$ for the combined therapy versus all other treatments). Subsequent Western blotting analysis showed that Bcl-2 expression was markedly decreased in tumors treated with antisense Bcl-2 ODN plus irradiation compared with controls (Fig. 3C, PC-3-Bcl-2 tumors); this was confirmed by immunostaining (Fig. 4). PC-3-Bcl-2 tumors treated with irradiation alone expressed higher levels of Bcl-2 than did mock-treated tumors (Fig. 3C).

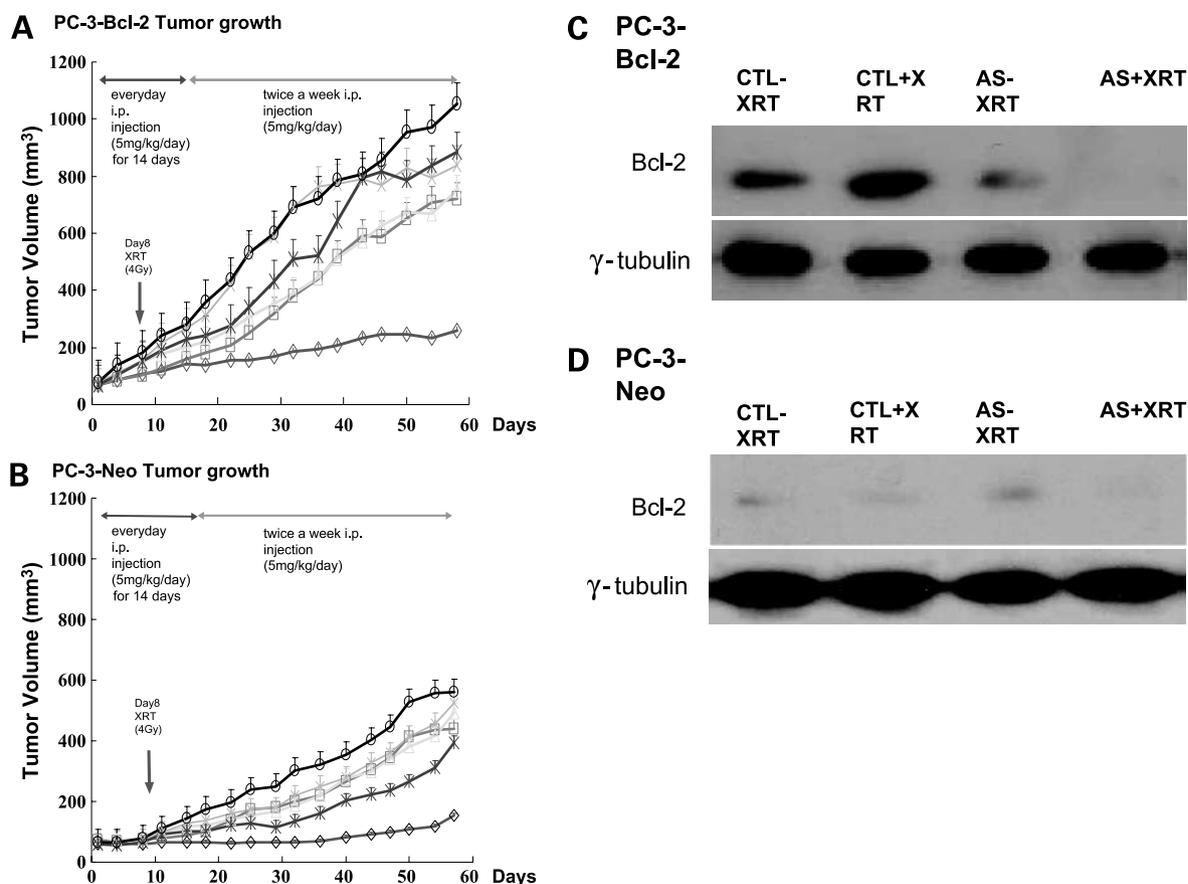
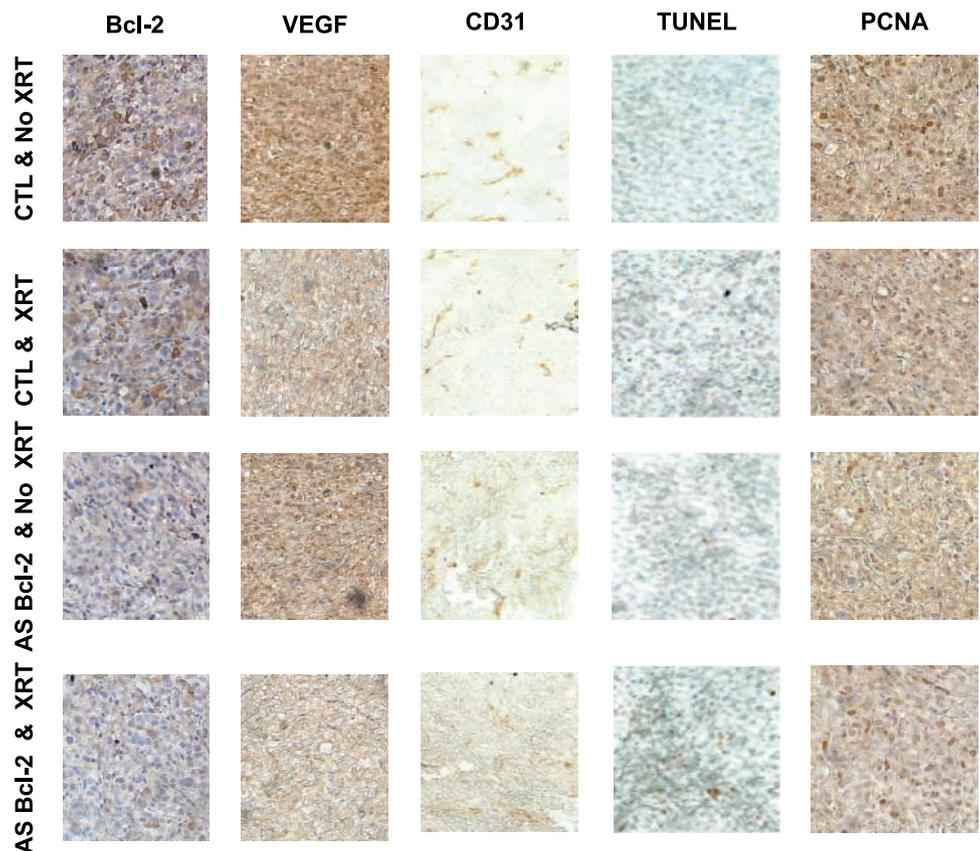


Figure 3. Effect of therapy on growth of PC-3-Bcl-2 (A) and PC-3-Neo (B) xenografts. Therapy was initiated when tumors grew to ~ 65 mm³. Mice were randomized and received a daily i.p. injection of 5 mg/kg/d G3139 AS Bcl-2 ODN, 5 mg/kg/d reverse CTL ODN, or mock only for 14 consecutive days, then twice weekly for the duration of the study. On day 8, the mice were randomized, and half of the mice in each of the three groups had their tumors irradiated (4 Gy). Tumor volumes were then measured at the time points indicated as described in Materials and Methods. Mice were maintained for 7 weeks after the initiation of treatment. Ten mice in each group completed treatment. Points, mean tumor volumes; bars, SE; mock no XRT (—○—), mock + XRT (—*—), CTL ODN no XRT (—×—), CTL ODN + XRT (—△—), AS Bcl-2 ODN no XRT (—□—), AS Bcl-2 ODN + XRT (—◇—). C and D, Western blot analysis of Bcl-2 expression in PC-3-Bcl-2 and PC-3-Neo tumors after the above treatment. γ -Tubulin was used as the loading control. Treatment with AS Bcl-2 ODN in addition to irradiation markedly reduced Bcl-2 protein expression in xenograft tumors (micrograph represents tumors from day 17 of treatment).

Figure 4. Immunohistochemical staining for *Bcl-2*, VEGF, CD31, TUNEL, and PCNA. PC-3-*Bcl-2* cells grown in the subcutis of athymic male mice were treated with daily i.p. injections of 5 mg/kg/d of G3139 AS *Bcl-2* ODN, 5 mg/kg/d of reverse CTL ODN, or mock only for 14 consecutive days, then twice weekly until study completion. On day 17, two mice from each group were sacrificed, and their tumors resected and processed for immunohistochemical analysis. Tissue sections were stained for *Bcl-2*, VEGF, CD31, TUNEL, and PCNA (as described in Materials and Methods). Down-regulation of *Bcl-2* was associated with an increased rate of apoptosis and decreased immunoreactivity of VEGF, CD31, and PCNA in PC-3-*Bcl-2* tumors and only decreased immunoreactivity of PCNA in PC-3-Neo tumors (data not shown for days 10, 24, or 56 in PC-3 *Bcl-2* tumors or in PC-3-Neo tumors).



This finding is consistent with other data from our laboratory demonstrating that the expression of cell survival proteins increases in cells subjected to an external stressor (30).

Antisense *Bcl-2* ODN and Irradiation Inhibit the Expression of *Bcl-2*, VEGF, and Angiogenesis

As shown by immunohistochemical staining and analysis of PC-3-*Bcl-2* and PC-3-Neo tumor xenografts, *Bcl-2* and VEGF protein expression was altered significantly in *Bcl-2*-expressing tumors treated with antisense *Bcl-2* ODN alone or antisense *Bcl-2* ODN plus irradiation as opposed to control mock treatment ($P = 0.029$ and 0.014 , respectively; Table 1). In addition, MVD was significantly decreased in mice treated with both antisense *Bcl-2* ODN and irradiation than in control mice (Fig. 4; Table 1). PC-3-Neo tumors expressed extremely low levels of *Bcl-2*. *Bcl-2* expression was decreased after antisense *Bcl-2* ODN and irradiation treatment. Angiogenesis, as depicted by CD31 expression and VEGF, were affected minimally by the addition of antisense *Bcl-2* ODN, irradiation, or both (data not shown for PC-3-Neo tumors).

Enhancement of Apoptosis and Inhibition of Proliferation by Treatment with Antisense *Bcl-2* and Irradiation

The effects of antisense *Bcl-2* ODN and irradiation on cellular proliferation and apoptosis in PC-3-*Bcl-2* and PC-3-Neo xenograft tumors was evaluated by PCNA and TUNEL assays, respectively (Fig. 4). In both xenograft

tumor types, the number of PCNA-positive cancer cells counted per $\times 200$ field was significantly lower after treatment with antisense *Bcl-2* ODN or irradiation alone than after treatment with mock only. Specifically, the combination of antisense *Bcl-2* ODN and irradiation inhibited the proliferation of PC-3-*Bcl-2* and PC-3-Neo tumors significantly more than did either modality alone ($P = 0.012$). However, only PC-3-*Bcl-2* xenografts showed a significant increase in the number of apoptotic cancer cells counted per $\times 200$ field in response to treatment with antisense *Bcl-2* ODN alone, and an even greater increase in apoptotic cells in response to combination treatment with antisense *Bcl-2* ODN and irradiation (Table 2). Conversely, PC-3-Neo xenografts treated with antisense *Bcl-2* ODN or irradiation alone or in combination showed no increase in apoptosis (data not shown).

Induction of Endothelial Cell Apoptosis after Treatment with Antisense *Bcl-2* ODN and Irradiation

Immunofluorescent double staining for TUNEL/CD31 and *Bcl-2*/CD31 showed that the vasculature in PC-3 *Bcl-2* xenograft tumors expressed both CD31 and *Bcl-2*, and that treatment with antisense *Bcl-2* ODN decreased *Bcl-2* expression in these cells (Fig. 5). In PC-3-*Bcl-2* xenografts, the proportion of CD31-expressing endothelial cells undergoing apoptosis was highest in tumors treated with the combination of antisense *Bcl-2* ODN and irradiation (Fig. 5; Table 3). PC-3-Neo xenografts did not show increased *Bcl-2*

Table 1. Protein expression levels and MVD in xenografts recovered from mice treated with AS Bcl-2 ODN, irradiation, or in combination after s.c. implantation of human PC-3-Bcl-2 prostate cancer cells

Treatment group	Protein expression*		MVD (per ×200 field) [†]
	Bcl-2	VEGF	
Mock no XRT	100	100	78 ± 22
Mock + XRT	108	73	64 ± 19
CTL no XRT	118	96	56 ± 24
CTL + XRT	152	53 [‡]	37 ± 2.5 [‡]
AS Bcl-2 ODN no XRT	52 [‡]	75	16 ± 19 [‡]
AS Bcl-2 ODN + XRT	48 [‡]	29 [‡]	15 ± 2.4 [‡]

*The intensity of the cytoplasmic immunostaining was measured by an image analyzer in three different areas of each sample to yield an average measurement. Results for each group were expressed as the average of the five areas of highest intensity identified within a single ×200 field (26).

[†]Microvessel density was evaluated by CD31 immunostaining. Values are expressed as the average of the five areas of highest density identified within a single ×200 field ± SD.

[‡]*P* < 0.05 versus mock (Mann-Whitney statistical comparison).

expression in the tumor vasculature and, conversely, did not show an increase in the rate of tumor endothelial cell apoptosis (data not shown).

Discussion

Researchers have recently shown that prostate tumors of certain stages and grades are resistant to radiation doses of <72 Gy (31), and that many intermediate- and high-risk prostate tumors still become resistant to radiation at doses >72 Gy (32). Therefore, new radiotherapeutic techniques or new radiation-sensitizing strategies must be developed if the outcome of patients treated with radiation therapy is to improve.

Our group previously showed that forced overexpression of PTEN down-regulates Bcl-2 expression in prostate cancer cells and xenograft tumors, ultimately rendering them sensitive to radiation (20, 21). In prostate cancer, overexpression of Bcl-2 is associated with tumor aggressiveness (33). In addition, tumor epithelial cells that overexpress Bcl-2 also overexpress and secrete other chemokines, specifically VEGF; VEGF then stimulates angiogenesis and tumor growth (34). The inhibition of Bcl-2 expression not only increased apoptotic rates, but decreased proliferation and decreased angiogenesis. The broad expression of Bcl-2 in tumors, coupled with its role in resistance to chemotherapy- and radiation therapy-induced apoptosis, make Bcl-2 a rational target for anticancer therapy.

Oblimersen (Genasense, G3139) is an 18-base synthetic ODN strand that is designed to hybridize to the first six codons of the Bcl-2 RNA transcript. Upon hybridization, the ODN-RNA hybrid is flagged to attract endogenous RNase H, which mediates scission of the Bcl-2 RNA and, thereby, inhibits Bcl-2 protein expression. Thus, this reagent has the potential to inhibit the production of Bcl-2 protein. Oblimersen is resistant to cleavage by intracellular

and extracellular nucleases and exhibits greater *in vivo* stability compared with native ODN because a sulfur is substituted for non-bridging oxygen atoms in oblimersen's phosphate backbone. Several phase I studies and one phase II study have shown the feasibility of administering antisense Bcl-2 ODN to patients with cancers (23, 24, 35–37).

Leung et al. showed that the down-regulation of Bcl-2 expression resulted in sensitization of LNCaP xenograft tumors to chemotherapy (38). Our present results confirm this finding. We showed that Bcl-2-overexpressing tumors are more aggressive than non-Bcl-2-overexpressing tumors, and that the Bcl-2-overexpressing tumors are more resistant to irradiation. We also showed in clonogenic assays and a xenograft model that down-regulation of Bcl-2 sensitized these once radiation-resistant tumors to radiation. This has been reported by others (39, 40).

However, we showed that the down-regulation of Bcl-2 expression in combination with irradiation was associated with a reduction in tumor growth *in vivo*, increased rates of apoptosis and decreased proliferation and angiogenesis in our Bcl-2 *in vivo* model. Furthermore, the addition of radiation allowed the use of lower dosages of antisense Bcl-2 ODN without a reduction in the therapeutic efficacy, compared with previous studies (22–24).

The novel aspect of our study is that we were able to show decreased rates of angiogenesis when Bcl-2 xenograft tumors were treated with antisense Bcl-2 ODN. In our xenograft model, PC-3-Bcl-2 tumors were more tumorigenic, more densely vascularized, and overexpressed Bcl-2 in their vasculature. All of these effects could be due to elevated levels of VEGF in the xenografts (Fig. 4). To determine whether this was so, we conducted ELISAs for VEGF in the culture media of PC-3-Bcl-2 and PC-3-Neo prostate cancer cells and found that the Bcl-2-overexpressing cells did secrete more VEGF than did the PC-3-Neo cells (data not shown).

Table 2. Apoptosis and cell proliferation indices for PC-3-Bcl-2 xenografts

Treatment group	Apoptotic index*	PCNA index*	Apoptosis/PCNA ratio [†]
Mock no XRT	7 ± 4	287 ± 8	0.02
Mock + XRT [‡]	3 ± 1	106 ± 38	0.01
CTL no XRT	5 ± 2	296 ± 24	0.02
CTL + XRT [‡]	8 ± 2	154 ± 108	0.05
AS Bcl-2 ODN no XRT [‡]	30 ± 20	160 ± 18 [‡]	0.43
AS Bcl-2 ODN + XRT [§]	51 ± 25 [‡]	65 ± 12 [‡]	1.27

NOTE: Tumors growing in athymic mice were treated with AS Bcl-2 ODN alone, irradiation alone, or in combination.

*The apoptotic index in cancer cells was evaluated by the TUNEL assay and cell proliferation by immunohistochemical PCNA staining. Data are expressed as the average of the five areas of highest intensity identified within a single ×200 field. Values are mean ± SD per ×200 field (26).

[†]Apoptosis/PCNA ratio: mean percentage of the number of apoptotic cells divided by the number of PCNA-positive cells ± SD.

[‡]*P* < 0.05 against mock (Mann-Whitney statistical comparison).

[§]*P* < 0.05 against mock, XRT only, and AS Bcl-2 ODN only.

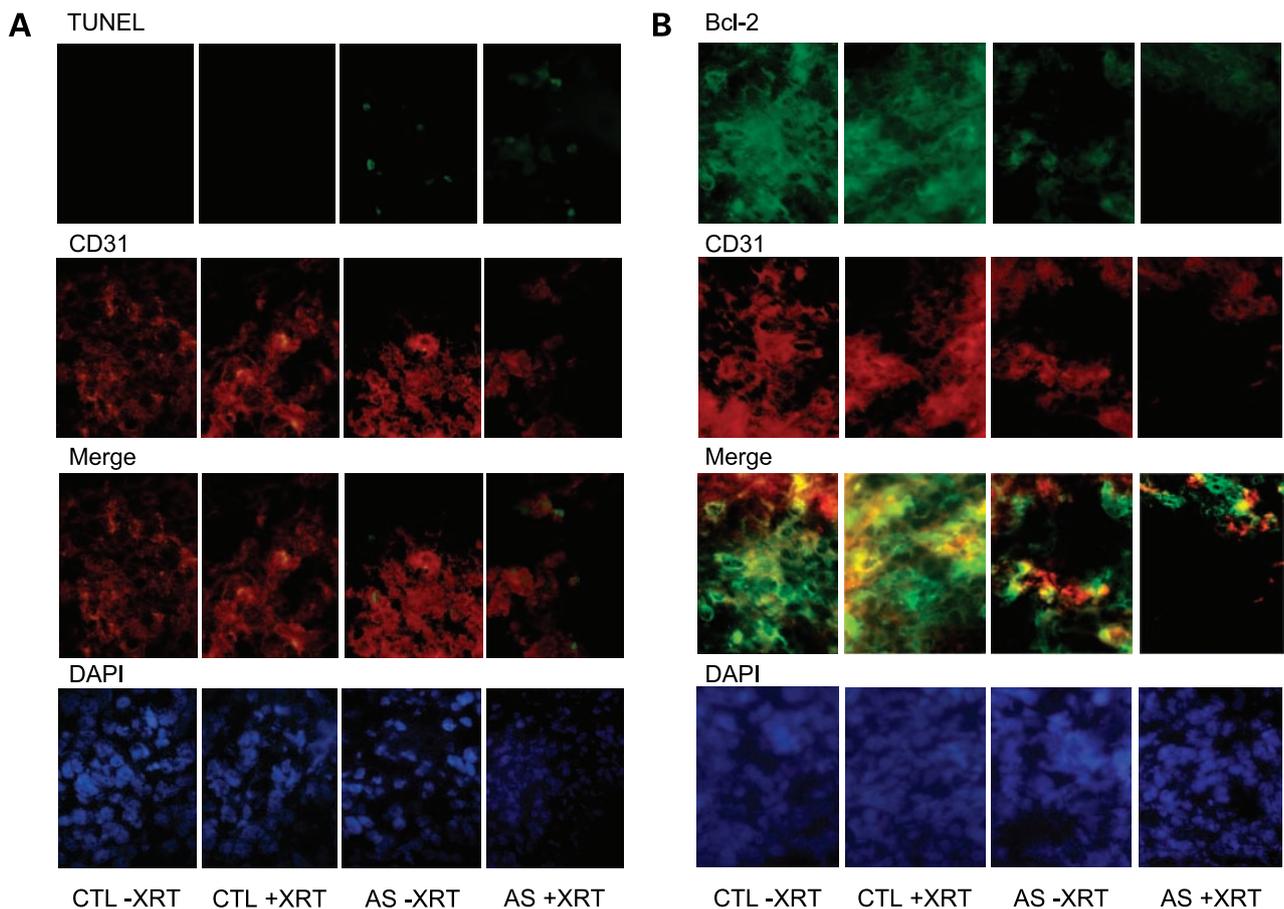


Figure 5. Double immunofluorescent staining for TUNEL/CD31 and *Bcl-2*/CD31. PC-3-*Bcl-2* cells grown in the subcutis of nude male mice were treated with daily i.p. injections of 5 mg/kg of G3139 AS *Bcl-2* ODN, 5 mg/kg of reverse CTL ODN, or mock for 14 consecutive days, then twice weekly until study completion. On days 10, 17, 24, and 56, mice from each group were sacrificed, and their tumors resected and processed for immunofluorescent analysis. Tissue sections were stained for TUNEL, CD31, and *Bcl-2* (as described in Materials and Methods). **A**, apoptotic CD31-expressing cells in tumors were seen in sections from mice treated with the combination of AS *Bcl-2* ODN and irradiation. **B**, CD31-expressing cells were noted to overexpress *Bcl-2*. Treatment with AS *Bcl-2* ODN caused down-regulation of *Bcl-2* in CD31-expressing cells compared with controls. Treatment with AS *Bcl-2* ODN plus irradiation also caused down-regulation of *Bcl-2* in CD31-expressing cells (day 17 tumors). PC-3-Neo tumors were not noted to express increased levels of *Bcl-2* in the tumor vasculature.

VEGF is a proangiogenic factor that has been shown to induce the proliferation of endothelial cells, increase vascular permeability, increase endothelial cell survival, and induce the production of other proangiogenic factors (41–45). In addition, VEGF has been shown to protect endothelial cells from ionizing radiation, antineoplastic agents, and antiangiogenic factors (46). One possible mechanism by which VEGF can mediate endothelial cell survival may involve *Bcl-2* expression (47). Down-regulation of *Bcl-2* expression has previously been associated with the down-regulation of VEGF and the growth inhibition of human xenografts in mice via an antiangiogenic mechanism (34). When combined in our study, antisense *Bcl-2* ODN and irradiation decreased VEGF expression and decreased MVD in PC-3-*Bcl-2* xenografts. VEGF signaling may inhibit apoptotic cell death in endothelial cells by several mechanisms including the induction of the antiapoptotic protein nuclear factor κ B

(48), Mcl1 (43), and phosphorylation of phosphatidylinositol 3-kinase (39). The effects of antisense *Bcl-2* on VEGF secretion and on the tumor vasculature is an interesting concept and further studies assessing apoptotic and angiogenic pathways are currently under way in our model.

Nevertheless, preliminary data from our laboratory lead us to believe that antisense *Bcl-2* ODN treatment might affect not only *Bcl-2* expression but also the expression of other key molecules (i.e., carbonic anhydrase 9, pAkt, and IL-8). PC-3-Neo xenograft tumors did express *Bcl-2*, albeit at low levels. Thus, the down-regulation of *Bcl-2* expression in PC-3-Neo xenograft tumors by antisense *Bcl-2* ODN therapy may have produced tumoricidal effects in addition to decreasing proliferation, as evidenced by decreased immunoreactivity for PCNA.

In summary, our preclinical experiments in the present study show that antisense *Bcl-2* enhances the antitumor

Table 3. Apoptosis of endothelial cells in xenografts treated with AS Bcl-2 ODN alone, irradiation alone, or in combination after s.c. implantation of human prostate PC-3-Bcl-2 cancer cells

Treatment group	Apoptotic index for endothelial cells*
Mock no XRT	0.26 ± 0.07
Mock + XRT	0.24 ± 0.09
CTL no XRT	0.53 ± 0.20
CTL + XRT	0.32 ± 0.17
AS Bcl-2 ODN no XRT	1.24 ± 0.45
AS Bcl-2 ODN + XRT	3.81 ± 0.77

*The apoptotic index for endothelial cells of microvessels (as measured by immunofluorescent double staining) is expressed as the average immunofluorescence of apoptotic cells (TUNEL assay) against all endothelial cells (CD31 immunofluorescence) present in the five areas of highest microvessel density identified within a single ×200 field ± SD.

effect of radiation in both tumors which overexpress Bcl-2 and tumors that do not. The improved response to this sensitization strategy seems to be the result of decreased proliferation, enhanced induction of apoptosis of Bcl-2-overexpressing epithelial and endothelial cells, and decreased rates of angiogenesis in Bcl-2-overexpressing xenograft tumors, compared with only decreased proliferation in tumors that do not overexpress Bcl-2. Together, our results warrant the clinical evaluation of this novel form of combination therapy in patients with prostate cancer.

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Mol Cancer Ther 2007;6:101-111.

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