Angiogenic Potential of Prostate Carcinoma Cells Overexpressing bcl-2

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Background: Tumors commonly outgrow their blood supply, thereby creating hypoxic conditions, which induce apoptosis and increase expression of angiogenic growth factors. The bcl-2 oncogene inhibits apoptosis induced by a variety of stimuli, including hypoxia. On the basis of bcl-2’s role in regulating apoptosis in response to hypoxia, we hypothesized that this oncogene might affect other responses to hypoxia, such as the expression of angiogenic growth factors. Methods: Three prostate carcinoma cell lines, PC3, LNCaP, and DU-145, were stably transfected with a bcl-2 complementary DNA (cDNA), and transfectants were analyzed in vitro for the expression of angiogenic factors after exposure to either normoxic (19% O₂) or hypoxic (1% O₂) conditions. The in vivo angiogenic potential of the transfected cells was determined by analyzing vessel density in xenografts derived from them and by measuring the ability of these xenografts to induce neovascularization when implanted in mouse corneal micropockets. Statistical tests were two-sided. Results: When exposed to hypoxic conditions, prostate carcinoma cells overexpressing bcl-2 expressed statistically significantly higher levels of vascular endothelial growth factor (VEGF), an angiogenic factor, than control-transfected cells (P = .001 for PC3, P = .04 for DU-145 after 48 hours). This effect of bcl-2 was independent of its antiapoptotic activity because increased expression of VEGF was detected in PC3 cells overexpressing bcl-2 even though PC3 cells are inherently resistant to hypoxia-induced apoptosis. In vivo, xenograft tumors derived from the bcl-2-overexpressing prostate carcinoma cell lines displayed increased angiogenic potential and grew more aggressively than tumors derived from the control cell lines (P = .03 for PC3).

Treatment of bcl-2-overexpressing and control tumors with the antiangiogenic drug TNP-470 neutralized the aggressive angiogenesis in bcl-2-overexpressing tumors (P = .04 for PC3, P = .004 for DU-145) and the moderate angiogenesis in control tumors (P = .01 for PC3, P = .05 for DU-145), resulting in similar growth rates for both tumors. Conclusions: bcl-2 may play a dual role in tumorigenesis by suppressing apoptosis and by stimulating angiogenesis. [J Natl Cancer Inst 2001;93: 208–13]
Materials and Methods

Cells

Human prostate carcinoma cell lines PC3, LNCaP, and DU-145 were stably transfected with a spleen focus-forming virus-based expression vector that either contained or lacked the complementary DNA (cDNA) encoding human bcl-2, as described previously (21, 22). Single-cell clones were selected after growing the transfected cells in G418 to select for the neomycin (neo) resistance gene contained on the vector and were expanded in culture to generate clonal populations of cells. Cells were cultured in Dulbecco’s modified Eagle medium (JRH Biosciences, Lenexa, KS) and were supplemented with 10% fetal bovine serum, 0.29 mg/mL glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 10% CO2 incubator.

Cell viability measurements were performed before injection in the tumor growth experiments. The number of viable cells was determined by use of a hemocytometer to count trypsinized cells that excluded the vital dye trypan blue.

Cell Incubation Under Normoxic and Hypoxic Conditions

Cells were incubated in hypoxic (10% CO2, 1% O2, and 89% N2) or normoxic (10% CO2 and 19% O2) conditions for various times. Oxygen concentrations were maintained at the desired levels by injecting the appropriate amounts of nitrogen gas into the incubators.

Enzyme-Linked Immunosorbent Assay for Vascular Endothelial Growth Factor Analysis

Conditioned media were collected from cells grown in culture, and, following centrifugation at 4000g for 5 minutes at 4°C, the supernatants were frozen at −20°C. The levels of vascular endothelial growth factor (VEGF) protein were measured in conditioned media samples by use of the Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Measurement of Apoptosis

Cells were washed twice with phosphate-buffered saline (PBS), treated with trypsin, and washed twice more with PBS. After the washes, the extent of apoptosis was determined by measuring Annexin V-fluorescein isothiocyanate binding to the cells by fluorescence-activated cell-sorting analysis by use of an Annexin V detection kit from Pharmingen (San Diego, CA), according to the manufacturer’s instructions.

Western Blotting for Detection of Hypoxia-Induced Transcription Factor HIF-1α

Cell lysates were prepared by adding 100 μL lysis buffer (i.e., 120 mM NaCl, 50 mM Tris, and 0.5% Nonidet P-40) to 0.5 × 106 cells and incubating them on ice for 20 minutes. Protein concentration was determined by the Bio-Rad DC Protein assay (Bio-Rad Laboratories, Hercules CA). Protein samples (50 μg) were boiled in sodium dodecyl sulfate (SDS) gel-loading sample buffer (i.e., 50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), separated on 8% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Biorad, Hercules NY). Nitrocellulose membranes were blocked in PBS containing 0.05% Tween 20 and 3% nonfat dry milk and then incubated with a mouse monoclonal anti-HIF-1α antibody (Affinity Bioreagents, Inc., Golden, CO) diluted 1:2000. Following three 15-minute washes in PBS with 0.05% Tween 20, blots were incubated with horseradish-peroxidase-conjugated rabbit anti-rat antibody at a 1:10,000 dilution. Bands were visualized by chemiluminescence, and relative levels of HIF-1α were determined by densitometric scanning and normalization against an actin control.

Xenograft Tumor Growth

Prostate carcinoma cells stably transfected with either empty vector (designated by the suffix, “neo”) or vector containing the bcl-2 cDNA (designated by the suffix, “-bcl-2”) were injected in the dorsal subcutaneous space of severe combined immunodeficiency (SCID) mice at a dose of 106 cells per mouse, and subsequent tumor growth was assessed twice a week. When tumors reached approximately 100 mm3, mice were randomly assigned to control (four mice) and experimental (five mice) groups. Mice in the experimental group received therapy with the angiogenesis inhibitor TNP-470 (30 mg/kg every other day by subcutaneous injection) or the cytotoxic drug mitomycin C (1 mg/kg once a week by intraperitoneal injection). The width (W) and length (L) of each tumor was measured biweekly on the mice with calipers. Tumor volume was calculated according to the formula \( V = \frac{4}{3} \pi \times \frac{W^2 \times L^2}{2} \). All animal studies have been approved by the animal use committee at Children’s Hospital and adhered to institutional guidelines for animal care.

Mouse Corneal Tumor Angiogenesis Assay

The procedure is a modification of that described by Muthukkaruppan et al. (23). Briefly, mice were anesthetized by intraperitoneal injection of 750 mg/kg Avertin (Aldrich, St. Louis, MO) and topical application of procarcinotic HCl (Allergan, Irvine, CA). An intrastromal linear keratotomy was made by use of a number 10 surgical blade angled at 30 degrees. A corneal micropocket was dissected toward the temporal limbus by use of a modified von Graefe knife #3 (2 × 30 mm). Untreated, subcutaneous xenograft tumors derived from injections of PC3-neo and PC3-bcl-2 cells were excised from carrier mice, and a fragment of approximately 0.3 × 0.3 × 0.3 mm was removed from the tumor while it was viewed under a dissecting microscope. This fragment was placed in the corneal micropocket and advanced to 1 mm from the limbus by use of the von Graefe knife. Topical erythromycin was applied once to the eye that had surgery. Neovascularization of the tumor fragment within the eye that had surgery was assessed at weekly intervals by use of a slit lamp for illumination and magnification.

Immunohistochemical Analysis of CD31 Expression

Sections from paraffin-embedded tumors derived from prostate carcinoma-bcl-2 or -neo cells were incubated overnight with the rat anti-mouse CD31 (an endothelial cell-specific antigen) monoclonal antibody (Pharmingen, San Diego, CA) in TNB (i.e., 0.1 M Tris–HCl [pH 7.5], 0.15 M NaCl, and 0.5% blocking reagent from the tyramide signal amplification (TSA) indirect amplification kit (Du Pont NEN, Boston, MA)) plus 10% rabbit serum. After incubation with a rabbit anti-rat secondary antibody (Vector Laboratories, Inc., Burlingame, CA), the antibody–antigen complexes were visualized by use of a TSA indirect amplification kit and the Vectastain ABC-AP kit (Vector Laboratories, Inc.). Stained tumor sections were scanned at low magnification to identify areas of high vessel density. For each section, CD31-positive cells within three areas of high vessel density were counted while viewed at high magnification (×100). From each group of tumors (-neo and -bcl-2), two tumors were selected and the number of stained cells on five sections were counted from each one.

Statistical Analysis

P values for the enzyme-linked immunosorbent assay experiments described in the text and in the legend for Fig. 1 were determined by use of a two-tailed Student’s t test of data collected from two independent experiments performed in triplicate. For the tumor experiments, the volumes determined from measurements taken 1 day after the final treatment were analyzed by a two-tailed Student’s t test. All P values are two-sided.

Results

Effect of bcl-2 Overexpression on Production of Angiogenic Factors

To examine the effect of bcl-2 overexpression on VEGF production, three different prostate carcinoma cell lines cells were stably transfected with vector only or with vector bearing the bcl-2 cDNA. Control PC3 transfectants (PC3-neo) and PC3 cells overexpressing bcl-2 (10-fold increase in bcl-2 protein compared with vector control; data not shown) were cultured in either normoxic (19% O2) or hypoxic (1% O2) conditions, and the VEGF protein levels were measured in the conditioned media. After 72 hours of culture in normoxic conditions, there was a small but statistically significant increase (P = 0.04) in the amount of VEGF secreted by PC3-bcl-2 cells (32 pg/105 cells; 95% confidence interval [CI] = 31 to 33 pg/105 cells) relative to that secreted by PC3-neo cells (20 pg/105 cells; 95% CI = 19.2 to 20.8 pg/105 cells) (Fig. 1, A). However, when cells were cultured in hypoxic conditions, there was a larger (2.3-fold) increase in the amount of VEGF secreted by the PC3-bcl-2 cells (91.5 pg/105 cells; 95% CI = 86 to 96 pg/105 cells) compared with that secreted by the PC3-neo cells (38.9 pg/105 cells; 95% CI = 37 to 41 pg/105 cells) (Fig. 1, A). The differ-
A similar association between bcl-2 expression and VEGF secretion was detected in LNCaP cells that were stably transfected with vector only (LNCaP-neo) or with the bcl-2 cDNA (LNCaP-bcl-2) and cultured in hypoxic conditions. LNCaP-neo and LNCaP-bcl-2 cells secreted similar levels of VEGF when cultured for 48 hours in normoxic conditions (30 and 24 pg/10^4 cells; 95% CI = 24 to 36 pg/10^4 cells and 14 and 34 pg/10^4 cells, respectively). After 48 hours of exposure to hypoxic conditions, LNCaP-bcl-2 cells secreted statistically significantly (P = .04) more VEGF (106 pg/10^4 cells; 95% CI = 98 to 114 pg/10^4 cells) than did LNCaP-neo cells (63 pg/10^4 cells; 95% CI = 52 to 74 pg/10^4 cells).

By contrast, analysis of DU-145 cells stably transfected with vector (DU-145-neo) or with the bcl-2 cDNA (DU-145-bcl-2) revealed that DU-145-bcl-2 transfectants secreted statistically significantly (P = .001) higher levels of VEGF in normoxic conditions (226 pg/10^4 cells; 95% CI = 218 to 234 pg/10^4 cells at 24 hours) than did DU-145-neo transfectants (106 pg/10^4 cells; 95% CI = 102 to 110 pg/10^4 cells). Culture in hypoxic conditions resulted in the rapid death of DU-145-neo cells, thus preventing the analysis of their VEGF secretion behavior under hypoxia. It is interesting to note that, in normoxic conditions, the three cell lines responded differently to bcl-2 overexpression: bcl-2 overexpression caused a strong increase in VEGF secretion in the DU-145 cells, caused a moderate increase in VEGF secretion in PC3 cells, and had no effect on VEGF secretion in LNCaP cells. These observations suggest that bcl-2 interacts with another factor or factors to regulate VEGF secretion, and that these factors are expressed at different constitutive levels in different tumor cells.

To establish whether bcl-2 overexpression could affect the secretion of angiogenic factors other than VEGF, we measured the levels of basic fibroblast growth factor (bFGF) in the culture media of stably transfected PC3 cells. PC3-neo and PC3-bcl-2 cells were cultured for 72 hours in normoxic or hypoxic conditions. bFGF in the culture media was measured by ELISA. Open bars = normoxia; hatched bars = hypoxia. Data points for both experiments represent the average of three measurements. Error bars = 95% confidence intervals.

Induction of VEGF expression in response to hypoxia requires the activation of the transcription factor HIF-1α (24,25). To determine whether the differences in VEGF expression detected between the PC3-derived cell lines were mediated by the induction of the expression of HIF-1α, we used western blotting to examine the levels of HIF-1α in cells that were cul-
tured in normoxic and hypoxic conditions. HIF-1α was expressed at similar levels in the PC3-bcl-2 and the PC3-neo cells cultured in normoxic conditions (data not shown). Exposure to hypoxic conditions resulted in a similar induction of HIF-1α in PC3-neo (2.4-fold) and PC3-bcl-2 (2.2-fold) cells (data not shown). This observation suggests that a mechanism other than the regulation of the levels of HIF-1α is responsible for the stimulatory effects of bcl-2 on VEGF secretion in PC3 cells under hypoxic conditions.

Tumor Growth and Vessel Density of bcl-2 Overexpressing Xenografts

To test whether the enhanced expression of VEGF in response to hypoxia in the bcl-2 overexpressing cells in vitro would result in higher levels of neovascular activity in vivo, we inoculated PC3-neo and PC3-bcl-2 cells subcutaneously into SCID mice. Tumors derived from PC3-bcl-2 cells grew at approximately twice the rate of tumors derived from PC3-neo cells (Fig. 3). At day 37 after inoculation, tumors were removed and analyzed for microvessel density by measuring the expression of the endothelial cell-specific marker CD31. The PC3-bcl-2-derived tumors had, on average, twice as many CD31-positive cells than the PC3-neo-derived tumors (134 cells [95% CI = 118 to 150 cells] versus 57 cells [95% CI = 49 to 65 cells] per ×100 field, respectively; Fig. 4, A and B). Immunohistochemical analysis of tumors derived from DU-145-bcl-2 and DU-145-neo cells revealed a similar difference in the number of CD31-positive cells (76 CD31-positive cells [95% CI = 66 to 86 CD31-positive cells] and 34 CD31-positive cells [95% CI = 28 to 40 CD31-positive cells] per ×100 field, respectively; Fig. 4, C and D).

A connection between the observed increases in tumor vascularization and the ability of bcl-2-overexpressing cells to produce higher levels of VEGF under hypoxia was also supported by the immunohistochemical analysis of VEGF expression in tumors. This analysis showed numerous clusters of cells positive for VEGF expression in the bcl-2-overexpressing tumors but only sporadic positive cells in the tumors derived from control cells (data not shown).

Induction of Angiogenesis in bcl-2-Overexpressing Tumors Implanted in a Cornea Micropocket

To determine the angiogenic potential of PC3-neo and PC3-bcl-2 cells, we measured the ability of tumors derived from each cell line to induce neovascularization after implantation into corneal micropockets. One week after tumor implantation, corneal angiogenesis (i.e., as assayed by the presence of vessels at least 1 mm long sprouting from the limbus vessel into the cornea) was detected in six of the eight corneas implanted with tumors derived from PC3-bcl-2 cells. In that same time period, none of the eight corneas implanted with tumors derived from PC3-neo cells revealed corneal angiogenesis. At 5 weeks after tumor implantation, vessels were detectable in 25% of the corneas.
implanted with tumors derived from PC3-neo cells. Whereas tumors derived from both the PC3-neo and PC3-bcl-2 cells induced morphologically similar vessels in this assay, tumors derived from the PC3-bcl-2 cells did so at a faster rate. Thus, the differences in induced neovascularization were due to the stronger angiogenic potential of the PC3-bcl-2 tumors and not to decreased viability of the PC3-neo-derived cells.

**Treatment of bcl-2-Overexpressing Tumors With Antiangiogenic Therapy and Conventional Chemotherapy**

Because tumors derived from PC3-bcl-2 cells stimulated angiogenesis, we examined whether angiogenesis inhibitors could effectively treat bcl-2-overexpressing tumors. PC3-neo and PC3-bcl-2 cells were injected into SCID mice to induce tumor formation. Once the tumors reached a volume of 100 mm³, the mice were treated with the antiangiogenic drug TNP-470. As shown in Fig. 3, A, TNP-470 strongly inhibited the growth of tumors derived from both PC3-bcl-2 and PC3-neo cells. The untreated PC3-bcl-2-derived tumors grew, on average, to a final volume roughly twice that of the untreated PC3-neo-derived tumors (1301 mm³ [95% CI = 1158 to 1543 mm³] and 587 mm³ [95% CI = 520 to 654 mm³], respectively; \(P = .03\)). However, treatment with TNP-470 reduced the final volume of both types of tumors to a similar level (206 mm³ [95% CI = 173 to 239 mm³]; \(P = .04\)) for PC3-bcl-2-derived tumors and 191 mm³ [95% CI = 174 to 208 mm³; \(P = .01\)] for PC3-neo-derived tumors. TNP-470 was also effective in decreasing the mean volume of tumors derived from both DU-145-bcl-2 cells (from 1043 mm³ [95% CI = 930 to 1158 mm³] for untreated tumors to 271 mm³ [95% CI = 187 to 355 mm³; \(P = .004\)] for treated tumors) and DU-145-neo cells (from 439 mm³ [95% CI = 320 to 568 mm³] for untreated tumors to 253 mm³ [95% CI = 146 to 360 mm³; \(P = .05\)] for treated tumors).

In contrast to their sensitivity to the antiangiogenic treatment, tumors derived from PC3-bcl-2 cells were completely resistant to the chemotherapeutic agent mitomycin C (the mean volume of untreated tumors was 950 mm³ [95% CI = 853 to 1047 mm³], while that of treated tumors was 997 mm³ [95% CI = 878 to 1116 mm³; \(P = .05\)]) for PC3-bcl-2 cells. This observation suggests that bcl-2 overexpression affects specific angiogenic factors in response to hypoxia.

**DISCUSSION**

Our results demonstrate that, when exposed to hypoxic culture conditions, PC3 prostate cancer cells that overexpress bcl-2 secrete more VEGF than do control cells. We interpret the increased production of VEGF by PC3-bcl-2 cells as resulting from a direct effect of bcl-2 on VEGF expression rather than as an indirect consequence of bcl-2’s effects on cell survival. Indeed, overexpression of bcl-2 did not change cell survival, because the PC3-bcl-2 and PC3-neo cells were equally resistant to hypoxia-induced apoptosis. Of interest, although more VEGF was secreted by PC3 cells overexpressing bcl-2 cultured in hypoxic conditions than by control cells, the levels of hypoxia-induced bFGF were similar in PC3-neo and PC3-bcl-2 cells. This observation suggests that bcl-2 overexpression affects specific angiogenic factors in response to hypoxia.

The regulation of VEGF secretion by bcl-2 is a new function for this oncogene, with important implications concerning bcl-2’s ability to support tumor progression. Critical steps in tumor progression include increased proliferation, induction of angiogenesis, inhibition of apoptosis, activation of telomerase, and independence from growth and survival factors (1). In this context, single genetic alterations that have the ability to confer several of these changes will have stronger effects on tumor progression than will those that affect only one step. Genes that are altered in such ways could thus be considered as more effective oncogenes than those with alterations that affect only one of these processes. Our results suggest that bcl-2 is an oncogene that has the ability to contribute to the tumor progression process at multiple levels, that is, through both the inhibition of apoptosis and the induction of angiogenesis.

It is interesting to note that the overexpression of bcl-2 caused an increase in VEGF secretion in PC3 and DU-145 cells that were cultured in normoxic conditions but not in LNCaP cells cultured in normoxic conditions. Because bcl-2 was expressed at comparable levels in these three cell lines, these results suggest that the effect of bcl-2 on VEGF expression may require a factor (or factors) that is expressed constitutively in some cell lines.
(PC3 and DU-145), but only in response to hypoxia in others (LNCaP).

Tumors derived from cells that overexpress bcl-2 grow more aggressively in vivo than tumors derived from cells that do not overexpress bcl-2. This phenomenon has been attributed to the antiapoptotic properties of bcl-2 (26). However, our observations suggest that bcl-2-overexpressing tumors may grow faster because they also induce more neovascularization. This possibility is supported by our TNP-470 treatment data. Tumors derived from PC3-neo and PC3-bcl-2 cells grew at equal rates when treated with TNP-470, suggesting that this angiogenesis-inhibiting drug eliminated the growth advantage bcl-2 overexpression usually confers on tumors. Together, these data support the hypothesis that increased VEGF production and the increased vascularization associated with this increased expression are essential components of the in vivo growth advantage conferred to tumors by overexpression of bcl-2.

Bcl-2 overexpression is associated with the development of androgen-independent prostate carcinomas as well as with increased resistance of such tumors to chemotherapy (12,27,28). One new therapeutic approach that has been proposed to treat prostate tumors is to inhibit their expression of bcl-2, which would restore the sensitivity of such tumors to apoptosis (29,30). Our results indicate that such an approach might have a dual effect on prostate tumors, by inhibiting angiogenesis as well as resensitizing them to apoptosis. In addition, our data suggest that therapies that inhibit angiogenesis may be effective tools in the treatment of prostate cancer.

REFERENCES


NOTES

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