Bcl-2 Acts in a Proangiogenic Signaling Pathway through Nuclear Factor-κB and CXC Chemokines

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Abstract

Vascular endothelial growth factor (VEGF) induces expression of Bcl-2 in tumor-associated microvascular endothelial cells. We have previously reported that up-regulated Bcl-2 expression in microvascular endothelial cells is sufficient to enhance intratumoral angiogenesis and to accelerate tumor growth. We initially attributed these results to Bcl-2–mediated endothelial cell survival. However, in recent experiments, we observed that conditioned medium from Bcl-2–transduced human dermal microvascular endothelial cells (HDMEC-Bcl-2) is sufficient to induce potent neovascularization in the rat corneal assay, whereas conditioned medium from empty vector controls (HDMEC-LXSN) does not induce angiogenesis. These results cannot be attributed to the role of Bcl-2 in cell survival. To understand this unexpected observation, we did gene expression arrays that revealed that the expression of the proangiogenic chemokines interleukin-8 (CXCL8) and growth-related oncogene-α (CXCL1) is significantly higher in HDMEC exposed to VEGF and in HDMEC-Bcl-2 than in controls. Inhibition of Bcl-2 expression with small interfering RNA-Bcl-2, or the inhibition of Bcl-2 function with small molecule inhibitor BL-193, down-regulated CXCL8 and CXCL1 expression and caused marked decrease in the angiogenic potential of endothelial cells without affecting cell viability. Nuclear factor-κB (NF-κB) is highly activated in HDMEC exposed to VEGF and HDMEC-Bcl-2 cells, and genetic and chemical approaches to block the activity of NF-κB down-regulated CXCL8 and CXCL1 expression levels. These results reveal a novel function for Bcl-2 as a proangiogenic signaling molecule and suggest a role for this pathway in tumor angiogenesis. (Cancer Res 2005; 65(12): 5063-9)

Introduction

Angiogenesis, the process of sprouting new capillaries from existing blood vessels, is fundamental for the pathogenesis of cancer (1). Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis that induces endothelial cell migration, differentiation, and vascular permeability (2, 3). VEGF was shown to mediate endothelial cell survival by inducing Bcl-2 expression in a pathway that requires its binding to VEGFR2 and activation of PI3K-Akt signaling (4, 5). However, the role of Bcl-2 in mediating VEGF-induced effects on microvascular endothelial cells remains poorly understood.

Bcl-2 is the founding member of a protein family composed of regulators of cell death (6, 7). Bcl-2 is a prosurvival multidomain protein that regulates apoptosis by preventing the release of proapoptogenic factors from the mitochondria (e.g., cytochrome *c*) and subsequent caspase activation (7, 8). In addition to promoting cell survival, Bcl-2 has been implicated in the differentiation of several cell types, including neuronal, epithelial, and hematopoietic cells (9, 10). Up-regulation of Bcl-2 expression in microvascular endothelial cells is sufficient to enhance tumor progression in carcinoma and sarcoma cancer models (11). However, it is unclear whether the effects of Bcl-2 on microvascular endothelial cells are mediated solely through its prosurvival activity or if there are additional activities induced by Bcl-2 that contributed to these findings.

Bcl-2 has been shown to activate nuclear factor-κB (NF-κB) in ventricular myocytes and in breast cancer cells through a mechanism that is dependent on I-κB kinase β (IKKβ) activity and I-κB phosphorylation (12–14). NF-κB is a transcriptional factor that regulates expression of genes involved in inflammation, angiogenesis, and cell survival (15, 16). Antiapoptotic signals via NF-κB have been also implicated in cell fate specification, molecular differentiation, and resistance to tumor necrosis factor (TNF)-α–induced cell death (17, 18). In addition, NF-κB regulates the expression of chemokines, which are small, secreted chemotactic cytokines.

The CXC chemokines play a critical role in the regulation of angiogenesis during many pathologic processes, such as tumor growth, ischemia, and wound healing (19). The ELR motif has been implicated in the regulation of angiogenesis by CXC chemokines. ELR^- chemokines (e.g., IP-10) have angiostatic functions, whereas the ELR^+ chemokines, such as CXCL8 and CXCL1, are proangiogenic (19, 20). CXCL8 and CXCL1 are 43% identical in amino acid sequence (21), bind to the CXC receptor 2 (CXCR2; ref. 20), and can be transcriptionally regulated by NF-κB (22, 23).

In previous studies, we observed that VEGF induces Bcl-2 expression in human microvascular endothelial cells (5) and that up-regulated Bcl-2 expression in tumor-associated endothelial cells enhances tumor progression (11). It is also known that Bcl-2–transduced endothelial cells are highly angiogenic *in vivo* (5, 24), which was initially believed to be due to the antiapoptotic effects of Bcl-2. Here, we report that Bcl-2 has a proangiogenic activity that is independent on its ability to enhance endothelial cell survival. We show that Bcl-2 can function as a proangiogenic signaling molecule through its ability to activate the NF-κB signaling pathway and to induce expression of the proangiogenic CXCL8 and CXCL1 chemokines in endothelial cells.

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Materials and Methods

Plasmids, cells, reporter assays, and ELISA. NF-κB activity was analyzed after cotransfection of 990 ng of NF-κB luciferase reporter and 10 ng Renilla reporter into 2×10^5 human dermal microvascular endothelial cells (HDMEC; Clonetics, San Diego, CA) stably transduced with Bcl-2 (HDMEC-Bcl-2; refs. 5, 11) or empty vector controls HDMEC-LXSN, as described (25). One day after transfection, cells were lysed in Reporter Lysis buffer (Promega, Madison, WI) and luciferase activity was measured in a luminometer. Data were represented as firefly luciferase activity normalized by Renilla luciferase. The expression of CXCL8 and CXCL1 were evaluated by ELISA (R&D Systems, Minneapolis, MN) 24 hours after treatment with BL193 (26) or IKK inhibitor peptide (Calbiochem, San Diego, CA). Alternatively, we transfected 2×10^5 HDMEC-Bcl-2 or HDMEC-LXSN using 1 μg SR-IκB, dnIKKβ, or pcDNA3 plasmid using Lipofectin (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Affymetrix microarrays. Ten micrograms of total RNA from HDMEC-Bcl-2 or HDMEC-LXSN were amplified and biotin-labeled according to GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Fragmented cRNA was hybridized with human gene chip U133A (Affymetrix); chips were washed and stained with streptavidin R-phycoerythrin (Molecular Probes, Eugene, OR). The chips were scanned and the data were analyzed with Microarray Suite and Data Mining Tool (Affymetrix). The data presented here is representative of microarrays done with three independent pools of G418-selected HDMEC-Bcl-2 and HDMEC-LXSN cells (5, 11).

Capillary sprouting assays. HDMEC (5×10^4) were seeded 1.5 mL type I collagen (Vitrogen 100; Cohesion Technologies, Palo Alto, CA). When indicated, cells were exposed to 1 μg/mL monoclonal antihuman CXCR2 antibody (MAB331; R&D Systems) or to 1 μg/mL mouse anti-IgG2A isotype Control (R&D Systems). Alternatively, cells were exposed to 50 ng/mL VEGF (R&D Systems) for 5 days and then to 50 ng/mL VEGF in presence of 0 to 5 μmol/L BL193 (26) thereafter. The number of sprouts in six random fields was counted daily in triplicate wells per condition at ×100.

Rat corneal micropocket assay. The angiogenic activity of HDMEC-Bcl 2 and HDMEC-LXSN conditioned medium was evaluated in the rat corneal micropocket assay as described (11).

Electrophoretic mobility shift assay. Nuclear extracts were prepared from HDMEC-LSXN, HDMEC-Bcl-2, or HDMEC exposed to 0 to 50 ng/mL VEGF for 24 hours or to 10 ng/mL TNF-α for 30 minutes. Aliquots of nuclear extracts were preincubated with 1 mg poly(deoxyinosinic-deoxycytidylic acid) in binding buffer [10 mmol/L Tris (pH 7.7), 50 mmol/L NaCl, 20% glycerol, 1 mmol/L DTT, and 0.5 mmol/L EDTA] for 10 minutes at room temperature. Approximately 20,000 cpm of ^{32}P-labeled DNA probe for NF-κB (p65) were added and reaction binding proceeded for 15 minutes. The sequence of the probe used here is 5′-CAG GGC TGG GGA TTC CCC ATC TCC ACA GTT TCA CTT-3′. The complexes were separated on a 5% polyacrylamide gel and exposed to an X-ray film for autoradiography. To confirm DNA binding specificity, nuclear proteins for HDMEC-Bcl-2 or HDMEC exposed to TNF-α were preincubated with polyclonal rabbit anti-NF-κB p65 (RelA; Rockland Immunochemicals, Gilbertsville, PA) for 10 minutes at 37°C and then incubated with ^{32}P-labeled DNA probe.

Small interfering RNA-Bcl-2 assays and semiquantitative reverse transcription-PCR. HDMEC (2×10^5) were transfected using Lipofectin (Invitrogen) with SureSilencing Human small interfering RNA (siRNA)-Bcl-2 (Superarray, Frederick, MD) or negative control siRNA-NC (Superarray) according to the manufacturer's instructions. Total RNA was extracted with Trizol Reagent (Invitrogen) and purified with RNeasy Mini kits (Qiagen, Valencia, CA) and RNase-Free DNase Set (Qiagen). cDNA synthesis and PCR amplification were done in a single tube using simultaneously a human Bcl-2 and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set with SuperScript one-step reverse transcription-PCR (RT-PCR) with

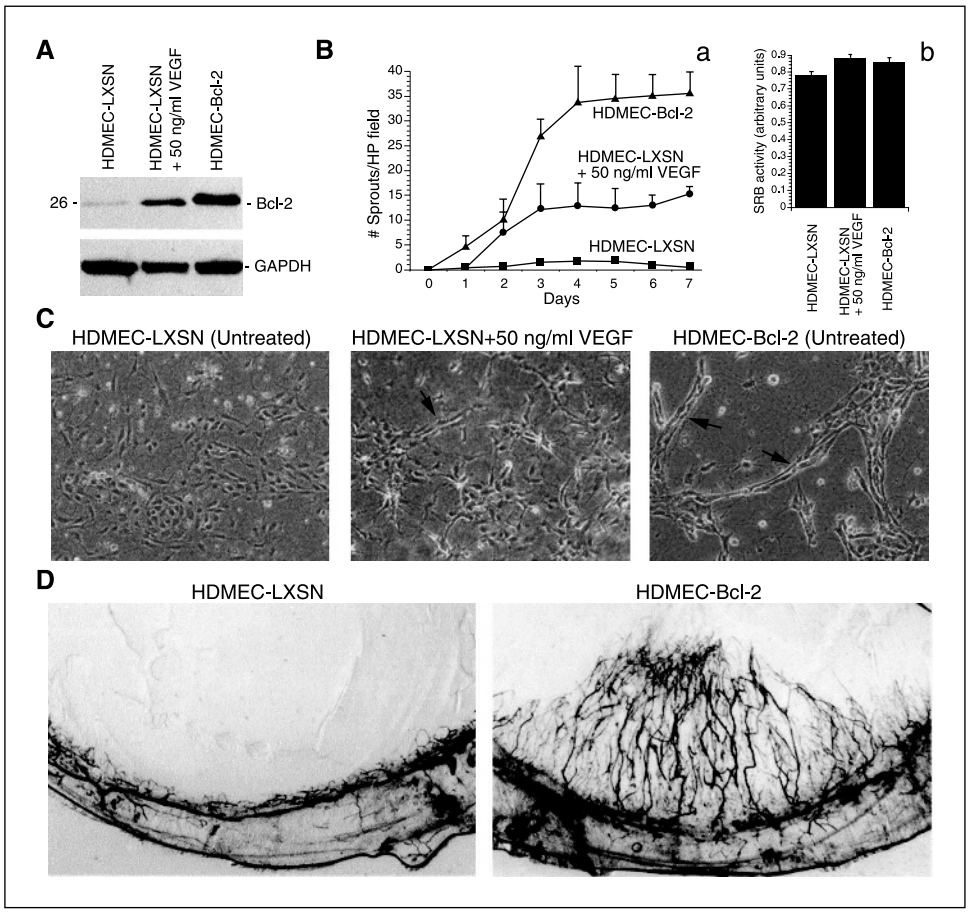

Figure 1. Bcl-2–transduced endothelial cells are highly angiogenic. *A,* expression of Bcl-2 analyzed by immunoblotting of lysates obtained from HDMEC-Bcl-2 compared with HDMEC-LXSN (empty vector control) or HDMEC-LXSN exposed to 50 ng/mL VEGF for 24 hours. *B,* Bcl-2 induces capillary sprouting, but not proliferation of endothelial cells. *a,* capillary sprouting assays with HDMEC-Bcl-2 or HDMEC-LXSN exposed to 0 to 50 ng/mL VEGF plated on type I collagen. At daily intervals, the number of sprouts was counted in six random microscopic fields (×100) from triplicate wells per condition. The data presented is representative of three independent experiments. *b,* SRB assays were done with HDMEC-Bcl-2, HDMEC-LXSN, or HDMEC-LXSN exposed to 50 ng/mL VEGF for 72 hours to evaluate relative cell number per condition. *C,* representative microscopic fields (×200) of untreated HDMEC-LXSN, HDMEC-LXSN exposed to 50 ng/mL VEGF or untreated HDMEC-Bcl-2. Arrows point to capillary sprouting. *D,* angiogenesis induced by HDMEC-Bcl-2–conditioned medium *in vivo*. Representative images of colloidal carbon-perfused rat corneas 7 days after implantation of Hydron pellets containing 24-hour–conditioned medium from HDMEC-Bcl-2 or from HDMEC-LXSN cells. HDMEC-Bcl-2–conditioned medium induced potent angiogenesis. In contrast, implants containing HDMEC-LXSN–conditioned medium showed no evidence of angiogenesis.

Platinum Taq kit (Invitrogen). The Bcl-2 primers used here were as follows: sense, CTGCGAAGAACCTTGTGTGA and antisense TGTCCCTACCAACCAGAAGG. The GAPDH primers were as follows: sense, CATGGCCTCCAAGGAGTAAG and antisense, AGGGGTCTACAGGCAACTG. The RT-PCR products were analyzed by electrophoresis on 1% agarose gels containing ethidium bromide. The density of the bands correspondent to Bcl-2 mRNA were measured with the Image J software (NIH, Bethesda, MD) and normalized against the density of the bands for GAPDH.

Sulforhodamine B assay. HDMEC-Bcl-2 (2×10^3) were exposed to 50 ng/mL VEGF, 1 μg/mL anti-CXCR2, or 1 μg/mL IgG, or to 0 to 5 μmol/L BL193. After 24 to 72 hours, cells were fixed with 10% trichloroacetic acid, stained with 0.4% sulforhodamine B (SRB) solution, and the plate was read in a microplate reader at 565 nm (TECAN, Salzburg, Austria). Triplicate wells per condition were evaluated and the data presented is representative of three independent experiments.

Western blot analysis. HDMEC-LXSN exposed to 0 to 50 ng/mL VEGF for 24 hours and HDMEC-Bcl-2 whole cell lysates were resolved by PAGE and membranes were probed overnight at 4°C with a 1:1,000 dilution of hamster antihuman Bcl-2 monoclonal antibody (BD Biosciences). Blots were exposed to appropriate peroxidase-coupled secondary antibodies and proteins were visualized with ECL (Amersham, Sunnyvale, CA).

Results and Discussion

Bcl-2 acts in a proangiogenic signaling pathway through CXC chemokines. To understand the effect of Bcl-2 in angiogenesis, we did capillary sprouting assays using primary HDMECs stably transduced with Bcl-2 (HDMEC-Bcl-2; ref. 5) and with empty vector control cells (HDMEC-LXSN) untreated or exposed to VEGF (Fig. 1*A*). We observed that untreated HDMEC-Bcl-2 spontaneously developed capillary-like sprouts, whereas HDMEC-LXSN did not (Fig. 1*B*, *a* and *C*). These results were reproducible using two additional independent pools of Bcl-2–transduced endothelial cells (data not shown). Notably, overexpression of Bcl-2 induced more sprouting than exposure of endothelial cells to the potent proangiogenic factor VEGF (Fig. 1*B*, *a* and *C*). No difference in cell number was observed when HDMEC-Bcl-2 and HDMEC-LXSN cultures were compared (Fig. 1*B*, *b*). Therefore, the increase in sprouting was not simply a consequence of increased cell number in HDMEC-Bcl-2 cultures.

To evaluate the effect of Bcl-2–induced proangiogenic signaling on neovascularization *in vivo*, we collected conditioned medium from HDMEC-Bcl-2 and HDMEC-LXSN and did the rat corneal assay. We observed that conditioned medium from HDMEC-Bcl-2 induced potent angiogenesis in the cornea, whereas conditioned medium from HDMEC-LXSN did not (Fig. 1*D*). These results clearly cannot be attributed to the role of Bcl-2 as a prosurvival factor. The ability of supernatant from HDMEC-Bcl-2 to induce migration and differentiation of endothelial cells from the limbus of the rat eye

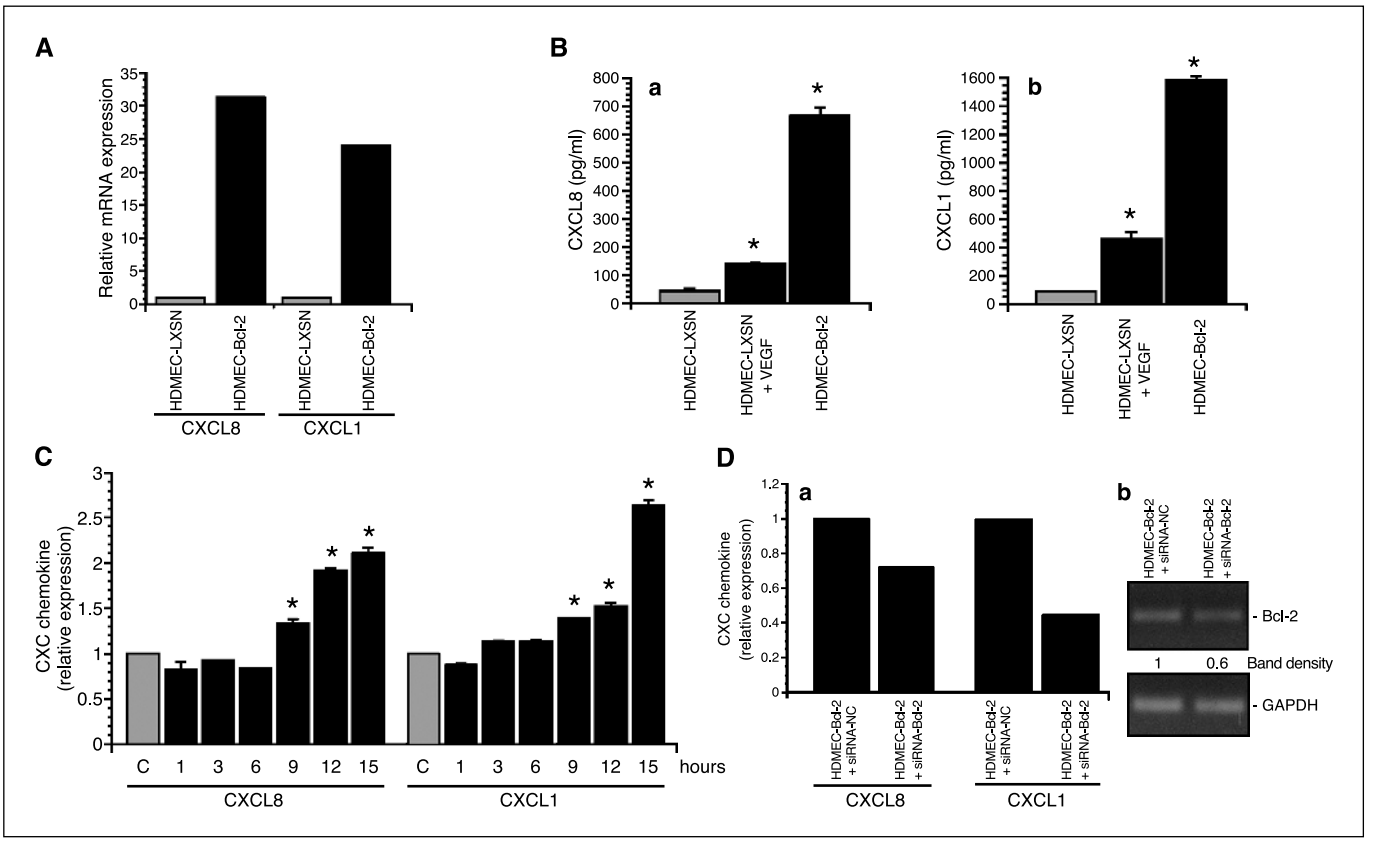

Figure 2. Bcl-2 up-regulates CXCL8 and CXCL1 expression in endothelial cells. *A,* relative expression of CXCL8 and CXCL1 analyzed by Affymetrix microarray. Data presented is representative of three independent microarrays with three independent pools of stably transduced HDMEC-Bcl-2 and HDMEC-LXSN cells. *B,* ELISA for evaluation of CXCL8 (*a*) and CXCL1 (*b*) expression in untreated HDMEC-Bcl-2 or HDMEC-LXSN exposed to 0 to 50 ng/mL VEGF for 24 hours. *C,* ELISA for evaluation of CXCL8 and CXCL1 expression 1 to 15 hours after transient transfection of HDMEC with $pcDNA_3$ or $pcDNA_3$-Bcl-2-flag. Control (*C*) represents the expression of CXCL8 or CXCL1 15 hours after transfection with $pcDNA_3$. Data is presented as relative expression (i.e., expression of CXCL8 and CXCL1 induced by transfection with $pcDNA_3$-Bcl-2-flag divided by the expression induced by $pcDNA_3$ at each time point). *D, a,* ELISA for evaluation of CXCL8 and CXCL1 expression 24 hours after transient transfection of HDMEC-Bcl-2 with siRNA-Bcl-2 or siRNA-NC. Data is presented as relative expression using HDMEC-Bcl-2 transfected with siRNA-NC as reference. *b,* RT-PCR analysis of Bcl-2 expression in HDMEC-Bcl-2 transfected with siRNA-Bcl-2 or siRNA-NC (negative control). Band densities for Bcl-2 were measured, normalized by GAPDH band densities, and described numerically.

toward the avascular cornea suggests the existence of a potent chemotactic activity within the panel of growth factors and cytokines secreted by these cells that is absent in control endothelial cells. Soluble factors secreted by endothelial cells could function via an autocrine pathway (which would explain the enhanced sprouting observed in HDMEC-Bcl-2 *in vitro*) and via a paracrine pathway (which would be capable of inducing corneal neovascularization *in vivo*).

To address this hypothesis, we searched for angiogenic factors that were up-regulated in HDMEC-Bcl-2 cells by microarray gene assays using HDMEC-LXSN as control. We observed that the chemokines CXCL8 and CXCL1 were up-regulated 31-fold and 24-fold, respectively, in HDMEC-Bcl-2 cells compared with HDMEC-LXSN (Fig. 2*A*). We also assayed the conditioned medium from HDMEC-Bcl-2 and HDMEC-LXSN by ELISA. These experiments showed that HDMEC-Bcl-2 cells secreted significantly more CXCL8 and CXCL1 than control cells (Fig. 2*B*, *a* and *b*). Because VEGF was shown to induce Bcl-2 expression in endothelial cells (5), we exposed endothelial cells to VEGF and observed a significant increase in CXCL8 and CXCL1 expression levels (Fig. 2*B*, *a* and *b*). To confirm that the increase expression of CXCL8 and CXCL1 in HDMEC-Bcl-2 was not related to viral transduction and selection of cells stably overexpressing Bcl-2, we transiently transfected HDMEC with Bcl-2 and measured CXCL8 and CXCL1 expression over time after transfection. We observed an increase in CXCL8 and CXCL1 expression by 9 hours after transfection with Bcl-2 plasmid, but not with control plasmid (Fig. 2*C*). However, the Bcl-2–mediated induction of CXCL8 and CXCL1 observed in the transient transfection was less pronounced than that observed with HDMEC stably expressing Bcl-2. This is likely due to the relatively low transfection efficiency normally observed with primary endothelial cells. To evaluate the specificity of the effect of Bcl-2 in CXCL8 and CXCL1 expression, we down-regulated Bcl-2 expression in HDMEC-Bcl-2 cells with siRNA-Bcl-2. We observed that transient transfection of siRNA-Bcl-2 into primary endothelial cells transduced with Bcl-2 resulted in a 40% decrease in Bcl-2 mRNA expression levels (Fig. 2*D*, *b*) and a correspondent decrease in CXCL8 and CXCL1 expression (Fig. 2*D*, *a*).

CXCR2 is a receptor for both CXCL8 and CXCL1 and has been implicated in the angiogenic signaling mediated by these chemokines (27). To evaluate if CXCL8 and CXCL1 are functionally involved in Bcl-2–mediated angiogenesis via an autocrine signaling pathway, we did capillary sprouting assays with neutralizing anti-CXCR2 antibody. Notably, HDMEC-Bcl-2 exposed to anti-CXCR2 antibody lost their ability to sprout spontaneously in collagen matrices (Fig. 3*A* and *D*). Whereas blockade of CXCR2 signaling mediated a 2-fold reduction in the number of HDMEC-Bcl-2 (Fig. 3*B*), it was correlated with a 10-fold reduction in the number of sprouts at day 7 in the HDMEC-Bcl-2 cultures (Fig. 3*A*). Our interpretation of these data is that when Bcl-2 is up-regulated, the endothelial cells become a source of the CXCL8 and CXCL1 that can be used via an autocrine pathway to enhance their angiogenic phenotype. Taken together, these data show that Bcl-2 has an effect on angiogenesis that is independent from its effect on endothelial cell survival.

Nuclear factor-κB mediates Bcl-2–induced CXCL8 and CXCL1 expression. Because Bcl-2 was shown to activate NF-κB in myocytes and breast cancer cells (12–14), and knowing that *CXCL8* and *CXCL1* are NF-κB target genes (22, 23), we decided to investigate the activity of this pathway in endothelial cells. To evaluate the activation of NF-κB in HDMEC-Bcl-2 and in HDMEC exposed to VEGF, we assayed the DNA binding activity of NF-κB in endothelial cell extracts by electrophoretic mobility shift assay (EMSA). Gel shift assays showed DNA binding activity of NF-κB in HDMEC-Bcl-2 cells (Fig. 4*A*, *a*; *lane 2*), as well as in HDMEC exposed to VEGF or to TNF-α (Fig. 4*A*, *a*; *lanes 4* and *5*), but not in control HDMEC-LXSN or unstimulated HDMEC (Fig. 4*A*, *a*; *lanes 1* and *3*). Supershifting assays done by incubating HDMEC-Bcl-2 extracts with anti-p65 antibody showed the specificity of this response (Fig. 4*A*, *a*; *lane 6*). We confirmed these results using NF-κB reporter assays. HDMEC-Bcl-2 showed 8-fold increase in NF-κB activity compared with HDMEC-LXSN (Fig. 4*A*, *b*). Similar results were observed in HDMEC exposed to VEGF (Fig. 4*A*, *b*). Phosphorylation of I-κB is necessary for NF-κB nuclear translocation and activation (15). Notably, HDMEC-Bcl-2 showed enhanced I-κB phosphorylation compared with empty vector control cells (Fig. 4*B*).

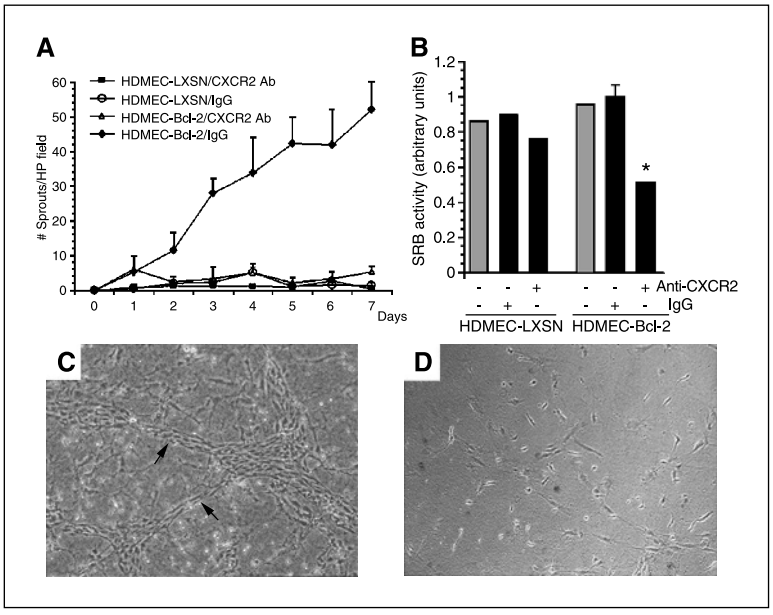

Figure 3. Blockade of CXCR2 inhibits the spontaneous sprouting of Bcl-2–transduced endothelial cells. *A,* capillary sprouting assays with HDMEC-Bcl-2 or HDMEC-LXSN either to 1 μg/mL anti-CXCR2 antibody or 1 μg/mL IgG. At daily intervals, the number of sprouts was counted in six random microscopic fields (×100) from triplicate wells per condition. The data presented is representative of three independent experiments. *B,* SRB assays were done with HDMEC-Bcl-2 or HDMEC-LXSN exposed to 1 μg/mL anti-CXCR2 antibody or 1 μg/mL IgG to evaluate relative cell number per condition. *C* and *D,* representative microscopic fields (×200) of untreated HDMEC-Bcl-2 (*C*) or HDMEC-Bcl-2 exposed to 1 μg/mL anti-CXCR2 antibody for 5 days (*D*). Arrows point to capillary sprouting.

To confirm that NF-κB activity mediates the ability of Bcl-2 to induce CXCL8 and CXCL1 in endothelial cells, we inhibited this pathway using both genetic and chemical approaches. The dnIKKβ works as a dominant-negative inhibitor that blocks IKKβ kinase activity and activation of NF-κB (25). The superrepressor form of I-κBα (SR-I-κB) prevents phosphorylation at the specific serine residues (S32 and S36) by IKK, which also prevents I-κB phosphorylation and NF-κB nuclear translocation. HDMEC-Bcl-2 cells transfected with either SR-I-κB or dnIKKβ showed a significant inhibition of CXCL8 (Fig. 4*C*, *a*) and CXCL1 expression (Fig. 4*D*, *a*). Chemical inhibitors of I-κB bind to its phosphorylation sites (Ser^{32} and Ser^{36}) preventing the phosphorylation of these serine residues and, therefore, blocking activation of NF-κB (28). We observed that treatment of HDMEC-Bcl-2 with an I-κB phosphorylation inhibitor peptide led to a significant decrease in Bcl-2–induced CXCL8 (Fig. 4*C*, *b*) and CXCL1 expression (Fig. 4*D*, *b*). Taken together, these results show that Bcl-2–mediated CXCL8 and CXCL1 expression in endothelial cells is dependent upon IKKβ kinase activity, phosphorylation of I-κB, and NF-κB activity. They also suggest that Bcl-2 might induce other cellular responses mediated through additional NF-κB target genes that were not evaluated here. For example, it is known that Bcl-2 induces matrix metalloproteinase-9 (MMP-9) through a NF-κB–dependent pathway in breast cancer cells (14). MMP-9–deficient mice have defective tumor growth (29), and release and activation of MMP-9 is necessary for matrix remodeling and mobilization of marrow-derived stem cells during tissue revascularization (30). Further studies are needed to test whether Bcl-2 mediates a signaling pathway that results in MMP-9 up-regulation in tumor-associated microvascular endothelial cells leading to enhanced recruitment of circulating progenitor cells to the tumor endothelium.

Blockade of the function of Bcl-2 with a small molecule inhibitor prevents Bcl-2–induced CXC chemokine up-regulation and endothelial cell sprouting. To further understand the proangiogenic effect of Bcl-2, we tested if blockade of Bcl-2 function with the small molecule inhibitor BL193 (26) prevents Bcl-2–induced CXCL8 and CXCL1 expression and affects the angiogenic potential of endothelial cells. We observed that both CXCL8 and CXCL1 were down-regulated upon exposure to BL193 in a dose-dependent fashion (Fig. 5*A* and *B*). Moreover, HDMEC exposed to VEGF in the presence of BL193 showed less sprouting in collagen than HDMEC exposed to VEGF alone (Fig. 5*C*). Importantly,

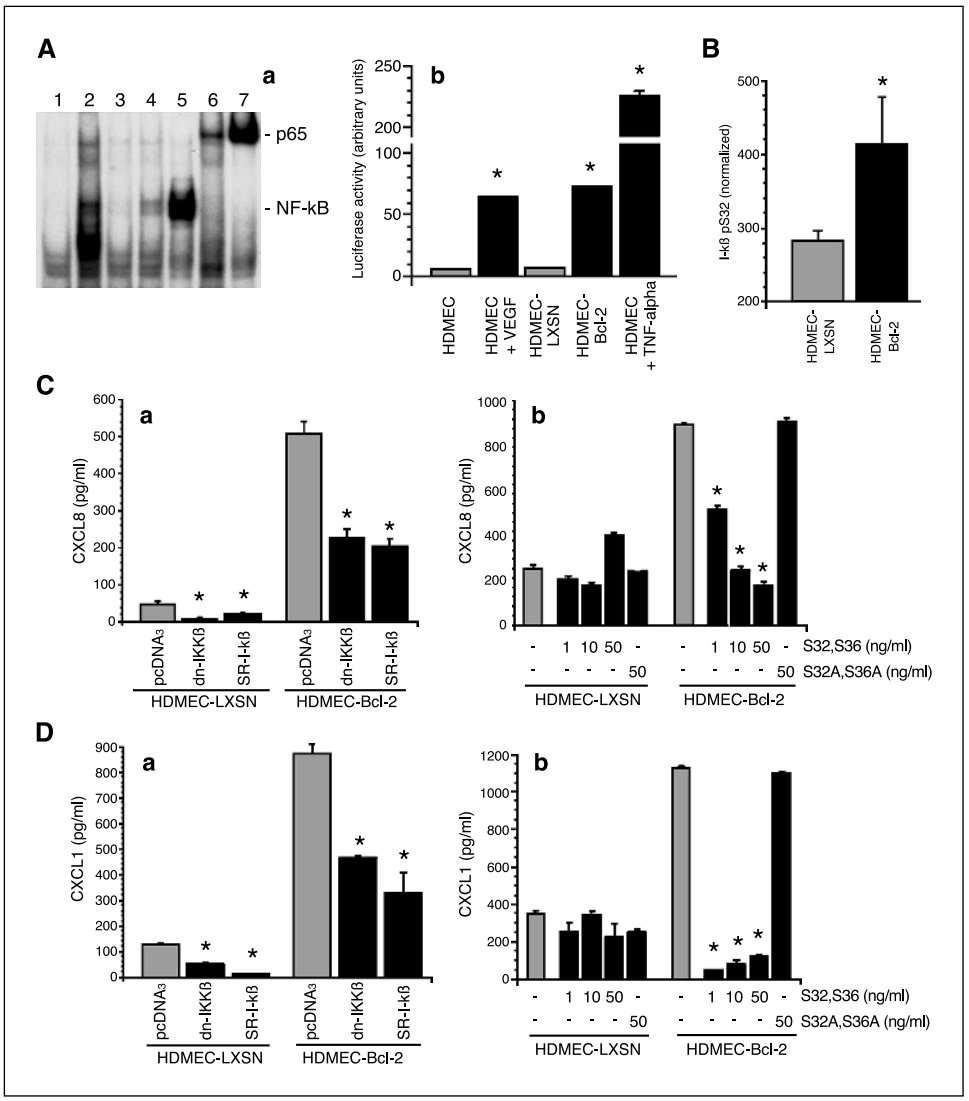

Figure 4. The NF-κB signaling pathway is activated in Bcl-2–transduced endothelial cells and in HDMEC exposed to VEGF. *A, a,* EMSA of nuclear extracts prepared from HDMEC (*lane 1*), HDMEC-Bcl-2 (*lane 2*), HDMEC-LXSN (*lane 3*), and HDMEC-LXSN (*lane 4*) exposed to 50 ng/mL VEGF; or to 10 ng/mL TNF-α (*lane 5*). Supershifting with anti-p65 antibody of nuclear extracts from HDMEC-Bcl-2 (*lane 6*) or HDMEC-LXSN (*lane 7*) exposed to 10 ng/mL TNF-α. *b,* luciferase assays of HDMEC-Bcl-2, HDMEC-LXSN exposed to either 50 ng/mL VEGF or 50 ng/mL TNF-α. Data for NF-κB activity was normalized for transfection efficiency with a Renilla reporter gene and is representative of five independent experiments. *B,* ELISA for I-κB phosphorylation in HDMEC-Bcl-2 and HDMEC-LXSN. Data presented for phosphorylated I-κB was normalized by total I-κB levels in each cell lysate. *C,* ELISA for evaluation of (*a*) CXCL8 expression 24 hours after transient transfection of HDMEC and HDMEC-Bcl-2 with SR-IκB, dnIKKβ, or $pcDNA_3$; and (*b*) CXCL8 expression 24 hours after exposure to 0 to 50 ng/mL I-κB phosphorylation inhibitor peptide (S32, S36), or the inactive control peptide (S32A, S36A). *D,* ELISA for evaluation of (*a*) CXCL1 expression 24 hours after transient transfection of HDMEC and HDMEC-Bcl-2 with SR-IκB, dnIKKβ, or $pcDNA_3$; and (*b*) CXCL1 expression 24 hours after exposure to 0 to 50 ng/mL I-κB phosphorylation inhibitor peptide (S32, S36) or the inactive control peptide (S32A, S36A). Experiments were done in triplicate and data were normalized by cell number. Statistical significance was determined at $P \leq 0.05$.

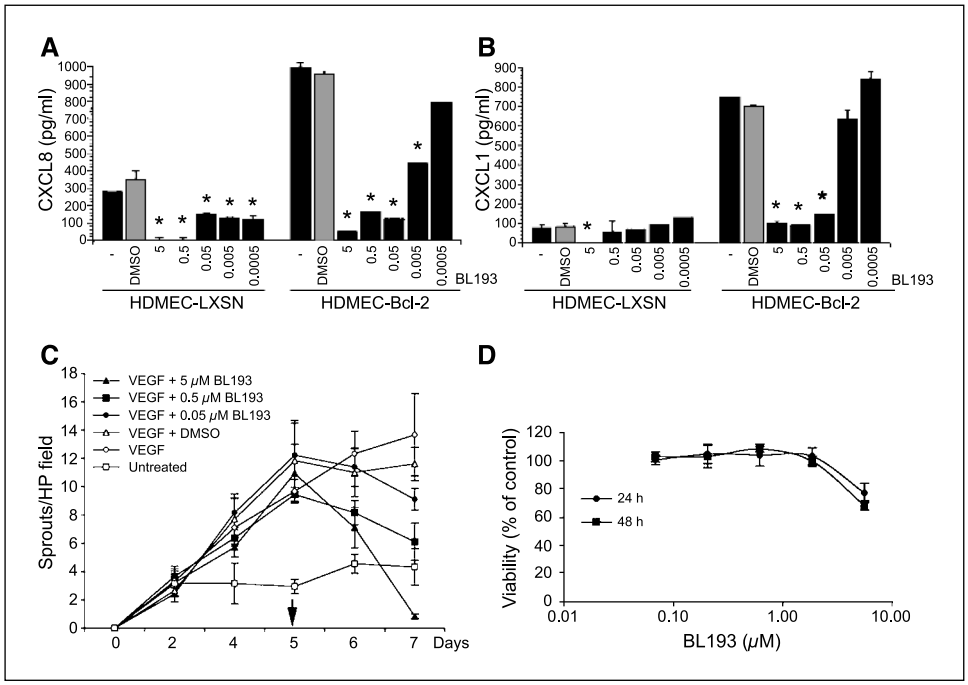

Figure 5. The small molecule inhibitor BL193 prevents Bcl-2–induced CXC chemokine up-regulation and endothelial cell sprouting. *A* and *B*, ELISA for evaluation of CXCL8 and CXCL1 expression in HDMEC-Bcl-2 or HDMEC-LXSN exposed to 0 to 5 μmol/L BL193 for 24 hours. The results were normalized by cell number and are representative of three independent experiments. *C*, effect of BL193 on endothelial cell sprouting. HDMEC were cultured on type I collagen with 50 ng/mL VEGF to induce sprouting. Starting on the 5th day and continuing thereafter, cells were exposed to 0 to 5 μmol/L BL193 in presence of 50 ng/mL VEGF. At daily intervals, the number of sprouts was counted in six random fields from triplicate wells per condition. *D*, SRB assays for evaluation of HDMEC cell viability after exposure to 0 to 5 μmol/L BL193. Data is presented as percentage of vehicle (i.e., DMSO)-treated controls from triplicate wells per condition. Statistical significance (*) was determined at $P \leq 0.05$.

submicromolar concentrations of BL193 did not affect the viability of HDMEC cells (Fig. 5*D*), demonstrating that the decrease in sprouting observed when cells were exposed to BL193 was not simply caused by drug-induced cytotoxicity and cell death.

Taken together, these data show that VEGF induces Bcl-2 expression, and that once Bcl-2 is up-regulated in endothelial cells it initiates a NF-κB–dependent pathway that results in elevated CXC chemokine expression levels. This pathway can also be initiated by endogenous levels of VEGF secreted by tumor cells (data not shown). These data led us to propose a model (Fig. 6) in which Bcl-2 expression in endothelial cells regulates two distinct, and perhaps synergistic, signaling pathways that may have direct consequences in tumor angiogenesis. First, Bcl-2 enhances endothelial cell survival by inhibiting caspase-mediated apoptotic signaling (4, 5, 11). Given the significant stresses that blood vessels withstand in the tumor microenvironment, the prosurvival input mediated by VEGF is essential for the maintenance of the tumor vasculature, as shown by elegant experiments described by Jain et al. (31). Second, Bcl-2 induces expression of at least two potent proangiogenic chemokines that can function in an autocrine pathway potentiating the angiogenic phenotype of endothelial cells locally. Bcl-2–induced chemokines may also function in a paracrine

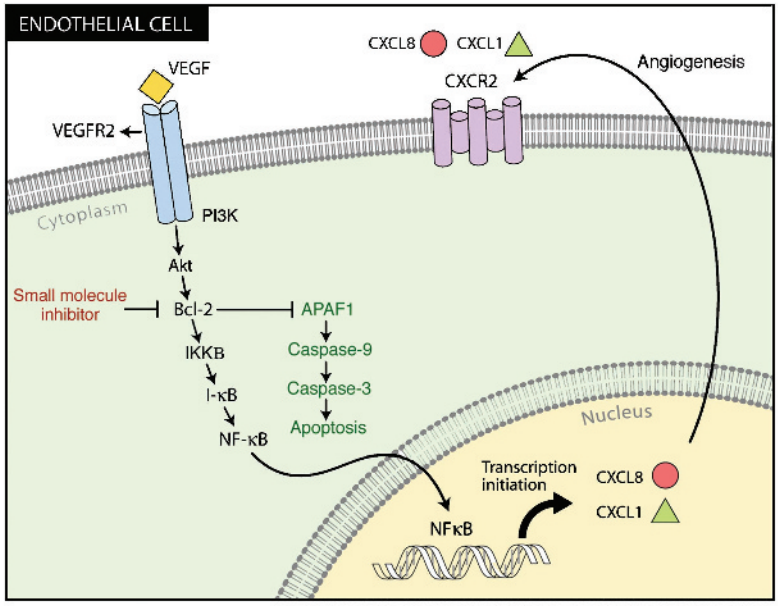

Figure 6. Bcl-2 functions as a proangiogenic signaling molecule and as a prosurvival protein in endothelial cells. This schematic model depicts the dual function of Bcl-2 in angiogenesis, as a proangiogenic and a prosurvival molecule. We and others have shown that VEGF induces Bcl-2 expression via the VEGFR2, PI3K/Akt signaling pathway, and that Bcl-2 expression enhances the survival of endothelial cells (4, 5). Here, we show that Bcl-2 also signals a proangiogenic cascade that is mediated by IKKβ kinase activity, phosphorylation of I-κB, activation of NF-κB, and expression of the proangiogenic chemokines CXCL8 and CXCL1. These chemokines are secreted and function via an autocrine pathway mediated by CXCR2 that results in enhanced angiogenesis. Inhibition of Bcl-2 function with a small molecule inhibitor (i.e., BL193) resulted in inhibition of proangiogenic CXC chemokine synthesis at concentrations 100× lower than the concentration required to induce endothelial cell apoptosis. The proposed model suggests the hypothesis that inhibition of Bcl-2 function might be an effective antiangiogenic strategy for patients with cancer.

pathway in the process of recruitment of circulating progenitor cells. Furthermore, these chemokines can directly affect tumor cell proliferation and metastasis because a large number of tumors express the receptors and respond to CXCL8- and CXCL1-mediated mitogenic and chemotactic signaling (32).

The classic function of Bcl-2 is that of a prosurvival protein (7, 33). The results of this study show a novel role for Bcl-2 as a molecule that can initiate a signaling cascade that results in the induction of angiogenesis. We have shown that up-regulation of Bcl-2 is sufficient to induce expression of the proangiogenic chemokines CXCL8 and CXCL1 through a NF-κB–mediated pathway. Importantly, we have also shown that is possible to block this pathway with small molecule inhibitors, which strengthen the rationale for exploiting this pathway as a therapeutic target for treatment of angiogenesis-dependent diseases. We conclude that Bcl-2 has multiple roles in endothelial cell physiology that can contribute to the neovascularization observed in response to tumor cell–derived proangiogenic stimuli.

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