Attenuation of retinal endothelial cell migration and capillary morphogenesis in the absence of bcl-2

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Kondo S, Tang Y, Scheef EA, Sheibani N, Sorenson CM. Attenuation of retinal endothelial cell migration and capillary morphogenesis in the absence of bcl-2. Am J Physiol Cell Physiol 294: C1521–C1530, 2008. First published April 16, 2008; doi:10.1152/ajpcell.90633.2007.—Aptosis plays a critical role during development and in the maintenance of the vascular system. B-cell leukemia lymphoma 2 (bcl-2) protects endothelial cells (EC) from apoptosis in response to a variety of stimuli. Previous work from this laboratory demonstrated attenuation of postnatal retinal vascular development and retinal neovascularization during oxygen-induced ischemic retinopathy in bcl-2-deficient (bcl-2−/−) mice. To gain further insight into the function of bcl-2 in the endothelium, we isolated retinal EC from bcl-2+/+ and bcl-2−/− mice. Retinal EC lacking bcl-2 demonstrated reduced cell migration, tenascin-C expression, and adhesion to vitronectin and fibronectin. The bcl-2−/− retinal EC also failed to undergo capillary morphogenesis in Matrigel. In addition, using an ex vivo angiogenesis assay, we observed reduced sprouting from aortic rings grown in culture from bcl-2−/− mice compared with bcl-2+/+ mice. Furthermore, reexpression of bcl-2 was sufficient to restore migration and capillary morphogenesis defects observed in bcl-2−/− retinal EC. Mechanistically, bcl-2−/− cells expressed significantly less endothelial nitric oxide synthase, an important downstream effector of proangiogenic signaling. This may be attributed to increased oxidative stress in the absence of bcl-2. In fact, incubation of retinal EC or aortic rings from bcl-2−/− mice with the antioxidant N-acetylcysteine rescued their capillary morphogenesis and sprouting defects. Thus, bcl-2-mediated cellular functions play important roles not only in survival but also in proangiogenic phenotype of EC with a significant impact on vascular development and angiogenesis.

angio genesis; apoptosis; endothelial nitric oxide; oxidative stress

APPTOSIS PLAYS AN INTEGRAL role during development, remodeling, and regression of the vascular system (9, 11, 34). Apoptosis is tightly regulated by a balanced production of pro- and antiapoptotic factors including the bcl-2 family of proteins. Bcl-2 family members affect cell death in either a positive or negative fashion. The founding family member, bcl-2, inhibits apoptosis. Although some redundancy exists between family members, expression of certain family members in an organ-specific manner may be important during development and have specific functions. Transgenic mice that lack one or more of the bcl-2 family members have provided valuable insight into the function of these genes and their tissue-specific function.

The bcl-2-deficient (bcl-2−/−) mice are viable but exhibit marked lymphocyte, neuronal, and intestinal apoptosis, develop renal hypoplasia/cystic dysplasia, and are hypopigmented (for review, see Ref. 40). The role bcl-2 plays during vascular development and angiogenesis is not completely understood. We recently demonstrated significant defects in postnatal retinal vascular development and retinal neovascularization during oxygen-induced ischemic retinopathy in bcl-2−/− mice. The retinas from the bcl-2−/− mice exhibited decreased branching and formation of major vessels concomitant with reduced number of endothelial cells (EC), pericytes, and capillaries, as well as an inability to undergo ischemia-driven neovascularization (47). Thus, expression of bcl-2 is important during vascular development and angiogenesis.

Recent studies using EC in culture have also demonstrated that modulation of bcl-2 expression is central to the activity of pro- and antiangiogenic factors. Angiogenic factors such as basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) mediate their effects, at least in part, through enhanced expression of bcl-2 (14, 21, 25, 29, 30). In contrast, most antiangiogenic factors, including thrombospondin 1 (TSP-1) and endostatin, inhibit angiogenesis by inducing EC apoptosis through downregulation of bcl-2 expression (7, 17). In addition, overexpression of bcl-2 in EC not only enhances formation of blood vessels, but also promotes progressive maturation of vasculature by recruitment of vascular smooth muscle cells/pericytes (19, 28). Therefore, bcl-2 may function not only as a survival factor, but also as an important modulator of vascular homeostasis.

Apoptosis of EC plays an important role during angiogenesis, and it is tightly regulated by expression of pro- and antiapoptotic factors (31, 46, 48). However, the identity of these factors and, specifically, the role bcl-2 plays during these processes require further investigation. To this end we have isolated retinal EC from bcl-2+/+ and bcl-2−/− mice. We showed that lack of bcl-2 has a great impact on retinal EC proliferation, adhesion, migration, and capillary morphogenesis. This is mediated, at least in part, through uncoupling of important signaling pathways downstream of VEGF and its receptor (VEGFR-2), including endothelial nitric oxide synthase (eNOS), which promote a proangiogenic phenotype. Thus, our results support an important role for bcl-2 in modulation of EC proangiogenic phenotype.

MATERIALS AND METHODS

Experimental animals and cell cultures. All animal studies were carried out in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic
and Vision Research and were approved by the Institutional Animal Care Committee of the University of Wisconsin School of Medicine and Public Health. Immortomice expressing a temperature-sensitive SV40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA). Bcl-2--/-- mice were crossed with the Immortomice and screened as previously described (52). Eyes from six to seven pups 4-wk-old wild-type and bcl-2--/-- Immortomice were enculturated and hemisected. The retinas were dissected out aseptically under a dissecting microscope and kept in serum free-Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing penicillin/streptomycin (Sigma, St. Louis, MO). Retinas (12 to 14 from one litter) were pooled together, rinsed with DMEM, minced into small pieces in a 60-mm tissue culture dish using sterilized razor blades, and digested in 5 ml of collagenase type I (Worthington, Lakewood, NJ; 1 mg/ml) in serum-free DMEM for 30–45 min at 37°C. Following digestion, DMEM with 10% fetal bovine serum (FBS) was added and cells were pelleted. The cellular digests were then filtered through a double layer of sterile 40-μm nylon mesh (Sefar America, Hanover Park, IL), centrifuged at 400 g for 10 min to pellet cells, and then cells were washed twice with DMEM containing 10% FBS. The cells were resuspended in 1.5 ml medium (DMEM with 10% FBS) and incubated with sheep anti-rat magnetic beads (Invitrogen) precoated with anti-platelet endothelial cell adhesion molecule (PECAM)-1 antibody (MEC13.3, BD Biosciences, Bedford, MA), as described previously (43). After affinity binding, magnetic beads were washed six times with DMEM with 10% FBS, and bound cells in EC growth medium were plated into a single well of a 24-well plate precoated with 2 μg/ml of human fibronectin (BD Biosciences). EC were grown in DMEM containing 20% FBS, 2 mM l-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml, endothelial growth supplement 100 μg/ml (Sigma), and murine recombinant interferon-γ (R & D, Minneapolis, MN) at 44 U/ml. Cells were maintained at 33°C with 5% CO2. Cells were progressively passed to larger plates, maintained, and propagated in 1% gelatin-coated 60-mm dishes. The experiments described here were performed with three separate isolations of cells, with similar results.

Cell proliferation and apoptosis. The cell proliferation assays were performed by plating cells in 60-mm tissue culture dishes and counting the number of cells every other day for 2 wk. Cells (10,000) were plated in triplicate in multiple sets on 60-mm tissue culture plates. Cells were fed every other day, and the cell number in one set of plates was determined by counting on each indicated day (32). As an apoptotic stimulus, bcl-2-/+ and bcl-2--/-- retinal EC were incubated with 1 mM 5-fluorouracil (5-FU, Sigma) or serum-free DMEM for 48 h. Apoptotic cells were determined by in situ monitoring of caspase-3 activity using the CaspACE FITC-VAD-FMK in situ marker (Promega, Madison, WI) (38).

Scratch wound and transwell migration assays. For scratch wound assays, cells (4 × 10^5) were plated on 60-mm tissue culture dishes and allowed to reach confluence (2–3 days). After aspiration of the medium, cell layers were wounded using a 1-ml micropipette tip. Plates were then rinsed with phosphate-buffered saline (PBS; Sigma) and fed with growth medium, and wound closure was monitored by phase microscopy and photographed at 0, 24, 48, and 72 h in digital format. The distance migrated as percentage of total distance was determined for quantitative assessments as described previously (23). Similar assays were performed in the presence of 5-FU (1 μM) to rule out the potential contribution of differences in cell proliferation to wound closure (6). These experiments were repeated at least twice, with similar results.

The transwell migration assays were performed as recently described (35). Briefly, the bottom side of 8-μm pore size Costar transwell (Corning, Acton, MA) was coated with 2 μg/ml of fibronectin in PBS overnight at 4°C. The bottom of the transwell was then rinsed with PBS and blocked with bovine serum albumin (BSA, 2% in PBS) for 1 h at room temperature. Cells were trypsinized, resuspended in serum-free DMEM, plated at 1 × 10^5 cells/0.1 ml on top of the transwell membrane, and incubated for 3 h at 37°C. Cells were fixed with paraformaldehyde (PFA 4% in PBS; Electron Microscopy Sciences, Hatfield, PA) for 15 min at room temperature and stained with hematoxylin/eosin, and the membrane was mounted on a glass slide. The mean number of cells migrated through the filter was determined by counting 10 high-power fields (×100).

Capillary morphogenesis in Matrigel. Matrigel (10 mg/ml) (BD Biosciences) was applied at 0.5 ml/35-mm tissue culture dish and incubated at 37°C for at least 30 min to harden. Cells were removed using trypsin-EDTA, washed with growth medium once, and resuspended at 1.0 × 10^5 cells/ml in serum-free growth medium. In some cases, cells were incubated for 16 h in medium containing 1 mM N-acetylcyesteine (NAC; Sigma) before being plated on Matrigel. Cells (2 ml), in the presence or absence of NAC, were gently added to the Matrigel-coated plates, incubated at 37°C, monitored for 6–24 h, and photographed using a Nikon microscope equipped with a digital camera. For quantitative assessment of the data, the mean number of branch points in 10 high-power fields (×100) was determined after 24 h. A longer incubation of the cells did not result in further branching morphogenesis (43).

Cell adhesion assays. Cell adhesion to various matrix proteins was performed as previously described (32). Briefly, varying concentrations of fibronectin, vitronectin, collagen type I, and laminin (BD BioSciences) were prepared in Tris-buffered saline [20 mM Tris, 150 mM NaCl, pH 7.6; with Ca^2+ and Mg^2+ (2 mM each; TBS with Ca/Mg)] were coated on 96-well plates overnight at 4°C. Control wells were coated with 1% BSA. The next day, plates were rinsed with TBS with Ca/Mg and blocked with 1% BSA. Cells were removed by dissociation solution (Sigma), washed with TBS, and resuspended at 5 × 10^5 cells/ml in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.6, and 4 mg/ml BSA). After blocking, plates were rinsed with TBS with Ca/Mg, 50 μl of cell suspension was added to each well containing 50 μl of TBS with Ca/Mg, and the cells were allowed to adhere to the plate for 1.5 h at 37°C. The nonadherent cells were removed by gently washing the plate with TBS containing Ca/Mg until no cells was left in wells coated with BSA. The number of adherent cells in each well was quantified by measuring the cellular phosphatase activity as previously described (52). All samples were done in triplicate.

Western blot analysis. Cells were plated at 4 × 10^5 in 60-mm dishes coated with 1% gelatin and were allowed to reach nearly 90% confluence (2 days). The cells were then rinsed once with serum-free medium and incubated with serum-free DMEM for 48 h. Conditioned medium was then collected and clarified by centrifugation. The equal volumes of samples were mixed with appropriate volume of 6× SDS sample buffer and analyzed by 4–20% SDS-PAGE (Invitrogen). In some cases, total protein lysates were prepared from the cells in a modified RIPA buffer [142.5 mM KCl, 5 mM MgCl2, 10 mM HEPES, pH 7.4, 2 mM orthovanadate, 2 mM sodium fluoride, 1% Nonidet P-40, and a complete protease inhibitor cocktail (Roche, Mannheim, Germany)]. The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with a rabbit anti-rat fibronectin polyclonal antibody (Invitrogen), a rabbit anti-chicken tenascin-C polyclonal antibody (Chemicon, Temecula, CA), anti-β1-catenin (Sigma), anti-phospho-Akt, anti-Akt, anti-heat shock protein 90 (HSP-90), anti-phospho-e-NOS (Cell Signaling Technology, Danvers, MA), and anti-eNOS (Santa Cruz, Santa Cruz, CA), at a dilution recommended by the supplier. The blot was washed, incubated with appropriate secondary antibody (1:10,000 dilution), and developed using enhanced chemiluminescence (Amersham, Piscataway, NJ) (38, 42).

FACScan analysis. FACScan analysis was performed essentially as previously described (23). The cells were washed once with PBS containing 0.04% EDTA and incubated with 2 ml of dissociation
solution (Sigma) to remove the cells from the plate. The cells (10^6) were washed with TBS, blocked in TBS containing 1% goat serum on ice for 20 min, and incubated with the primary antibody at dilutions recommended by the supplier: anti-PECAM-1, anti-αv-integrin (01521 D), anti-α5-integrin (BD Biosciences), anti-vascular endothelial (VE)-cadherin (Alexis Biochemical, San Diego, CA), B4-lectin (Sigma), anti-β1-integrin, anti-α5-integrin (MAB1949), anti-β3-integrin (MAB1957), anti-αvβ3-integrin (MAB1976Z), or control IgG (Chemicon). For antibodies that required cell permeabilization, cells were removed from the dish, washed with PBS, fixed with 2% PFA on ice for 30 min, washed with PBS, and resuspended in PBS containing 0.1% Triton-X-100 and 0.1% BSA containing appropriate dilution of primary antibody. The cells were washed with TBS containing 1% BSA and then incubated with the appropriate secondary antibody (1:200; Pierce) on ice for 30 min. Following the incubation, cells were washed twice with TBS containing 1% BSA and resuspended in 0.5 ml of TBS containing 1% BSA. FACScan analysis was performed on a FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Reexpression of bcl-2 in retinal EC. Retinal EC (5 × 10^4) were plated on a 60-mm culture dish. The next day, cells were rinsed twice with serum-free medium and transfected with pcDNA3:bcl-2 expression vector or pcDNA3 (Invitrogen) as a control vector in the presence of 0.1 ml of Lipofectin (Invitrogen) as previously described (37). Following incubation, plates were rinsed with growth medium to remove Lipofectin solution and fed with growth medium. To confirm the effectiveness of transfection, the levels of bcl-2 expression were analyzed by Western blotting of protein lysates 2 days after transfection as previously described (38). Bcl-2-transfected bcl-2−/− retinal EC were used in migration and capillary morphogenesis assays as described above.

Aortic ring culture. Thoracic aortas were removed from mice, immediately transferred to a 50-ml tubes with 40 ml of ice-cold serum-free DMEM, and washed by shaking the tube for 15 s. The periaortic fibro adipose tissue was carefully removed with fine micro-dissecting forceps and iridectomy scissors, with special attention paid to not damage the aortic wall. Aortic rings (1 mm long, 8 per aorta) were sectioned and rinsed extensively in three consecutive washes of DMEM. The mouse aorta pieces were then embedded in Matrigel (10 mg/ml)-plated wells (0.3 ml/well) in 12-well plates on ice with up to six aortic rings per well. The Matrigel containing the aortic rings was incubated in 37°C incubator for 30 min to harden. Then 1 ml DMEM containing 1% FBS was added into each well. In some cases, 1 mM NAC was added to the cultures. The cultures were kept at 37°C in a humidified environment and fed every other day. Five-day cultures were photographed using a Nikon microscope equipped with a digital camera. For quantitative assessment of sprouting, the areas of sprouting per millimeter of tissue was assessed using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij).

Statistical analysis. Statistical differences between groups were evaluated with Student’s unpaired t-test (two-tailed). Data are shown as means ± SE. P values <0.05 were considered significant.

RESULTS

Isolation and characterization of bcl-2+/+ and bcl-2−/− retinal EC. We previously demonstrated that bcl-2−/− mice have significant defects in normal postnatal retinal vascularization and retinal neovascularization during oxygen-induced ischemic retinopathy (47). Thus, expression of bcl-2 is required for both appropriate development of retinal vasculature and retinal neovascularization. To determine the role of bcl-2 in cellular angiogenic processes, including EC migration and capillary morphogenesis, we isolated retinal EC from bcl-2+/+ and bcl-2−/− mice. We first examined the morphology and expression of EC markers in isolated retinal EC to confirm that these cells maintain their EC characteristics. Both bcl-2+/+ and bcl-2−/− retinal EC expressed VE-cadherin and PECAM-1 and were positive for B4-lectin (a mouse EC-specific lectin; Fig. 1A). Figure 1B shows that the morphologies of retinal EC prepared
from bcl-2+/+ and bcl-2−/− mice were similar when plated on gelatin-coated plates.

**Bcl-2−/− retinal EC demonstrated increased proliferation and apoptosis.** We have previously shown increased proliferation and apoptosis in the developing retinal vasculature of bcl-2−/− mice (47). We next addressed whether bcl-2−/− retinal EC demonstrate accelerated cell death following an apoptotic stimulus. Bcl-2+/+ and bcl-2−/− retinal EC were incubated with 1 mM 5-FU for 48 h, and apoptotic cells were identified by in situ monitoring of caspase activity (Fig. 1C). We observed a 1.7-fold increase in the percentage of apoptotic cells in bcl-2−/− retinal EC compared with bcl-2+/+ retinal EC (34 ± 12% apoptotic cells in bcl-2+/+ vs. 58 ± 2% apoptotic cells in bcl-2−/−; P < 0.05). There was no significant difference in the rates of apoptosis in the absence of 5-FU (P > 0.05). Next, the rates of cell proliferation were determined by counting the number of cells every other day for 2 wk. Figure 1D shows that bcl-2−/− retinal EC proliferated at a significantly faster rate (P < 0.01; days 8–14) than the bcl-2+/+ retinal EC. These data are consistent with our previous results in which we observed accelerated cell death in bcl-2−/− thymocytes and ureteric bud cells following an apoptotic stimulus (38, 44), as well as an antiproliferative effect with bcl-2 expression (Dr. J. E. French, personal communication).

**Attenuation of capillary morphogenesis in bcl-2−/− retinal EC.** Capillary morphogenesis is fundamental in vascular development and remodeling. Retinal EC rapidly organize into capillary-like networks in response to vascular endothelial growth factor (VEGF) and other factors (43). To determine whether capillary morphogenesis is affected in the absence of bcl-2, bcl-2+/+, and bcl-2−/−, retinal EC were plated on Matrigel. Figure 2A shows that bcl-2+/+ retinal EC form a well-branched capillary-like network by 24 h. In contrast, bcl-2−/− retinal EC ability to organize into a capillary-like network was severely compromised. The quantitative assessment of the data is shown in Fig. 2B. A longer incubation of the cells did not result in further branching morphogenesis. Thus, lack of bcl-2 was associated with the inability of EC to undergo capillary morphogenesis.

**Attenuation of sprouting of aorta ex vivo cultures from bcl-2−/− mice.** We next used the aortic ex vivo angiogenesis assay to examine the impact lack of bcl-2 has on vascular sprouting. Aortic rings were prepared and cultured in Matrigel as described in MATERIALS AND METHODS. We observed a significant decrease in the number of outgrowths/sprouting in the absence of bcl-2 (Fig. 2C). The quantitative assessment of the data is shown in Fig. 2D (P < 0.05). Thus, the ability of vascular cell sprouting was also significantly diminished in the absence of bcl-2.

**Bcl-2−/− retinal EC were less migratory.** Cell adhesion and migratory properties impact the ability of EC to form branched structures. The inability of bcl-2−/− retinal EC and aortas to undergo capillary morphogenesis may indicate suboptimal migration in the absence of bcl-2. We used a scratch wound assay to evaluate the migration characteristics of retinal EC. The confluent monolayer of bcl-2+/+ and bcl-2−/− retinal EC were wounded as described in MATERIALS AND METHODS and returned to 37°C in the presence of 5-FU (1 μM) to prevent the impact of differences in the rate of cell proliferation on wound closure (6). Bcl-2−/− retinal EC migrated and closed a significant area of the wound after 48 h (Fig. 3A). However, in bcl-2−/− retinal EC, a significant area of the wound remained uncovered after 48 h (Fig. 3A). The quantitative assessment of the data is shown in Fig. 3B (P < 0.05). Similar results were observed using a transwell migration assay (data not shown; Fig. 6E).
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Thus, bcl-2−/− retinal EC are less migratory compared with bcl-2+/+ retinal EC.

Altersations in production of extracellular matrix proteins in bcl-2−/− retinal EC. Extracellular matrix (ECM) proteins are implicated in regulation of angiogenesis including EC adhesion and migration. In particular, fibronectin, tenascin-C, osteopontin, and TSP-1 play important roles in the EC migratory and proliferative events as well as regulation of angiogenesis (5, 22, 24, 37, 51). We next determined whether lack of bcl-2 impacts production of these ECM proteins in retinal EC. Serum-free conditioned medium was prepared from bcl-2+/+ and bcl-2−/− retinal EC and evaluated by Western blot analysis. Figure 4 shows bcl-2−/− retinal EC secreted decreased amounts of tenascin-C. A modest decrease in fibronectin and a modest increase in TSP-1 expression, compared with bcl-2+/+

Bcl-2−/− retinal EC, was also observed. Osteopontin expression was similar (Fig. 4). Neither cell line expressed thrombospondin 2 (TSP-2) (data not shown). These results are consistent with the reduced migratory and diminished proangiogenic characteristics of bcl-2−/− retinal EC.

Bcl-2−/− retinal EC were less adherent on fibronectin and vitronectin despite a similar pattern of integrin expression. Integrins are cell surface receptors that play critical roles in directing EC behavior such as adhesion, migration, and capillary morphogenesis through binding to the ECM proteins (15). Bcl-2−/− retinal EC showed altered migration characteristics, capillary morphogenesis, and secretion of ECM proteins. We next examined bcl-2+/+ and bcl-2−/− retinal EC ability to adhere to various ECM proteins including fibronectin, vitronectin, collagen type I, and vitronectin (Fig. 5A). Bcl-2+/+ retinal EC adhered well to fibronectin and vitronectin. In contrast, bcl-2−/− retinal EC did not adhere well to either fibronectin or vitronectin. Minimal adhesion was observed on collagen type I or laminin for either bcl-2+/+ and bcl-2−/− retinal EC. Thus, lack of bcl-2 impacts retinal EC adhesion.

The difference in adhesion properties of these cells suggested alterations in expression and/or activity of integrins expressed on the surface of these cells. We determined expression of various integrins on the surface of retinal EC by FACScan analysis. Bcl-2+/+ and bcl-2−/− retinal EC expressed similar levels of α3, β3, α5, αv, β1, and β3-integrins on their surface. Neither bcl-2+/+ nor bcl-2−/− retinal EC expressed the α1-integrin (Fig. 5B). Thus, the alterations observed in the absence of bcl-2 are independent of changes in the expression levels of integrins and may be dependent on the alterations in the affinity and/or avidity of these integrins.

Reexpression of bcl-2 restores capillary morphogenesis of bcl-2−/− retinal EC. We next examined whether reexpression of bcl-2 in bcl-2−/− retinal EC was sufficient to restore capillary morphogenesis. Bcl-2−/− retinal EC were transiently

Fig. 3. Bcl-2−/− retinal EC are less migratory. Cell migration was determined by scratch wounding of the retinal EC monolayers, and wound closure was monitored by photography. A: representative experiment. Note that bcl-2−/− retinal EC cover a significant portion of the wound within 48 h. In contrast, a significant portion of the wound remains uncovered in bcl-2−/− retinal EC. B: quantitative assessment of the data. Note that bcl-2−/− retinal EC are significantly less migratory compared with bcl-2−/− retinal EC (*P < 0.05). These experiments were repeated with two different isolation of EC, with similar results.
transfected with the empty expression vector or a vector encoding murine bcl-2 cDNA. The expression of bcl-2 in bcl-2−/− retinal EC was confirmed with Western blot analysis and was comparable to the levels seen in the wild-type cells [data not shown (38)]. Bcl-2+/+ cells organized on Matrigel and formed a capillary-like network, whereas minimal capillary morphogenesis was observed in bcl-2−/− cells expressing empty vector (Fig. 6A). Reexpression of bcl-2 in bcl-2−/− retinal EC restored their ability to form processes and organize into a capillary-like network (Fig. 6A). The quantitative assessment of the data is shown in Fig. 6B. Thus, the expression of bcl-2 in bcl-2−/− retinal EC significantly improved their ability to undergo capillary morphogenesis (P < 0.05).

We next evaluated the impact of bcl-2 expression on migration of bcl-2−/− retinal EC using scratch wound and transwell assays. Bcl-2+/+ and bcl-2−/− retinal EC expressing empty vector migrated similar to that observed in Fig. 3A. Bcl-2+/+ retinal EC expressing empty vector effectively closed a significant area of the wound after 48 h. In contrast, in bcl-2−/−

Fig. 6. Reexpression of bcl-2 restores the migration and capillary morphogenesis defects observed in bcl-2−/− retinal EC. Bcl-2+/+ and bcl-2−/− retinal EC were transfected with the empty pcDNA3 vector and bcl-2−/− retinal EC transfected with the pcDNA3 vector expressing murine bcl-2. A: capillary morphogenesis of bcl-2−/− retinal EC transfected with the empty pcDNA3 vector or the pcDNA3 vector expressing bcl-2 were evaluated on Matrigel. C: quantitative assessment of the data. Note the significant attenuation of bcl-2−/− retinal EC capillary morphogenesis compared with bcl-2+/+ cells (⁎P < 0.05), whereas bcl-2−/− cells expressing bcl-2 underwent significant capillary morphogenesis compared with vector-transfected bcl-2−/− cells (⁎⁎P < 0.05). C: bcl-2+/+ and bcl-2−/− retinal EC transfected with the empty pcDNA3 vector or the pcDNA3 vector expressing bcl-2 were evaluated in a scratch wound migration assay. D: quantitative assessment of the data. Note the significant attenuation of wound closure in bcl-2−/− retinal EC expressing empty vector compared with bcl-2+/+ cells (⁎P < 0.05), whereas a significant area of the wound is closed in bcl-2−/− cells expressing bcl-2 compared with vector expressing bcl-2−/− cells (⁎⁎P < 0.05). E: bcl-2+/+ and bcl-2−/− retinal EC transfected with the empty pcDNA3 vector or the pcDNA3 vector expressing bcl-2 were evaluated in a transwell migration assay. Note the significant decrease in migration in bcl-2−/− retinal EC expressing vector compared with bcl-2+/+ cells (⁎⁎P < 0.05), whereas bcl-2−/− cells expressing bcl-2 migrated significantly faster than bcl-2−/− cells expressing vector (⁎⁎⁎P < 0.05). These experiments were repeated with two different isolations of retinal EC with similar results.
retinal EC expressing empty vector, a significant area of the wound remained uncovered. However, expression of bcl-2 in bcl-2−/− retinal EC significantly improved their ability to cover the wound (Fig. 6C). The quantitative assessment of the data is shown in Fig. 6D (P < 0.05). Similar results were observed using the transwell migration assay (Fig. 6E). Thus, reexpression of bcl-2 in bcl-2−/− retinal EC significantly improved their migration.

**Decreased expression of eNOS in the absence of bcl-2.**

VEGF is a major player in retinal vascular development and angiogenesis (2, 26, 36, 49). A major pathway used by VEGF to promote angiogenesis is through activation of Akt1 and eNOS (3, 13, 18). Therefore, we then examined expression and/or phosphorylation of eNOS in retinal EC. Western blot analysis was performed to determine expression levels of phosphorylated (active) and total eNOS, as well as its associated protein, HSP-90. HSP-90 expression was similar in both bcl-2+/+ and bcl-2−/− retinal EC. However, bcl-2−/− retinal EC demonstrated a significant decrease in the total amount of eNOS compared with bcl-2+/+ cells (Fig. 7A). Bcl-2+/+ cells expressed a significant amount of phospho-eNOS, whereas bcl-2−/− cells expressed undetectable levels of phospho-eNOS (Fig. 7A). The expression of eNOS was also significantly reduced in retinal vasculature of bcl-2−/− mice (Fig. 7B, arrowheads). We also examined the expression of Akt1 and phosphorylated Akt1 in lysates from bcl-2+/+ and bcl-2−/− retinal EC by Western blot analysis. Figure 7C demonstrates that the expression of Akt1 and phospho-Akt1 was similar in retinal EC from bcl-2+/+ and bcl-2−/− mice. We were unable to detect any inducible NOS or neuronal NOS in these cells (data not shown).

**Incubation with NAC restores capillary morphogenesis and sprouting of aortic ex vivo cultures in the absence of bcl-2.**

Bcl-2 plays a central role in maintaining the mitochondrial oxidative homeostasis (53). Thus, lack of bcl-2 may contribute to increased intracellular oxidative stress and inhibition of angiogenesis. This notion is further supported by the alterations in eNOS expression and activity in bcl-2−/− retinal EC (Fig. 7, A and B). We next used NAC as an antioxidant and evaluated its impact on capillary morphogenesis of bcl-2−/− retinal EC. We observed that bcl-2−/− retinal EC undergo minimal capillary morphogenesis in Matrigel (Figs. 2A, 6A, and 8A). However, incubation of bcl-2−/− retinal EC with NAC significantly enhanced their ability to undergo capillary morphogenesis in Matrigel (Fig. 8A). The quantitative assessment of the data is shown in Fig. 8B (P < 0.05). The incubation of bcl-2+/+ retinal EC with NAC had no stimulatory effect on their capillary morphogenesis (data not shown). The NAC incubation of aortic ex vivo cultures also resulted in enhanced sprouting in the absence of bcl-2 (Fig. 8C). The quantitative assessment of the data is shown in Fig. 8D (P < 0.05). Thus, lack of bcl-2 in the endothelium may result in increased reactive oxygen species, aberrant eNOS activity, and inhibition of capillary morphogenesis and sprouting.

**DISCUSSION**

Angiogenic signals promote EC cytoskeletal reorganization, migration, proliferation, survival, and differentiation into a patent vessel (27). We recently demonstrated that lack of bcl-2 has severe consequences on mouse retinal physiological and pathological angiogenesis (47). Here we prepared retinal EC from bcl-2+/+ and bcl-2−/− mice to determine how lack of bcl-2 impacts their proangiogenic properties. We believe that the lack of bcl-2 disrupts the interrelationship between cell survival and proangiogenic signaling, preventing capillary morphogenesis. We show that bcl-2−/− retinal EC I) exhibit increased rates of apoptosis and proliferation; 2) fail to undergo
capillary morphogenesis in vitro and ex vivo; 3) are less migratory; 4) exhibit alterations in expression of ECM proteins; 5) are less adherent on vitronectin and fibronectin independent of changes in integrin expression levels; and 6) express reduced levels of eNOS independent of changes in expression and activity of Akt1. In addition, reexpression of bcl-2 or incubation with the antioxidant NAC was sufficient to restore migration and capillary morphogenesis defects observed in bcl-2−/− retinal EC. Thus, bcl-2-mediated cell functions play important role(s) in modulation of endothelial cellular oxidative state impacting vascular development and angiogenesis.

Apoptosis plays a critical role during development and in the maintenance of vascular system. In vitro, bcl-2 protects EC from apoptosis due to loss of cell adhesion or growth factor deprivation (12, 21). VEGF-antiapoptotic effect is mediated through upregulation of bcl-2 expression in EC (8, 28, 29). The increased expression of bcl-2 in EC can result in enhanced angiogenesis independent of its survival activity (29). In contrast, TSP-1 and transforming growth factor-β downregulate bcl-2 expression inducing EC apoptosis, inhibiting angiogenesis (17, 45). We observed increased apoptosis in bcl-2−/− retinal EC in the presence of 5-FU (Fig. 1C). This was compensated for, in part, by an increase in the rate of proliferation in bcl-2−/− retinal EC (Fig. 1D). Thus, bcl-2 plays a central role in EC survival and angiogenesis.

The identification of molecular and cellular pathways by which bcl-2 mediates cell survival is essential for determination of its role during vascular development and angiogenesis. Bcl-2 may facilitate survival of precursor cells or play a more active role during morphogenesis through its interactions with other proteins. We have previously demonstrated that bcl-2 directly interacts with paxillin, a focal adhesion protein with important roles in cell adhesion, migration, and survival (41). We believe this is an important function of bcl-2 during morphogenesis and/or organogenesis, when three-dimensional structures are forming. The interaction of bcl-2 with paxillin may bypass the need for integrins for survival in cells that need to detach from their basement membrane during morphogenesis. This is consistent with significant ureteric bud and retinal vasculature branching defects observed in bcl-2−/− mice (41, 47), as well as alterations in retinal EC adhesion observed in the present study. We have shown that interaction of paxillin with bcl-2 occurs through the BH4 domain of bcl-2, the domain essential for antiapoptotic activity of bcl-2 (41). Thus, survival signaling provided by bcl-2 may play a crucial role during capillary morphogenesis by actively impacting cell adhesive and cell migratory functions.

Dynamic interactions between EC and components of their surrounding ECM are necessary for their invasion, migration, and survival during angiogenesis (16). Furthermore, response of EC to anti- and proangiogenic factors is influenced by the composition of their ECM (1). Lack of bcl-2 in retinal EC was associated with changes in expression of ECM proteins and adhesion to fibronectin and vitronectin. Bcl-2−/− retinal EC secreted decreased amounts of tenascin-C and fibronectin and increased levels of TSP-1 compared with bcl-2+/+ retinal EC. Tenascin-C and fibronectin expression are generally associated with a more migratory proangiogenic phenotype, whereas TSP-1 is antimigratory and inhibits angiogenesis. Thus, bcl-2 may impact proangiogenic characteristics of EC through modulation of ECM proteins produced by EC impacting their behavior. Unfortunately, how the presence or lack of bcl-2 affects the expression of these ECM proteins requires investigation.
Incubation of human dermal microvascular EC with VEGF results in upregulation of bcl-2 in a VEGF receptor dependent manner. This process may capillary morphogenesis of EC through activation of nuclear factor-κB (NF-κB) and production of CXC chemokines (19, 20). It is presently not clear whether similar pathways are functional in vivo and/or in retinal EC. However, production of chemokines may provide an additional argument for bcl-2-mediated proangiogenic functions independent of its prosurvival activity. The production of these proangiogenic chemokines may also promote production of ECM proteins that support a proangiogenic state. NF-κB is one the major redox-sensitive transcription factors, which is activated in an oxidant-dependent manner (4, 10, 30). It is presently not clear how bcl-2 activates NF-κB in EC promoting expression of these chemokines. Recent studies suggest that bcl-2 plays an active role in maintaining mitochondrial oxidative homeostasis through its direct interaction with glutathione (53). We showed in the present study that the majority of proangiogenic defects observed in bcl-2−/− retinal EC are restored in the presence of the antioxidant NAC. Thus, alterations in intracellular oxidative state in the absence of bcl-2 may impact NF-κB activity and its downstream effectors.

VEGF signaling through its receptor (VEGFR-2) also results in activation of Akt1. eNOS is a downstream target of Akt1. The phosphorylation of eNOS by Akt1 results in its activation and production of nitric oxide, which mediates the proangiogenic phenotype of bcl-2−/− retinal EC. Here the changes in eNOS expression and activity were, however, independent of Akt1 expression and/or activation in retinal EC. Thus, in the absence of bcl-2, the expression and/or activity of eNOS may be uncoupled from that of Akt1. In addition, incubation of bcl-2−/− retinal EC with NAC did not significantly impact expression and/or phosphorylation of eNOS (data not shown). However, whether incubation of bcl-2−/− retinal EC with NAC impacts eNOS function and/or NO production remain to be determined.

In summary, we demonstrated that bcl-2 is an important modulator of retinal EC adhesion, migration, and capillary morphogenesis. Mice deficient in bcl-2 or retinal EC from these mice exhibit defects in proangiogenic signaling and fail to undergo angiogenesis. Strategies that target the bcl-2-related functions in retinal EC may permit the development of new therapeutic methods for the inhibition of progressive retinopathies.

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REFERENCES


38. Sorenson CM. Bcl-2 AND RETINAL ENDOTHELIAL CELLS


