BIM Deficiency Differentially Impacts the Function of Kidney Endothelial and Epithelial Cells through Modulation of their Local Microenvironment

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Running title: BIM deficient kidney endothelial and epithelial cells

Keywords: Angiogenesis; Apoptosis; Capillary Morphogenesis; Extracellular Matrix Proteins, Organogenesis
ABSTRACT

The extracellular matrix (ECM) acts as a scaffold for kidney cellular organization. Local secretion of the ECM allows kidney cells to readily adapt to changes occurring within the kidney. In addition to providing structural support for cells, the ECM also modulates cell survival, migration, proliferation and differentiation. Although aberrant regulation of ECM proteins can play a causative role in many diseases, it is not known whether ECM production, cell adhesion and migration are regulated in a similar manner in kidney epithelial and endothelial cells. Here we demonstrate that lack of BIM expression differentially impacts kidney endothelial and epithelial cell ECM production, migration and adhesion, further emphasizing the specialized role of these cell types in kidney function. *bim* /- kidney epithelial cells demonstrated decreased migration, increased adhesion, and sustained expression of osteopontin and thrombospondin-1 (TSP1). In contrast, *bim* /- kidney endothelial cells demonstrated increased cell migration, and decreased expression of osteopontin and TSP1. We also observed a 5-fold increase in VEGF expression in *bim* /- kidney endothelial cells consistent with their increased migration and capillary morphogenesis. These cells also had decreased eNOS activity and NO bioavailability. Thus, kidney endothelial and epithelial cells make unique contributions to the regulation of their ECM composition with specific impact on adhesive and migratory properties that are essential for their proper function.
INTRODUCTION

Bcl-2 is the founding member of a family of proteins that influence apoptosis. Family members contain conserved regions denoted as Bcl-2 homology (BH) domains. Pro-apoptotic members are divided into those that only contain a BH3 domain and those that contain multiple BH domains (14). BIM is a BH3 only containing pro-apoptotic protein. Anoikis is a form of cell death resulting from matrix detachment. BIM is a critical mediator of anoikis in epithelial cells, acting as a sensor of integrin and growth factor signals to the Erks pathway (17). Although it is well established that extracellular matrix (ECM) expression can impact cell survival, less is known as to whether modulation of proteins that influence apoptosis can impact ECM production and tissue homeostasis in a cell type specific manner.

The mammalian kidney is a complex organ that contains over 25 different cell types. It is a highly vascularized organ in which the various segments of the vascular tree accomplish specialized regional functions (5). The microenvironment of the kidney consists of epithelial, vascular, fibroblast and smooth muscle cells embedded in a complex network of ECM proteins, which enhances the complexity of this microenvironment. The ECM is locally secreted and acts as a scaffold for tissue organization, regulating growth factor and cytokine availability to aid tissue homeostasis. ECM composition adapts to the changing conditions within the organ, including injury. In addition to providing structural support for cells, the ECM also modulates several cell functions including cell survival, migration, proliferation and...
differentiation (3). Thus, changes in the ECM milieu can affect kidney structure/function through aberrant modulation of cell function such as cell survival.

Cell-cell and cell-matrix interactions impact cell proliferation, migration, differentiation and apoptosis. The ability of the cell to sense their three dimensional location through interaction with the ECM and neighboring cells is essential for tissue homeostasis. Altered ECM expression can impact cell adhesive mechanisms influencing tissue architecture and function. Disruption of this delicately balanced microenvironment can also lead to many disease states. However, it is not well understood whether this delicate balance is regulated similarly in all cell types housed within this microenvironment or how they may vary in their responses. Previous work from this laboratory demonstrated that bcl-2 not only regulates apoptosis, but also influences the ECM milieu, with significant impact on adhesion and migration characteristics of kidney epithelial cells. Loss of bcl-2 expression resulted in precocious down regulation of thrombospondin -1 (TSP1) and osteopontin, increased cell migration and decreased cell adhesion (28).

To begin to address whether loss of a specific pro-apoptotic protein, BIM, in kidney epithelial and endothelial cells would have a similar impact on the microenvironment, we prepared and characterized kidney cells from weanling wild-type and bim -/- mice. Kidney epithelial cells demonstrated sustained expression of TSP1 and osteopontin, while kidney endothelial cells demonstrated decreased expression. These changes corresponded with decreased migration and increased adhesion to fibronectin,
vitronectin and collagen IV in bim-/- kidney epithelial cells. In contrast, bim-/- kidney endothelial cells demonstrated increased migration, enhanced capillary morphogenesis, decreased phosphorylated endothelial nitric oxide synthase (p-eNOS) expression, a 2-fold decrease in nitric oxide (NO) production and a 5-fold increase in VEGF expression. Thus, loss of bim expression differentially impacts kidney endothelial and epithelial cell function through modulation of their responses to their distinct local microenvironment.
MATERIALS AND METHODS

Experimental animals and cell cultures

The mice used for these studies were maintained and treated in accordance with our protocol approved by the University of Wisconsin animal care and use committee. Immortomice expressing a temperature-sensitive SV40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA). *bim* -/- mice (Jackson Laboratory, Bar Harbor, ME) were crossed with the Immortomouse and screened as previously described (7, 28). To isolate kidney endothelial cells, kidneys from 2-3 pups 4 week-old wild-type and *bim* -/- Immortomice were dissected out aseptically and placed in serum free-Dulbecco’s Modified Eagle Medium (DMEM) containing penicillin/streptomycin (Sigma, St. Louis, MO). The kidneys 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 (1 mg/ml in serum free DMEM, Worthington, Lakewood, NJ) for 30-45 minutes 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 Inc., Hanover Park, IL), centrifuged at 400xg for 10 min to pellet cells and then the 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 pre-coated with anti-PECAM-1 antibody (MEC13.3, BD Biosciences, Bedford, MA), as described previously (22). After affinity binding, magnetic beads were washed six times with DMEM with 10% FBS and the bound cells plated into a single well of a 24 well plate pre-coated with 2 µg/ml of human fibronectin (BD Biosciences) in endothelial growth
medium. Endothelial cells were grown in DMEM containing 10% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 100 µg/ml streptomycin, 100 U/ml penicillin, heparin at 55 U/ml (Sigma, St. Louis, MO), endothelial growth supplement 100 µg/ml (Sigma, St. Louis, MO) and murine recombinant interferon-γ (R & D, Minneapolis, MN) at 44 units/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. Kidney endothelial cells were positive for B4-lectin (a mouse endothelial cell specific lectin) and expressed PECAM-1 and VE-cadherin as previously described (7, 10). The experiments described here were performed with three separate isolations of cells with similar results.

To isolate collecting duct epithelial cells (referred subsequently to as kidney epithelial cells), both kidneys from four week-old wild-type and bim -/- Immortomice were minced into small pieces in a 60 mm tissue culture dish using sterile razor blades and digested in 5 ml of collagenase type I (1mg/ml in serum free DMEM, Worthington, Lakewood, NJ) for 30-45 min at 37°C (28). Following digestion, DMEM containing 10% fetal bovine serum (FBS) was added, the cells were pelleted and rinsed twice in DMEM containing 10% FBS. The cells were resuspended in growth medium (DMEM:F12 (Invitrogen, Carlsbad, CA) supplemented with 1% FBS, 5X MITO (BD BioSciences, Franklin Lakes, NJ), 44 units/ml γ-interferon (R&D, Minneapolis, MN), 2 mM glutamine, 50 µg/ml streptomycin/ 50U/ml penicillin (Sigma, St Louis, MO), 50 µg/ml gentamicin (Invitrogen) and 50 unit/ml nystatin (Sigma) and plated on a 35 mm dish pre-coated with Matrigel (1:400 in serum-free DMEM:F12 medium). The cells were plated, grown to
near confluence and expanded in 60 mm dishes coated with Matrigel. Cells from two 60 mm dish were harvested by incubation with 2 mM EDTA in Tris buffered saline containing 0.05% bovine serum albumin for 10 min and scraping. The cells were rinsed with serum free DMEM:F12 and incubated with magnetic beads pre-coated with *Dolichos biflorus* agglutinin (DBA) (28). After binding, the magnetic beads were washed 6 times with DMEM containing 10% FBS and the bound cells were plated into a single well of a 24 well plate pre-coated with Matrigel (1:400) in growth medium. The cells were maintained at 33°C with 5% CO₂. Cells were progressively passed to larger plates, maintained and propagated on Matrigel (1:400) coated 60 mm plates. The selection process was repeated twice. Collecting duct epithelial cells expressed aquaporin 2 and calbindin and were DBA positive (mouse collecting duct specific lectin) as we previously described (20, 28).

**Cell apoptosis assays**

As an apoptotic stimulus, cells were incubated with 5-fluorouracil (5-FU) (epithelial cells 1 mM; endothelial cells 5 mM) or growth medium for 48 hours at 37°C. The rate of apoptotic cells was determined by *in situ* monitoring of caspase activity using the CaspACE FITC-VAD-FMK *in situ* marker (Promega, Madison, WI) or Caspase 3/7 Glo (Promega), as recommended by the supplier (20).

**Scratch Wound Assay**

Cells (4x10⁵) were plated in 60 mm tissue culture dishes and allowed to reach confluence (2-3 days). After aspirating the medium, cell layers were wounded using a 1
ml micropipette tip. Plates were then rinsed with PBS, fed with growth medium containing 100 ng/ml of 5-FU, to rule out potential contribution of differences in cell proliferation and incubated at 37°C for the duration of the experiment. The wounds were observed and photographed up to 72 hours. The distance migrated was determined as percent of total distance for quantitative assessments as described previously (7). These experiments were repeated at least twice with two different isolations with similar results.

**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 minutes to harden. Cells were removed using trypsin-EDTA, washed with growth medium once and resuspended at 1x10^5 cells per ml in serum free growth medium. Cells (2 ml) 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 (x100) was determined after 24 h. A longer incubation of the cells did not result in further branching morphogenesis (22).

**Cell adhesion assays**

Cell adhesion to various matrix proteins was performed as previously described (18). Briefly, varying concentrations of fibronectin, vitronectin, collagen I, collagen IV and laminin (BD BioSciences) prepared in TBS with Ca^{2+} and Mg^{2+} (2 mM each; TBS with
Ca/Mg) were coated on 96-well plates (50 μl per well; Nunc Maxisorbe plates, Fisher Scientific) overnight at 4°C. As a control, wells were coated with 1% BSA. Plates were rinsed 4 times with 200 μl of TBS with Ca/Mg and blocked with 200 μl of 1% BSA prepared in TSB with Ca/Mg for at least 1 h at room temperature. Cells were removed by dissociation solution (Sigma), washed with TBS, and resuspended at 5x10^8 cells/ml in HBS (20 mM HEPES, 150 mM NaCl, pH 7.6, and 4 mg/ml BSA). After blocking, plates were rinsed with TBS with Ca/Mg once, 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 hours at 37°C. The non-adherent cells were removed by gently washing the plate four times with TBS with Ca/Mg, or until no cells were 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 (28). All samples were done in triplicate.

**Western blot analysis**

Cells were plated at 4x10^5 in 60 mm dishes coated with 1% gelatin (endothelial cells) or Matrigel (epithelial cells) and allowed to reach nearly 90% confluence in 2 days. The cells were then rinsed once with serum-free medium and incubated with serum-free DMEM for 48 hours at 37°C. Then, conditioned medium (3.5 ml) was collected and clarified by centrifugation. The 40 μl of sample was mixed with appropriate volume of 6X SDS buffer and analyzed by SDS-PAGE (4-20% Tris glyidine gel; Invitrogen, Carlsbad, CA). In some cases, total protein lysates were prepared from these cells in a modified RIPA buffer (142.5 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.4, 2 mM orthovandate...
and 2 mM sodium difluoride, 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 an anti-fibronectin (Sigma), a rabbit anti-chicken tenascin C polyclonal antibody (Millipore;AB19013, Billerica, MA), anti-TSP1 monoclonal antibody (Clone A6.1, Neo Marker, Fremont, CA), anti-collagen IV (Millipore;AB756P), anti-osteopontin (R&D, Minneapolis, MN), anti-β-catenin (Sigma), anti-HSP90 (Cell Signaling Technology), anti-Akt (Cell Signaling) anti-phospho-Akt (Cell Signaling), anti-β-actin (Sigma), anti-phospho-eNOS (Cell Signaling), and anti-eNOS (Santa Cruz Technology, Santa Cruz, CA). The blot was washed, incubated with appropriate secondary antibody and developed using ECL (Amersham, Piscataway, NJ) (7, 10).

**FACScan analysis**

FACScan analysis was performed as previously described (10). 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 minutes and incubated with the appropriate dilution of primary antibody; anti-PECAM-1 (BD Pharmingen), anti-vascular endothelial (VE)-cadherin (Alexis Biochemical, San Diego, CA), B4-lectin (Sigma), anti-calbindin (Cell Signaling Technology, Danvers, MA), anti-aquaporin 2 (Cell Signaling Technology), DBA (Vector), anti-β1 (Millipore), anti-α5 (MAB1949; Millipore), anti-αv (01521 D; BD Pharmingen), anti-α1 (BD Pharmingen), anti-β3 (MAB1957; Millipore), anti-αvβ3 (MAB1976Z; Millipore) or control IgG (Millipore). For antibodies that
required cell permabilization, cells were removed from the dish, washed with PBS, fixed
with 2% paraformaldehyde on ice for 30 min, washed with PBS, and resuspended in
PBS containing 0.1% Triton-X-100 and 0.1% BSA containing the appropriate dilution of
primary antibody. The cells were washed with TBS containing 1% BSA and then
incubated with the appropriate secondary antibody (1:200) on ice for 30 minutes. After
the incubation, the 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).

Transwell Assay

Transwell filters (Costar 3422) were coated with 1% gelatin (EC) or 200 μg/ml of
Matrigel (CD), rinsed with PBS and then blocked with 2% BSA in PBS. 500 μl of serum
free DMEM:F12 medium was added to the bottom of each well and 1 X 10^5 cells in 100
μl of medium was added to the top of each well. Each condition was done in duplicate.
Following 4 hours (endothelial cells) or 16 hours (epithelial cells) in a 33°C tissue culture
incubator, the cells and medium were aspirated and the upper side of the membrane
wiped with a cotton swab. The cells that had migrated through the membrane were fixed
with 2% paraformaldehyde and stained with hematoxylin and eosin. Ten fields of cells
were counted for each condition and the average and standard deviations were
determined.
VEGF analysis

VEGF protein levels were determined from condition medium prepared from kidney endothelial or epithelial cells, utilizing a mouse VEGF Immunoassay kit (R&D, Minneapolis, MN). Briefly, kidney endothelial cells were grown for 2 days in serum free medium at 37°C. The conditioned medium (50 µl) was used in the VEGF Immunoassay, which was performed in triplicates as recommended by the manufacturer and was normalized to the number of cells. The assay was repeated twice using two different isolations of endothelial cells with similar results.

Nitric oxide analysis

Kidney endothelial cells were plated in black wall clear bottom Microtest TM 96 well plates (BD #35 3948; 5 x 10^3 cells in 100 µl). The next morning the medium was changed to EC medium containing 30 µM DAF-FM diacetate (Invitrogen; D-23842) and 5 µg/ml of Cell trackerRed (Invitrogen; C34552). Following a 40 min incubation at 33°C, fresh EC medium was placed on the cells and the incubation continued for 20 min. The wells were washed with TBS and the cells in each well were resuspended in 100 µl of TBS. The absorbance was read at 495/515 nm using a fluorescence plate reader (7). These experiments were performed in triplicates and repeated twice with similar results.

RNA Purification and qPCR

The total RNA from cells was extracted by mirVana PARIS kit (Ambion) according to the manufacturer’s instructions. cDNA synthesis was performed from 1 µg of total RNA using Sprint RT Complete-Double PrePrimed kit from (Clontech). 1 µl of
each cDNA (dilution 1:10) was used as template in qPCR assays, performed in triplicate of three biological replicates on Mastercycler Realplex (Eppendorf) using the SYBR qPCR Premix (Clontech). Amplification parameters were as follows: 95°C for 2min; 40 cycles of amplification (95°C for 15 sec, 60°C for 40 sec); dissociation curve step (95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec).

Standard curves were generated from known quantities for each target gene of linearized plasmid DNA. Ten times dilution series were used for each known target, which were amplified using SYBR-Green qPCR. The linear regression line for ng of DNA was determined from relative fluorescent units (RFU) at a threshold fluorescence value (Ct) to quantify gene targets from cell extracts by comparing the RFU at the Ct to the standard curve, normalized by the simultaneous amplification of RpL13A which was used as a housekeeping gene to normalize all samples. The primer sequences are listed in Table 1.

**Processing of Kidneys for Histological Studies and Immunochemistry**

Following surgical removal from mice, kidneys from P20 wild-type and bim -/- mice were fixed with formalin overnight and processed for paraffin sectioning. For immunohistochemical staining, paraffin sections were deparaffinized with xylene and rehydrated. Antigen unmasking was performed using antigen-unmasking solution (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. The sections were then washed in phosphate buffered saline (PBS) and incubated for 15 min in PBS blocking buffer (PBS containing 1% bovine serum albumin, 0.3% Triton X-100 and 0.2% skim milk powder). The sections were incubated overnight with anti-
PECAM-1 (1:150; R&D Systems, Minneapolis, MN). The sections were then incubated with indocarbocyanine (CY3)-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA) and photographed. Vascular density was determined by counting the number of capillaries and tubules on at least 10 high magnification fields (x400). The data are represented as number of capillaries per tubule.

Statistical analysis

Statistical differences between groups were evaluated with the student's unpaired t-test (two-tailed). Mean ± standard deviations are shown. P values <0.05 were considered significant.
RESULTS

*Bim -/- kidney endothelial and epithelial cells show decreased apoptosis when challenged*

To determine the role bim plays in kidney function, we isolated kidney epithelial and endothelial cells from wild type and *bim -/-* mice as previously described (7, 10, 11, 20, 28). Both kidney endothelial and epithelial cells express significant levels of BIM with epithelial cells expressing the highest level (Figure 1A). We next examined expression of epithelial and endothelial markers, to ensure these cells retained normal characteristics. Wild-type and *bim -/-* kidney epithelial cells were DBA positive (mouse collecting duct specific lectin) and expressed modest levels of aquaporin 2 and calbindin (Figure 1B), while endothelial cells expressed VE-cadherin and PECAM-1 and were positive for B4-lectin (a mouse microvascular EC specific lectin; Figure 1C) as we previously described (10, 11, 20, 28).

The morphology of wild-type and *bim -/-* kidney epithelial cells were similar when plated on Matrigel-coated plates (Figure 1D). The morphology of wild-type and *bim -/-* kidney endothelial cells also had a similar appearance when plated on gelatin-coated plates (Figure 1E). Since BIM is a proapoptotic bcl-2 family member, we next addressed whether loss of BIM expression affected the level of apoptosis. *bim -/-* kidney epithelial (Figure 2 A) and endothelial cells incubated with 5-FU for 48 h (Figure 2 B), demonstrated decreased numbers of apoptotic cells compared to wild-type cells. There were no significant differences in the basal rates of apoptosis in the absence of 5-FU.
Sustained osteopontin and TSP1 expression in bim -/- epithelial cells

Altered ECM expression can influence cell adhesion and migration. We next examined whether lack of BIM differentially impacts ECM production in kidney epithelial and endothelial cells. Serum free conditioned-medium was prepared and evaluated by Western blot analysis. We observed sustained expression of TSP1 and osteopontin in bim -/- epithelial cells, while collagen IV and fibronectin expression was similar (Figure 3A). In contrast, TSP1 and osteopontin expression was down-regulated in bim -/- endothelial cells compared to wild-type, while fibronectin and tenascin C expression was similar (Figure 3B).

Loss of bim expression differentially impacts migration of kidney epithelial and endothelial cells

Cell migratory and adhesive properties impact the ability of epithelial and endothelial cells to form branched structures. We next examined cell migration characteristics using a scratch wound assay. A confluent monolayer of wild-type and bim -/- kidney epithelial and endothelial cells were wounded and returned to 37°C in the presence of 5-FU (100 ng/ml) to prevent cell proliferation. Bim -/- epithelial cells migrated slower than their wild-type counterpart (Figure 4A). In contrast, bim -/- endothelial cells migrated faster than their wild-type counterpart, completely covering the wounded area (Figure 4B). The quantitative assessment of the data is shown in Figures 4C, D. Similar results were obtained using a transwell migration assay. bim -/- epithelial cells demonstrated a 2.5-fold decrease in the number of cells that migrated through the membrane compared to their wild-type counterpart (Figure 4E). In contrast, we observed a 2-fold increase in the number of bim -/- endothelial cells that migrated through the membrane compared to
their wild-type counterpart (Figure 4 F). Thus, loss of BIM expression differentially impacts migration of kidney epithelial and endothelial cells.

*Bim -/- epithelial cells are more adherent*

Changes in migration of *bim -/-* kidney epithelial and endothelial cells could be due to altered cell adhesion. We next examined wild-type and *bim -/-* cells ability to adhere to various ECM proteins including fibronectin, laminin, collagen I, collagen IV and vitronectin (Figures 5 A, B). *bim -/-* epithelial cells displayed increased adhesion to fibronectin, vitronectin and collagen IV compared to wild-type cells, while wild-type and *bim -/-* endothelial cells adhered similarly well to fibronectin, vitronectin and collagen IV. Minimal adhesion was observed on collagen I or laminin for wild-type or *bim -/-* cells. Thus, lack of BIM expression differentially influenced adhesion of kidney epithelial and endothelial cells.

We next analyzed integrin expression on the surface of kidney epithelial and endothelial cells by FACScan analysis (Figures 6 A, B). Wild-type and *bim -/-* epithelial cells expressed similar levels of α2, α6, β1 and αvβ3 integrins. Wild-type and *bim -/-* endothelial cells expressed similar levels of α4, α5, αvβ3, β1 and β3 integrins on their surface. Thus, the increased adhesion noted in *bim -/-* epithelial cells may be independent of significant changes in the expression levels of integrins and may be due to alterations in the affinity and/or avidity of these integrins.
Wild-type and bim -/- cells undergo tubular morphogenesis in Matrigel

We next determined whether the changes observed in cell migration and adhesion impacted the ability of kidney epithelial and endothelial cells to undergo tubular morphogenesis, in the absence of BIM (Figures 7 A, B). Wild-type and bim -/- kidney epithelial and endothelial cells plated on Matrigel formed an extensive network within 24 h. Longer incubation of the cells did not result in further branch formation. The quantitative assessment of the data shown in Figures 7 C, D demonstrated that lack of bim expression in endothelial cells resulted in a 2-fold increase in branch points. Similar numbers of branch points were noted in wild-type and bim -/- epithelial cells.

Decreased p-eNOS expression in bim -/- kidney endothelial cells

VEGF promotes angiogenesis through activation of Akt1 and endothelial nitric oxide synthase (eNOS) (1, 4, 9). Here, we examined expression and phosphorylation of eNOS in kidney endothelial cells, as well as its associated protein, HSP90. HSP90 expression was similar in wild-type and bim -/- kidney endothelial cells. However, bim -/- kidney endothelial cells demonstrated a significant decrease in levels of p-eNOS expression compared to wild-type cells (Figures 8 A). Total eNOS levels were similar in wild-type and bim -/- endothelial cells. Consistent with the decreased p-eNOS expression, NO production decreased approximately 2-fold in bim -/- kidney endothelial cells (Figure 8 B). VEGF expression increased 5-fold in bim -/- kidney endothelial cells compared to wild-type kidney endothelial cells (Figure 8 C). VEGF expression also increased in bim -/- kidney epithelial cells compared to their wild-type counterpart (Figure 8 D). We next examined the expression of Akt1 and phosphorylated Akt1 in
lysates from wild-type and $bim^{-/-}$ endothelial cells by Western blot analysis (Figure 8A). Expression of Akt1 and phospho-Akt1 was similar among the endothelial cells examined here. Thus, enhanced migration of $bim^{-/-}$ kidney endothelial cells may be mediated by increased production of VEGF, concomitant with decreased eNOS activity and NO bioavailability.

**Increased kidney vascular density in the absence of bim**

To determine whether increased capillary morphogenesis *in vitro* correlated with increased renal vascular density, we immunostained kidney sections from P28 wild-type and $bim^{-/-}$ mice with anti-PECAM-1. Wild-type and $bim^{-/-}$ mice demonstrated significant PECAM-1 staining (Figure 9). However, calculation of the ratio of peritubular capillaries per tubule demonstrated an increased number of peritubular capillaries in kidneys from $bim^{-/-}$ mice compared to their wild-type counterpart (Figure 9). Thus, enhanced capillary morphogenesis of $bim^{-/-}$ kidney endothelial cells, correlated with increased numbers of capillaries in kidneys from these mice.
DISCUSSION

The kidney is a highly vascularized organ in which the various segments of the vascular tree accomplish specialized regional functions, potentially influencing renal epithelial cells in the surrounding area (5). Here, we examined whether loss of bim expression had a similar impact on kidney endothelial and epithelial cell function. bim-/- kidney endothelial cells demonstrated increased migration and capillary morphogenesis. This proangiogenic phenotype of bim -/- kidney endothelial cells was associated with a 5-fold increase in VEGF expression and down-regulation of TSP1. Phosphorylation of eNOS and increased NO production can mediate the proangiogenic activity of VEGF. However, bim -/- kidney endothelial cells exhibited decreased phospho-eNOS and NO production, indicating that the proangiogenic activity of VEGF was not mediated through eNOS in these cells and may be responsible for suppression of TSP1 expression (23). These results are consistent with our data in bim -/- lung endothelial cells, in which increased VEGF expression and a proangiogenic phenotype was independent of changes in eNOS/NO activity (7). TSP1 has been recently shown to inhibit NO mediated angiogenesis through a cGMP dependent and independent manner (8). Thus, in the absence of TSP1 angiogenesis may proceed without a need for excess NO.

Vascular homeostasis is maintained by a tightly balanced production of pro- and anti-angiogenic factors. The rigid control of vascular homeostasis, however, is abrogated in many disease states resulting in an array of vasculopathies. This may be accomplished by up-regulation of proangiogenic and/or down-regulation of angiostatic...
factors. TSP1 is an endogenous inhibitor of angiogenesis (6) which inhibits angiogenesis *in vivo* and migration of capillary endothelial cells *in vitro*. The molecular mechanisms by which TSP1 and/or its angiostatic peptides inhibit vascularization *in vivo* or endothelial cell migration *in vitro* are evolving. Mutant mice which lack TSP1, exhibit a significant increase in blood vessel density in many organs (12, 13), thus providing further evidence for the important role of this molecule in the regulation of angiogenesis *in vivo*.

TSP1 is synthesized and secreted by a number of renal cell types, including glomerular mesangial cells and renal tubule cells (15, 16, 27). TSP1 activates latent TGF-β, and is thought to play a role in the pathogenesis of diabetic tubular hypertrophy, glomerular expansion and fibrotic renal disease. Peptide antagonists of TSP1-mediated TGF-β activation block glucose-induced activation of TGF-β and expression of fibronectin, type IV collagen and osteopontin (15). Renal tubule hypertrophy and mesangial expansion are inhibited by interference with this pathway (2, 19, 29).

Although increased TSP1 expression is associated with tubular hypertrophy and mesangial expansion, which has a negative impact on kidney function (15, 27), its expression in the endothelium is essential for maintenance of the differentiated state of the vasculature. Thus, loss of TSP1 expression in the endothelium can be associated with activation of endothelial cells and vasculopathies. Although suppression of TSP1 expression may improve renal tubular hypertrophy and mesangial expansion, renal vascular rarefaction could be an unwanted side effect. Thus, understanding how
manipulation of a targeting molecule impacts its various cellular functions is essential for
effective development therapeutic regimens.

Osteopontin, like TSP1, is a matricellular protein that plays important roles in
normal physiology and in pathological states such as fibrosis. Osteopontin mediates cell
adhesion, migration and survival of several cell types including endothelial, renal
epithelial, smooth muscle and inflammatory cells. Increased osteopontin expression in
bim -/- kidney epithelial cells may contribute to increased adhesion to ECM proteins and
decreased migration. In bim -/- kidney endothelial cells, decreased expression of
osteopontin and TSP1 correlated with increased migration and capillary morphogenesis.
Thus, in the absence of BIM, modulation of TSP1 and osteopontin expression occur in
opposing fashion in kidney endothelial and epithelial cells, concurrent with opposing
changes in migration. We had previously shown that decreased endothelial cell
migration correlated with decreased capillary morphogenesis and vascular density (7,
11, 24). In contrast, decreased migration of bim -/- kidney epithelial cells did not affect
tubular morphogenesis in Matrigel. Decreased migration of bim -/- epithelial cells
correlated with increased TSP1 and osteopontin expression and cell adhesion. Thus,
our data suggest that modulation of ECM expression, migration and adhesion
differentially impacts endothelial and epithelial branching morphogenesis.

Kidney tubules have been proposed to be an angiogenic soup (26). Our data
support this supposition with wild-type kidney epithelial cells expressing 5-fold higher
levels of VEGF than their endothelial cell counterpart. Interestingly, loss of BIM
expression in kidney endothelial cells increased VEGF levels to that observed in wild-type kidney epithelial cells. Kidney epithelial cells \textit{in vitro} also express anti-angiogenic factors such as TSP1 and osteopontin in a developmentally regulated fashion (Sorenson, unpublished data; (28)). Thus, it is tempting to speculate that epithelial cell dysfunction due to disease, may influence epithelial cell secretion of growth factors and ECM proteins, further impacting kidney endothelial cell and vascular function.

Studies from our laboratories have consistently shown that when endothelial cell migration is decreased, capillary morphogenesis and vascular development is significantly compromised (7, 11, 24). Enhanced endothelial cell migration does not negatively impact capillary morphogenesis but instead leads to enhanced vascular density (7, 22, 25). Here we also show increased migration, capillary morphogenesis and vascular density in the absence of BIM. Unlike endothelial cells, decreased cell migration of \textit{bim} -/- epithelial cells did not adversely impact tubular morphogenesis in Matrigel. Previously, studies from our laboratories demonstrated that enhanced kidney epithelial migration led to an inability to undergo tubular morphogenesis in Matrigel and branching morphogenesis \textit{in vivo} (21). The impact modulation of migration has on capillary/tubular morphogenesis in Matrigel appears to be regulated differently in endothelial and epithelial cells. In summary, the studies presented here emphasize the importance of considering all cell types within an organ when designing treatment modalities with minimal off target effects.
ACKNOWLEDGMENTS

This work was supported by grants from the University of Wisconsin Department of Pediatrics Research and Development Fund and University of Wisconsin Medical School Research Committee (CMS). CMS was funded, in part, by DK067120 from the National Institutes of Health and AHA research award (0950057G). NS is supported by NIH grants EY016995, EY018179, and RC4 EY 021357 (NS), P30 CA014520 UW Paul P. Carbone Cancer Center support grant, P30 EY016665, and an unrestricted departmental award from Research to Prevent Blindness. NS is a recipient of a Research Award from American Diabetes Association (1-10-BS-160) and Retina Research Foundation. MEM is a recipient of a Senior Thesis Grant from the College of Letters and Science at the University of Wisconsin-Madison. SYP is a recipient of a pre-doctoral studentship from AstraZeneca. The authors wish to thank Robert Gordon for his assistance with the graphics.
REFERENCES


FIGURE LEGENDS

Figure 1. Wild-type and bim -/- cells have similar morphology. BIM expression was determined by qPCR in kidney epithelial and endothelial cells (Panel A) prepared from wild-type mice. In Panel B, kidney epithelial cells prepared from wild-type and bim -/- mice were examined for expression of aquaporin 2, calbindin and DBA. In Panel C, kidney endothelial cells prepared from wild-type and bim -/- mice were examined for expression of PECAM-1, VE-cadherin and B4-lectin by FACScan analysis. The shaded areas show staining in the presence of control IgG. Please note similar expression of these cell specific markers in all cells. Wild-type and bim -/- kidney epithelial (Panel D) and endothelial (Panel E) cells were cultured on gelatin or Matrigel-coated plates, respectively. Cells were photographed using a phase microscope in digital format at low magnification (X40). These experiments were performed at 37°C.

Figure 2. Decreased apoptosis in bim -/- cells. Wild-type and bim -/- kidney epithelial (Panel A) and endothelial (Panel B) cells were incubated with solvent control or 5-FU (1 mM epithelial cells; 5 mM endothelial cells) for 48 h. Apoptotic cells were determined by in situ monitoring of caspase activity. The percentage of apoptotic cells was determined as the percentage of positive cells relative to total number of cells per five high power field (x100;*P≤0.05,n=5). Please note that no significant difference in the steady state rate of apoptosis was observed. Please note bim -/- cells incubated with 1 mM 5-FU showed decreased amounts of apoptosis. These experiments were performed at 37°C.
Figure 3. Altered expression of ECM proteins in bim-/- kidney cells. Wild-type and bim-/- kidney epithelial (Panel A) and endothelial (Panel B) cells were grown for 2 days in serum free medium. The medium was harvested, clarified and Western blotted for extracellular matrix proteins as noted. Please note bim-/- kidney endothelial cells secreted decreased amounts of TSP1 and osteopontin. These experiments were repeated twice with similar results at 37°C.

Figure 4. Bim-/- kidney epithelial and endothelial cells demonstrate opposing changes in migration. Cell migration was determined by scratch wounding of kidney epithelial (Panel A) or endothelial (Panel B) cell monolayers and wound closure at 37°C was monitored by photography. A representative experiment is shown here. Please note that wild-type epithelial cells migrate faster than bim-/- epithelial cells. In contrast, wild-type kidney endothelial cells migrated slower than bim-/- endothelial cells. The quantitative assessment of the data is shown in Panels C&D (*P≤0.05, n=3). Transwell assays were performed at 33°C with wild-type and bim-/- kidney epithelial (Panel E) and endothelial (Panel F) cells (*P≤0.05, n=3). Please note that the transwell assay confirmed the migration results observed in the scratch wound assay. These experiments were repeated twice with two different isolations of cells with similar results.

Figure 5. Bim-/- kidney epithelial cells are more adherent on fibronectin and vitronectin. Adhesion of kidney wild-type (■) and bim-/- (♦) epithelial (Panel A) and wild-type (♦) and bim-/- (■) endothelial (Panel B) cells to fibronectin, vitronectin, collagen type I, collagen IV or laminin was determined as described in Methods at 37°C. Please
note bim -/- epithelial cells had increased adherence to fibronectin, vitronectin, and collagen type IV. These experiments were repeated twice with two different isolations of cells.

**Figure 6.** Wild-type and bim -/- kidney epithelial and endothelial cells had similar integrin expression. Expression of α2, α6, β1 and β3 integrin was examined in wild-type and bim -/- kidney epithelial (Panel A) cells while α1, α5, αvβ3 and β3 integrin expression was determined in wild-type and bim -/- kidney endothelial (Panel B) cells using FACSan analysis as described in Methods at 37°C. The shaded graphs show staining in the presence of control IgG. These experiments were repeated twice.

**Figure 7.** Bim -/- kidney endothelial cells undergo enhanced capillary morphogenesis. Wild-type and bim -/- kidney epithelial (Panel A) and endothelial (Panel B) cells were plated in Matrigel, incubated at 33°C and photographed in digital format. The quantitative assessment of the data is shown in Panels C,D. The data are the mean number of branch points from 10 high power fields (x100) ± SD (*P≤0.05, n=3). These experiments were repeated twice with similar results.

**Figure 8.** Decreased phospho-eNOS and increased VEGF expression in bim -/- kidney endothelial cells. Protein lysates (20 μg) from wild-type and bim -/- kidney endothelial cells incubated at 37°C were analyzed by Western blot analysis for expression of phospho-eNOS, total eNOS, HSP90, phospho-Akt1 and total Akt1. β-actin expression was assessed as a loading control (Panel A). In Panel B, the intracellular NO production
was determined as analyzed by DAF-FM fluorescence. An immunoassay was used to
determine VEGF levels (pg/ml) in wild-type and bim -/- endothelial cells (Panel C) and
epithelial cells (Panel D) incubated at 37°C (*P ≤ 0.05, n=3). Experiments were repeated
twice with two different isolations of endothelial cells with similar results. Please note
decreased levels of phospho- eNOS and NO levels and increased VEGF levels in
bim -/- kidney endothelial cells.

**Figure 9. Increased number of peritubular capillaries in kidneys from bim -/- mice.**

Fluorescence photomicrographs of kidneys P28 wild-type and bim -/- mice stained with
anti-PECAM-1. The peritubular capillary density was calculated as described in the
Materials and Methods (*P ≤ 0.05, n=5). Please note increased vascular density in
kidneys from bim -/- mice. Sections are representative of > 4 mice of each genotype. A
representative image is shown.
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<tr>
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<th>Forward 5’ to 3’</th>
<th>Reverse 5’ to 3’</th>
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<td>TCTCAAGGTTGTCGGCTGAA</td>
<td>GCCAGACGCCAAGGTA</td>
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Table 1
Figure 2

A

Mean Number of Apoptotic Cells (% of Total)

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<tr>
<th></th>
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<th>WT 5-FU</th>
<th>bim -/-</th>
<th>bim -/- 5-FU</th>
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<td>bim -/-</td>
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<tr>
<td>bim -/- 5-FU</td>
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B

Caspase 3/7 activity (Luminiscence)

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<th></th>
<th>WT</th>
<th>WT 5-FU</th>
<th>bim -/-</th>
<th>bim -/- 5-FU</th>
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<td>bim -/-</td>
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<tr>
<td>bim -/- 5-FU</td>
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* Indicates significant difference.
Figure 4

A

WT  bim -/

0 hr  48 hr

B

WT  bim -/

0 hr  72 hr

C

% Wound Closure

WT  bim -/

D

% Wound Closure

WT  bim -/

E

Mean Number of Cells/Field

WT  bim -/

F

Mean Number of Cells/Field

WT  bim -/
Figure 5

A

- Fibronectin (µg/ml)
- Vitronectin (µg/ml)
- Collagen IV (µg/ml)
- Collagen I (µg/ml)
- Laminin (µg/ml)

B

- Fibronectin (µg/mL)
- Vitronectin (µg/mL)
- Collagen IV (µg/mL)
- Collagen I (µg/mL)
- Laminin (µg/mL)
Figure 7

A  WT  bim -/-

B  WT  bim -/-

C  Mean Number Branch Points/Field
WT  bim -/-

D  Mean Number Branch Points/Field
WT  bim -/-

*
Figure 8

A

WT  bim -/-

p-eNOS

total eNOS

β-actin

p-AKT

total AKT

β-actin

HSP90

β-actin

B

DAF-FM Fluorescence

WT  bim -/-

C

VEGF (pg/ml)

WT  bim -/-

D

VEGF (pg/ml)

WT  bim -/-
Figure 9

WT   bim -/-

![Images showing WT and bim -/- conditions](image)

![Bar chart showing mean number of peritubular capillaries per tubule](chart)

Mean number of peritubular capillaries per tubule

WT  bim -/-