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Tumorigenesis and Neoplastic Progression

Bovine Lactoferricin Inhibits Basic Fibroblast Growth Factor- and Vascular Endothelial Growth Factor$_{165}$-Induced Angiogenesis by Competing for Heparin-Like Binding Sites on Endothelial Cells

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Angiogenesis is a complex process whereby new blood vessels form from pre-existing vasculature in response to proangiogenic factors such as basic fibroblast growth factor (bFGF) and the 165-kd isoform of vascular endothelial growth factor (VEGF$_{165}$). Angiogenesis inhibitors show considerable potential in the treatment of cancer because angiogenesis is necessary for tumor growth beyond a few millimeters in diameter because of the tumor's need for oxygen and nutrient supply, as well as waste removal. Bovine lactoferricin (LfcinB) is a peptide fragment of iron- and heparin-binding lactoferrin obtained from cow's milk. Here we provide *in vivo* and *in vitro* evidence that LfcinB has potent antiangiogenic activity. LfcinB strongly inhibited both bFGF- and VEGF$_{165}$-induced angiogenesis in Matrigel plugs implanted in C57BL/6 mice. In addition, LfcinB inhibited the *in vitro* proliferation and migration of human umbilical vein endothelial cells (HUVECs) in response to bFGF or VEGF$_{165}$ but was not cytotoxic to HUVECs. Rather, LfcinB complexed with heparin-like structures on the HUVEC surface that are involved in the binding of bFGF and VEGF$_{165}$ to their respective receptors, thereby preventing receptor-stimulated angiogenesis. These findings suggest that LfcinB may have utility as an antiangiogenic agent for the treatment of human cancers. ***(Am J Pathol 2006, 169:1753–1766; DOI: 10.2353/ajpath.2006.051229)***

An urgent need exists for innovative forms of cancer treatment that avoid the serious problem of chemoresistance caused by the inherent genetic instability of cancer cells and the cellular heterogeneity of tumors.[1] This quest has led researchers to focus attention on an array of angiogenesis inhibitors that target the genetically stable, untransformed endothelial cells comprising the tumor vasculature.[2] Angiogenesis, which is the process by which new blood vessels develop from pre-existing vessels, is governed by a very complex network of opposing signals that, under normal physiological conditions, are elicited by various highly regulated angiogenesis stimulators and inhibitors.[3] Angiogenesis is essential for tumor growth beyond a few millimeters in diameter because of the tumor's requirement for a network of blood vessels to deliver oxygen and nutrients and to remove waste products of metabolism. During tumor-associated angiogenesis, the balance of angiogenesis stimulators and inhibitors is tipped in favor of angiogenesis by hypoxia-inducible factor-1 gene expression.[4] The result is a microenvironment that is rich in proangiogenic factors produced by tumor cells, as well as by host cells (eg, macrophages) that are recruited to the tumor site.[5] These proangiogenic factors, in combination with basement membrane degradation by proteolytic enzymes, trigger endothelial cell proliferation, tube formation, and migration toward the tumor site.

Basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF) are two of the principal soluble stimulators of angiogenesis.[6,7] bFGF is a ubiquitously expressed polypeptide growth factor that is normally sequestered in the extracellular matrix of healthy tissues.[8] bFGF is also expressed by many human cancer cells, including prostate carcinoma and melanoma cells and is believed to be important for the formation of tumor

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vasculature.[9,10] VEGF is a specific mitogen for vascular endothelial cells that is produced by a variety of cell types, including activated macrophages and cancer cells.[10–12] Alternate mRNA splicing of the VEGF gene product gives rise to four different VEGF isoforms,[13] including the $VEGF_{165}$ isoform that binds heparan sulfate.[14] Both bFGF and $VEGF_{165}$ must interact with heparan sulfate proteoglycans at the cell surface for these proangiogenic factors to bind to and signal through their respective receptors.[15,16]

Lactoferrin is an 80-kd iron-binding single-chain glycoprotein that is present in the secretory granules of neutrophils and is also found at significant levels in several biological fluids, including saliva, tears, and milk.[17] Lactoferrin is considered to be a major component of anti-microbial host defense,[18] in addition to playing important roles in the regulation of cell growth and differentiation.[19] Bovine lactoferricin (LfcinB) is a 25-amino acid cationic peptide with an amphipathic, anti-parallel β-sheet structure that is obtained by acid-pepsin hydrolysis of the N-terminal region of lactoferrin from cow's milk.[20] LfcinB accounts for the iron-independent, anti-microbial action of bovine lactoferrin because the peptide is cytotoxic for a diverse range of gram-positive and gram-negative bacteria,[21] as well as showing anti-viral[22] and anti-fungal activity.[23] There is evidence that LfcinB also possesses potent *in vitro* and *in vivo* anti-cancer activity,[24,25] which is likely related to the ability of LfcinB to disrupt the plasma membrane of neoplastic cells.[26] Recently, we have shown that LfcinB selectively induces apoptosis in a range of human leukemia and carcinoma cell lines via the reactive oxygen species-dependent loss of mitochondrial transmembrane potential and the sequential activation of caspase-2, -9, and -3.[27] Interestingly, LfcinB treatment of tumor-bearing mice leads to a reduction in the number of tumor-induced blood vessels,[25] suggesting a possible antiangiogenic role for LfcinB. However, whether this effect is a consequence of LfcinB-induced apoptosis of endothelial cells or LfcinB-mediated inhibition of tumor blood vessel development remains to be determined.

In the present study, we used both *in vivo* and *in vitro* approaches to investigate the putative antiangiogenic activity of LfcinB. The Matrigel plug assay, which is a well-established method of assessing the *in vivo* activity of antiangiogenic factors,[28] was used to determine the effect of LfcinB on bFGF- and $VEGF_{165}$-induced blood vessel development in mice. We also investigated the effect of LfcinB on the *in vitro* proliferation and migration of human umbilical vein endothelial cells (HUVECs) in response to bFGF and $VEGF_{165}$. Endothelial cell proliferation and migration induced by proangiogenic factors are crucial steps in the development of tumor vasculature.[5] Because LfcinB is derived from bovine lactoferrin, and both molecules exhibit heparin-binding activity,[29,30] we also determined the ability of LfcinB to bind heparin-like molecules that are involved in bFGF and $VEGF_{165}$ interactions with their respective receptors.[15,16]

Materials and Methods

Animals

Adult (6 to 8 weeks old) C57BL/6 mice were purchased from Charles River Canada (Lasalle, QC, Canada) and housed in the Carleton Animal Care Facility of Dalhousie University, Halifax, NS, Canada. Mice were maintained on a diet of standard rodent chow and water supplied *ad libitum*. Animal use was in accordance with protocols consistent with the Canadian Council on Animal Care guidelines and was approved by the Dalhousie University Committee on Laboratory Animals.

Materials

HUVECs that were isolated by standard protocols were kindly provided by Dr. A. Issekutz (Dalhousie University). LfcinB (amino acid sequence: FKCRRWQWRMKKLGAPSITCVRRAF) and biotinylated LfcinB were synthesized in linear form by Sigma Genosys (The Woodlands, TX) with a purity of greater than 95%. Scrambled LfcinB (amino acid sequence: KRWFCKWQRGMRLKASTPICRVRFA) and biotinylated scrambled LfcinB were synthesized in linear form by Dalton Chemical Laboratories Inc. (Toronto, ON, Canada) with a purity of greater than 95%. Lyophilized peptides were dissolved in serum-free RPMI 1640 medium (Sigma-Aldrich Canada, Oakville, ON, Canada), and stock aliquots (1 mg/ml) were stored at −70°C. Heparin, hematoxylin, eosin, 1,2-phenylenediamine substrate, bovine serum albumin (BSA), bovine lactoferrin, Hoechst 33342 trihydrochloride dye, chondroitinase ABC, phosphoinositide-specific phospholipase-C (PI-PLC), heparinase I, II, and III (all purified from *Flavobacterium heparinum*), *N*-hydroxysuccinimidobiotin, *N,N*-dimethyl formamide, and endothelial cell growth supplement were purchased from Sigma-Aldrich Canada. $VEGF_{165}$, bFGF, and nonheparin-binding epidermal growth actor (EGF) were obtained from Peprotech Inc. (Rocky Hill, NJ). Growth factor-reduced Matrigel was purchased from BD Biosciences (Bedford, MA). Streptavidin-Texas Red and streptavidin-horseradish peroxidase (HRP) were from Jackson ImmunoResearch (West Grove, PA). Rabbit IgG antibody against human factor VIII-associated antigen (von Willebrand factor), which cross-reacts with murine von Willebrand factor, was purchased from DAKO Corp. (Copenhagen, Denmark). Rabbit IgG was from Cedarlane Laboratories (Hornby, ON, Canada). Biotinylated goat anti-rabbit IgG was from Invitrogen Corp. (Burlington, ON, Canada).

Cell Culture

HUVECs were maintained at 37°C in a 5% CO_2 humidified atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES buffer (pH 7.4) (all from Invitrogen Corp.), 25 μg/ml endothelial cell growth supplement, and 45 μg/ml heparin.

Matrigel Plug Assay

Mice were injected at four different sites along the dorsal midline with growth factor-reduced Matrigel (0.3 ml/site) plus sterile distilled water (vehicle for LfcinB), LfcinB alone (200 μg/ml), bFGF (1 μg/ml), $VEGF_{165}$ (5 μg/ml), nonheparin-binding EGF (2 μg/ml) alone, or LfcinB (200 μg/ml) in combination with bFGF (1 μg/ml), $VEGF_{165}$ (5 μg/ml), or nonheparin-binding EGF (2 μg/ml). After 6 days, mice were sacrificed and Matrigel plugs were surgically excised, fixed in Carnoy's fixative, and sectioned. Sections were blocked for endogenous peroxidase activity and nonspecific antibody-binding and then stained using rabbit IgG (negative control) or rabbit IgG antibody (1:100 dilution in 1% BSA solution) that recognizes both mouse and human factor VIII-associated antigen (von Willebrand factor), which is a selective stain for endothelial cells,[31] followed by sequential treatments with biotinylated goat anti-rabbit IgG (1:3000 dilution in 1% BSA solution) and streptavidin-HRP. Von Willebrand factor-specific staining in Matrigel sections was developed with aminoethylcarbazole and visualized by light microscopy ($\times$20 magnification). The area of individual Matrigel plug sections that stained positive for von Willebrand factor relative to the unstained area was determined by computer analysis. As an alternative to staining for von Willebrand factor, blood vessel density in hematoxylin and eosin (H&E)-stained Matrigel plug sections was determined on the basis of the number of mature lumens per field of view (n = 10, $\times$200 magnification). Mature lumens were defined by the presence of erythrocytes surrounded by an identifiable endothelial cell layer.

HUVEC Proliferation

HUVECs were plated in quadruplicate in flat-bottomed 96-well (4×10^3 cells/well) microtiter plates that were previously coated with 2% gelatin and allowed to adhere overnight at 37°C in a 5% CO_2 humidified atmosphere. Culture medium was then replaced with RPMI 1640 medium containing 0.5% FCS without or with LfcinB (200 μg/ml). Plates were then incubated for an additional 15 minutes. bFGF (10 ng/ml), $VEGF_{165}$ (100 ng/ml), or nonheparin-binding EGF (20 ng/ml) was then added, and the plates were incubated for 18 hours at 37°C in a 5% CO_2 humidified atmosphere. HUVEC cultures were then pulsed with 1 μCi/ml of tritiated thymidine ([^{3}H]TdR, specific activity 60 Ci/mmol; MP Biomedicals, Irvine, CA), and 6 hours later DNA was harvested onto glass fiber filter mats using a multiple sample harvester (Skatron Instruments, Sterling, VA). [^{3}H]TdR incorporation into DNA was determined by liquid scintillation counting.

HUVEC Migration

Modified Boyden chambers and Costar 12-μm pore transwell inserts (Corning, Acton, MA) precoated with growth factor-reduced Matrigel were used to assess the effect of LfcinB on HUVEC migration. HUVECs were suspended in RPMI 1640 medium containing 0.1% BSA, and 5×10^5 cells were added to the upper chamber of triplicate wells. bFGF (10 ng/ml) or $VEGF_{165}$ (100 ng/ml) without or with LfcinB (200 μg/ml) in RPMI 1640 medium containing 0.1% BSA was added to the bottom chamber. After incubation for 2 or 4 hours at 37°C in a 5% CO_2 humidified atmosphere, filters were fixed in ethanol and stained for 10 minutes with hematoxylin. HUVECs were scraped from the upper chamber, and cells that had migrated through the filter were then counted at $\times$400 magnification in three nonoverlapping fields by light microscopy.

Cell Viability Assays

The effect of LfcinB on HUVEC viability was assessed by ^{51}Cr release from the intracellular compartment[32] and Hoechst 33342 trihydrochloride dye staining of nuclear material.[33] For ^{51}Cr release, HUVECs were labeled for 1 hour with 100 μCi of $Na_2{}^{51}CrO_4$ (MP Biomedicals). HUVECs were then washed extensively with phosphate-buffered saline (PBS), resuspended in RPMI 1640 medium containing 0.5% FCS, and plated in triplicate in a 96-well (4000 cells/well) V-bottom microtiter plate that was precoated with 2% gelatin. HUVECs were allowed to adhere for 1 hour, after which medium or LfcinB (200 μg/ml) without or with bFGF (10 ng/ml) or $VEGF_{165}$ (100 ng/ml) was added, and plates were incubated for an additional 6 hours at 37°C in a 5% CO_2 humidified atmosphere. ^{51}Cr release into cell-free culture supernatants was then determined by gamma counting. Percent ^{51}Cr release was then determined in comparison to HUVECs lysed by 10% sodium dodecyl sulfate. For Hoechst staining, HUVECs were cultured at 37°C in a 5% CO_2 humidified atmosphere in the absence or presence of LfcinB (200 μg/ml) for 24 hours. HUVECs were then washed with PBS, and resuspended in 50 μl of 4% paraformaldehyde in PBS. HUVECs were then placed on silinated microscope slides and allowed to dry overnight, after which the HUVECs were stained for 10 minutes at room temperature with Hoechst 33342 trihydrochloride dye (10 μg/ml). Slides were then rinsed with distilled water and allowed to air-dry in the dark. Chromatin condensation and nuclear fragmentation were then assessed at $\times$200 magnification by UV microscopy.

Solid Phase Heparin-Binding Assay

LfcinB binding to plastic-immobilized heparin was determined using a modification of the method described by Silvestri and Sundqvist.[34] In brief, 10 μg/ml of heparin in 15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$ (pH 9.2), and 3 mmol/L NaN_3 was added to 96-well flat-bottom microtiter plates that were then incubated for 18 hours at 4°C to allow heparin to bind to the plastic. Plates were then washed, and 1% (w/v) BSA in blocking buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 5 mmol/L $CaCl_2$) was added to wells to block

nonspecific binding sites. After incubation at room temperature for 2 hours, plates were washed repeatedly with 0.04% Tween 20 in PBS. In some experiments bFGF or $VEGF_{165}$ (both at 100 ng/ml) was immobilized on plastic instead of heparin. Biotinylated LfcinB (50 μg/ml) was added to replicate heparin-coated wells alone or in combination with increasing concentrations of bFGF (5, 10, or 20 ng/ml), $VEGF_{165}$ (50, 100, or 200 ng/ml), or nonheparin-binding EGF (10, 20, or 40 ng/ml as a negative control) in blocking buffer. After incubation for 2 hours at 4°C, plates were washed repeatedly with 0.04% Tween 20 in PBS, and streptavidin-HRP (1:1000) was added to wells. After an additional 2-hour incubation at 4°C, plates were again washed repeatedly with 0.04% Tween 20 in PBS, and 1,2-phenylenediamine substrate (0.4 mg/ml) was added to the wells. Absorbance was measured at 492 nm using a microplate autoreader (Bio-Tek Instruments, Winooski, VT).

Biotinylation of Lactoferrin, bFGF, and $VEGF_{165}$

Bovine lactoferrin (50 μg), $VEGF_{165}$ (40 μg), or bFGF (50 μg) were resuspended in 0.1 ml of PBS and combined with 0.1 ml of borate buffer. *N*-Hydroxysuccinimido-biotin was resuspended at 1 mg/ml in *N*,*N*-dimethyl formamide, and the resulting solution was slowly added on ice to the proteins such that a 40-fold molar excess of biotin to lactoferrin, bFGF, or $VEGF_{165}$ was achieved. The proteins were then incubated on ice for 8 hours, after which each protein was added to the upper chamber of a 10K Nanosep centrifugal device (Pall Life Sciences, Ann Arbor, MI) and centrifuged for 10 minutes at 10,000 $\times$ *g* and 4°C. Biotinylated lactoferrin, bFGF, and $VEGF_{165}$ were then eluted with 0.02 ml of PBS from the upper chamber, which retained 90% of the protein. Aliquots of biotinylated lactoferrin, bFGF, and $VEGF_{165}$ were stored at −20°C.

Colorimetric HUVEC-Binding Assay

HUVECs were plated in quadruplicate in flat-bottomed 96-well (4000 cells/well) microtiter plates that were previously coated with 2% gelatin and allowed to adhere overnight at 37°C in a 5% CO_2 humidified atmosphere. Some cultures were then treated with chondroitinase ABC, heparinase I, heparinase II, heparinase III (all at 1.5×10^{-2} U/ml), or PI-PLC (40 U/ml) for 2 hours at 37°C in a 5% CO_2 humidified atmosphere to remove cell-surface glycosaminoglycans. Heparinase treatment did not cause cells to be lost from HUVEC monolayers. Culture medium was replaced with RPMI 1640 medium containing 0.5% FCS without or with biotinylated LfcinB (50 μg/ml) plus increasing concentrations of bFGF (5, 10, or 20 ng/ml), $VEGF_{165}$ (50, 100, or 200 ng/ml), or nonheparin-binding EGF (10, 20, or 40 ng/ml). Alternatively, RPMI 1640 medium containing 0.5% FCS without or with biotinylated bFGF (10 ng/ml) or biotinylated $VEGF_{165}$ (100 ng/ml) plus increasing concentrations of LfcinB (10, 25, or 50 μg/ml) was added to HUVEC cultures. After incubation for 2 hours at 37°C in a 5% CO_2 humidified atmosphere, wells were washed repeatedly with 0.04% Tween 20 in PBS, and streptavidin-HRP (1:1000 dilution) was added to each well. After an additional 2-hour incubation at 4°C followed by extensive washing, 1,2-phenylenediamine substrate (0.4 mg/ml) was added to the wells. Absorbance at 492 nm was determined using a microplate autoreader.

Fluorescent Microscopy

HUVECs were placed on coverslips (5×10^4 cells/coverslip) precoated with 2% gelatin and cultured overnight at 37°C in a 5% CO_2 humidified atmosphere. Medium was then removed and replaced with RPMI 1640 medium containing 0.5% FCS without or with biotinylated LfcinB (50 μg/ml) alone or in combination with bFGF (20 ng/ml) or $VEGF_{165}$ (200 ng/ml). After a 2-hour incubation at 37°C in a 5% CO_2 humidified atmosphere, coverslips were washed with PBS, and fixed with 4% paraformaldehyde. Coverslips were then air-dried overnight at room temperature, incubated with periodate-lysine-paraformaldehyde solution for 5 minutes, blocked with 2% BSA in PBS for 45 minutes, washed with PBS and incubated with streptavidin-Texas Red (1:1000) in the dark for 1 hour at room temperature. After washing, coverslips were mounted and LfcinB binding to HUVECs was visualized by fluorescent microscopy at ×200 magnification.

Results

LfcinB Inhibits in Vivo *Angiogenesis*

The *in vivo* antiangiogenic activity of LfcinB was assessed by the subcutaneous implantation into C57BL/6 mice of Matrigel plugs that contained vehicle (water), LfcinB, bFGF, or $VEGF_{165}$ alone, or LfcinB in combination with bFGF or $VEGF_{165}$. After 6 days, the Matrigel plugs were removed, sectioned, and stained for von Willebrand factor, which is an endothelial cell marker.[31] Figure 1a shows representative histological images of Matrigel plug sections that were stained with von Willebrand factor-specific antibody. Figure 1b shows the percentage of von Willebrand factor-positive area in sections of replicate Matrigel plugs ($n = 8$). Matrigel plug sections containing only vehicle or LfcinB did not show substantial staining for von Willebrand factor, whereas Matrigel plug sections containing bFGF or $VEGF_{165}$ stained strongly for von Willebrand factor. Importantly, staining for von Willebrand factor in Matrigel plug sections containing LfcinB plus bFGF or $VEGF_{165}$ was reduced by 35 and 45% ($P < 0.001$), respectively, in comparison with sections containing growth factors alone. In contrast, von Willebrand factor staining in Matrigel plug sections containing nonheparin-binding EGF alone or in combination with LfcinB was equivalent (data not shown). To exclude the possibility that LfcinB simply decreased von Willebrand factor expression by endothelial cells, we determined blood vessel density (number of mature lumens/field of view) in H&E-stained Matrigel plug sections. Figure 2a shows representative his-

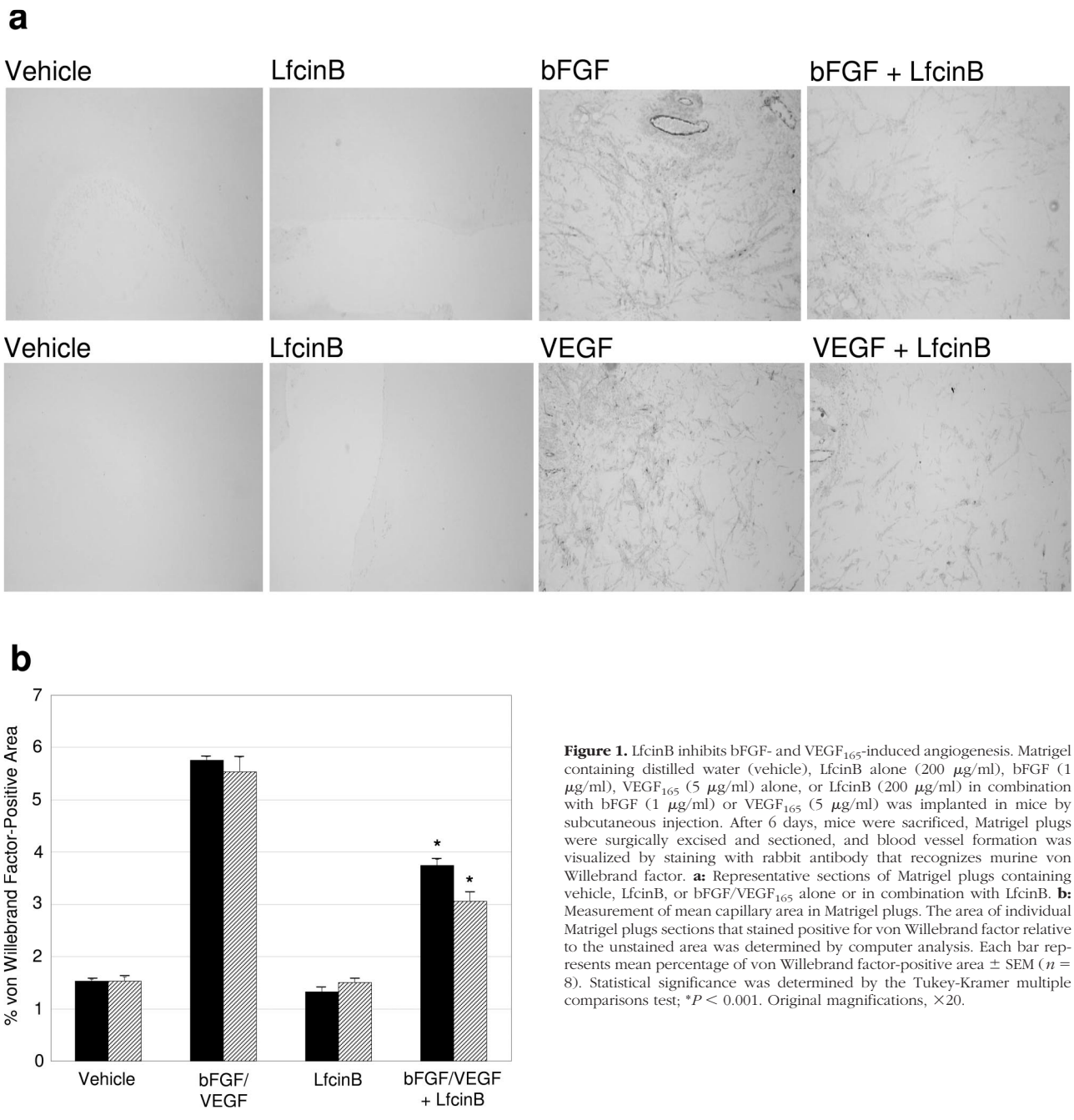

Figure 1. LfcinB inhibits bFGF- and $VEGF_{165}$-induced angiogenesis. Matrigel containing distilled water (vehicle), LfcinB alone (200 μg/ml), bFGF (1 μg/ml), $VEGF_{165}$ (5 μg/ml) alone, or LfcinB (200 μg/ml) in combination with bFGF (1 μg/ml) or $VEGF_{165}$ (5 μg/ml) was implanted in mice by subcutaneous injection. After 6 days, mice were sacrificed, Matrigel plugs were surgically excised and sectioned, and blood vessel formation was visualized by staining with rabbit antibody that recognizes murine von Willebrand factor. **a:** Representative sections of Matrigel plugs containing vehicle, LfcinB, or bFGF/$VEGF_{165}$ alone or in combination with LfcinB. **b:** Measurement of mean capillary area in Matrigel plugs. The area of individual Matrigel plugs sections that stained positive for von Willebrand factor relative to the unstained area was determined by computer analysis. Each bar represents mean percentage of von Willebrand factor-positive area ± SEM (n = 8). Statistical significance was determined by the Tukey-Kramer multiple comparisons test; *$P < 0.001$. Original magnifications, ×20.

tological images (×100 and ×400 magnification) of H&E-stained Matrigel plug sections containing bFGF or $VEGF_{165}$ alone or in combination with LfcinB. Figure 2b shows that the number of vessels in sections of replicate Matrigel plugs (n = 8) that developed in response to bFGF or $VEGF_{165}$ was reduced by 61% ($P < 0.002$) and 67% ($P < 0.001$), respectively, in the presence of LfcinB. In contrast, LfcinB did not significantly affect blood vessel development in response to nonheparin-binding EGF. Taken together, these data suggested that LfcinB inhibited endothelial cell migration and tube formation in response to heparin-binding growth factors.

LfcinB Inhibits bFGF- and $VEGF_{165}$-Induced HUVEC Proliferation and Migration

It is well known that angiogenesis involves the proliferation and migration of endothelial cells.[5] We therefore used HUVEC-based *in vitro* assays to determine whether LfcinB inhibition of bFGF- and $VEGF_{165}$-induced angiogenesis in Matrigel plugs might be accounted for by an inhibitory effect of LfcinB on the proliferation and/or migration of endothelial cells. Figure 3a shows that LfcinB (200 μg/ml) strongly inhibited ($P < 0.001$) the *in vitro* proliferation of HUVECs in

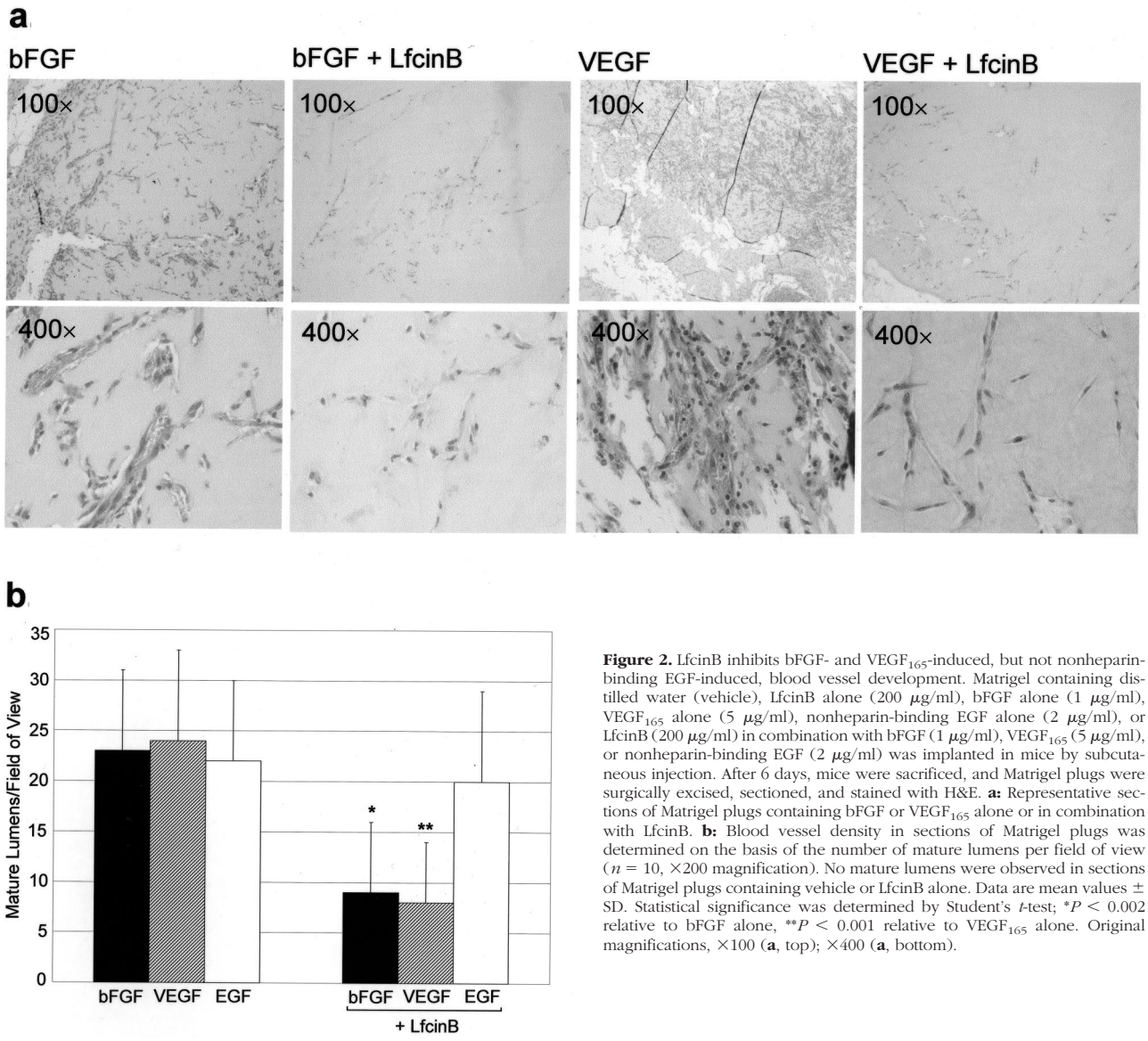

Figure 2. LfcinB inhibits bFGF- and $VEGF_{165}$-induced, but not nonheparin-binding EGF-induced, blood vessel development. Matrigel containing distilled water (vehicle), LfcinB alone (200 μg/ml), bFGF alone (1 μg/ml), $VEGF_{165}$ alone (5 μg/ml), nonheparin-binding EGF alone (2 μg/ml), or LfcinB (200 μg/ml) in combination with bFGF (1 μg/ml), $VEGF_{165}$ (5 μg/ml), or nonheparin-binding EGF (2 μg/ml) was implanted in mice by subcutaneous injection. After 6 days, mice were sacrificed, and Matrigel plugs were surgically excised, sectioned, and stained with H&E. **a:** Representative sections of Matrigel plugs containing bFGF or $VEGF_{165}$ alone or in combination with LfcinB. **b:** Blood vessel density in sections of Matrigel plugs was determined on the basis of the number of mature lumens per field of view ($n = 10$, ×200 magnification). No mature lumens were observed in sections of Matrigel plugs containing vehicle or LfcinB alone. Data are mean values ± SD. Statistical significance was determined by Student's *t*-test; $^{*}P < 0.002$ relative to bFGF alone, $^{**}P < 0.001$ relative to $VEGF_{165}$ alone. Original magnifications, ×100 (**a**, top); ×400 (**a**, bottom).

response to bFGF or $VEGF_{165}$. In contrast, HUVEC proliferation induced by nonheparin-binding EGF was not affected by LfcinB (200 μg/ml). All subsequent experiments used LfcinB at 200 μg/ml because lower concentrations of the peptide did not significantly inhibit bFGF or $VEGF_{165}$-induced proliferation of HUVECs (data not shown). LfcinB dramatically reduced ($P < 0.001$) the migration of HUVECs in response to bFGF or $VEGF_{165}$ during 2- and 4-hour periods of time (Figure 3b). Collectively, these data indicated that LfcinB inhibited the bFGF- and $VEGF_{165}$-induced proliferation and migration of endothelial cells.

LfcinB Does Not Affect HUVEC Viability

Because LfcinB induces apoptosis in a variety of human cancer cell lines as early as 1 hour after exposure to the peptide,[24,27] we addressed the possibility that the antiangiogenic activity of LfcinB was the result of a cytotoxic effect by LfcinB on endothelial cells. Figure 4a shows that ^{51}Cr-labeled HUVECs that were exposed to medium, bFGF, $VEGF_{165}$, or LfcinB for 6 hours released similar amounts of ^{51}Cr into culture supernatant. Moreover, ^{51}Cr release was not increased in the presence of combined LfcinB and bFGF or $VEGF_{165}$. Figure 4b shows that HUVECs cultured for 24 hours in the presence of LfcinB without or with bFGF or $VEGF_{165}$ did not exhibit chromatin condensation or nuclear fragmentation by Hoechst staining. We concluded that LfcinB was not inhibiting angiogenesis via a cytotoxic effect on resting or activated endothelial cells.

LfcinB Binding to Immobilized Heparin Is Inhibited by bFGF or $VEGF_{165}$

Both bovine lactoferrin and its derivative LfcinB possess heparin-binding activity.[29,30] Figure 5 demonstrates that

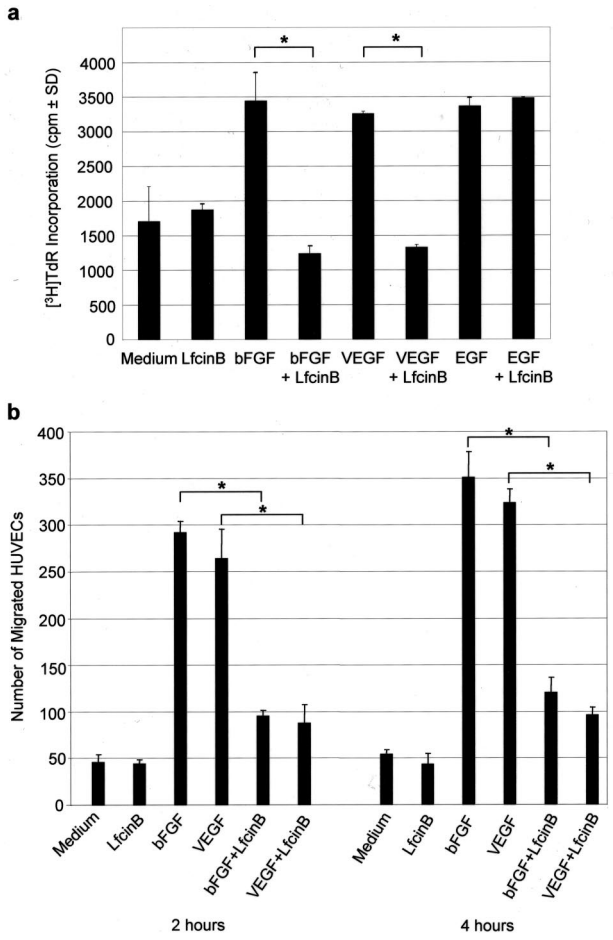

Figure 3. LfcinB inhibits bFGF- and $VEGF_{165}$-induced HUVEC proliferation and migration. **a:** HUVECs (4×10^3 cells/well) were cultured for 24 hours in the presence of medium, LfcinB (200 μg/ml), bFGF (10 ng/ml), $VEGF_{165}$ (100 ng/ml), or nonheparin-binding EGF (20 ng/ml) alone or with bFGF (10 ng/ml), $VEGF_{165}$ (100 ng/ml), or EGF (20 ng/ml) in combination with LfcinB (200 μg/ml). DNA synthesis was measured by [^{3}H]TdR incorporation. Data are shown as mean cpm ± SD of quadruplicate determinations. Statistical significance was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.001$. **b:** HUVECs (5×10^5 cells) were added to the upper chamber. Medium, LfcinB (200 μg/ml), bFGF (10 ng/ml), or $VEGF_{165}$ (100 ng/ml) alone or bFGF (10 ng/ml) or $VEGF_{165}$ (100 ng/ml) in combination with LfcinB (200 μg/ml) was added to the bottom chamber. After 2- and 4-hour incubations, filters were fixed and stained with hematoxylin, and HUVECs that had migrated across the filter were enumerated by light microscopy. Data are shown as mean number of migrated HUVECs ± SD of triplicate determinations. Statistical significance was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.001$.

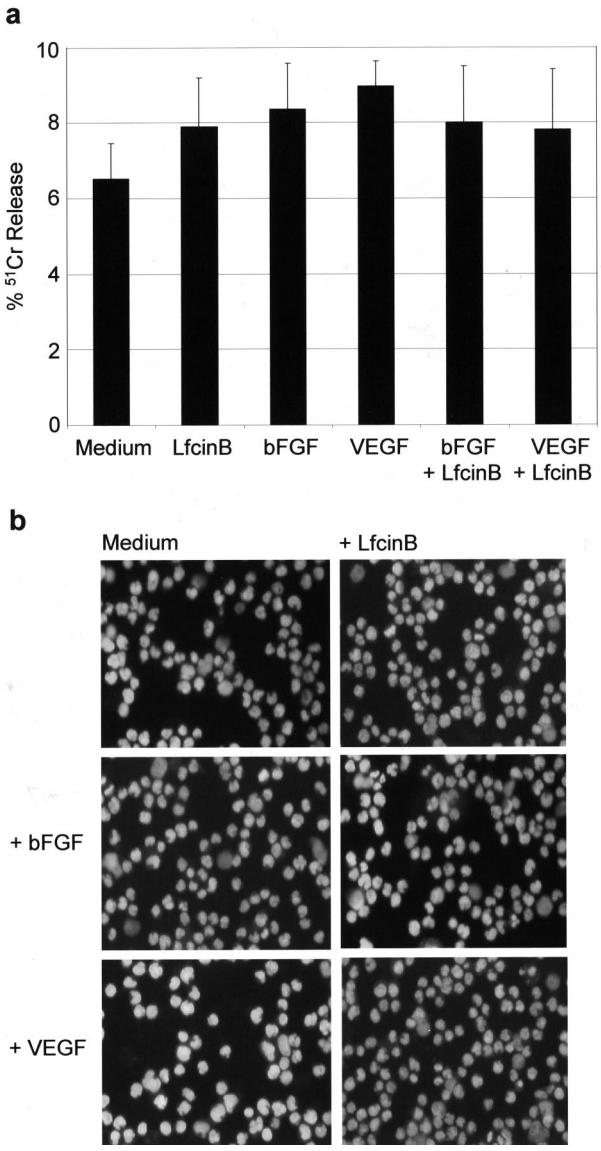

Figure 4. LfcinB treatment does not affect HUVEC viability. **a:** Effect of LfcinB on cell membrane integrity. HUVECs were labeled with ^{51}Cr, washed extensively, and cultured for 6 hours in the presence of medium, LfcinB (200 μg/ml), bFGF (10 ng/ml), or $VEGF_{165}$ (100 ng/ml) alone, or bFGF (10 ng/ml) or $VEGF_{165}$ (100 ng/ml) in combination with LfcinB (200 μg/ml). ^{51}Cr present in cell-free supernatants at the end of the incubation period was determined by gamma counting. Data are expressed as percentage of ^{51}Cr release ± SD of triplicate determinations. **b:** Identification of apoptotic cells by Hoechst staining. HUVECs were cultured for 24 hours in the presence of medium, LfcinB (200 μg/ml), bFGF (10 ng/ml), or $VEGF_{165}$ (100 ng/ml) alone, or bFGF (10 ng/ml) or $VEGF_{165}$ (100 ng/ml) in combination with LfcinB (200 μg/ml). HUVECs were then fixed and stained with Hoechst 33342 trihydrochloride dye to detect chromatin condensation and nuclear fragmentation. Original magnifications, ×200.

LfcinB bound plastic-immobilized heparin, suggesting that the heparin-binding activity of bovine lactoferrin was localized to the LfcinB fragment. Moreover, both bFGF and $VEGF_{165}$ inhibited LfcinB binding to immobilized heparin in a dose-dependent manner, whereas there was no inhibitory effect by nonheparin-binding EGF on LfcinB binding to heparin. LfcinB failed to bind to immobilized bFGF or $VEGF_{165}$ (data not shown), excluding any direct interaction between LfcinB and these growth factors. Because both bFGF and $VEGF_{165}$ must interact with heparin-like heparan sulfate proteoglycans for binding and signaling through their respective receptors,[15,16] these data suggested that LfcinB might interfere with endothelial cell responses to bFGF and $VEGF_{165}$ by competing with these growth factors for the same binding sites on cell-surface heparan sulfate proteoglycans.

LfcinB Binding to HUVECs Is Heparin-Dependent and Inhibited by bFGF and $VEGF_{165}$

We next determined whether LfcinB was able to bind to HUVEC monolayers. Biotinylated LfcinB bound strongly to HUVECs, as determined by a colorimetric

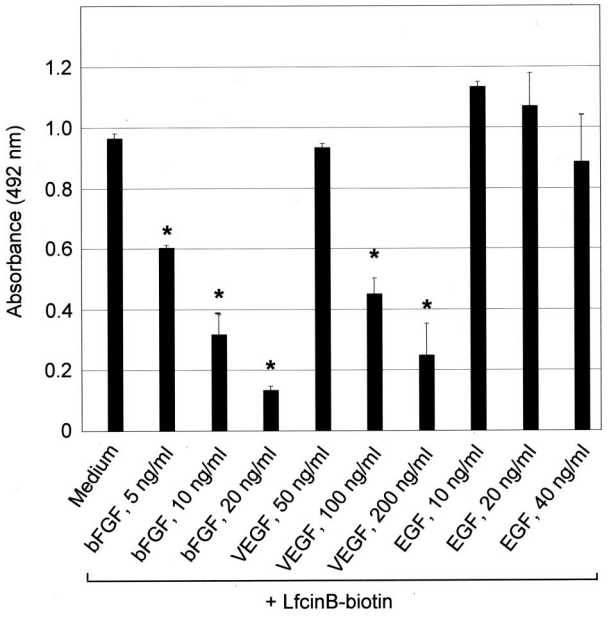

Figure 5. bFGF and $VEGF_{165}$ interfere with LfcinB binding to immobilized heparin. Biotinylated LfcinB (50 μg/ml) alone or in combination with the indicated concentrations of bFGF, $VEGF_{165}$, or nonheparin-binding EGF was added to heparin-coated wells of a flat-bottom tissue culture plate and incubated for 2 hours. Plates were then washed, and streptavidin-HRP was added to wells. After 2 hours, plates were washed and 1,2-phenylenediamine substrate was added. Absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.043 ± 0.001. Statistical significance relative to the LfcinB control was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.001$.

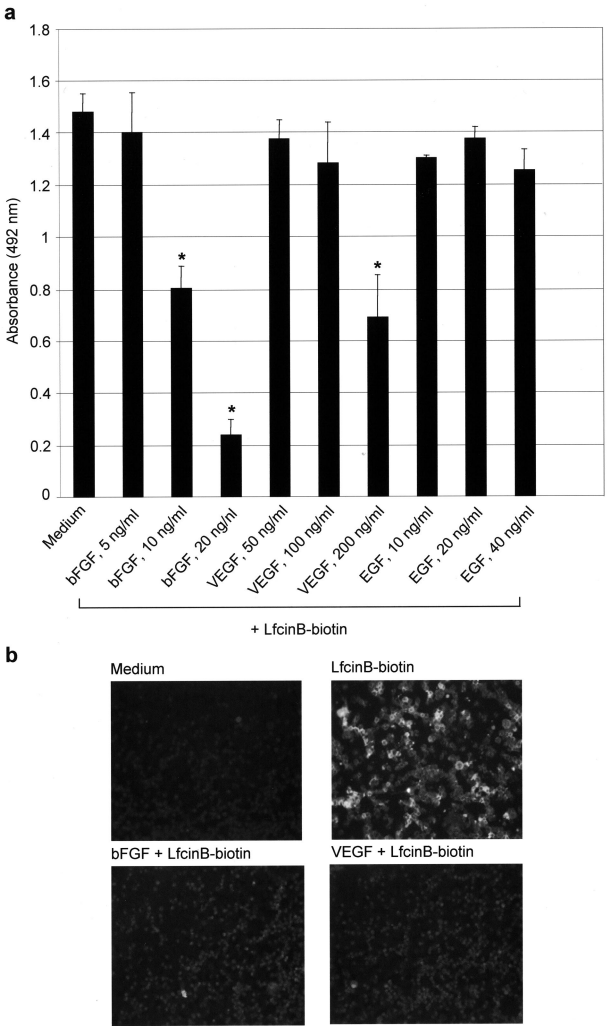

Figure 6. bFGF and $VEGF_{165}$ inhibit LfcinB binding to HUVECs. **a:** Colorimetric analysis of the effect of growth factors on LfcinB-HUVEC interactions. HUVEC monolayers were exposed to biotinylated LfcinB (50 μg/ml) in the absence or presence of the indicated concentrations of bFGF, $VEGF_{165}$, or nonheparin-binding EGF for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.043 ± 0.001. Statistical significance relative to the LfcinB control was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.001$. **b:** Determination by fluorescent microscopy of the effect of bFGF or $VEGF_{165}$ on LfcinB-HUVEC interactions. HUVEC monolayers were exposed to medium alone or to biotinylated LfcinB (50 μg/ml) in the absence or presence of bFGF (20 ng/ml) or $VEGF_{165}$ (200 ng/ml) for 2 hours. HUVEC monolayers were then washed and incubated with streptavidin-Texas Red. After additional washes, LfcinB binding to HUVECs was visualized by fluorescent microscopy. Original magnifications, ×200.

assay (Figure 6a). Furthermore, the heparin-binding growth factors bFGF and $VEGF_{165}$ had a dose-dependent inhibitory effect on LfcinB binding to HUVECs. Similar results were obtained when fluorescence microscopy was used to determine the effect of bFGF or $VEGF_{165}$ on the interaction of LfcinB with HUVECs (Figure 6b). In contrast, nonheparin-binding EGF did not interfere with LfcinB binding to HUVEC monolayers (Figure 6a). To determine whether LfcinB was binding to heparin-like structures on HUVECs, we treated HUVEC monolayers with heparinase I, heparinase II, or heparinase III to remove heparin-like molecules, including heparan sulfate proteoglycans, from the cell surface before exposing the HUVECs to biotinylated LfcinB. Heparinase treatment did not cause cells to be lost from HUVEC monolayers (data not shown). Figure 7a demonstrates that biotinylated LfcinB did not bind to heparinase-treated HUVECs. Although not shown here, heparinase-treated HUVECs also failed to bind biotinylated bFGF or $VEGF_{165}$, consistent with previously published work showing that bFGF and $VEGF_{165}$ must interact with heparin-like heparan sulfate proteoglycans to bind and signal through their respective receptors.[15,16] In addition, LfcinB exhibited dramatically reduced ($P < 0.01$) binding to HUVECs treated with PI-PLC to strip them of glycosylphosphatidylinositol (GPI)-anchored proteins (Figure 7b), suggesting that LfcinB interacted with GPI-anchored heparan sulfate proteoglycans. In contrast, treatment with chondroitinase ABC to remove chondroitin sulfate proteoglycans from HUVECs did not significantly alter LfcinB-HUVEC binding (Figure 7b), nor did chondroitinase ABC treatment affect bFGF or $VEGF_{165}$ binding to HUVEC monolayers (data not shown). Figure 8 shows that LfcinB inhibited, in a dose-dependent manner, bFGF (Figure 8a) and $VEGF_{165}$ (Figure 8b) binding to HUVEC monolayers. Collectively, these data suggested that LfcinB interacted with HUVECs via the same heparin-like heparan sulfate proteoglycans that are required for bFGF and $VEGF_{165}$ interactions with their respective cell-surface receptors, thereby ac-

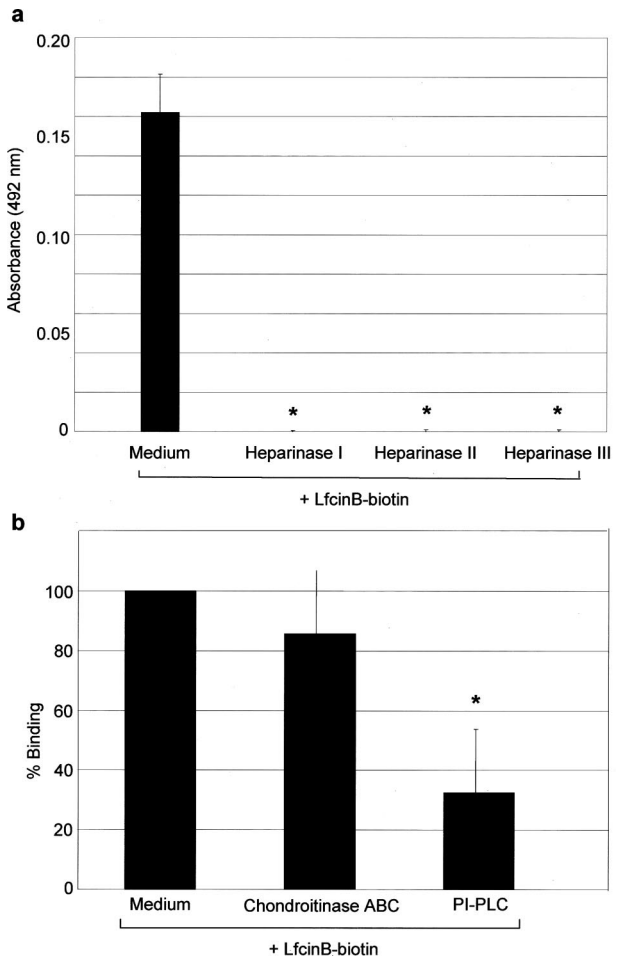

Figure 7. Reduced binding of LfcinB to heparinase- or PI-PLC-treated HUVECs. **a:** HUVEC monolayers were treated with medium, heparinase I, heparinase II, or heparinase III (all at 1.5×10^{-2} U/ml), washed, and then incubated with biotinylated LfcinB (50 μg/ml) for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added, and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.063 ± 0.001. Statistical significance relative to the medium control was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.001$. **b:** HUVEC monolayers were treated with medium, chondroitinase ABC (1.5×10^{-2} U/ml), or PI-PLC (40 U/ml), washed, and then incubated with biotinylated LfcinB (50 μg/ml) for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added and absorbance was measured at 492 nm. Pooled data from replicate experiments ($n = 3$) are shown as mean percent LfcinB binding ± SEM relative to the medium control. Statistical significance relative to the medium control was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.01$.

counting for the inhibitory effect of LfcinB on bFGF- and $VEGF_{165}$-induced angiogenesis.

Scrambled LfcinB Binds Poorly to HUVECs and Does Not Inhibit Binding of bFGF and $VEGF_{165}$ to HUVECs

Finally, we determined whether the positive charge of LfcinB and/or its amino acid sequence accounted for the peptide's inhibitory effect on the binding of bFGF and $VEGF_{165}$ to HUVECs. Figure 9 shows that LfcinB with a scrambled amino acid sequence that retained the net

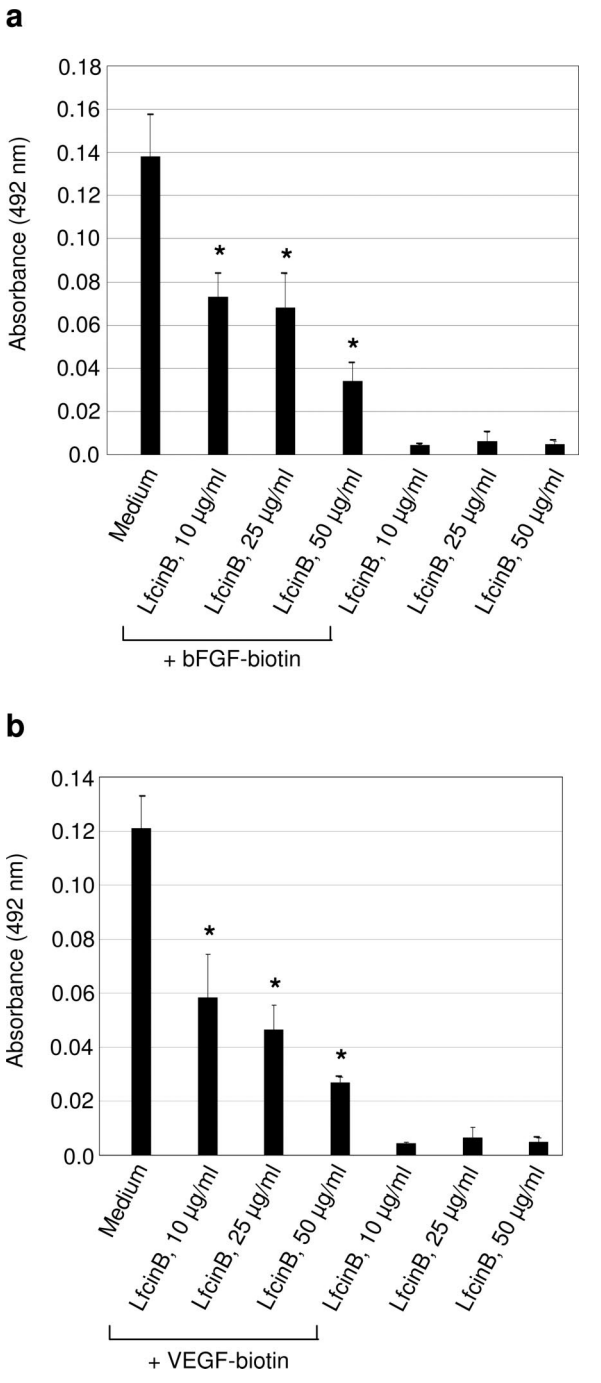

Figure 8. LfcinB interferes with bFGF and $VEGF_{165}$ binding to HUVECs. **a:** Colorimetric analysis of the effect of LfcinB on bFGF binding to HUVECs. HUVEC monolayers were exposed to biotinylated bFGF (10 ng/ml) in the absence or presence of the indicated concentrations of LfcinB for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added, and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.043 ± 0.002. Statistical significance relative to the bFGF control was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.001$. **b:** Colorimetric analysis of the effect of LfcinB on $VEGF_{165}$ binding to HUVECs. HUVEC monolayers were exposed to biotinylated $VEGF_{165}$ (100 ng/ml) in the absence or presence of the indicated concentrations of LfcinB for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added, and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Statistical significance relative to the $VEGF_{165}$ control was determined by the Tukey-Kramer multiple comparisons test; $^*P < 0.001$.

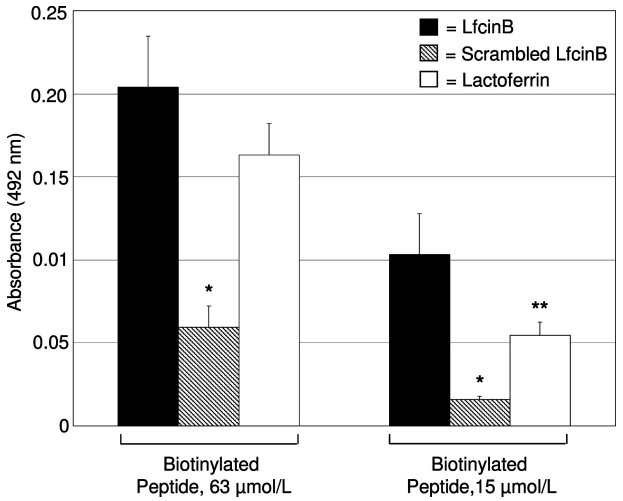

Figure 9. The HUVEC-binding capacity of LfcinB is superior to that of scrambled LfcinB or bovine lactoferrin. HUVEC monolayers were exposed to the indicated concentrations (63 and 15 μmol/L correspond to 200 and 50 μg/ml LfcinB, respectively) of biotinylated LfcinB, biotinylated scrambled LfcinB, or biotinylated lactoferrin for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added, and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.05 ± 0.0005. Statistical significance relative to the LfcinB control was determined by the Tukey-Kramer multiple comparisons test; $^{*}P < 0.001$, $^{**}P < 0.01$.

positive charge of unscrambled LfcinB was markedly inferior to native LfcinB in its ability to bind HUVEC monolayers. Interestingly, unlike native LfcinB (Figure 7a), scrambled LfcinB bound to heparinase-treated HUVECS nearly as well as the scrambled peptide bound to untreated HUVECs (data not shown). In addition, bovine lactoferrin, from which LfcinB is derived,[20] bound to HUVECs almost as well as unscrambled LfcinB at the higher concentration (63 μmol/L), although there was a significant reduction ($P < 0.01$) in the binding capacity of bovine lactoferrin compared with unscrambled LfcinB at the lower concentration (15 μmol/L). These data suggested that the structure dictated by the amino acid sequence, and to a lesser extent the cationic nature, of LfcinB was involved in the binding of LfcinB to HUVECs.

We also compared the ability of native LfcinB and scrambled LfcinB to interfere with the binding of bFGF and $VEGF_{165}$ to HUVEC monolayers. As shown in Figure 10a, scrambled LfcinB did not prevent the interaction of bFGF or $VEGF_{165}$ with HUVECs, whereas an equivalent concentration of unscrambled LfcinB had a significant inhibitory effect on bFGF and $VEGF_{165}$ binding to HUVEC monolayers. Conversely, neither bFGF nor $VEGF_{165}$ interfered with the ability of scrambled LfcinB to interact with HUVECs, albeit at a greatly reduced level in comparison with native LfcinB, whereas both bFGF and $VEGF_{165}$ inhibited the binding of unscrambled LfcinB to HUVEC monolayers (Figure 10b). These findings indicated that the structure of LfcinB rather than its positive charge was the major determinant of LfcinB specificity for heparin-like structures involved in bFGF and $VEGF_{165}$ interactions with HUVECs.

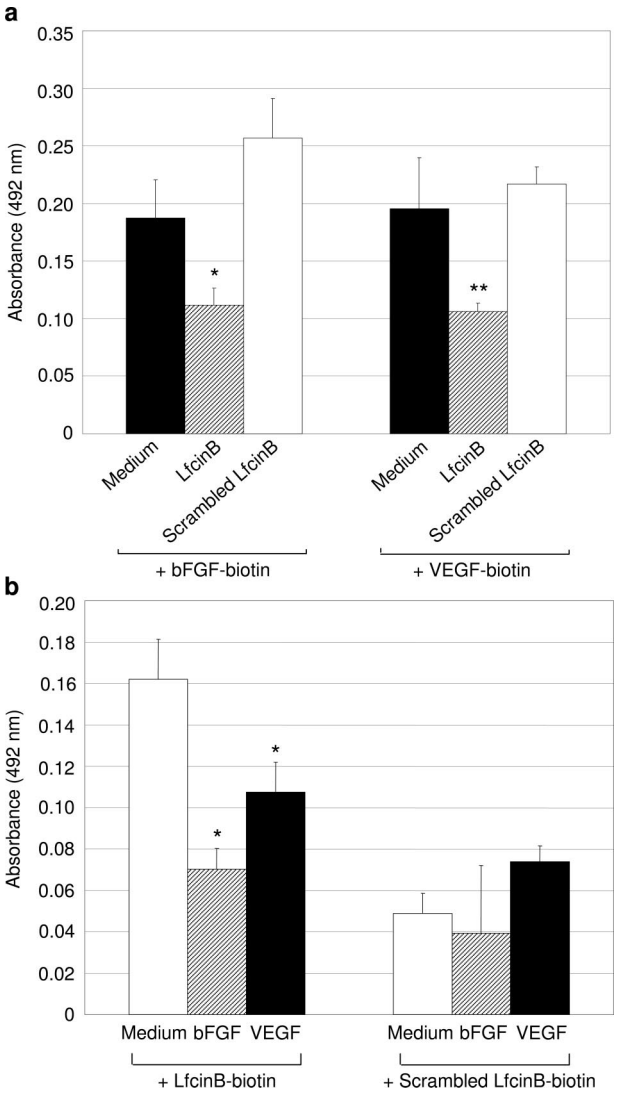

Figure 10. Scrambled LfcinB does not compete with bFGF and $VEGF_{165}$ for binding sites on HUVECs. **a:** HUVEC monolayers were exposed to biotinylated bFGF (10 ng/ml) or biotinylated $VEGF_{165}$ (100 ng/ml) in the absence or presence of LfcinB or scrambled LfcinB (both at 50 μg/ml) for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added, and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.038 ± 0.001. Statistical significance relative to the medium control was determined by the Tukey-Kramer multiple comparisons test; $^{*}P < 0.05$, $^{**}P < 0.01$. **b:** HUVEC monolayers were exposed to biotinylated LfcinB or biotinylated scrambled LfcinB (both at 50 μg/ml) in the absence or presence of bFGF (10 ng/ml) or $VEGF_{165}$ (100 ng/ml) for 2 hours. HUVEC monolayers were then washed and incubated for 2 hours with streptavidin-HRP. After additional washes, 1,2-phenylenediamine substrate was added, and absorbance was measured at 492 nm. Data are shown as mean absorbance ± SD of quadruplicate determinations. Background absorbance was 0.063 ± 0.001. Statistical significance relative to the medium control was determined by the Tukey-Kramer multiple comparisons test; $^{*}P < 0.001$.

Discussion

New cancer treatment strategies based on the use of angiogenesis inhibitors have gained considerable attention in recent years because of the inherent advantages that antiangiogenesis therapy has over conventional chemotherapy: Selective targeting of tumor-associated vasculature results in relatively few adverse side effects,

endothelial cells that form the tumor vasculature are easily accessible to antiangiogenic agents delivered via the blood, and resistance to antiangiogenic agents is unlikely to occur because endothelial cells are genetically stable, diploid, and homogenous.[2] Many different angiogenesis inhibitors are currently undergoing clinical trials or are being introduced into clinical practice. Nevertheless, the search continues for new antiangiogenic agents that might prove useful in the treatment of human cancers. In this regard, a possible antiangiogenic role for LfcinB is suggested by the finding that systemic administration of LfcinB to tumor-bearing mice causes a reduction in the number of tumor-induced blood vessels.[25] The same study shows that a similar result is obtained when tumor-bearing mice are treated with bovine lactoferrin. In addition, oral administration of bovine lactoferrin has been reported to inhibit VEGF-induced angiogenesis in a mesenteric-window assay in rats,[35] as well as Lewis lung carcinoma-induced angiogenesis in a dorsal air sac assay in mice.[36] The fact that LfcinB inhibited bFGF- and $VEGF_{165}$-induced angiogenesis in the *in vivo* Matrigel assay and inhibited bFGF- and $VEGF_{165}$-induced *in vitro* proliferation and migration of HUVECs suggests that the antiangiogenic activity of bovine lactoferrin (previously demonstrated by others in mouse and rat tissues after systemic treatment with bovine lactoferrin[25,35,36]) may reside within the LfcinB sequence located proximal to N terminus of lactoferrin. This is consistent with the observation that LfcinB exhibited a capacity to bind to HUVECs that was at least equivalent to that of bovine lactoferrin.

It is noteworthy that substantial amounts of LfcinB are produced in the stomach of rats and humans after ingestion of bovine lactoferrin.[37,38] Although a recent study failed to demonstrate the presence of dietary bovine lactoferrin or functional fragments of bovine lactoferrin such as LfcinB in the portal blood of rats,[39] the systemic antiangiogenic activity of ingested bovine lactoferrin in both rats and mice[35,36] suggests that transfer of LfcinB to circulating blood is likely to in fact take place. However, it is important to realize that a systemic distribution of LfcinB at the concentration (200 μg/ml) that showed antiangiogenic activity in our *in vivo* and *in vitro* assays cannot be attained by the normal consumption of dairy products containing bovine lactoferrin as a source of LfcinB because cow's milk, for example, contains less than 100 mg/L lactoferrin.[40]

Endothelial cell proliferation and migration are important components of the angiogenic process, which is normally tightly regulated by a balance of pro- and antiangiogenic factors, but may become dysregulated under pathological conditions such as tumor growth.[3] Growth factors that promote angiogenesis include bFGF and $VEGF_{165}$,[6,7] both of which are produced by neoplastic cells.[9,10,12] LfcinB inhibited bFGF- and $VEGF_{165}$-induced, but not nonheparin-binding EGF-induced, angiogenesis in the *in vivo* Matrigel plug assay. Although it is possible that trace amounts of undefined proangiogenic factors that remain in growth factor-reduced Matrigel might have influenced our results, we believe this to be unlikely because very little endothelial cell infiltration was detected in Matrigel plugs that did not contain exogenous bFGF or $VEGF_{165}$. However, it is important to recognize that the Matrigel angiogenesis assay used in our studies is limited by the use of exogenous growth factors rather than tissue (eg, tumor tissue) to serve as a more physiologically relevant source of endogenous pro- and/or antiangiogenic factors. We are therefore in the process of examining the effect of LfcinB on angiogenesis induced *in vivo* in Matrigel implants containing breast cancer cells as an endogenous source of proangiogenic factors such as VEGF.[12]

LfcinB also had a potent inhibitory effect on bFGF- and $VEGF_{165}$-induced proliferation and migration of HUVECs but did not affect the ability of nonheparin-binding EGF to stimulate HUVEC proliferation. Cultures of endothelial cells derived from the human umbilical vein are a well-established model system in which to study various *in vitro* aspects of angiogenesis.[41] Although endothelial cells originating from large vessels such as the umbilical vein and microvascular endothelial cells (the principal cells involved in tumor-associated angiogenesis) show different gene expression patterns,[42] we considered it appropriate to use HUVECs in our *in vitro* studies because HUVECs and microvascular endothelial cells show similar dose response profiles to bFGF and VEGF.[43] Moreover, HUVECs have been used to model sprout formation, which is an important step of angiogenesis seen *in vivo*.[44] LfcinB did not exhibit any cytotoxic activity against HUVECs, excluding the possibility that LfcinB simply caused HUVECs to undergo apoptosis, as occurs when various cancer cell lines are exposed to LfcinB.[24,27] Because both bovine lactoferrin and LfcinB are known to bind heparin,[29,30] our findings led us to hypothesize that LfcinB competed with bFGF and $VEGF_{165}$ for heparin-like binding sites on heparan sulfate proteoglycans on the surface of HUVECs. Heparan sulfate proteoglycans are required for bFGF and $VEGF_{165}$ binding and signaling through their respective cell-surface receptors.[15,16] Our finding that bFGF and $VEGF_{165}$ failed to bind to heparinase-treated HUVECs was consistent with these earlier reports. We also observed that LfcinB bound to immobilized heparin, consistent with earlier published findings.[30] However, LfcinB did not bind to immobilized bFGF or $VEGF_{165}$, suggesting that a direct interaction between LfcinB and bFGF or $VEGF_{165}$ was not responsible for the antiangiogenic effect of LfcinB. Importantly, LfcinB binding to heparin was reduced in the presence of bFGF or $VEGF_{165}$, suggesting that LfcinB, bFGF, and $VEGF_{165}$ might all interact with the same heparin-like binding sites on cell-surface heparan sulfate proteoglycans. In addition, LfcinB and bFGF or $VEGF_{165}$, but not nonheparin-binding EGF, showed competitive binding to the surface of HUVECs. The observation that LfcinB failed to bind to HUVECs that were pretreated with heparinase I, heparinase II, or heparinase III provided compelling evidence that LfcinB interacted with heparan sulfate proteoglycans on the surface of HUVECs. In this regard, heparinase I preferentially cleaves heparin over heparan sulfate (3:1), whereas heparinase II is less effective at cleaving heparin in comparison with heparan sulfate (1:2) and heparinase III exclusively cleaves heparan sulfate.[45,46] In contrast to the results obtained with hepari-

nase-treated HUVECs, LfcinB binding to HUVEC monolayers was unaffected by chondroitinase ABC-mediated removal of chondroitin sulfate proteoglycans from HUVECs. Collectively, these findings support our contention that LfcinB exerts its antiangiogenic activity by interfering with heparan sulfate proteoglycan-dependent bFGF and $VEGF_{165}$ binding and signaling through their respective cell-surface receptors. Our findings are also consistent with reports that LfcinB interferes with the ability of herpes simplex virus and adenovirus to infect cells by competing for heparan sulfate proteoglycans that function as viral attachment sites.[47,48]

It is noteworthy that LfcinB showed a reduced capacity to bind to HUVECs that had been pretreated with PI-PLC to remove cell-surface GPI-anchored proteins. This finding leads us to suggest that glypican-1, a GPI-anchored heparan sulfate proteoglycan that is present on endothelial cells and potentiates $VEGF_{165}$ or bFGF binding to their respective receptors,[49,50] may function as a binding partner for LfcinB. Interestingly, glypican-1 is overexpressed by human breast and pancreatic cancer cells,[51,52] which might promote the binding of LfcinB to these tumor cell types. We therefore speculate that the selective cytotoxic activity that LfcinB exhibits against several different human breast carcinoma cell lines[27] may be, at least in part, attributable to interactions between LfcinB and cell-surface glypican-1.

Cell-surface heparan sulfate proteoglycans, which function as co-receptors for soluble ligands such as growth factors (eg, bFGF and $VEGF_{165}$) and for insoluble ligands such as extracellular matrix molecules, are produced by covalent linkage of the glycosaminoglycan heparan sulfate to a protein core.[53] Although the exact mechanism by which LfcinB interacts with heparin-like molecules has not yet been elucidated, it is known that LfcinB has a net positive charge of 7.85 at pH 7.0,[54] whereas both heparin and heparan sulfate are negatively charged molecules.[55,56] It was therefore possible that the affinity that LfcinB displayed for heparin-like structures was the result of electrostatic interactions, which would be in line with the recent finding that $VEGF_{165}$ interacts with long stretches of anionic residues in heparan sulfate molecules.[57] However, a comparison of the HUVEC-binding capacity of native LfcinB and LfcinB with a scrambled amino acid sequence that retained the net positive charge of native LfcinB revealed that the scrambled peptide showed greatly decreased binding to HUVEC monolayers. In addition, scrambled LfcinB bound to a similar extent, albeit at a minimal level, to untreated and heparinase-treated HUVECs. Importantly, unlike native LfcinB, scrambled LfcinB did not interfere with bFGF or $VEGF_{165}$ binding to HUVECs. Moreover, neither bFGF nor $VEGF_{165}$ inhibited the minimal binding of scrambled LfcinB to HUVECs, whereas both bFGF and $VEGF_{165}$ interfered with the binding of native LfcinB to HUVEC monolayers. Taken together, these findings strongly suggest that the structure conferred on LfcinB by its amino sequence rather than the positive charge of LfcinB was a major factor in the selectivity of LfcinB for heparin-like structures involved in bFGF and $VEGF_{165}$ interactions with their respective receptors on the surface of HUVECs. However, the fact that scrambled LfcinB still bound to HUVECs, albeit at a modest level, suggests that electrostatic interactions between LfcinB and anionic heparan sulfate molecules may contribute to the HUVEC-binding activity of LfcinB. At this time, it is not clear why LfcinB that bound to HUVECs did not have the same cytotoxic effect that the peptide exerts on neoplastic cells.[24,26,27] Perhaps LfcinB that complexed with cell-surface heparan sulfate proteoglycans was not in close enough proximity to the endothelial cell surface to destabilize the cell membrane and trigger apoptosis. Interestingly, in contrast to normal vascular endothelium, anionic phospholipids such as phosphatidylserine are exposed on the surface of tumor endothelium.[58] LfcinB may therefore be able to bind anionic phospholipids on the plasma membrane of tumor-associated vascular endothelial cells *in vivo* in close enough proximity and sufficient quantity to cause membrane destabilization and apoptosis, as well as blocking angiogenesis induced by heparin-binding growth factors. Ongoing studies seek to determine whether this is in fact the case.

In summary, we have shown that LfcinB interfered with the interaction of the heparin-binding growth factors bFGF and $VEGF_{165}$ with their receptors on the surface of endothelial cells, resulting in decreased endothelial cell proliferation and migration and, ultimately, diminished angiogenesis. These findings indicate that the possible application of LfcinB as an antiangiogenic agent for the treatment of human cancers warrants further investigation. The possibility that LfcinB might be able to interfere with tumor-associated angiogenesis caused by multiple heparin-binding growth factors represents a considerable advantage over current antibody-based antiangiogenic agents that target only a single growth factor receptor. However, it is important to note that systemic administration of unmodified LfcinB may not be optimal for antiangiogenic therapy because cationic peptides with anti-cancer activity are known to be susceptible to enzymatic digestion and inactivation through interactions with anionic serum components.[59] One possible solution to this problem may be to use an all-D-amino acid analogue of LfcinB because an all-D-amino acid analogue of magainin 2, another cationic peptide with anti-cancer activity, exhibits enhanced stability in serum.[60] Alternatively, systemically administered LfcinB might be delivered to tumor sites via liposomes to preserve its antiangiogenic activity. Targeted liposomal delivery of antiangiogenic LfcinB to tumor sites in rodents is already under investigation in our laboratory.

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