b-FGF Induces Corneal Blood and Lymphatic Growth in a Spatially Distinct Pattern

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Abstract

Purpose—To study the spatial variances in ligand expression and angiogenic effect in response to the inflammatory response induced by b-FGF.

Methods—b-FGF micropellets (80ng) were implanted in the temporal side of the cornea of Balbc/c mice. On days 1, 3, and 7 blood (heme) and lymph-angiogenesis were observed by immunofluorescence staining of corneal flat mounts with LYVE-1 and CD31 to identify lymphatic and blood vessels, respectively. A second group of corneas were harvested for quantitative RT-PCR. Each cornea was divided in two different area defines as (i) pre-pellet area and (ii) opposite-pellet area. Expression of VEGF ligands were evaluated using Real-time PCR in each respective zone.

Results—Blood vessels grew into the cornea from the pre-pellet area while corneal lymphatic vessels grew from the opposite-pellet area toward the center of the cornea. VEGF-A was upregulated in the pre-pellet while VEGF-D expression was mostly observed in the opposite-pellet area. VEGF-C level increased simultaneously in both areas.

Conclusion—A single inducing factor, i.e., b-FGF, may simultaneously provoke heme-and lymph-angiogenesis in different locations of the cornea through differential expression of VEGF ligands. This distinctive spatial pattern should be considered while evaluating the corneal predilection for inflammation beyond that which is directly visible by slit lamp examination.

Keywords

Corneal angiogenesis; fibroblast growth factor; vascular endothelial growth factor

Introduction

The cornea is devoid of both blood and lymphatic vessels. However, many pathologic conditions of the cornea are associated with the ingrowth of new vessels from the pre-existing vasculature of the limbus with resultant adverse consequences [1–2]. Several key angiogenic factors, including fibroblast growth factor (FGF),[3] vascular endothelial growth factor (VEGF),[4–6] platelet-derived growth factor (PDGF),[7] and angiopoietin[8–11] families regulate angiogenesis through multiple and overlapping molecular pathways in various physiological and pathological processes[12]. While each of these growth factors has been

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described to play a dominantly pro-hemangoigenic (*e.g.* VEGF-A[13]) or pro-lymphangiogenic (*e.g.* VEGF-C and VEGF-D[13]) function, their in vivo effect is more complex since the function of these factors is affected by the microenviroment in which they are expressed.

Investigation of the distinctive molecular and cellular mechanisms underlying the growth of corneal blood, as opposed to lymphatic, vessels has been particularly challenging as the majority of the pathologic conditions and animal models stimulate coincident growth of both lymphatic and blood vessels [14]. Multiple groups have defined strategies to selectively induce blood or lymphatic growth in the cornea. One example is basic FGF (b-FGF) which was initiallycharacterized as an inducer of hemangiogenesis, while intrastromal micropellet implantation of very low doses of b-FGF (12.5 ng) was subsequently shown to be a selective lymphangiogenic stimulator. In addition, we and others have shown that systemic blockade of VEGFR3[15–16] and α5 integrin[17] predominantly inhibit corneal lymphangiogenesis.

Very little is known about the relative contribution of blood versus lymphatic vessels to various pathological conditions of the cornea. This is relevant since pathological angiogenesis of the cornea, as appreciated in clinical cases, is highly heterogeneous, not just from eye to eye, but also *within* the same cornea. Here we study the spatial variances in ligand expression and angiogenic effects in response to the same inflammatory stimulus induced by b-FGF.

Material and methods

Animals

Male 6- to 8-week-old Balb/c mice were used in all experiments. Animals were anesthetized by intraperitoneal injection of ketamine (120 mg/kg) and xylazine (20mg/kg) before any surgery and were treated in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments described herein were conducted under institutional animal care and use committee approval.

Pellet Implantation

b-FGF micropellets (80 ng/pellet) were prepared as described previously. [18] To implant the pellets, half thickness linear incisions were made at the center of cornea using a disposable 30°microknife (F.S.T., Foster City, CA, USA). Lamellar pocket incisions were then made parallel to the corneal plane using a Von Graefe knife (F.S.T., Foster City, CA, USA) and advanced to the temporal limbus at the lateral canthal area. The pellets were positioned into the pocket 1.0 mm apart from the limbal vascular arcade in the temporal side, and tetracycline ophthalmic ointment was applied to the eye after pellet implantation.

Immunohistochemistry and Morphometry of HA and LA

After taking photographs under the slit lamp, five mice per group were sacrificed 1, 3, and 7 days after implantation, and freshly enucleated eyes were prepared into corneal flat mounts. Immunohistochemical staining was performed with FITC-conjugated CD31/PECAM-1 (rat-anti–mouse antibody; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Staining against LYVE-1 (lymphatic endothelium-specific hyaluronic acid receptor; goat-anti–rabbit antibody; 1:400; Abcam, Cambridge, MA) was also performed with purified antibody followed by rhodamine-conjugated secondary antibody. To quantify the level of blood vessel (CD31^{high}/LYVE-1^{low}) or lymphatic vessel (CD31^{low}/LYVE-1^{high}) formation low magnification (2X) micrographs were captured, and the area covered by each type of vessels was calculated (NIH ImageJ software 1.34; developed by Wayne Rasband, National

Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) and expressed as the percentage of total corneal area (%).

Quantitative Real-Time PCR (qRT-PCR)

Corneas were harvested on days 1, 3, 7 post-implantation. In a group of animals, each cornea was divided based on the proximity to the pellet into 2 different areas: pre-pellet area (PA) or temporal cornea, and the opposite-pellet area (OA) or the nasal part of the cornea. RNA was isolated from either the whole or different regions of the cornea by RNeasy Micro Kit (Qiagen, Valencia, CA) and reverse transcribed (Superscript III Kit; Invitrogen Life Technologies, Carlsbad, CA). Real-time PCR was performed (TaqMan Universal PCR MasterMix; Applied Biosystems, Foster City, CA), and primers were preformulated for VEGF-A (assay ID Mm00437304_ml), VEGF-C (assay ID Mm00437313_ml), VEGF-D (assay ID Mm00438965_ml), (Applied Biosystems). PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 35 cycles of 15 seconds at 95°C and 60°C for 1 minute using an ABI PRISM 7900 HT (Applied Biosystems). The results were analyzed by comparative threshold cycle method. The relative expression level of each sample was expressed compare to 10^5 of GAPDH using Microsoft Office Excel 2003.

Statistical Analysis

Error bars displayed in the figures were calculated from the standard error of the mean (±SEM). Statistical analysis of blood and lymphatic vessel area was performed using a one-way ANOVA. Student's t test was performed to compare the level of mRNA in qPCR. P < 0.05 was considered statistically significant.

Results

b-FGF induces corneal angiogenesis and up-regulates VEGF ligand expression

Hemangiogenic properties of b-FGF have long been studied. However its recently identified pro-lymphangiogenic properties have not been well-characterized. Using immunostaining for specific markers of blood and lymphatic vessel, we showed that implantation of b-FGF micropellets results in extensive corneal hemangiogenesis and lymphangiogenesis (Figure 1-B). As the pro-angiogenenic effects of b-FGF are believed to be mediated through VEGF[19], we examined the expression levels of VEGF ligands at different time points following corneal implantation of the b-FGF micropellets. As shown in Figure 1-C, corneal mRNA expression of all three principal VEGF ligands, VEGF-A, VEGF-C and VEGF-D, were significantly up-regulated after b-FGF micropellet implantation compared to normal levels, implicating these VEGF species in b-FGF induced corneal neovascularization.

b-FGF promotes a spatially distinct pattern of lymph- and hem-angiogenesis

As illustrated in Figure 2, we observed a robust hemangiogenic response in the limbal-peripheral cornea closest to the pellet (pre-pellet area [PA]), while lymphatics grew more primarily in the limbal-peripheral cornea opposite to the pellet (opposite area [OA]) toward the center. This distinct spatial pattern of blood and lymphatic growth was consistently observed at different time points after implantation of b-FGF micropellets. Quantification of the relative area of the corneas covered by each vessel type (Figure 3) showed that on the PA (92%) of the corneas are blood vessels, and only 8 % lymphatic vessels. In contrast on the OA there was nearly 3 times as much of the cornea covered by lymphatics (77%) as compared to blood vessels (23%).

b-FGF induces VEGF ligands in a spatially distinct pattern

Further to evaluate the relative expression of VEGF ligands in different areas of cornea by b-FGF micropellet we used qRT=PCR of VEGF ligands in PA and OA. Consistent with our in situ results on lymphatic and blood vessel growth induced by b-FGF micropellet, we observed that VEGF ligands were upregulated in a spatially distinct manner in the cornea (Figure 4A–C). VEGF-A and VEGF-C ligands were mainly upregulated in the PA, while in the OA there were significant increases in VEGF-D and VEGF-C, and to the lesser extent in VEGF-A compared to the normal state. This pattern of VEGF ligand upregulation was consistent with the 'phenotype' observed in the corneal immunofluorescent staining for lymphatic and blood vessel growth.

Discussion

Angiogenesis is an intricate process that involves many cell types through a diverse group of growth factors and signaling pathways. FGF is a potent angiogenic factor[19–22] which contributes to the pathogenesis of corneal inflammatory diseases through interactions with other angiogenic factors including VEGF. Here we evaluated the pattern of angiogenesis induced by FGF micropellet, and demonstrate that it results in a spatially distinct modulation of VEGF ligand expression and resultant growth of lymphatic versus blood vessels in the cornea.

Accumulating evidence is challenging the original characterization of angiogenic phenotypes through receptor-ligand specific interactions. Angiogenesis is not interrupted in the double knockout of FGF1 and FGF2, indicating extensive redundancy of the ligand system [23–25]. On the other hand, while exogenous FGF2 is able to induce vascular structure formation in normal embryos, VEGF-R2 −/− embryos fail to develop vessels in the presence of b-FGF [26]. These findings support the idea that FGF may act upstream to VEGFs. Supporting this concept is our finding that all VEGF ligand are over-expressed in the presence of b-FGF stimulation in the cornea.

The novel finding in this study is the observed high lymphangiogenic response noted following b-FGF stimulation in the cornea on side opposite to where the FGF pellet is implanted. More importantly, we observed differential pattern of VEGF stimulation by b-FGF micropellet in the various parts of the cornea, with high levels of VEGF-D expression on the OA.

Our findings have several interesting implications for our assessment of immunity in the setting of corneal inflammatory angiogenesis. First, given that the majority of cases of corneal angiogenesis are sectoral rather than diffuse, it is provocative to hypothesize that ample lymphatics may well be present in areas of the cornea directly opposite from the clinically visible blood vessels and adjacent area of stromal inflammation. Given that corneal lymphatics which cannot be seen directly by slit lamp examination, play a crucial role in the induction and amplification of alloreactive[28] and autoimmune[29] responses to corneal antigens, these findings would suggest that continued induction of immune reactivity could be ongoing *away from the site of inflammation* even in a setting where the therapy has led to blood vessel regression. Put another way, inflammatory changes that induce even sectoral corneal angiogenesis likely lead to more 'global' changes in corneal immunity. This concept is indirectly supported by the definition of risk factors for rejecting a corneal graft, which induces as little as 1–2 quadrants of neovascularization in the host bed[30]. Additionally, given the development of in vivo imaging technologies that allow for imaging and monitoring of corneal lymphatics[31] our data imply that the area likely requiring our focus for assessment of lymphatics also included the area opposite from the site of inflammation.

Here we show for the first time that both processes of blood and lymphatic growth can potentially happen at the same time but in different locations in the cornea in response to differential expression of VEGF ligands, suggesting more global changes in corneal immunity and predilection for inflammation beyond that which is directly visible by slit lamp examination.
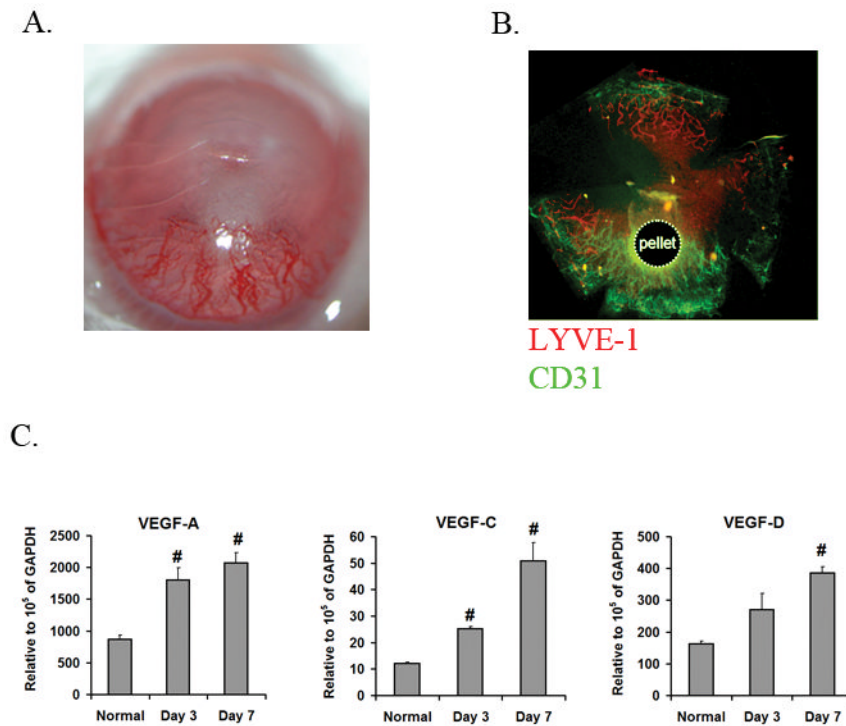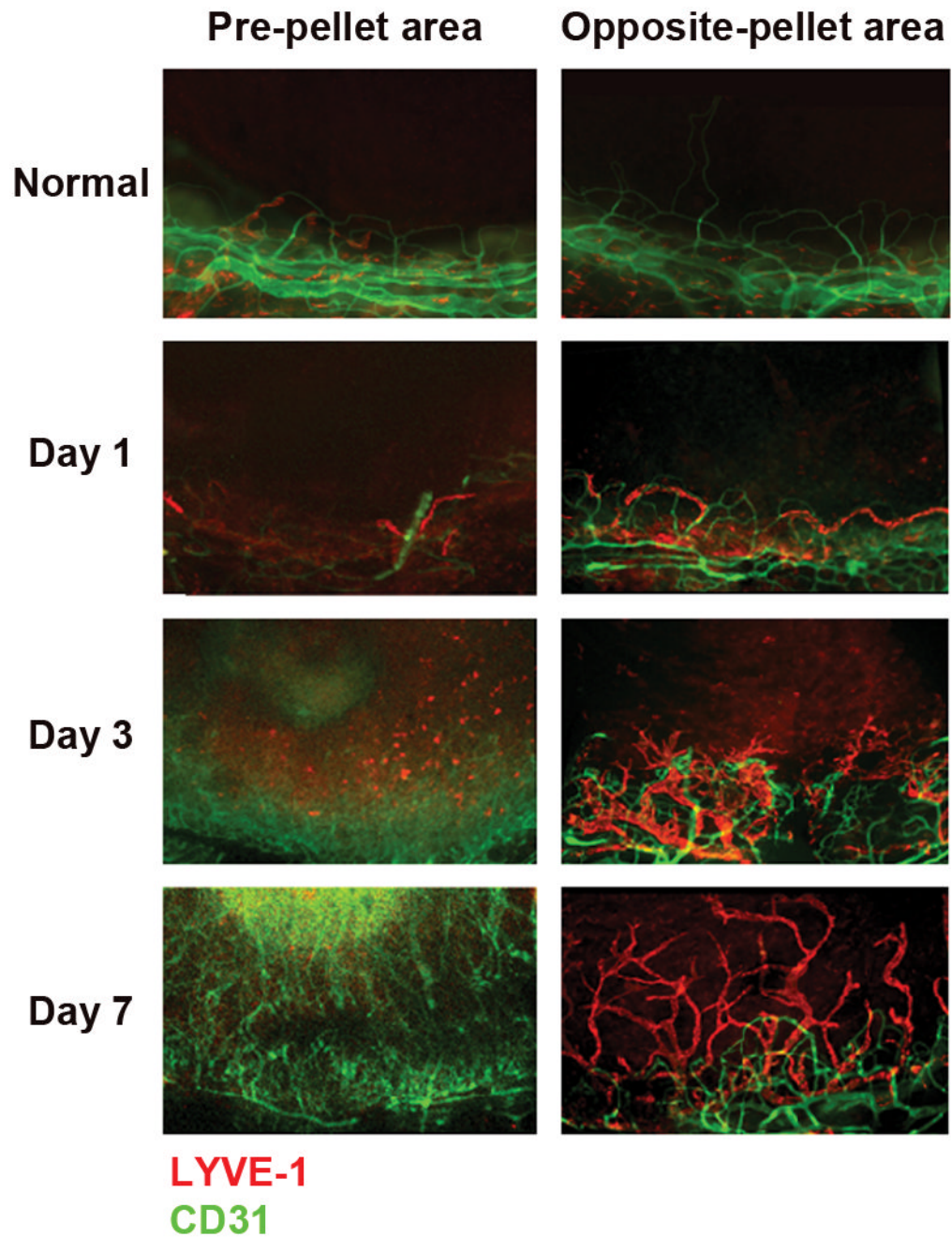
Acknowledgments

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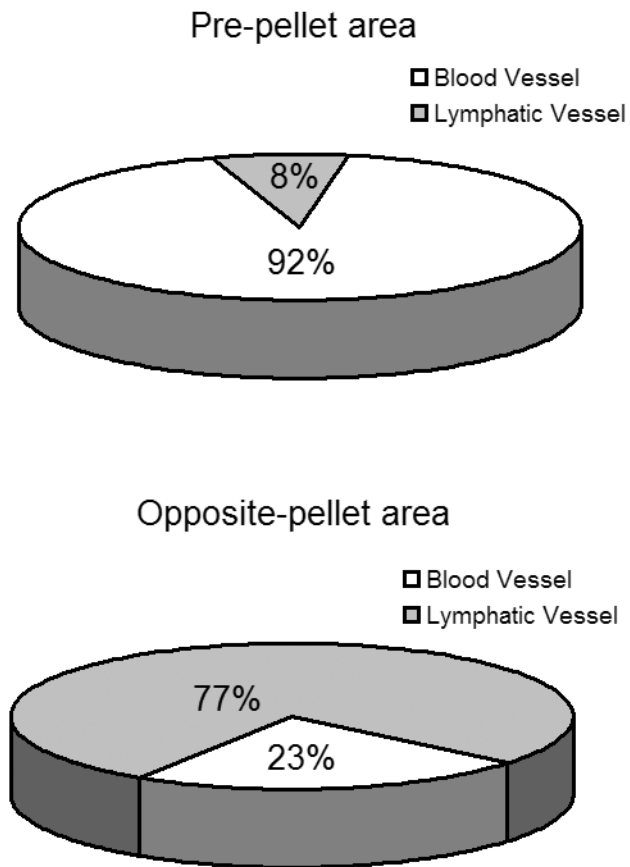
A.

B.

LYVE-1
CD31

C.

Figure 1.
b-FGF induces both blood and lymphatic vessel growth. **A.** Representative photographs of a cornea at 7 days after implantation of b-FGF micropellet demonstrating extensive neovascularization. **B.** Immunostaining of a corneal flat mount at 7 days after b-FGF micropellet implantation with CD31 (green) and LYVE-1 (red) antibodies demonstrates the development of blood (CD31^{high}/LYVE-1^{low}) and lymphatic (CD31^{low}/LYVE-1^{high}) vessels. **C.** VEGF ligands mRNA expression levels were determined by qRT-PCR in corneas of normal and b-FGF micropellet implanted mice. Corneal expression of all VEGF ligands are increased 7 days after b-FGF micropellet implantation (#: $P<0.01$, compared to normal corneas).

Pre-pellet area | Opposite-pellet area
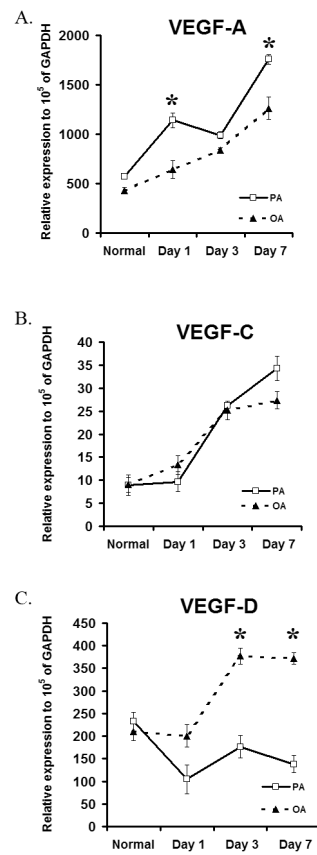
Normal

Day 1

Day 3

Day 7

LYVE-1
CD31

Figure 2.
Corneal flat mounts staining demonstrates a spatially distinct pattern of hemangiogenesis-and lymphangiogenesis at 1, 3 and 7 days following implantation of b-FGF pellet (80 ng) into the corneal stroma. Extensive blood vessels growth was observed on the limbal-peripheral cornea closest to the pellet (pre-pellet area [PA]), while lymphatics grew more primarily in the limbal-peripheral cornea opposite to the pellet (opposite area [OA]).

Pre-pellet area

☐ Blood Vessel
☐ Lymphatic Vessel

8%

92%

Opposite-pellet area

☐ Blood Vessel
☐ Lymphatic Vessel

77%

23%

Figure 3.
Percentage of the corneal area covered by blood and lymphatic vessels is significantly different in the pellet *vs.* opposite side of the cornea. Data are representative of 5 corneas.

Figure 4.
Spatial differences in mRNA expression of VEGF ligands in normal and b-FGF micropellet implanted mice were observed. **A–C.** The expression levels of VEGF- A increases most at pre-pellet area (PA) over time, while VEGF-D increased in opposite-pellet area (OA) of the pellet. VEGF-C shows a consistent pattern of up-regulation in all three areas over time. Graphs represent mean values (± SEM) (n=5) (*: P<0.01, PA compared to OA levels observed in the corneas in each time point).