VEGF-Dependent Conjunctivalization of the Corneal Surface

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PURPOSE. To investigate the mechanisms governing corneal neovascularization and the appearance of goblet cells in a murine model of limbal insufficiency.

METHODS. The spatial and time-dependent relationship between corneal neovascularization and goblet cell density was analyzed in corneal flatmounts. Immunohistochemical detection of the vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR1) was performed in paraffin-embedded sections. A transgenic mouse that expresses the reporter gene lacZ targeted to the Flt-1 locus through homologous recombination was used to analyze corneal expression of Flt-1. The presence of soluble and membranous goblet cell Flt-1 mRNA and protein content was assessed with Northern and Western blot analyses, respectively. Finally, systemic adenoviral expression of a soluble Flt-1/Fc construct was used to study the effect of inhibition of VEGF bioactivity on the appearance of goblet cells and neovascularization.

RESULTS. Corneal neovascularization preceded the appearance of goblet cells, although both processes overlapped temporally. Flt-1 was abundant in the conjunctival-like epithelium covering the cornea, as well as in the goblet cells, invading leukocytes, and vasculature. A similar expression pattern was observed in the transgenic mice expressing the lacZ gene downstream from the Flt-1 promoter. Isolated human and rat goblet cells in culture expressed Flt-1 mRNA and protein, as did freshly isolated human conjunctiva. The systemic inhibition of VEGF bioactivity potently suppressed both corneal neovascularization (8.3% ± 8.1% vs. 41.1% ± 15.3% corneal area; P < 0.001) and corneal goblet cell density (1.6% ± 2.5% vs. 12.2% ± 2.4% corneal area; P < 0.001).

CONCLUSIONS. Two important features of corneal conjunctivalization, the appearance of goblet cells and neovascularization, are regulated by VEGF. Both processes are probably mediated, in part, through the Flt-1 receptor. Taken together, these data indicate that an anti-VEGF therapeutic approach may limit the visual loss associated with conjunctivalization of the corneal surface. (Invest Ophthalmol Vis Sci. 2003;44:117-125) DOI: 10.1167/iovs.01-1277

The tight regulation of corneal neovascularization helps maintain the transparency and immune privilege of the cornea.1 The destruction of the limbal cells through infection, injury, or immunologic disease frequently results in the conjunctivalization of the corneal surface. Conjunctivalization refers to coverage of the cornea by conjunctival epithelium, replete with goblet cells and vessels, resulting in corneal opacity and loss of vision. Although it has been proposed that the healthy limbus maintains a barrier between the corneal and conjunctival epithelia,2 it is still not fully understood why conjunctival epithelial overgrowth is closely associated with the development of corneal neovascularization.3 In 1988, Huang et al.1 provided the first evidence that the two phenomena may be mechanistically linked. Photodynamic ablation of corneal neovascularization has been shown to trigger the loss of goblet cells and the adoption of a more cornea-like phenotype by the conjunctival epithelium, a process some term “transdifferentiation.”

The corneal neovascularization that characterizes conjunctivalization is vascular endothelial growth factor (VEGF)-dependent. In a model of limbal and corneal epithelial debridement, the inhibition of VEGF bioactivity effectively suppressed corneal neovascularization.4 Vascular endothelial growth factor (VEGF) is thought to act directly on the vasculature and signals through at least two high-affinity receptor tyrosine kinases: VEGFR-1 (Flt-1; fms-like tyrosine kinase) and VEGFR-2 (KDR; kinase domain region or Flk-1 in the rodent). Of the two VEGF receptors, Flt-1 has a higher affinity for VEGF, and a naturally produced soluble form of Flt-1 efficiently neutralizes the bioactivity of VEGF.5

The extent to which another aspect of conjunctivalization, the appearance of goblet cells, is VEGF-dependent remains unknown. In the present study, the mechanistic link between VEGF-dependent corneal neovascularization and the appearance of goblet cells was examined. Specifically, the spatial and temporal relationship between the two processes was characterized. In subsequent experiments, the bioactivity of VEGF was inhibited with a systemically administered adenovirus coding for a soluble Flt-1/Fc receptor chimera. The effect of inhibition of VEGF on neovascularization and goblet cell density was then quantified.

MATERIALS AND METHODS

Animals

Male C57 BL/6 mice, weighing 20 to 25 g. (Jackson Laboratories, Bar Harbor, ME) were used in the experiments. All experiments followed the guidelines of the ARVO Statement for the Use of Animals in
Ophthalmic and Vision Research and were approved by the Animal Care and Use Committees of the Children’s Hospital and Massachusetts Eye and Ear Infirmary. All procedures were performed with animals under general anesthesia induced by intramuscular xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (55 mg/kg). The animals had free access to food and water. A 12-hour day-night cycle was maintained.

**Induction of Corneal Neovascularization**

After general anesthesia and topical application of lidocaine (Alcon, Fort Worth, TX), corneal epithelial and limbal debridement was performed by application of 1.5 μL of 0.15 mM NaOH to the central cornea. Inter- and intraexperimental variability was reduced by using mice of the same strain, gender, and age and by having the same investigator (AMJ) perform the standardized epithelial debridement. With these methods, 360° neovascularization was achieved in all animals; however, the degree of neovascularization varied between experiments, as can be the case with this model.6 The corneal epithelium and limbal epithelium were removed with a blunt von Graefe knife (Geuder, Heidelberg, Germany). After debridement, the eyes received a single application of antibiotic ointment consisting of neomycin sulfate 3.5 IE/mg, bacitracin 0.3 IE/mg, and polymyxin B sulfate 7.5 IE/mg (Polyspectran; Alcon). After epithelial debridement, the animals were randomized to the treatment groups.

**Corneal Neovascularization Quantitation**

Mayer’s hematoxylin (Sigma, St. Louis, MO) stains endothelial cells when injected intravenously. After the induction of deep anesthesia, the chest was carefully opened and a 20-gauge canula was placed into the left ventricle. Mayer’s hematoxylin (1:1 diluted with PBS) was injected at 80 mm Hg, reaching a total volume of 200 μL/kg over approximately 5 minutes. The corneas were then removed in toto 1 hour after induction of anesthesia. For one set of experiments, to correlate neovascularization with the appearance of goblet cells, the extent of limbal debridement was varied to gain various degrees of neovascularization (described later).

Flatmounts were prepared, and images were captured using a charge-coupled device (CCD) camera (CD-330; Dage-MTI, Inc., Michigan City, IN) attached to a microscope (MZ FLIII; Leica Microsystems, Wetzlar, Germany) for 1 hour. After general anesthesia and topical application of lidocaine (Alcon), corneal epithelial and limbal debridement was performed as described earlier and incubated with a polyclonal anti-mouse Flt1 Ab (RBI, Natick, MA) at a concentration of 1:500 in PBS at 4°C overnight. After they were washed, the sections were incubated with an affinity-purified biotinylated anti-rabbit secondary antibody (1:500 in PBS; Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. After three PBS washes, the sections were incubated with avidin-biotin reagent (ABC; Vector Laboratories, Burlingame, CA), and the peroxidase reaction was developed with diaminobenzidine. Omission of the primary antibody was used to assess the specificity of the staining.

**Flt-1/LacZ Transgenic Mouse**

Trangenic mice that possessed the lacZ gene inserted into the Flt-1 locus through homologous recombination were used.7 To enhance sensitivity in the paraffin-embedded tissue, in which enzymatic activity is very low, β-galactosidase immunohistochemistry was performed. On day 21 after corneal and limbal debridement, eyes were enucleated, fixed in formalin, and processed for paraffin embedding. The sections were prepared as described earlier and incubated with a polyclonal rabbit anti-β-galactosidase antibody (Cortex Biochem, San Leandro, CA) at a concentration of 1:500 in PBS at 4°C overnight. After three washes in PBS, they were incubated with an affinity purified biotin-conjugated anti-rabbit secondary antibody (diluted 1:500 in PBS supplemented with 2% rabbit serum; Jackson Immunoresearch) for 1 hour at room temperature. After three PBS washes, the sections were incubated with the avidin-biotin reagent (ABC; Vector Laboratories), and the peroxidase reaction was developed with diaminobenzidine.

**Treatment with Adenovirus Expressing Soluble Flt-1/Fc**

VEGF bioactivity was blocked by the systemic administration of an adenovirus that expresses the soluble form of the VEGF receptor Flt-1 fused to the Fc portion of IgG (Ad-Flt-1). Two days before corneal epithelial debridement, animals received intravenous injections of 1 × 10^9 plaque-forming units (PFU) Ad-Flt-1. Control mice received 1 × 10^9 PFU Ad-GFP, an adenovirus coding for green fluorescent protein (GFP).

**RT-PCR for Flt-1**

Total RNA was isolated from rat and human goblet cells and human conjunctiva with extraction reagent (TRIzol; GibcoBRL, Grand Island, NY). Two milligrams of RNA was used for first strand of cDNA synthesis with oligo-dT primer and a PCR kit (Superscript II; GibcoBRL) and subsequently 0.5 μg of cDNA was used as template for PCR amplification. The primers used were as follows: for membranous Flt-1: forward (5′-AAG GTC TAC AGC ACC AAG-3′) and reverse (5′-CAT TAC CTC ACT TGC ACC-3′); for soluble Flt-1: forward (5′-AGC AGA CAA GTC TCT ACT TGC ACC-3′) and reverse (5′-CAT TAC TTT GTG TGG CAC AAC CAC TCC-3′).

PCR reactions of 50 μL were prepared with the use of Taq polymerase (ExTaq (PanVera, Madison, WI) and processed in a thermocycler (model 480; Applied Biosystems, Foster City, CA) under the following conditions: 94°C for 4 minutes/94°C for 1 minute, 52°C for 1 minute, 72°C for 2 minutes) × 25/72°C for 5 minutes. For comparison,

For spatial analyses of corneal goblet cells and neovascularization, images from each cornea, highlighting goblet cells and vessels, respectively, were digitally overlapped. The goblet cells were enhanced for improved visualization with a digital red-pink pseudocolor.
GAPDH cDNA was also amplified for 30 cycles with respective primers. The products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator.

**Western Blot Analysis for Flt-1**

Rat and human goblet cells were isolated as previously described.

Normal human conjunctiva was obtained from surgical patients after they provided informed consent. The samples were lysed for 30 minutes on ice in lysis buffer (50 mM Tris-HCl [pH 8.0], containing 120 mM NaCl and 1% Igepal; Chem Associates, North East, PA), supplemented with a mixture of proteinase inhibitors (Complete; Roche Molecular Biochemicals, Indianapolis, IN). The samples were cleared by centrifugation (14,000 rpm for 30 minutes at 4°C) and assessed for protein concentration with the bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12%; Invitrogen Corp., Carlsbad, CA) was performed (30 μg of protein per lane), and the proteins were electroblotted onto nylon membranes. After a 1-hour incubation in blocking solution (20% IgG-free normal horse serum in PBS-Tween: 0.5% Tween 20 [Sigma] in PBS), the membranes were exposed to primary antibody (1:500 dilution for anti-Flt-1, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After a wash in PBS supplemented with 1% Triton (Sigma), peroxidase-labeled secondary antibodies were added at a concentration of 1:10,000 (anti-mouse Amersham Pharmacia Biotech, Piscataway, NJ), or 1:5,000 (anti-goat, Santa Cruz) for 40 minutes at room temperature. The proteins were visualized with an enhanced chemiluminescence technique (Amersham Pharmacia Biotech).

**Statistics**

To analyze the differences between treated and control eyes, as well as within the treatment groups, an unpaired *t-*test with two tailed probability or ANOVA (for multiple comparisons) was used, as appropriate. Results are presented as the mean ± SD. *P* < 0.05 was deemed significant.

**RESULTS**

**Corneal Neovascularization and the Appearance of Goblet Cells**

After complete corneal and limbal epithelial debridement, the cornea was fully reepithelialized by day 3 (Fig. 1A), the time point at which inflammation was most prominent. Corneal neovascularization was visible by day 3, and progressed through day 21. Starting at day 7, a few goblet cells began appearing within the epithelium, and occupied a larger surface area by day 14. When the neovascularization and goblet cells were quantified over time (Fig. 1B), it was apparent that the onset of corneal neovascularization preceded the appearance of goblet cells and that the final area of neovascularization occupied a larger corneal surface area than the final area of goblet cell coverage.
Corneal Neovascularization and Goblet Cell Density

To correlate neovascularization with the appearance of goblet cells, the extent of limbal debridement was varied to obtain various degrees of neovascularization \((n = 11\) eyes; Figs. 2A, 2B).\(^2\) Preliminary experiments demonstrated that the degree of limbal deficiency and subsequent conjunctivalization could be varied as a function of duration of exposure to NaOH (30 seconds to 5 minutes) and debridement pressure. Increasing the concentration of NaOH beyond 0.15 M resulted in corneal perforation; therefore, all debridements were performed with 0.15 M NaOH. Our preliminary experiments also demonstrated that a 30-second exposure to 0.5 M NaOH, combined with vigorous corneal and limbal debridement, resulted in a complete absence of corneal and limbal cells on histopathologic examination (data not shown). This indirect method of assessing the presence of limbal stem cells was used because no consensus currently exists regarding murine stem cell markers. The neovascularization and goblet cell areas were quantified 14 days after debridement. A significant correlation between neovascularization and goblet cell area was observed (Fig. 2C). In some eyes, neovascularization was accompanied by very few goblet cells; however, all eyes with goblet cells had neovascularization (data not shown). Goblet cells frequently, but not always, colocalized to areas of neovascularization. (Figs. 2A, 2B).

Flt-1 in Conjunctivalized Epithelium

Flt-1 immunohistochemistry revealed a relative absence of staining in the normal uninjured corneal epithelium (Fig. 3). However, Flt-1 was evident in the conjunctivalized epithelium after corneal and limbal epithelial debridement. Flt-1 localized to the endothelial cells of new corneal vessels (internal positive control) and to the inflammatory cells invading the cornea. The later-appearing goblet cells demonstrated the strongest staining along their cell margins.

To further confirm the presence of Flt-1 in the goblet cells and conjunctivalized epithelium, transgenic mice with a lacZ reporter cassette in the Flt-1 gene locus were studied (Fig. 4). These animals, after corneal and limbal epithelial debridement, confirmed the presence of Flt-1 in the conjunctivalized epithelium, including the goblet cells. The pattern of staining mirrored that of Flt-1 immunohistochemistry.

Expression of Soluble and Membranous Flt-1 mRNA and Protein

RT-PCR was performed on cultured rat and human goblet cells, as well as human conjunctiva (Fig. 5A). mRNAs for the soluble and membranous forms of Flt-1 were detected in all three specimens. Relative PCR quantitation indicated that the conjunctiva bore less Flt-1 mRNA than pure populations of goblet cells. Western blot analysis of rat goblet cells confirmed the presence of soluble and membranous Flt-1 protein (Fig. 5B).

Effect of Inhibition of VEGF

Animals were randomized to groups that received a systemic injection of adenovirus that expresses the soluble form of Flt-1 (Ad-Flt-1) or GFP (Ad-GFP) or received no treatment at all (control). Two days later, total limbal and corneal epithelial debridement was performed. On day 7 after limbal and corneal epithelial debridement, the vascularized area in Ad-GFP-injected mice was 25.9% ± 10.3% versus 17.78% ± 17.7% for the Ad-Flt-1-treated mice \((P > 0.05)\). Untreated control animals showed a mean vascularized area of 22.7% ± 10.1% \((P > 0.05\) versus Ad-GFP). The goblet cell area measured 4.6% ± 4.3% in the Ad-GFP-treated mice, 1.7% ± 1.1% in the Ad-Flt-1-treated mice \((P < 0.005)\) and 8.5% ± 3.1% in the untreated control mice \((P < 0.001; n = 8\) for each group; Fig. 6, top). On day 14, the vascularized area in the Ad-GFP injected mice was 41.1% ± 15.3% versus 8.3% ± 8.1% for the Ad-Flt-1-treated mice \((n = 8\) vs. \(n = 10\), respectively; \(P < 0.001\)). The untreated control showed a mean vascularized area of 40.4% ± 17.1% \((n = 6)\). The goblet cell area measured 12.2% ± 2.4% in the Ad-GFP-treated mice, 1.6% ± 2.5% in the Ad-Flt-1-treated mice, \((P < 0.001)\) and 10.3% ± 9.53% in untreated control mice \((P < 0.001; \text{Fig. 6, bottom})\).
VEGF inhibition, per se, does not cause regression of established vessels; however, it can lead to the regression of newly formed vessels. The latter appeared to occur, to some degree, between days 7 and 14. Benjamin et al. have called this period of susceptibility to VEGF withdrawal the "plasticity window."

**DISCUSSION**

The present study demonstrates that VEGF is required for both corneal neovascularization and the appearance of goblet cells after extensive limbal injury. As such, VEGF is causally linked to two important facets of the conjunctivalization process. Huang et al. demonstrated that selective photothermolysis of corneal neovascularization triggers transdifferentiation of the conjunctivalized cornea. The present study complements and extends those findings by demonstrating that conjunctivalization can be directly prevented through the blockade of a specific molecule, VEGF. These data also highlight the increasingly evident pleiotropic effects of VEGF.

VEGF is an angiogenesis- and permeability-enhancing factor. Its two major high-affinity tyrosine kinase receptors, Flt-1 and Flk-1 (VEGFR1 and VEGFR2, respectively), are primarily confined to the vasculature. The presence of Flt-1 on nonendothelial cells in vivo is rare, but has been described in cells of hematopoietic origin, as well as in normal and inflamed corneal epithelium. When goblet cell Flt-1 was observed by immunohistochemistry in the present study, its presence in Flt-1lacZ knockin mice was sought for additional confirmation. The identification of Flt-1 mRNA and protein in isolated goblet cells and fresh conjunctiva provided further proof of its presence.

The mechanism by which VEGF promotes conjunctivalization is only partially understood. Because VEGF is an endothelial cell mitogen and migration factor and because Flt-1 and Flk-1 are expressed on vessels, VEGF most likely acts directly to trigger the growth of new corneal vessels. The mechanism by which VEGF triggers the appearance of goblet cells is less well understood. The present study demonstrates that both the soluble and transmembrane forms of Flt-1 are made by goblet cells. The identification of Flt-1 on goblet cells suggests that VEGF may directly mediate goblet cell migration and/or appearance. Flt-1 mediates monocyte migration in vitro, so it is conceivable that is has the same function in goblet cells. Others have shown that endothelial Flt-1 can act as a decoy receptor, sequestering VEGF and limiting binding to the Flk-1 receptor. The function of the Flt-1 receptor in goblet cells remains to be determined. VEGF levels peak in the first days after corneal injury, well before goblet cells appear. Because the appearance of goblet cells was correlated with neovascularization, it is possible that a blood-borne factor triggered the appearance of goblet cells.

**FIGURE 3.** Flt-1 immunohistochemistry of normal and conjunctivalized corneas. Cytoplasmic and membranous staining was observed in the corneal epithelium as early as day 3. By day 7, stromal leukocytes and vascular endothelium were positive for Flt-1. By day 21, goblet cells showed a rim of intense cytoplasmic staining (arrow).

**FIGURE 4.** Cornea stained for β-galactosidase from a transgenic mouse possessing a lacZ reporter cassette downstream from the Flt-1 promoter. After corneal and limbal epithelial debridement, intense cytoplasmic staining was observed in the conjunctivalized epithelium, goblet cells (arrowhead), and vascular endothelium (asterisk) (A). The conjunctiva (positive control) demonstrated positive staining within the conjunctival epithelium (B). Staining of a wild-type normal cornea (negative control) demonstrated an absence of any specific immunoreactivity (C). The tissues were counterstained with hematoxylin.
subsequent appearance and maintenance of the goblet cells. Further, the data of Huang et al.\textsuperscript{1,16} in which photothrombosis triggered transdifferentiation also argues for an indirect effect. However, it is also possible that the appearance of goblet cells is dependent in part on the production of local cytokines.

The subtle effects of inhibition of VEGF on corneal wound healing remain to be determined. In the present study, epithelia healed in all treatment groups by day 3. However, a more understated delay in wound healing would not be identified by the present study design. A potential effect on wound healing is suggested by the presence of Flt-1 in the non-goblet-cell conjunctivalized epithelium. The ability of a VEGF-targeted therapy to reverse conjunctivalization was also not addressed in this study, but it is an important clinical question. Because inhibition of VEGF alone does not cause regression of established corneal vessels (Amano S, Joussen AP, Adamis AM, et al., unpublished observations, 1997), anti-VEGF monotherapy may not be sufficient to trigger transdifferentiation. This hypothesis is currently undergoing testing.

These caveats notwithstanding, the current data clearly demonstrate that the conjunctivalization is in part VEGF dependent. Both neovascularization and the appearance of goblet cells were suppressed when VEGF bioactivity was inhibited. An angiogenic factor, VEGF, promotes a pathologic corneal epithelial phenotype. As such, these data provide the first molecular target for the treatment of this clinically intractable cause of blindness.

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