Blockade of Prolymphangiogenic VEGF-C suppresses Dry Eye Disease

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Abstract

Objective—To determine if blocking prolymphangiogenic factors like VEGF-C would suppress alloimmunity in dry eye disease (DED) using a murine model.

Methods—The effects of intraperitoneal injections of 400 μg of anti-VEGF-C antibody (treated group) and intraperitoneal normal saline (untreated group) were studied in murine dry eyes induced by exposing mice to high-flow desiccated air in the Controlled Environment Chamber (CEC). Growth of lymphatic vessels and infiltration of macrophages was evaluated by immunohistochemistry using CD31 (pan-endothelial marker), LYVE-1 (lymphatic endothelial marker) and CD11b (monocytes/macrophages marker). Real time PCR was performed to quantify expression of different inflammatory cytokine transcripts in the conjunctiva and lymph nodes, and vascular endothelial growth factors and their receptors (VEGF-A, C, D/R2, R3) in the cornea.

Results—Blocking VEGF-C led to significant reduction in lymphatic caliber (P=0.025) and lymphatic area (P=0.006) in the corneas of DED mice. In addition to significantly decreasing (P=0.005) CD11b+ cells, anti-VEGF-C treatment significantly decreased transcript levels of VEGF-C (P=0.002), VEGF-D (P=0.014) and VEGFR-3 (P=0.023) in the corneas of treated group. Significant decrease in expression of inflammatory cytokines in the conjunctiva (IL1-α, P= 0.003; IL1-β, P= 0.025 and IL-6, P= 0.005) and lymph nodes (IFN-γ, P= 0.008; and IL-17, P= 0.003) was also seen with anti-VEGF-C treatment.

Conclusions—Treatment with anti-VEGF-C led to significant improvement in DED reflected by decrease in inflammation at the clinical, molecular, and cellular levels.

Clinical Relevance—Targeting prolymphangiogenic growth factors or their receptors could inhibit the trafficking of antigen presenting cells to the draining lymph nodes and hence prove to be a potential therapeutic target for dry eye disease.

INTRODUCTION

Dry eye disease (DED) is a complex, multifactorial, immune-mediated disorder of the ocular surface affecting about 5 million Americans above the age of 50 years.1, 2 Millions more suffer from manifestations which are precipitated under adverse environmental conditions such as low humidity. DED severely impacts the vision-related quality of life and the symptoms can be both psychologically and physically debilitating.1 The current therapeutic
options for DED are limited, mostly palliative and costly. Currently, topical cyclosporine-A is the only approved treatment for this disease.

The ocular surface inflammation in DED is sustained by ongoing activation and infiltration of pathogenic immune cells primarily of the CD4+ T cell compartment. Recently, we have demonstrated that lymphangiogenesis, without concurrent growth of blood vessels (hemangiogenesis), occurs in the DED cornea. Interestingly, these lymphatics not only grow toward the central cornea, but also show significantly increased caliber compared to those restricted to the limbal areas of normal corneas. Furthermore, DED corneas show a significant up-regulation of pro-lymphangiogenic specific vascular endothelial growth factor (VEGF)-C and VEGF-D, along with their receptor VEGFR-3, confirming that the low-grade inflammation seen in DED is selectively conducive for lymphangiogenesis.

Lymphangiogenesis is linked to a number of pathological conditions including lymphedema and cancer. Correlative studies with human tumors and functional studies using animal tumor models show that increased levels of VEGF-C or VEGF-D in tumors promotes metastasis to regional lymph nodes. Also, it is now well established that VEGF is accountable for many ocular pathologies involving angiogenesis including age-related macular degeneration, diabetic retinopathy, neovascular glaucoma, and corneal transplantation. Over the past several years, agents targeting VEGF-A have been developed for intraocular use and have revolutionized ophthalmological care for many of these potentially blinding conditions. Here, we tested the hypothesis that a strategy targeting the primarily pro-lymphangiogenic VEGF-C may suppress the inflammation and epitheliopathy associated with DED, and could thus provide therapeutic value for the treatment of dry eyes.

METHODS

Experimental Dry Eye Murine Model

Eight to ten week-old female C57BL/6 mice (Charles River Laboratory, Wilmington, MA) were used in accordance with the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by the Schepens Eye Research Institute Animal Care and Use Committee. As described previously, dry eye was induced in the mice by placing them in a Controlled Environment Chamber (CEC). To achieve maximum ocular surface dryness, the conditions in the CEC were supplemented with topical application of 1% atropine sulfate (Falcon Pharma, Fort Worth, Texas) twice for the first 48 hours and subcutaneous injections of 0.1ml of 5mg/ml of scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) three times a day, for the entire duration of the experiment.

Corneal Surface Staining

Fluorescein staining of the corneal epithelium was used as a diagnostic tool to study the effect of desiccating stress on the ocular surface of the mice. Corneal fluorescein staining was performed at baseline (day -1, before placing the mice in the CEC) and subsequently at days 2, 5, 9 and 13. 0.7 μL of 2.5% fluorescein (Sigma-Aldrich, St. Louis, MO) was applied using a micropipette into the inferior conjunctival sac of the mice’s eye as previously described. After five minutes, punctate staining on the corneal surface was evaluated in a masked fashion with the help of a slit lamp biomicroscope using the National Eye Institute grading system (NEI).

Anti-VEGF-C antibody and treatment Regimen

A day before placing the mice in the CEC, they were randomly divided into two groups. The treatment group (n=5) received daily intraperitoneal injections of 400 μg of anti-VEGF-C
antibody (VGX-100; Vegenics, Circadian Technologies, Australia) in 100 μl of normal saline from day −1 to day 13. According to manufacturer (Vegenics), VGX-100 binds to and precipitates all forms of VEGF-C including full-length, partially-processed and mature forms, thus inhibiting VEGF-C binding and activation of VEGFR-2 and VEGFR-3. Another group (untreated group) placed in the CEC (n=5) received 100 μl of intraperitoneal normal saline for the same duration of the experiment. Age and sex matched mice not placed in CEC served as normal control. Mice were then euthanized on day 14 for cellular and molecular studies. All experiments were repeated three times.

**Immunohistochemistry**

The following primary antibodies were used for immunohistochemical staining: FITC-conjugated rat anti-mouse CD11b for monocytes/macrophages (BD Pharmingen, San Diego, California; isotype FITC-conjugated rat anti-mouse IgG2bk), FITC conjugated goat anti-mouse CD31 as pan-endothelial marker (Santa Cruz Biotechnology, Santa Cruz, California; isotype FITC-conjugated rat IgG2A) and purified rabbit anti-mouse LYVE-1 as lymphatic endothelial marker (Abcam, MA, USA; isotype rabbit IgG). Rhodamine conjugated goat anti-rabbit (BD Pharmingen, San Diego, California, 1:100) was the secondary antibody used.

Freshly excised corneas were washed in PBS, fixed in acetone for 15 minutes and then double stained with CD31 and LYVE-1 as described before. To analyze infiltration of CD11b+LYVE-1 cells, corneas from three mice from each group were taken and cells were counted in 5–6 areas in the periphery (0.5 μm area from the limbus) using epifluorescence microscope (model E800; Nikon, Melville, NY) at 40X magnification. The mean number of cells was obtained by averaging the total number of cells in all the areas studied and the result was expressed as the number of positive cells per mm².

**Quantification of Lymphatics in the Cornea**

Lymphatics were quantified by an automated image analysis program written using Matlab as described previously (The Mathworks, Inc., Natick, MA). In brief, lymphatics were isolated from digitized immunofluorescent micrographs and subsequently analyzed for lymphatic area (LA) and lymphatic caliber (LC). LA represents the total surface area of the lymphatic vessels and LC is a summary measure of the diameters of the lymphatic vessels present.

**RNA Isolation and Molecular analysis using Real Time Polymerase Chain Reaction**

Total RNA was isolated from the cornea, conjunctival and draining lymph nodes of mice from normal, and untreated and treated groups using the RNeasy microkit (Qiagen, Valencia, CA; catalog No. 74004). Equal amounts of RNA were used to synthesize cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA; catalog No.18080) according to the manufacturer’s recommendations. Real-Time PCR was performed using FAM-MGB dye labeled predesigned primers (Applied Biosystem, Foster City, CA) for GAPDH (Mm99999915_g1), IL-1α (Mm00439620_m1) IL-1β (Mm00434228_m1), IL-6 (Mm0046190_m1), IFN-γ (Mm00801778_m1), IL-17A (Mm00439619_m1), VEGF-A (Mm00437304_m1), VEGF-C (Mm00437313_m1), VEGF-D (Mm00438965_m1), VEGFR-2 (Mm00440099_m1), VEGFR-3 (Mm00433337_m1). 2.5 μL of cDNA was loaded in each well and assays were performed in duplicates. A non-template control was included in all the experiments to evaluate DNA contamination of the reagent used. The results were normalized by the cycle threshold (C_T) of GAPDH as an internal control.
Statistical Analysis

A two-tailed Student’s paired t-test was performed and P-values less than 0.05 were deemed statistically significant. Results are presented as the mean ± SEM of at least three experiments.

RESULTS

Effect of VEGF-C neutralization on lymphatics in dry eye corneas

There is in-growth of lymphatics from the limbus toward the center of the cornea with progression of DED.\textsuperscript{6} Corneal whole mounts were double stained for CD31 and LYVE-1 and quantified for lymphangiogenesis. Blood vessels were identified as CD31\textsuperscript{hi}/LYVE-1\textsuperscript{−} and lymph vessels were identified as CD31\textsuperscript{lo}/LYVE-1\textsuperscript{hi} (Fig-1a). The dry eye group treated with anti-VEGF-C antibody showed a significant (nearly 2-fold) reduction in caliber (LC; Fig-1b; P=0.025), and nearly 5-fold reduction in total area (LA; Fig-1c; P=0.006) of corneal lymphatic vessels compared to the untreated group.

Effect of VEGF-C neutralization on angiogenic molecules in the cornea

VEGF-C and VEGF-D are the characteristic lymphangiogenic factors and act by binding to their receptor VEGFR-3.\textsuperscript{7} VEGF-A indirectly contributes to lymphangiogenesis by recruiting VEGF-C and VEGF-D secreting macrophages.\textsuperscript{14, 15} Expression of different vascular endothelial growth factors and their receptors were quantified in the cornea using real time PCR. Anti-VEGF-C treatment significantly decreased transcript levels of VEGF-C (P=0.002), VEGF-D (P=0.014) and VEGFR-3 (P=0.023) in the corneas of treated mice (Fig-2). Though decreased levels of VEGF-A were seen in the anti-VEGF-C treated group, the changes were not statistically significant. No change in the expression levels (from the baseline levels in normal corneas) of the pro-hemangiogenic receptor VEGFR2 was observed in the anti-VEGF-C treated or the untreated dry eye groups.

Effect of VEGF-C neutralization on immuno-inflammatory markers in the conjunctiva and draining lymph nodes

There is ample evidence for increased levels of pro-inflammatory cytokines in the conjunctiva in dry eye.\textsuperscript{16, 17} Real time PCR was used to quantify mRNA expression levels of IL1-\(\alpha\), IL1-\(\beta\), IL-6 and IL-17 in the conjunctiva (Fig-3a). The group receiving anti-VEGF-C treatment showed a significant decrease in the levels of IL1-\(\alpha\) (\textasciitilde 5 fold; P=0.003), IL1-\(\beta\) (\textasciitilde 4 fold; P=0.025) and IL-6 (\textasciitilde 3 fold; P=0.005). Although a similar downward trend (\textasciitilde 3 fold) was seen in the levels of IL-17, statistical significance was not achieved due to high variance.

In DED, antigen-presenting cells migrate from the ocular surface to draining LNs where they stimulate T cells, leading to expansion of IL-17 secreting Th17 cells and interferon (IFN) - \(\gamma\)-secreting Th1 cells.\textsuperscript{18} Interestingly, treatment with anti-VEGF-C was paralleled by a significant reduction in IL-17 (P=0.008) levels and IFN-\(\gamma\) (P=0.003) in the draining LNs (Fig-3b).

Effect of VEGF-C neutralization on CD11b\textsuperscript{+} cells in dry eye corneas

Recruitment and activation of monocytic (CD11b\textsuperscript{+}) cells to the cornea is an important hallmark of DED.\textsuperscript{19} To evaluate changes to the CD11b\textsuperscript{+} cells as a result of VEGF-C blockade, at the end of study period (day 14), corneal whole mounts were immunostained for CD11b, and the CD11b\textsuperscript{+} cells were quantified under the confocal microscope. Therapy with anti-VEGF-C significantly decreased (P=0.005) CD11b\textsuperscript{+} cells in the treated vs. the untreated corneas; an overall 30% reduction in CD11b\textsuperscript{+} cells was seen in the treated group.
(Fig-4a and 4b) suggesting that immune activation in the cornea is blunted as a result of VEGF blockade.

**Effect of VEGF-C neutralization on clinical signs of dry eye disease**

To determine if treatment with anti-VEGF-C decreases the corneal signs of dry eye (surface epitheliopathy), mice were assessed clinically using fluorescein dye (Fig-5). Two days after induction of DED, no difference was seen amongst the untreated and anti-VEGF-C treated groups in the corneal fluorescein scores. However, starting at day 5 mice treated with anti-VEGF-C antibody began to show a significant decrease in corneal fluorescein staining (P=0.005) compared to the untreated group. Henceforth, this difference was significantly maintained for the entire duration of the experiment (Day 14).

**DISCUSSION**

Lymphatics play an important role in generating immuno-inflammatory responses in peripheral tissues by directing antigen presenting cells (APCs) from the periphery to the draining lymph nodes where T cells are primed and expanded.\(^7\, 20\, 21\) VEGF-C and VEGF-D are the archetypal lymphangiogenic factors and preferentially bind VEGFR-3, a tyrosine kinase receptor expressed by lymphatic endothelial cells and activated macrophages, to induce lymphangiogenesis.\(^7\, 8\) Targeting the VEGF-C/VEGF-D/VEGFR-3 pathway has proven beneficial in preclinical models of lymphedema and tumor metastasis.\(^22\) In our recent work where we provide evidence of selective lymphangiogenesis (but not blood vessel ingrowth) occurring in DED corneas, we demonstrate upregulation of VEGF-C, VEGF-D and VEGFR-3 in DED corneas.\(^6\)

Accordingly, in our study, systemic anti-VEGF-C treatment was initiated before induction of disease. Though lymphangiogenesis still occurred in the treated corneas in the setting of a desiccating environment, there was significant reduction in both LA and LC parameters compared to the untreated group. Lymphatics are potential conduits that provide access to APCs and antigens that migrate from the cornea to the draining LNs.\(^23\) Studies have shown that VEGF-A/VEGFR-2–induced lymphatic vessels differ in structure and function from those induced by VEGFR-3 ligands, exhibiting a relatively dilated, leaky, and poorly functional phenotype.\(^24\, 25\, 26\) Hence, in the current study it is possible that interrupting VEGF-C not only decreased the number and area of lymphatics but that the residual lymphatics could be functionally different and less efficient as carriers of APCs.

Inflammation is the main stimulus leading to the expression of VEGF-C and VEGF-D\(^27\). DED corneas are characterized by high levels of proinflammatory cytokines such as IL-1β and IL-17, which are also known to have strong angiogenic potential, in part by regulating the gene expression of various VEGF ligands and receptors.\(^27\, 28\, 29\) We have previously illustrated T cell activation in the regional LNs along with induction of autoimmunity in the draining LNs of dry eye mice due to generation of pathogenic Th17 cells.\(^30\, 31\) Current data in the LNs of anti-VEGF-C treated mice demonstrate decreased expression of IL-17 (Th17 cells) and IFN-γ (Th1 cells). Moreover in DED, the inflammatory milieu induces the recruitment and mobilization of activated CD11b+ cells in the cornea.\(^19\) In the current study, decrease in the inflammatory attractants at the ocular surface led to decreased recruitment of CD11b+ cells in the anti-VEGF-C treated corneas. This is significant as various studies have suggested that the CD11b+ macrophages play an important role in inflammation-induced lymphangiogenesis and induction of immunity, and that depletion of these macrophages suppresses these processes.\(^14\, 20\) Thus, disrupting the VEGF-C/R3 axis appears to interrupt the afferent arm of the immune cycle as was evident by decreased pro-inflammatory cytokines in both the ocular and lymphoid tissues.
In addition, anti-VEGF-treatment led to suppression of epithelial disease, as a result of desiccating stress, as seen by a significant decrease in corneal fluorescein uptake. This may be the result of decrease in expression of proinflammatory cytokines, as there is evidence that elevated cytokine levels in the tear film creates an environment in which terminal differentiation of the ocular surface epithelium is impaired, thereby impairing the epithelial surface production of mature surface protective molecules.32

In conclusion, the current study provides evidence that blocking VEGF-C helps to suppress inflammation and corneal epitheliopathy as a result of DED, and may form the basis for novel treatments targeting the lymphatic system in ocular surface disease.

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References

Figure 1. Effect of in vivo blockade of anti-VEGF-C on corneal lymphatics in DED

Representative micrographs of corneal wholemounts immunostained for CD31 (green) and LYVE-1 (red) expressions showing CD31<sup>lo</sup>LYVE-1<sup>hi</sup> lymphatic vessels (orange) in (a) normal, untreated and anti-VEGF-C treated corneas (100x magnification). Morphometric evaluation showing significant decrease in (b) LC (c) LA in anti-VEGF-C Ab treated group compared to normal and untreated corneas. (Lymphatics marked by arrows; C- Cornea; L- Limbus). Data from a representative experiment of three performed is shown as mean ± S.E.M; each group consisting of four to five mice.

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Figure 2. Effect of in vivo blockade of anti-VEGF-C on angiogenic markers in cornea
Real Time PCR analysis showing transcript levels of VEGF-A, VEGF-C, VEGF-D, VEGFR-2 and VEGFR-3 in corneas of different groups. Amongst group comparisons showed significant decreased expression levels of VEGF-C, VEGF-D and VEGFR-3 in the anti-VEGF-C Ab treated group compared to the untreated group.
Figure 3. Effect of in vivo blockade of anti-VEGF-C on inflammatory cytokines in the conjunctiva and lymph nodes.

Real Time PCR analysis showing transcript levels of (a) inflammatory cytokines IL1-α, IL1-β, IL-6 and IL-17 in the conjunctiva and (b) inflammatory cytokines IL-17 and IFN-γ in the lymph nodes. Anti-VEGF-C significantly decreased levels of cytokines in the ocular surface and lymph nodes compared to the untreated group, suppressing levels close to those observed in normal (non-dry) eyes and normal lymph nodes (defined as=1). Data from a representative experiment of three performed is shown as mean ± S.E.M; each group consisting of four to five mice.
Figure 4. Effect of in vivo blockade of anti-VEGF-C on infiltration of CD11b+ cells in DED corneas
(a) Representative confocal images of whole mount corneas showing CD11b+ cells (green) in normal, untreated, and anti-VEGF-C-treated eyes. (b) Anti-VEGF-C treatment significantly decreased the number of CD11b+ cells in the dry eye corneas compared to the normal and untreated groups. Data from a representative experiment of three performed is shown as mean ± S.E.M; each group consisting of 4–5 mice.
Figure 5. Effect of in vivo blockade of anti-VEGF-C on Corneal epitheliopathy in DED
Corneal fluorescein staining scores were significantly decreased in the group treated with anti-VEGF-C Ab at days 5, 9 and 13 vs. the untreated group. Data from a representative experiment of three performed is shown as mean ± S.E.M; each group consisting of five mice.