Evidence of Corneal Lymphangiogenesis in Dry Eye Disease

A Potential Link to Adaptive Immunity?

Sunali Goyal, MD; Sunil K. Chauhan, PhD; Jaafar El Annan, MD; Nambi Nallasamy, AB; Qiang Zhang, MD; Reza Dana, MD, MSc, MPH

Objective: To determine the effect of desiccating stress on corneal angiogenic responses in dry eye disease (DED) using a murine model.

Methods: Dry eye was induced in murine eyes using high-flow desiccated air. Corneas were double stained with CD31 (panendothelial marker) and LYVE-1 (lymphatic endothelial marker). Real-time polymerase chain reaction was performed to quantify expression of vascular endothelial growth factors (VEGF-A, VEGF-C, and VEGF-D) and their receptors (VEGFR-2 and VEGFR-3) in the cornea on days 6, 10, and 14. Enumeration of CD11b+/LYVE-1+ monocytic cells was performed in corneas with DED on day 14. Flow cytometric evaluation of the draining lymph nodes in normal mice and mice with DED was performed to determine whether DED is associated with homing of mature (major histocompatibility complex II+) antigen-presenting cells to the lymphoid compartment.

Results: Lymphatic vessels unaccompanied by blood vessels were seen growing toward the center of corneas with DED. Significant increases in lymphatic area (P < .001) and lymphatic caliber (P < .02) were seen on day 14 of disease. Lymphangiogenic-specific VEGF-D and VEGFR-3 levels increased earliest on day 6 followed by increased VEGF-C, VEGF-A, and VEGFR-2 levels. Increased recruitment of CD11b+/LYVE-1+ monocytic cells to the cornea and homing of mature CD11b+ antigen-presenting cells to the draining lymph nodes were also associated with DED.

Conclusion: Low-grade inflammation associated with DED is an inducer of lymphangiogenesis without accompanying hemangiogenesis.

Arch Ophthalmol. 2010;128(7):819-824

Dry Eye Disease (DED), once thought to be solely due to deficiency of tears, is increasingly being recognized as an immune-mediated disorder. It affects many millions of people with a wide spectrum of seminal features ranging from mild ocular discomfort to sight-threatening corneal complications, such as persistent epithelial defects and sterile stromal ulceration. In the United States alone, the vision-related quality of life of more than 3.2 million women and 1.6 million men older than 50 years is adversely affected by this potentially disabling disease.

Clinically significant DED is associated with ocular surface inflammation, although its precise immunopathogenesis is not known. There is strong evidence regarding T-cell involvement in the pathogenesis of DED in animal models and humans. Recently, T-cell activation was illustrated in the regional lymph nodes (LNs) of mice with DED, coincident with the acquisition of specific chemokine markers that help in the homing of T cells to the inflamed ocular surface. Furthermore, induction of autoimmunity was demonstrated in the draining LNs of mice with DED due to impaired Treg function and the generation of pathogenic Th17 cells. These Th17 cells were resistant to Treg-mediated suppression, leading to unrestrained generation of pathogenic T cells and sustained ocular surface inflammation. Accordingly, much of the work to date has focused on understanding immunologic phenomena occurring in the lymphoid compartment and the effector responses thereby generated, leaving unanswered the question of how naive T cells in the draining LNs get primed to the ocular surface antigen(s) that drives immunity in DED.

The draining LNs are critical sites for induction of immunity, and their role in the generation of alloimmunity has been well established in corneal transplantation. The enhanced survival rate of corneal transplants in mice with excised cervical LNs highlights the importance of the
functional flow of antigen-presenting cells (APCs) from the ocular surface to the draining lymphoid tissue as a necessary component of alloimmunity and graft rejection. However, little is known about the pathway that allows trafficking of corneal APCs to the draining LNs, where they prime naïve T cells to corneal antigens and generate autoimmune responses in dry eye.

Emphasis is now being given to the importance of pathologic angiogenesis (hemangiogenesis and lymphangiogenesis) in various corneal diseases, such as different forms of keratitis, chemical burns, and graft vs host disease, but, to our knowledge, there are no data regarding corneal angiogenesis in DED. A plausible reason could be that the previously mentioned conditions, except DED, are accompanied by ingrowth of clinically visible blood vessels in the cornea. Traditionally, it has been thought that lymphatic vessels and blood vessels, which serve as afferent and efferent arms of the immune response, respectively, always coexist in pathologic states. The present work provides the first evidence, to our knowledge, of selective lymphangiogenesis occurring in corneas of murine models with DED. Herein, we attempt to determine the growth of lymphatic vessels in the cornea with the progression of DED, and we discuss the pathophysiologic implications of corneal lymphangiogenesis in dry eye and the potential of antilymphangiogenic therapy for ameliorating DED.

**METHODS**

**EXPERIMENTAL DRY EYE MURINE MODEL**

Eight- to 10-week-old female C57BL/6 mice (Charles River Laboratory, Wilmington, Massachusetts) were used in accordance with the standards in the Association for Research in Vision and Ophthalmology 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. To achieve maximum ocular surface dryness, the conditions in the controlled environment chamber were supplemented with topical application of atropine sulfate, 1% (Falcon Pharma, Fort Worth, Texas), twice in the first 48 hours and subcutaneous injections of 0.1 mL of scopolamine hydrobromide, 5 mg/mL (Sigma-Aldrich Chemical Co, St Louis, Missouri), 3 times a day for the duration of the experiment.11

**RNA ISOLATION AND MOLECULAR ANALYSIS USING REAL-TIME POLYMERASE CHAIN REACTION**

Five mice (10 eyes) were included in each group. Two corneas were pooled together to equal 1 sample and were stored at −80°C in Trizol (catalog No. 15596026; Invitrogen, Carlsbad, California) until future use. Total RNA was isolated from these corneas using the RNeasy Micro Kit (catalog No. 74004; Qiagen, Valencia, California). Equal amounts of RNA were used to synthesize complementary DNA using SuperScript III Reverse Transcriptase (catalog No.18080; Invitrogen) according to the manufacturer’s recommendations. Real-time polymerase chain reaction was performed using FAM-MGB dye-labeled predesigned primers (Applied Biosystem, Foster City, California) for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (assay ID.Mm99999915_g1), vascular endothelial growth factor A (VEGF-A) (Mm00437304_m1), VEGF-C (Mm03437313_m1), VEGF-D (Mm00438965_m1), VEGFR-2 (Mm00440099_m1), and VEGFR-3 (Mm00433337_m1). Complementary DNA, 2.5 µL was loaded in each well, and assays were performed in duplicate. The GAPDH gene was used as the endogenous reference for each reaction. The results were normalized by the cycle threshold of GAPDH, and the relative messenger RNA level in the normal mice was used as the normalized control.

**IMMUNOHISTOCHEMICAL ANALYSIS**

The following primary antibodies were used for immunohistochemical staining: rat anti–mouse CD11b-FITC for monocytes and macrophages (BD Pharmingen, San Diego, California), diluted 1:100; goat anti–mouse CD31 FITC as panendothelial marker (Santa Cruz Biotechnology, Santa Cruz, California), diluted 1:100; and purified rabbit anti–mouse LYVE-1 as lymphatic endothelial marker (Abcam Inc, Cambridge, Massachusetts), diluted 1:400. Respective isotypes were used as negative controls. Rhodamine-conjugated goat anti-rabbit (BD Pharmingen), diluted 1:100, was the secondary antibody used.

Freshly excised corneas were washed in phosphate-buffered saline, fixed in acetone for 15 minutes, and then double stained with CD31 and LYVE-1 as described previously. To analyze infiltration of CD11b+LYVE-1 cells, corneas from 3 mice from each group were taken, and cells were counted in 3 to 6 areas in the peripheral (0.5-µm area from the limbus) of each cornea in a masked manner using an epifluorescence microscope (model E800; Nikon, Melville, New York) at ×40 magnification. The mean number of cells was obtained by averaging the total number of cells in all the areas studied, and the result is expressed as the number of positive cells per square millimeter.

**MORPHOMETRIC ANALYSIS OF LYMPHANGIOGENESIS IN THE CORNEA**

The morphologic features of lymphatic vessels were analyzed using an automated image analysis program written using Matlab (The MathWorks Inc, Natick, Massachusetts). Lymphatic vessels were isolated from digitized images using this program and standard computer vision techniques for image segmentation, including background isolation and subtraction, edge detection, and k-means clustering. This segmentation process generated binary images in which lymphatic vessels are represented by 1’s and all other image content is represented by 0’s. The resultant isolated lymphatic vessels were subsequently analyzed morphologically using 2 metrics: lymphatic area (LA) and lymphatic caliber (LC). The LA represents the total surface area of the lymphatic vessels when projected into the plane of the image. The LC is a summary measure of the diameters of the lymphatic vessels present. The LC was measured using a computational technique that generates the largest-diameter circle centered at each pixel inside a lymphatic vessel. The mean value across all pixels in lymphatic vessels was taken as an estimate of the mean LC for a given image.

**FLOW CYTOMETRY**

Draining LNs from mice with DED (day 10) and normal mice were collected. A single-cell suspension of LN cells was stained with anti–CD11b-FITC and anti-lab (MHC-II)-PE. Stained LN cells were then analyzed using an EPICS XL flow cytometer (Beckman Coulter Inc, Brea, California). All the antibodies with their matched isotype controls were purchased from eBioscience (San Diego).
STATISTICAL ANALYSIS

A 2-tailed paired t test was performed, and $P < .05$ was deemed statistically significant. Results are presented as the mean (SEM) of at least 3 experiments.

RESULTS

DEMONSTRATION AND QUANTIFICATION OF LYMPHATIC VESSELS IN CORNEAS WITH DED

To determine whether DED induces growth of lymphatic vessels in the cornea and whether lymphatic growth is paralleled by growth of blood vessels, corneal whole mounts were double stained for CD31 (panendothelial marker) and LYVE-1 (lymphatic vascular endothelial marker) on days 0, 6, 10, and 14 and were quantified for lymphangiogenesis. Blood vessels were identified as CD31hi/LYVE-1−, and lymphatic vessels were identified as CD31lo/LYVE-1hi. Morphometric analysis revealed small buds of lymphatic vessels arising from the limbal vascular arcade at an early time point (day 6), which increased in caliber (LC) and area (LA) and advanced toward the center of the cornea with DED progression (Figure 1 and Figure 2). A significant increase in LA was seen as early as day 6 ($P < .01$) and continued until day 14 ($P < .001$) (Figure 3A). However, LC was significantly increased from the normal cornea only by day 14 ($P < .02$) (Figure 3B). These lymphatic vessels were not accompanied by the growth of blood vessels at any given time point.

DIFFERENT VEGF AND VEGFR EXPRESSION LEVELS IN CORNEAS WITH DED

The development of lymphatic vessels is regulated by factors common to hemangiogenesis and lymphangiogenesis. The classic lymphangiogenic factors, VEGF-C and VEGF-D, act by binding to their receptors, VEGFR-2 and VEGFR-3, which are expressed on lymphatic endothelial cells.13,14 To determine the molecular mechanisms of lymphangiogenesis in DED, expression of different VEGFs and their receptors were quantified at indicated time points in
Among the VEGF species, lymphangiogenic-specific VEGF-D was not only the earliest to increase on day 6 (approximately 2-fold; P < .03) but also showed the maximum increase in expression on day 14 (approximately 3-fold; P < .03) (Figure 4A). Significant increased transcript expression of VEGF-A and VEGF-C was seen only by day 14 (P < .03 for both). Similarly, levels of lymphangiogenic-specific VEGFR-3 were first to show a significant increase on day 6 (approximately 4-fold; P < .01) and continued to rise until day 14 (approximately 8-fold; P < .01). Although an overall trend toward increased expression was noticed with VEGFR-2 (primarily specific for blood vessel growth), a significant increase was appreciated only by day 14 (P = .048) (Figure 4B).

**Enumeration of CD11b+/LYVE-1+–Positive Cells in Corneas with DED**

The normal cornea has a resident population of bone marrow–derived CD11b+ monocyte-macrophage lineage cells, and the development of DED increases the number of CD11b+ cells in the cornea. The role of macrophages in inflammatory lymphangiogenesis is well established. These CD11b+ macrophages may also express various lymphatic endothelial markers, such as LYVE-1. To determine what proportion of these CD11b+ cells had lymphangiogenic potential, we double-stained whole-mount corneal tissues with CD11b and LYVE-1 on day 14. There was a significant increase in the number of CD11b+ (P < .02) and CD11b+/LYVE-1+ (P < .001) cells in dry eye compared with normal corneas (Figure 5). The percentage of CD11b+ cells positive for LYVE-1 was approximately 23% in DED and only 4% in normal corneas.

**Homing of APCs to the Draining LNs of Mice with DED**

We next investigated whether corneal lymphangiogenesis in DED is associated with the increased homing of APCs in the draining LNs. Using flow cytometry, we analyzed the frequencies of mature APCs (MHC-II+CD11b+) in the draining LNs of normal mice and mice with DED (Figure 6). The data showed a significant increase in the frequency of MHC-II+CD11b+ APCs in the LN cells of mice with DED compared with those in the LNs of normal mice (range, 14.9%-19.5% vs 10%-13.5%; P < .04).

**Comment**

Lymphangiogenesis in the postnatal period is primarily a response to inflammation and is seen in various pathologic states as diverse as tumor metastasis, wound heal-
ing, and transplantation. Lymphatics play an important role in generating immunoinflammatory responses by directing the antigen-bearing immunocytes (eg, dendritic cells) from the periphery to the draining LNs, where T cells are primed and expanded. The normal human cornea is avascular, thus suppressing the afferent lymphatic and efferent vascular arms of the immune cycle. Inflammation, however, negates this “immune” and “angiogenic” privileged state of the cornea and gives it the potential to mount an immune response.

Angiogenesis in the cornea is now extensively being studied in various pathologic models, such as transplantation. Whereas corneal blood vessels have long been thought to be an important risk factor for immune rejection in corneal transplantation, it is only recently after the unveiling of new lymphatic-specific markers that the significance of lymphangiogenesis in corneal alloimmunity has been characterized. Despite recognizing the role of inflammatory angiogenesis in the eye, little has hitherto been studied regarding angiogenic mechanisms in DED. Desiccating stress in DED initiates an immune-based inflammatory response that is sustained by the ongoing interplay between the ocular surface and various pathogenic immune cells, primarily the CD4+ T cells in the conjunctiva and CD11b+ monocytic cells in the cornea.

Desiccating stress induces the secretion of inflammatory cytokines, especially interleukin (IL)−1, tumor necrosis factor, and IL-6, by ocular surface tissues, which facilitate the activation and migration of resident APCs toward the regional draining LNs. These data on frequencies of mature APCs in the LNs also suggest increased trafficking of mature APCs in the LNs of mice with DED (Figure 6). In the LNs, these APCs stimulate naïve T cells, leading to the expansion of IL-17-secreting T helper 17 cells and interferon γ-secreting T helper 1 cells. Once these effector cells are generated in the LNs, they migrate to the ocular surface and secrete effector cytokines. Recent studies provided evidence of the induction of T-cell–mediated autoimmune responses in the regional LNs of mice with DED. But what has remained unanswered is how corneal APCs can traffic to the draining lymphoid compartment to initiate the immune cycle in DED.

To date, no data have been published, to our knowledge, on this important facet of immunity in DED. The present data demonstrate the development of lymphatic vessels in the setting of DED. These lymphatic vessels increase in caliber and area while advancing toward the corneal center with the progression of DED. These lymphatic vessels are not accompanied by growth of blood vessels. Various spatiotemporal studies examining relationships between new blood and lymphatic vessels have led to the belief that a preexisting blood vascular bed is necessary to guide lymphangiogenesis. The present study refutes the general perception of wound-healing models in skin, where the growth of lymphatic vessels follows that of blood vessels by several days. This is also in contrast to other robust models of corneal inflammation where there is either parallel outgrowth of blood and lymphatic vessels or the blood vessels are precedent over the lymphatic vessels. To our knowledge, this is the first evidence of selective “natural” lymphangiogenesis in a disease model dissociated from hemangiogenesis.

Lymphangiogenesis is mediated primarily by the interaction of VEGF-C and VEGF-D on VEGFR-2 and VEGFR-3. Albeit indirectly, VEGF-A also contributes to lymphangiogenesis by recruiting VEGF-C– and VEGF-D–secreting macrophages. In the present study, dry eye induction led to the upregulation of all the VEGFs and their receptors. Although the rise in levels of VEGF-A, VEGF-C, and VEGF-2 occurred at later time points (day 14), it is noteworthy that VEGF-D and VEGFR-3 (which are largely specific to lymphangiogenesis) increased as early as day 6 of disease. The functional relevance of the early rise in VEGF-D levels is highlighted in a recent study in which VEGF-D, via its action on VEGFR-3, was shown to be a critical modulator of VEGF-C–driven early sprouting and migration of lymphatic endothelial cells. Macrophages also seem to play a crucial role in lymphangiogenesis. Under normal physiologic conditions, all ocular tissues except the central cornea are rich in bone marrow–derived LYVE-1+ macrophages, which may serve as precursor cells for de novo formation of lymphatic vessels. In the present study, we noticed significantly more CD11b+/LYVE-1+ cells in the peripheral corneas after exposure to desiccating stress, suggesting that these cells either infiltrate into or multiply from preexisting CD11b+/LYVE-1+ cells in the cornea and contribute to lymphangiogenesis. Alternatively, there is a possibility of upregul-
lation of LYVE-1 in previously LYVE-1− cells. This area is, however, not completely understood and is under investigation.25

In summary, we present herein novel evidence of the selective growth of lymphatic (but not blood) vessels in DED, providing new insights into the pathogenesis of the disease. These findings suggest that these newly formed corneal lymphatic vessels may serve as potential conduits for migration of corneal APCs to lymphoid tissues, where they generate autoreactive Th17 and Th1 cells in DED.3 This study not only provides a link between ocular surface inflammation and the generation of T-cell–mediated immunity in the lymphoid compartment but also offers an example of how lymphangiogenesis and hemangiogenesis can be naturally dissociated in a pathologic state. The severing of the “eye-lymphatic axis” in other immune-mediated conditions, such as transplant rejection, has been shown to hold promise as a strategy for suppressing alloimmunity without inhibiting needed innate host defense mechanisms.7,26 Similarly, a strategy targeting prolymphangiogenesis and hemangiogenesis can be naturally dissociated in a pathologic state. The severing of the “eye-lymphatic axis” in other immune-mediated conditions, such as transplant rejection, has been shown to hold promise as a strategy for suppressing alloimmunity without inhibiting needed innate host defense mechanisms.7,26 Similarly, a strategy targeting prolymphangiogenic factors, such as VEGF-C and VEGF-D, may prove effective in ameliorating DED.

Submitted for Publication: June 21, 2009; final revision received November 27, 2009; accepted December 2, 2009.

Correspondence: Reza Dana, MD, MSc, MPH, Schepens Eye Research Institute, 20 Stanford St, Boston, MA 02114 (reza.dana@schepens.harvard.edu).

Financial Disclosure: None reported.

REFERENCES