Increasing Lymphatic Microvessel Density in Primary Pterygia

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Objective: To examine lymphatic microvessel density (LMVD) in primary pterygia.

Methods: We included tissue samples from 88 excised primary (including 34 grade 1, 28 grade 2, and 26 grade 3) pterygia and from 7 nasal epibulbar conjunctivae segments used as control samples. Sections from each pterygium were immunostained with CD31 and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) monoclonal antibodies to evaluate LMVD and blood microvessel density. We used real-time polymerase chain reaction analysis to measure expression of vascular endothelial growth factor A (VEGF-A) and VEGF-C messenger RNA (mRNA) in the pterygia.

Results: A small number of CD31-positive LYVE-1–negative blood vessels and only a few CD31- and LYVE-1–positive lymphatic vessels were detected in the normal epibulbar conjunctiva segments. Lymphatic vessels were mildly increased in grade 1 pterygia but were dramatically increased in grades 2 and 3 pterygia. Lymphatic microvessel density correlated closely with blood microvessel density in grades 1, 2, and 3 pterygia (P < .05 for all). The width and area of pterygia were significantly correlated with LMVD. In addition, we found a significant relationship between VEGF-C mRNA expression and LMVD in grades 1, 2, and 3 pterygia, whereas VEGF-A mRNA expression correlated closely with LMVD only in grade 1 pterygia.

Conclusions: Lymphatic microvessel density increases dramatically in grades 2 and 3 pterygia. Transient up-regulation of VEGF-C might be responsible for the occurrence of lymphangiogenesis.

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phocytes and memory T lymphocytes), corneal lymphangiogenesis enables the exit of antigenic material, for example, antigen-presenting cells, from the cornea to the regional lymph node. It is well known that blood vessels play an important role in the formation and progression of pterygia. Markers for vascular endothelial cells, such as CD31, are increased in pterygia. Concentrations of many angiogenic factors are elevated in pterygia, including vascular endothelial growth factor (VEGF), thrombospondin-1, and substance P.

Findings from the present study may potentially broaden our understanding of immune mechanisms that can be instrumental in the pathogenesis of pterygia.

## METHODS

### PATIENTS

A total of 88 patients with a pterygium (39 men and 49 women) with a mean age of 60.9 (range, 35-81) years were enrolled in the study at the Department of Ophthalmology, Third Affiliated Hospital, and the China State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, at Sun Yat-Sen University from January 1, 2006, through June 30, 2010. Patients included in the study had a primary pterygium with an apex of at least 1 mm invading the cornea. Clinical evaluations were performed according to the grading systems described by Awdeh et al. Briefly, pterygia were graded preoperatively on the basis of objective signs, including vascularity, conjunctival congestion and edema, relative thickness of the fibrovascular lesion, and general eye redness, on a scale of 1 to 3, where 1+ indicates mild; 2+, moderate; and 3+, severe. The size of the pterygium, including the horizontal extension onto the cornea from the limbus and the width of the base at the limbus, was measured (in millimeters) with a slit lamp using a slit beam of light. The total area was calculated. Seven nasal epibulbar conjunctiva segments near the limbus, excised from 7 age-matched control patients who underwent surgery for strabismus, were used as control samples. Each excised tissue sample was divided equally into 3 pieces: 1 for immunohistochemistry, 1 for enzyme-linked immunosorbent assay, and 1 for realtime polymerase chain reaction. All patients and control subjects were informed of the experimental nature of this procedure, and signed consent was obtained beforehand. All procedures were conducted according to the principles expressed in the Declaration of Helsinki.

### IMMUNOHISTOCHEMISTRY

After being fixed in 10% neutral formalin for 24 hours, embedded in paraffin, serially sectioned (thickness, 4 µm), and rehydrated with graded ethanol-water mixtures, excised conjunctival segments were washed with distilled water. Endogenous peroxidase activity was blocked after being incubated with 30-mL/L hydrogen peroxide for 20 minutes. For antigen retrieval, tissue sections were then autoclaved at 121°C in 10mM citrate buffer (pH, 6.0) for 10 minutes. The sections were then allowed to cool at room temperature for 30 minutes. Subsequently, sections were incubated for 3 hours with mouse antihuman lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) monoclonal antibody (R&D Systems) or mouse antihuman CD31 (R&D Systems) and biotin-marked rabbit antimouse immunoglobulin as the secondary antibody. Streptavidin-biotin-peroxidase complex was used as the immune check system. The slides were visualized for peroxidase activity with diaminobenzidine and counterstained with hematoxylin.

### LYMPHATIC AND BLOOD MICROVESSEL DENSITY

Lymphatic microvessel density (LMVD) and blood microvessel density (BMVD) of human excised tissues were evaluated independently by 2 observers (S.L. and H.L.) without prior knowledge of the experimental details, and the tests were repeated once. Sections of vessels with CD31-positive and LYVE-1-negative findings were identified as blood vessels, whereas those with CD31- and LYVE-1-positive findings were recognized as lymphatic vessels. Each sample was excised into 40 sections. Then, sections were analyzed using standard light microscopy (Eclipse 200; Nikon). Under 100x magnification (0.78 mm²), the 3 most lymphovascularized areas were identified, and the number of immunostained lymphatic vessels were counted. Only vessels exhibiting typical morphology (having a lumen) were considered lymphatic microvessels. The LMVD for each case was expressed as the mean value (calculated as the total number of vessels in 200 microscopic fields divided by 200). Similarly, to calculate BMVD, all blood vessels in 200 fields of the 40 sections were summed and divided by 200.

### QUANTIFICATION OF VEGF-A AND VEGF-C PROTEINS

Each excised tissue was placed in 100 µL of lysis buffer (20mM imidazole hydrochloride, 10mM potassium chloride, 1mM magnesium chloride, 10mM ethyleneglycoltetraacetic acid, 1% nonionic surfactant [Triton X-100; Dow Chemical Company], 10mM sodium fluoride, 1mM sodium molybdate, and 1mM EDTA [pH, 6.8]) supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals),

### Table 1. Composition of Pterygia

<table>
<thead>
<tr>
<th>Patient Population</th>
<th>1 (n = 34)</th>
<th>2 (n = 28)</th>
<th>3 (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56.0 (0.0)</td>
<td>62.1 (8.4)</td>
<td>63.3 (6.9)</td>
</tr>
<tr>
<td>No. female/male</td>
<td>17/17</td>
<td>16/12</td>
<td>16/10</td>
</tr>
<tr>
<td>Width of pterygium, mm</td>
<td>3.9 (0.8)</td>
<td>4.1 (0.7)</td>
<td>5.5 (0.8)</td>
</tr>
<tr>
<td>Extension of pterygium, mm</td>
<td>1.9 (0.5)</td>
<td>2.3 (0.6)</td>
<td>3.1 (0.7)</td>
</tr>
<tr>
<td>Area of pterygium, mm²</td>
<td>7.0 (1.9)</td>
<td>7.7 (1.8)</td>
<td>10.9 (2.0)</td>
</tr>
</tbody>
</table>

*a Unless otherwise indicated, data are expressed as mean (SD).
*b P < .05 compared with grade 1.
*c P < .05 compared with grade 2.
*d P < .05 compared with grade 1.
*e P < .05 compared with grade 2.
which was homogenized with a plastic pestle (Geno Technology Inc) attached to a handheld drill. Tissues were homogenized in three 15-second bursts, and the suspension was incubated on ice for 10 minutes to allow lysis. The lysate was cleared of debris by centrifugation at 18,000 g for 15 minutes at 4°C, and the supernatant was assayed. Total protein content was determined by a commercial assay (BCA kit; Bio-Rad). Supernatant cytokine levels were determined by a sandwich enzyme-linked immunosorbent assay for VEGF-A and VEGF-C according to the manufacturer’s instructions (RapidBio) and were normalized to the total protein level.

RNA ISOLATION AND PURIFICATION

Total RNA was isolated from the samples using commercially available reagent (Trizol; Gibco-BRL Life Technologies). The RNA was prepared following the protocol from the manufacturer. The RNA pellets were washed with 75% ethanol, centrifuged, and dried. The residual DNA was removed by DNase I treatment. Pellets were resuspended in 30 µL of diethylpyrocarbonate-treated water followed by the addition of 50mM TRIS buffer (pH, 7.5), 10mM magnesium chloride, 20 U of RNase-free DNase I, and 20 U of ribonuclease inhibitor (RNasin; Promega Corporation) in a total volume of 60 µL. The samples were incubated at 37°C for 25 minutes. The RNA was then cleaned using commercially available purification kits (RNeasy Mini Kits; Qiagen) following the protocol provided by the manufacturer. The concentration and purity of RNA were determined by measuring optical density at 260 and 280 nm in a spectrophotometer.

REAL-TIME REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

Complementary DNA (cDNA) was generated from the total RNA samples by using a reverse transcription reagents kit (TaqMan; Applied Biosystems). To make the cDNA, the total RNA from each sample was first incubated at 25°C for 10 minutes and then reverse transcribed at 48°C for 30 minutes. Real-time reverse transcriptase–polymerase chain reaction was performed using a DNA-binding dye (SYBR Green; Applied Biosystems) with a sequence detection system (ABI PRISM 7900HT; Applied Biosystems). The primers for VEGF-A were 5'-GCAGATGTGAGATGACAA-3' (sense) and 5'-CTGGGATCTTGGACAAAC-3' (antisense) (GenBank No. NM009505). The primers of VEGF-C were 5'-GAAGTTCCTGAGGACAAAC-3' (sense) and 5'-GATCGGCA-CATGGTATCC-3' (antisense) (GenBank No. NM009506). The DNA polymerase was first activated at 95°C for 10 minutes, followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing/extension at 60°C for 1 minute according to the manufacturer’s protocol. The products were sequenced to ensure that the correct gene sequence was being amplified. All polymerase chain reactions were performed in triplicate. Relative quantitation of gene expression used the standard curve method (user bulletin 2 in the ABI PRISM 7700 sequence detection system). For comparison of the transcript levels between samples, standard curves were prepared for the target gene and an endogenous reference (18S ribosomal RNA). For each experimental sample, the amount of target and endogenous reference was determined from the appropriate standard
curves. The target amount was then divided by the endogenous reference amount to obtain a normalized target value. Each of the experimental normalized sample values was divided by the normalized control sample value to generate the relative expression levels. We repeated examinations for every sample (3 times for each) and then calculated the mean values for every sample.

**STATISTICAL ANALYSIS**

Analysis of significant differences between groups was performed using a paired t test (SPSS 12.0 statistical software; SPSS Inc). Pearson analysis was used to determine correlations among BMVD, VEGF-A messenger RNA (mRNA), VEGF-C mRNA, and LMVD. Values are presented as mean (SD). All reported P values are 2-tailed, and statistical significance was defined at the level of $\alpha = .05$.

**RESULTS**

**COMPOSITION OF THE PTERYGIA STUDY GROUP**

Of the 88 patients, a grade 1 pterygium was found in 34 (39%); grade 2, in 28 (32%); and grade 3, in 26 (30%). The extension of the pterygium onto the cornea ranged from 1.1 to 4.8 mm, with a mean of 2.4 (0.8) mm. The width ranged from 1.8 to 6.9 mm, with a mean of 4.5 (1.0) mm. The total area ranged from 1.4 to 14.2 mm², with a mean of 8.4 (2.5) mm² (Table 1).

**BMVD AND LMVD IN PTERYGIA**

Immunohistochemical analysis was performed on LYVE-1- and CD31-stained serial sections of human pterygium tissue. Because CD31 stains blood and lymphatic vessels and LYVE-1 stains the lymphatic endothelium, we could identify and distinguish corneal blood and lymphatic vessels in histological sections simultaneously. Compared with blood vessels, lymphatic vessels had a relatively larger lumen and did not contain erythrocytes. Our data showed a small number of CD31-positive and LYVE-1-negative blood vessels but only a few CD31- and LYVE-1-positive lymphatic vessels in normal epibulbar conjunctiva segments. Lymphatic vessels were mildly increased in grade 1 pterygia but were dramatically increased in grades 2 and 3 pterygia (Figure 1). Moreover, we examined the relationship between LMVD and BMVD and found that lymphatic vessels were associated closely with blood vessels in pterygia ($P < .01$ for all) (Figure 2). However, compared with blood vessels, LMVD was greater in grades 2 and 3 pterygia. The LMVD in grades 2 and 3 pterygia was approximately double and triple, respectively, that in grade 1 pterygia, whereas the increasing rate of BMVD in grade 2 pterygia was less than 20% in comparison with that in grade 1 pterygia (Table 2). This finding suggested that the outgrowth of lymphatic vessels (lymphangiogenesis) might play a more important role in more substantial pterygia.

**RELATIONSHIP BETWEEN LYMPHATIC VESSELS AND PTERYGIA**

To elucidate the relationship between lymphatic vessels and pterygia, we compared LMVD according to the degree of pterygia. First, we examined LMVD in pterygia of grades 1, 2, and 3 and in the normal control conjunctiva. Our data showed that the difference in LMVD was significant among the groups (normal conjunctiva was paired with the pterygia that were classified into grades
Compared with angiogenesis, lymphangiogenesis is poorly understood, partly because of the lack of specific lymphatic endothelium markers. This situation has been improved since the identification of LYVE-1.30 A hyaluronic receptor related to CD44 expression in lymph vessel endothelial cells of normal and neoplastic tissues and on the luminal and abluminal surfaces of the lymphatic endothelial cells, LYVE-1 is a powerful marker of lymphatic structure and function.31,32 By using LYVE-1 and CD31 double immunohistochemical analysis, we have distinguished lymphatic vessels from blood vessels, investigated new lymphangiogenesis, and elucidated the development of lymphatic vessels in pterygia.

The lymphatic system plays an important role in maintaining tissue fluid homeostasis by collecting and transporting protein-rich interstitial fluid via lymph nodes, large collecting lymphatic vessels, and lymphatic trunks (including the thoracic duct) and thereby back to the blood vascular circulation. The lymphatic system also plays an essential role in the immune response to infectious agents. Compared with angiogenesis, lymphatic vessels play a crucial role in eye immunity and may be more important in allograft rejection of normal- and high-risk corneal trans-
A pterygium is a pathologic alteration of the conjunctiva and cornea, with the immunopathologic mechanisms thought to play a role in its development. Because afferent lymphatic vessels are the route by which antigen-presenting cells migrate to the regional lymph nodes, which has been shown to be essential in promoting immunity of the eye,33 valuable knowledge of immunopathologic mechanisms in the formation and development of pterygia could be gained through the investigation of lymphangiogenesis in pterygia, which could have therapeutic implications.

In our study, we divided patients with pterygia into 3 groups. We examined lymphatic vessels in each excised specimen and discovered that lymphangiogenesis was associated closely with pterygium severity. We then compared the relationship between LMVD and the size of the pterygium among groups. Our analyses showed that lymphatic vessels developed in parallel with blood vessels faster than that of blood vessels in grades 2 and 3 pterygia, suggesting that lymphangiogenesis plays a key role in immunopathologic mechanisms and the development of pterygia.

We also examined the relationship between blood vessels and lymphatic vessels in pterygia. Recent evidence from animal and human vascularized corneas indicated that the degree of corneal lymphangiogenesis was significantly correlated with the degree of corneal hemangiogenesis.34,35 In a previous study, we also observed that corneal lymphatic vessels developed in parallel with blood vessels after corneal alkaline burns.26 In the present study, despite a significant relationship between BMVD and LMVD, we found that the outgrowth of lymphatic vessels was faster than that of blood vessels in grades 2 and 3 pterygia, suggesting that lymphangiogenesis plays a key role in immunopathologic mechanisms and the development of pterygia.
phatic vasculature, VEGF-C is thought to be the predominant lymphangiogenic factor. Direct evidence of the role of VEGF-C in promoting lymphangiogenesis comes from studies of transgenic mice overexpressing VEGF-C under the control of the keratin 14 promoter. These mice displayed a pronounced hyperplasia of cutaneous lymphatic vessels, whereas the growth of blood vessels was not affected. Conversely, lymphatic vessels regress in the skin transiently in the inner organ of keratin 14–controlled VEGFR3-1g mice. However, some studies have also argued that VEGF-A plays a more important role in lymphangiogenesis. Recently, Kajiyama et al. showed that exposure to UVA-B irradiation, which is the main cause of pterygia, results in prominent enlargement and an increasing number of lymphatic vessels of murine skin. Kajiyama et al. suggested that the expression levels of VEGF-A, but not of the known lymphangiogenic factor VEGF-C, are responsible for lymphangiogenesis in UVA-B-irradiated epidermis. Therefore, we examined the expression of VEGF-C and VEGF-A, and we compared expression levels with LMVD in pterygia. Expression of VEGF-C and VEGF-A mRNA correlated closely with LMVD in grade 1 pterygia, but only VEGF-C mRNA was significantly associated with LMVD in grades 2 and 3 pterygia. Recent evidence suggests that even VEGF-A can be lymphangiogenic via its receptors for VEGFR2, which is also expressed on lymphatic endothelial cells. This possibility might be partially explained by the close relationship between VEGF-C mRNA and LMVD in grade 1 pterygia. Besides VEGFR2, VEGF-C also binds to VEGFR3, which has been shown to be essential for the formation of lymphatic vessels. In our study, the relationship between VEGF-C mRNA and LMVD was significant not only in grade 1 but also in grades 2 and 3 pterygia, suggesting that such a VEGF-C–VEGFR3 pathway might be critical in lymphangiogenesis, especially in serious pterygia.

In summary, our study has revealed the development of lymphatic vessels in pterygia and has indicated that transient upregulation of VEGF-C might be responsible for the occurrence of lymphangiogenesis. Lymphatic vessels might accelerate immunological injury and play a key role in immunopathologic mechanisms resulting in the pathogenesis of pterygia. Strategies of antilymphangiogenic therapy might be investigated to improve the efficacy of pterygium interventions and the prognosis for these patients.

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