

# Increasing Lymphatic Microvessel Density in Primary Pterygia

Shiqi Ling, MD; Lingyi Liang, MD; Haotian Lin, MD; Weihua Li, MD; Jiangang Xu, MD

**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 nor-

mal 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.

*Arch Ophthalmol.* 2012;130(6):735-742

**P**TERYGIUM IS AN INVASIVE OCULAR surface disease characterized by proliferation, inflammatory infiltrates, fibrosis, angiogenesis, and extracellular matrix breakdown. It is a common disorder of the ocular surface, with a prevalence of 2% in temperate areas and up to 20% in tropical regions.<sup>1</sup> The pathogenesis of pterygia has intrigued researchers for centuries, but it is not completely understood. Studies have shown an increasing prevalence of pterygia with closer proximity to the equator, secondary to greater exposure to UV radiation.<sup>2,3</sup> Coroneo et al<sup>4-6</sup> proposed an initial alteration of limbal stem cells as a result of chronic UV exposure, with a resultant breakdown of the limbal barrier leading to conjunctivalization of the cornea. Recently, more direct proof of the hypothesis that pterygia develop from limbal epithelial progenitors has been provided with evidence that Fuchs islet cells have stem cell characteristics.<sup>7</sup> Other investigators have proposed neoplastic factors, focusing on the p53 tumor suppressor gene,<sup>8,9</sup> whereas some believe tear film abnormalities and allergic factors are significant contributors.<sup>10</sup>

More recently, immunopathologic mechanisms have been studied to deter-

mine their roles in the pathogenesis of pterygia. Pterygia samples have been shown to have increased levels of cell-signaling and adhesion molecules, such as vascular cellular adhesion molecule 1, intercellular adhesion molecule 1, and aberrant expression of HLA-DR.<sup>11,12</sup> Other signaling molecules, including E-cadherin and  $\beta$ -catenin, are upregulated and concentrated in the heads of pterygia.<sup>13</sup> Increased  $\beta$ -catenin levels have been shown to trigger certain cell cycle proteins and matrix metalloproteinases.<sup>14</sup> Stromal infiltrates of T cells with an increased ratio of helper to suppressor cells and abnormal deposits of IgE and IgG have been described in pterygia.<sup>15,16</sup> An increase in mast cells, lymphocytes, plasma cells, dendritic cells, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells in pterygia samples has also been documented, suggesting that cellular immunity and hypersensitivity contribute to pterygium formation.<sup>11,12,17</sup>

Recent studies in corneal neovascularization provide evidence that new corneal blood vessels and lymphatic vessels consist of the 2 arms of a potential immune reflex that could lead to immune responses.<sup>18-20</sup> Although the blood vessels provide a route of entry for immune effector cells (eg, CD4<sup>+</sup> alloreactive T lym-

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**Table 1. Composition of Pterygia<sup>a</sup>**

Patient Population	Pterygia Grade		
	1 (n = 34)	2 (n = 28)	3 (n = 26)
Age, y	58.0 (9.0)	62.1 (8.4) <sup>b</sup>	63.3 (6.9) <sup>c,d</sup>
No. female/male	17/17	16/12	16/10
Width of pterygium, mm	3.9 (0.8)	4.1 (0.7) <sup>b</sup>	5.5 (0.8) <sup>c,e</sup>
Extension of pterygium, mm	1.9 (0.5)	2.3 (0.6) <sup>c</sup>	3.1 (0.7) <sup>c,e</sup>
Area of pterygium, mm <sup>2</sup>	7.0 (1.9)	7.7 (1.8) <sup>b</sup>	10.9 (2.0) <sup>c,e</sup>

<sup>a</sup>Unless otherwise indicated, data are expressed as mean (SD).

<sup>b</sup> $P > .05$  compared with grade 1.

<sup>c</sup> $P < .05$  compared with grade 1.

<sup>d</sup> $P > .05$  compared with grade 2.

<sup>e</sup> $P < .05$  compared with grade 2.

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<sup>+</sup>, are increased in pterygia.<sup>21</sup> Concentrations of many angiogenic factors are elevated in pterygia, including vascular endothelial growth factor (VEGF), thrombospondin-1, and substance P.<sup>22,23</sup>

In previous studies, we demonstrated that there is a parallel relationship between angiogenesis and lymphangiogenesis in inflamed corneas.<sup>24-26</sup> Because it has been shown that angiogenesis contributes to pterygia development and growth and was regarded as an entry arm in immunity, we believed that further investigation was warranted on whether such a simultaneous appearance of blood and lymphatic vessels also occurs in pterygia. However, to our knowledge, few studies are available on the outgrowth of lymphatic vessels in pterygia.

The aims of the present study were to examine angiogenesis and lymphangiogenesis in primary pterygia and to discuss the molecular mechanisms of lymphangiogenesis. 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.<sup>27</sup> 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 cor-

nea from the limbus and the width of the base at the limbus, was measured (in millimeters) with a slitlamp 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 real-time 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

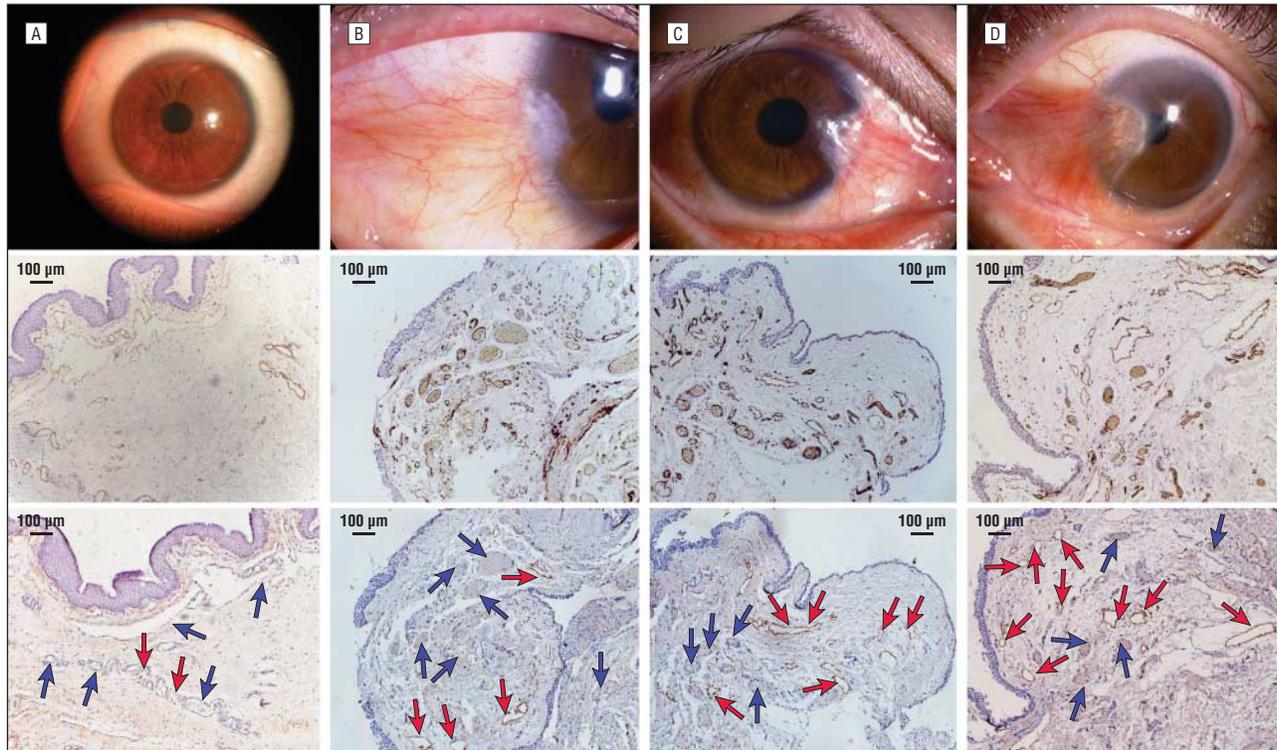
After being fixed in 10% neutral formalin for 24 hours, embedded in paraffin, serially sectioned (thickness, 4  $\mu$ m), and rehydrated with graded ethanol-water mixtures, excised conjunctive segments were washed with distilled water. Endogenous peroxidase activity was blocked after being incubated with 30-mL/L hydrogen peroxidase 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 100 $\times$  magnification (0.78 mm<sup>2</sup>), the 5 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  $\mu$ L of lysis buffer (20mM imidazole hydrochloride, 10mM potassium chloride, 1mM magnesium chloride, 10mM ethyleneglycoltetracetic 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),



**Figure 1.** Results of CD31 and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) immunohistochemistry for human normal conjunctiva and pterygia. A small number of blood vessels were positive for CD31 and negative for LYVE-1, but only a few lymphatic vessels were positive for CD31 and LYVE-1 in normal epibulbar conjunctivae segments. Lymphatic vessels were mildly increased in grade 1 pterygia but were dramatically increased in grades 2 and 3 pterygia. A, Control sample. B, Grade 1 pterygia. C, Grade 2 pterygia. D, Grade 3 pterygia. The top row shows photographs of the human eye; the middle row, CD31 immunohistochemistry; and the bottom row, LYVE-1 immunohistochemistry. Red arrows point to lymphatic vessels and blue arrows, blood vessels (original magnification  $\times 100$ ).

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 18000g 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  $\mu$ L 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  $\mu$ L. 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'-GCAGATGTGAATGCAGACCAAA-3' (sense) and 5'-CTGCGGATCTTGGACAAACA-3' (antisense) (GenBank No. NM009505). The primers of VEGF-C were 5'-CAATTATTA-GACGTTCTCTGCCAGC-3' (sense) and 5'-GCATCGGCA-CATGTAGTTATTCC-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  $\alpha = .05$ .

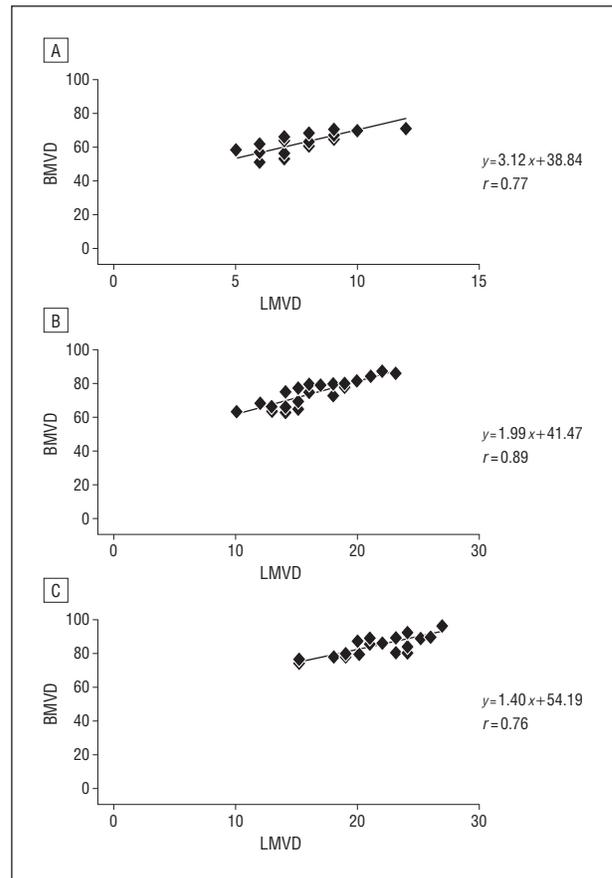
## RESULTS

### 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 mm<sup>2</sup>, with a mean of 8.4 (2.5) mm<sup>2</sup> (**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,<sup>28,29</sup> 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.



**Figure 2.** The relationship between lymphatic (LMVD) and blood microvessel density (BMVD) in pterygia. We found a close correlation between LMVD and BMVD in all grades of pterygia. A, Grade 1 pterygia. B, Grade 2 pterygia. C, Grade 3 pterygia. Densities are calculated as the total number of vessels in 200 microscopic fields divided by 200. The solid line in each graph represents the linear regression fit across all subjects. The slope and the Spearman rank correlation coefficient for each are shown.

**Table 2. LMVD and BMVD in Pterygia**

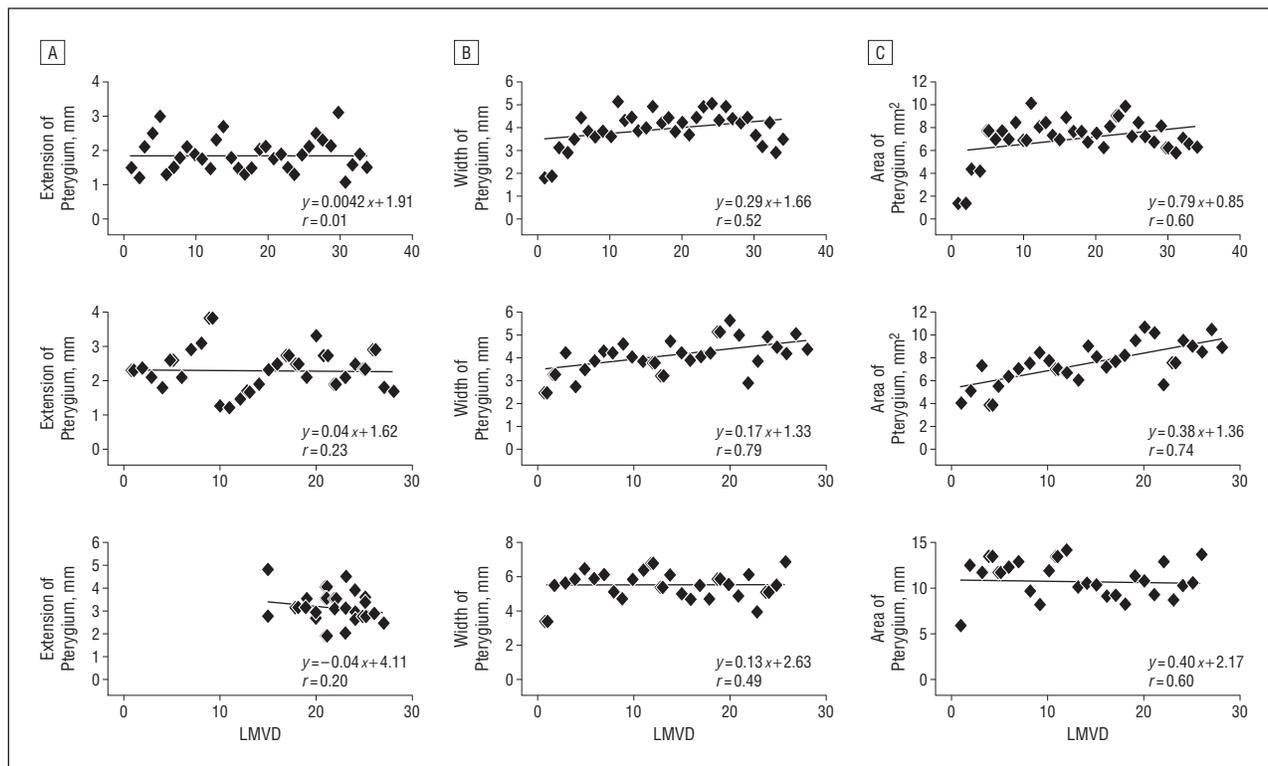
Pterygium Grade	Mean (SD) Density <sup>a</sup>	
	LMVD	BMVD
Grade 1 (n = 34)	7.88 (1.43)	63.44 (5.77)
Grade 2 (n = 28)	16.39 (3.44)	74.21 (7.67)
Grade 3 (n = 26)	21.73 (3.07)	84.65 (5.65)
Controls (n = 7)	3.71 (1.11)	45.57 (3.10)

Abbreviations: BMVD, blood microvessel density; LMVD, lymphatic microvessel density.

<sup>a</sup> Calculated as the total number of vessels in 200 microscopic fields divided by 200.

### 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



**Figure 3.** The relationship between lymphatic microvessel density (LMVD) and the size of the pterygium. Although there was no significant relationship between LMVD and the extension of the pterygium, LMVD was significantly correlated with the width and area of the pterygium. A, Relationship between LMVD and the extension of the pterygium. B, Relationship between LMVD and the width of the pterygium. C, Relationship between LMVD and the area of the pterygium. The top row involves grade 1 pterygia; the middle row, grade 2 pterygia; and the bottom row, grade 3 pterygia. Densities are calculated as the total number of vessels in 200 microscopic fields divided by 200. The solid line in each graph represents the linear regression fit across all subjects. The slope and the Spearman rank correlation coefficient for each are shown.

1, 2, or 3;  $P < .01$  for all). Subsequently, we examined the size of pterygia and analyzed the relationship between grades 1, 2, and 3 pterygia (ie, extension, width, and total area) and LMVD. Although the relationship was not significant between the extension and LMVD, we found that the other 2 indexes (ie, width and total area) correlated closely with LMVD, which indicated that greater LMVD was associated with larger pterygia (**Figure 3**).

#### EXPRESSION OF VEGF-A AND VEGF-C IN PTERYGIA

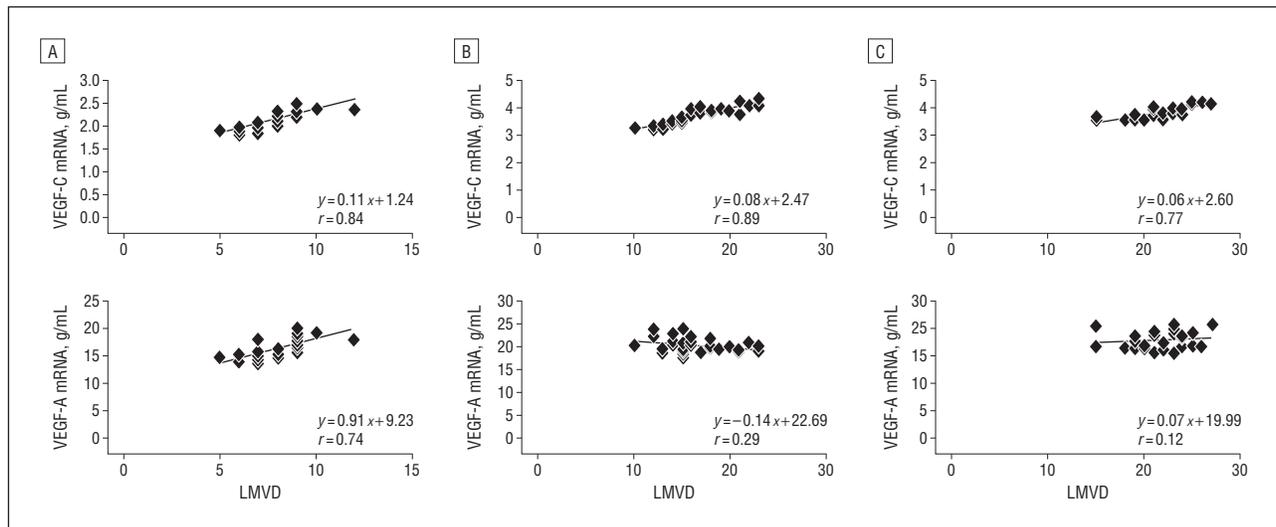
Compared with normal conjunctiva, the expression of proteins and mRNAs of VEGF-A increased dramatically in grade 1 pterygia. However, the expression of VEGF-A was upregulated moderately in grades 2 and 3 pterygia, and we found no significant difference in the expression of VEGF-A proteins between grades 2 and 3 pterygia ( $P > .05$ ) (**Figure 4**). The expression of VEGF-C increased mildly in grade 1 pterygia compared with normal conjunctiva, but expression was upregulated dramatically in grades 2 and 3 pterygia. The difference in VEGF-C mRNA was not significant between grades 2 and 3 pterygia. In addition, we examined the relationship between VEGF-C mRNA and LMVD and between VEGF-A mRNA and LMVD. Our data showed that there was a significant relationship between VEGF-C mRNA and LMVD in grades 1, 2, and 3 pterygia, whereas VEGF-A mRNA

was closely correlated with LMVD only in grade 1 pterygia (**Table 3**).

#### COMMENT

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.<sup>30</sup> A hyaluronan 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.<sup>31,32</sup> 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-



**Figure 4.** The relationship between expression of vascular endothelial growth factor C (VEGF-C) messenger RNA (mRNA) (top row) and VEGF-A mRNA (bottom row) and lymphatic microvascular density (LMVD). A, Grade 1 pterygia. B, Grade 2 pterygia. C, Grade 3 pterygia. We found a close correlation between VEGF-C mRNA and LMVD in grades 1, 2, and 3 pterygia. The relationship was significant between VEGF-A mRNA and LMVD in grade 1 pterygia but not grades 2 and 3 pterygia. Densities are calculated as the total number of vessels in 200 microscopic fields divided by 200. The solid line in each graph represents the linear regression fit across all subjects. The slope and the Spearman rank correlation coefficient for each are shown.

**Table 3. Expression of VEGF-A and VEGF-C in Pterygia**

Pterygium Grade	Mean (SD) Expression			
	VEGF-A		VEGF-C	
	mRNA, g/mL	Proteins, pg/mg	mRNA, g/mL	Proteins, pg/mg
Grade 1 (n = 34)	16.38 (1.76) <sup>a,b</sup>	14.98 (1.11) <sup>a,b</sup>	2.12 (0.19) <sup>a,b</sup>	2.14 (0.27) <sup>a,b</sup>
Grade 2 (n = 28)	20.43 (1.61) <sup>a</sup>	17.93 (1.92) <sup>a</sup>	3.74 (0.30) <sup>a</sup>	3.58 (0.34) <sup>a</sup>
Grade 3 (n = 26)	21.60 (1.92) <sup>a,b</sup>	18.90 (1.96) <sup>a,c</sup>	3.86 (0.23) <sup>a,c</sup>	3.77 (0.31) <sup>a,b</sup>
Controls (n = 7)	3.93 (0.31)	3.90 (0.50)	1.48 (0.21)	1.66 (0.34)

Abbreviations: mRNA, messenger RNA; VEGF, vascular endothelial growth factor.

<sup>a</sup>  $P < .05$  compared with controls.

<sup>b</sup>  $P < .05$  compared with grade 2.

<sup>c</sup>  $P > .05$  compared with grade 2.

plants.<sup>30</sup> 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,<sup>33</sup> 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 correlated closely with the width and total area of pterygia in all groups. Because of our finding that LMVD doubled in grades 2 and 3 pterygia compared with grade 1 pterygia, pterygia likely becomes more severe with more outgrowth of the lymphatic vessels. Moreover, 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.<sup>34,35</sup> In a previous study, we also observed that corneal lymphatic vessels developed in parallel with blood vessels after corneal alkaline burns.<sup>26</sup> 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. This finding was consistent with the notion that afferent lymphatics may be equal to or even more important than efferent blood vessels in corneal immunity.<sup>18</sup> Therefore, strategies of antilymphangiogenic therapy might be further investigated to improve the efficacy of pterygium interventions and the prognosis of these patients.

Although some other unknown factors may be involved in the establishment and maintenance of the lym-

phatic vasculature, VEGF-C is thought to be the predominant lymphangiogenic factor.<sup>36,37</sup> 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.<sup>38</sup> Conversely, lymphatic vessels regress in the skin transiently in the inner organ of keratin 14–controlled VEGFR3-Ig mice.<sup>39</sup> However, some studies have also argued that VEGF-A plays a more important role in lymphangiogenesis.<sup>40,41</sup> Recently, Kajiyama et al<sup>42</sup> showed that exposure to UV-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<sup>42</sup> suggested that the expression levels of VEGF-A, but not of the known lymphangiogenesis factor VEGF-C, are responsible for lymphangiogenesis in UV-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.<sup>43,44</sup> This possibility might be partially explained by the close relationship between VEGF-A 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.<sup>45,46</sup> 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.

**Submitted for Publication:** September 18, 2011; final revision received December 23, 2011; accepted January 6, 2012.

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**Author Contributions:** Drs Ling, Liang, and Lin contributed equally to this work.

**Financial Disclosure:** None reported.

**Funding/Support:** This study was supported by grants 81070711 and 30700927 from the China Natural Science Foundation and grant S2011010006061 from the Guangdong Natural Science Foundation.

**Additional Contributions:** Chaoyang Li, PhD, and

Chuangchao Xu, PhD, provided invaluable technical support.

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### Correction

**Error in Figure.** In the Clinical Sciences article titled "Intravitreal Ketorolac for Chronic Uveitis and Macular Edema: A Pilot Study" by Kim et al, published in the April issue of the *Archives* (2012;130[4]:456-460), an error occurred in Figure 3 on page 459. The 2 images for patient 9 on day 3 (B) were incorrect and should have been visibly different from the images for day 0 (A).