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 conjunctivea 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 epibular 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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TERGYGIUM 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. THE PATHOGENESIS OF PTERGYIA HAS INTRIGUED RESEARCHERS FOR CENTURIES, BUT IT IS NOT COMPLETELY UNDERSTOOD. STUDIES HAVE SHOWN AN INCREASING PREVALENCE OF PTERGYIA WITH CLOSER PROXIMITY TO THE EQUATOR, SECONDARY TO GREATER EXPOSURE TO UV RADIATION. CORONEO ET AL 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 PTERGYIA DEVELOP FROM LIMBAL EPITHELIAL PROGENITORS HAS BEEN PROVIDED WITH EVIDENCE THAT FUCHS ISLET CELLS HAVE STEM CELL CHARACTERISTICS. OTHER INVESTIGATORS HAVE PROPOSED NEOPLASTIC FACTORS, FOCUSING ON THE P53 TUMOR SUPPRESSOR GENE, WHEREAS SOME BELIEVE TEAR ABNORMALITIES AND ALLERGIC FACTORS ARE SIGNIFICANT CONTRIBUTORS.

More recently, immunopathologic mechanisms have been studied to determine 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 cell adhesion molecule 1, intercellular adhesion molecule 1, and aberrant expression of HLA-DR. Other signaling molecules, including E-cadherin and β-catenin, are upregulated and concentrated in the heads of pterygia. Increased β-catenin levels have been shown to trigger certain cell cycle proteins and matrix metalloproteinases. 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. An increase in mast cells, lymphocytes, plasma cells, dendritic cells, and CD4⁺ and CD8⁺ T cells in pterygia samples has also been documented, suggesting that cellular immunity and hypersensitivity contribute to pterygium formation.

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. Although the blood vessels provide a route of entry for immune effector cells (eg, CD4⁺ alloreactive T lymphocytes, CD8⁺ cytotoxic T lymphocytes, and plasma cells), they also play a role in the development of pterygia. This suggests that the immune response in pterygia is multifactorial, involving both innate and adaptive immune mechanisms.

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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.22,23

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.** 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 slitlamp using a slit beam of light. The total area was calculated. Seven nasal epibulbar conjunctival 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 10mL 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× magnification (0.78 mm²), 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 µL 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),
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’-GCAGATGTGAATGCAGACCAAA-3’ (sense) and 5’-CTGGGGATCTTGGACAAACA-3’ (antisense) (GenBank No. NM009505). The primers of VEGF-C were 5’-CAATTATAGTCGTTCTCAGCAAA-3’ (sense) and 5’-CTGGGGATCTTGGACAAACA-3’ (antisense) (GenBank No. NM009505). 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 \( p = .05 \).

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\(^2\), with a mean of 8.4 (2.5) mm\(^2\) (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,\(^28,29\) 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 pterygium 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.

Table 2. LMVD and BMVD in Pterygia

<table>
<thead>
<tr>
<th>Pterygium Grade</th>
<th>Mean (SD) Densitya</th>
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<tbody>
<tr>
<td></td>
<td>LMVD</td>
</tr>
<tr>
<td>Grade 1 (n = 34)</td>
<td>7.88 (1.43)</td>
</tr>
<tr>
<td>Grade 2 (n = 28)</td>
<td>16.39 (3.44)</td>
</tr>
<tr>
<td>Grade 3 (n = 26)</td>
<td>21.73 (3.07)</td>
</tr>
<tr>
<td>Controls (n = 7)</td>
<td>3.71 (1.11)</td>
</tr>
</tbody>
</table>

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

a 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...
1, 2, or 3; \( P < 0.01 \) 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 (\( P > 0.05 \)) (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. 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. 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-
grade 1 pterygia, pterygia likely becomes more severe with
LMVD doubled in grades 2 and 3 pterygia compared with
area of pterygia in all groups. Because of our finding that
lymphatic vessels correlated closely with the width and total
area of pterygia among groups. Our analyses showed that lym-
phatic vessels developed in parallel with blood vessels
in pterygia. Recent evidence from animal and human vascularized corneas indicated that the
degree of corneal lymphangiogenesis was significantly cor-
related with the degree of corneal hemangiogenesis.34,35
In a previous study, we also observed that corneal lym-
phatic vessels developed in parallel with blood vessels
after corneal alkaline burns.26 In the present study, de-
spite 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 pte-
ygia, suggesting that lymphangiogenesis plays a key role
in immunopathologic mechanisms and the develop-
ment 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 im-
munity.18 Therefore, strategies of antilymphangiogenic
therapy might be further investigated to improve the ef-
cacy of pterygium interventions and the prognosis of
these patients.

Although some other unknown factors may be in-
volved in the establishment and maintenance of the lymp-

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groups. We examined lymphatic vessels in each excised
specimen and discovered that lymphangiogenesis was as-
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these patients.

Although some other unknown factors may be in-
volved in the establishment and maintenance of the lymp-
phatic vasculature, VEGF-C is thought to be the predomi-
nant lymphangiogenic factor.36,37 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 dis-
played a pronounced hyperplasia of cutaneous lymphatic
vessels, whereas the growth of blood vessels was not af-
acted.38 Conversely, lymphatic vessels regress in the skin
transiently in the inner organ of keratin 14–controlled
VEGF3-1g mice.39 However, some studies have also ar-
gued that VEGF-A plays a more important role in lymph-
angiogenesis.9,40 Recently, Kajiya et al40 showed that ex-
posure to UV-B irradiation, which is the main cause of
pterygia, results in prominent enlargement and an increas-
ing number of lymphatic vessels of murine skin. Kajiya et
al40 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 epi-
dermis. 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 sug-
gests that even VEGF-A can be lymphangiogenic via its re-
ceptors for VEGFR2, which is also expressed on lymph-
atic endothelial cells.43,44 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 es-
sential for the formation of lymphatic vessels.45,46 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 path-
way might be critical in lymphangiogenesis, especially in
serious pterygia.

In summary, our study has revealed the develop-
ment of lymphatic vessels in pterygia and has indicated
that transient upregulation of VEGF-C might be respon-
sible for the occurrence of lymphangiogenesis. Lyn-
phatic vessels might accelerate immunological injury and
play a key role in immunopathologic mechanisms re-
sulting in the pathogenesis of pterygia. Strategies of an-
tilymphangiogenic therapy might be investigated to im-
prove the efficacy of pterygium interventions and the
prognosis for these patients.

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