·Clinical Research·

Relations between lymphangiogenesis and the size of pterygium

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Abstract

• AIM: To examine the relations between lymphangiogenesis and the size of pterygium.

• METHODS: Tissues from 88 primary and 34 recurrent pterygia were evaluated, and those from 7 nasal epibulbar conjunctiva segments were used as controls. Pterygium slices from each patient were stained with LYVE-1 monoclonal antibodies to identify lymphatic microvessel for calculating lymph-vascular area (LVA), lymph-microvascular density (LMD) and lymph-vascular luminal diameter (LVL). Also, the relations between lymphangiogenesis (measuring by LVA, LMD and LVL) and the size of pterygium (extension, width and area) were explored.

• RESULTS: There were a few LYVE-1 $^{(+)}$ lymphatic vessels in normal epibulbar conjunctiva segments. However, the number of lymphatic vessels slightly increased in primary pterygia and dramatically increased in recurrent pterygia. LVA, LMD and LVL significantly increased in recurrent pterygia in comparison with primary pterygia (all $P<0.05$). Both LMD and LVA were correlated with the width and area of pterygia (both $P<0.05$), and LVA was also correlated with the extension of pterygia($P<0.05$).

• CONCLUSION: Lymphangiogenesis is correlated with the size of pterygium. The outgrowth of lymphatic vessels might contribute to the development of pterygia.

• KEYWORDS: pterygia; lymphatic vessel; size

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INTRODUCTION

Pterygium is a neoformation characterized by the encroachment of a fleshy fibrovascular tissue from the bulbar conjunctiva onto the cornea [1-2]. It is a common disorder of the ocular surface, with a prevalence of 2% in temperate areas and up to 20% in tropical regions [3]. The pathogenesis of pterygium has intrigued researchers for centuries, but it has not been completely understood.

Recent studies have shown that immunological mechanisms contribute to the development of pterygium[4-7]. In a previous study, Cimpean *et al* [8] discovered denser lymphatic microvessel in primary pterygia. The lymphatic system plays a key role in maintaining tissue fluid homeostasis by collecting and transporting protein-rich interstitial fluid, back to the blood circulation via lymph nodes, large collecting lymphatic vessels and lymphatic trunks, including the thoracic duct. The lymphatic system also plays an essential role in the immune response to infectious agents [9,10]. Lymphangiogenesis in pterygia provides new evidence that immunological mechanisms is involved in the growth of pterygia.

On this basis, the current study focuses on the relationship between lymphangiogenesis and the size of primary and recurrent pterygium. To our knowledge, there is no other literature available about it. Findings from the present study may broaden our understanding of mechanisms for pterygia development.

MATERIALS AND METHODS

Subjects A total of 122 subjects, including 88 of primary pterygium (39 males and 49 females, average age, 47.5±12.1 years), and 34 of recurrent pterygium (15 males and 19 females, average age, 53.2±17.3 years) were enrolled in the study from January 2006 to June 2010 at the Department of

Ophthalmology, the Third Affiliated Hospital, and the State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University. The apex of the pterygium of patients with primary condition invaded the cornea for at least 1mm. The size of the pterygium, including the horizontal extension onto the cornea from the limbus, and the width of the base at the limbus, were measured (in millimeters) under a slit-lamp. The total area was then calculated. Controls were seven nasal epibulbar conjunctiva segments near the limbus excised from seven age-matched control patients undergoing strabismus surgery. All patients and controls were informed of the experimental nature of this procedure and signed consents were obtained beforehand. All procedures were conducted according to the principles stated in the Declaration of Helsinki.

Methods

Immunohistochemistry After being fixed in 10% neutral formalin for 24 hours, embedded in paraffin, serially sectioned into slices of 4-μm thick, and rehydrated with graded ethanol-water mixtures, excised conjunctiva segments were washed with distilled water. Endogenous peroxidase activity was blocked through incubation in 30mL/L hydrogen peroxidase for 20 minutes. For antigen retrieval, slices were then autoclaved at 121℃ in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes. Then the slices were allowed to cool at room temperature for 30 minutes. Subsequently, they were incubated for 3 hours with mouse anti human LYVE-1 monoclonal antibody (R&D systems, MN) and biotin-marked rabbit anti mouse immunoglobulin, the secondary antibody. Strept avidin biotin complex (SABC)-peroxidase was used as the immune check system. The slices were visualized for peroxidase activity with diaminobenzidine (DAB) and counterstained with hematoxylin.

Quantification of immunohistochemical staining Slices were viewed using a Zeiss Axioskop microscope and images were projected to a Sony PVM1440QM video monitor through Sony CCDIRIS video camera. Digitized images were captured with Fujix HC-1000 3CCD high resolution color camera. Following preliminary scanning of each slice at low power, five areas of high lymphvascular density were imaged at high power ($100\times$) and captured for further analysis by Axiovision 4.7.2 (Carl Zeiss, Jena, Germany), a digital image analysis software.

Lymph-vascular area (LVA) quantification Computer images were converted into a threshold raw binary format, highlighting the LYVE-1-stained lymphatic vessels with minimal highlighting of background tissue. These images were then analyzed using an in-house computer image analysis program that reported the proportion of the area occupied by immunostained lymphatic vessels.

Lymph-microvascular density (LMD) quantification The number of stained lymph-microvessels on computer images was counted manually, and each vessel was marked after being counted to avoid repetition. Vessel counts per field were represented as vessels per μm^2.

Lymph-vascular luminal diameter (LVL) quantification Maximum luminal diameter of stained microvessel with clear lumen was measured manually on computer images. Each vessel was marked after being measured to avoid repetition.

Statistical Analysis The significance of differences between the two groups was examined using paired Student's t-test (SPSS 12.0 statistical software, SPSS Inc., Chicago, Illinois, USA). Pearson's analysis was used to analyze the correlations between LVA, LMD, LVL, and the size of pterygium. Values were presented as mean ± SD. All reported P-values were 2-tailed, and statistical significance was defined at $\alpha=0.05$ level.

RESULTS

Size of Pterygia Of the 122 subjects, the extension of the pterygium onto the cornea ranged from 1.1mm to 4.8mm, with a mean of (2.4±0.78)mm. The width measured from 1.8mm to 6.9mm, averaging (4.5±1.04)mm. And the total area was between 1.4mm^2 and 14.2mm^2, with an average of (8.4±2.51)mm^2.

Immunohistochemical Staining Serial sections of human pterygium tissue were stained for LYVE-1. Compared with blood vessel, lymphatic vessel had a larger lumen and did not contain erythrocytes. Our data showed that there were a few LYVE-1 $^{(+)}$ lymphatic vessels in normal epibulbar conjunctiva segments. Lymphatic vessels moderately increased in primary pterygia, but considerably increased in recurrent pterygia (Figure 1). This suggests that the outgrowth of lymphatic vessels (lymphangiogenesis) might function in the development of pterygia.

Relationship between LVA, LMD, LVL and the Size of Pterygium To elucidate the relationship between lymphatic vessels and pterygia, we compared LVA, LMD and LVL between pterygium tissues of different sizes. First, we examined LVA, LMD and LVL in conjunctiva tissue of patients with pterygia and normal control. Our data showed that LVA, LMD and LVL significantly increased in pterygium in comparison with normal control conjunctiva (all $P<0.01$, Table 1). Subsequently, we examined the size of pterygia (i.e., extension, width and total area) and analyzed its relations with its number of lymphatic vessels. We found that both LMD and LVA were correlated with the width and area of pterygia, and LVA was also correlated with the extension of pterygia (Figure 2). These findings suggest that lymphatic vessels contribute to the development of pterygia.

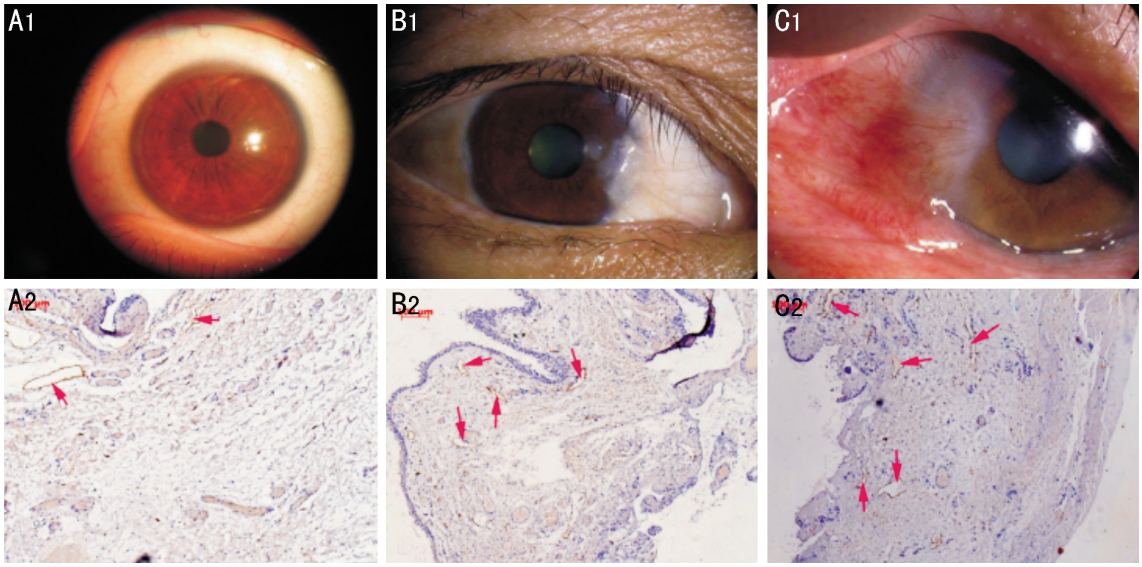

Figure 1 LYVE –1immunohistochemistry for normal human conjunctiva and pterygia There was a small number of LYVE-1 $^{(+)}$ lymphatic vessels in normal epibulbar conjunctivae segments (A). Lymphatic vessels moderately increased in primary pterygium (B), and dramatically increased in recurrent pterygium (C); Footnote 1: photograph of the human eye; Footnote 2: LYVE-1 immunohistochemistry. Red arrows: lymphatic vessels. Magnification for immunohistochemistry ×100.

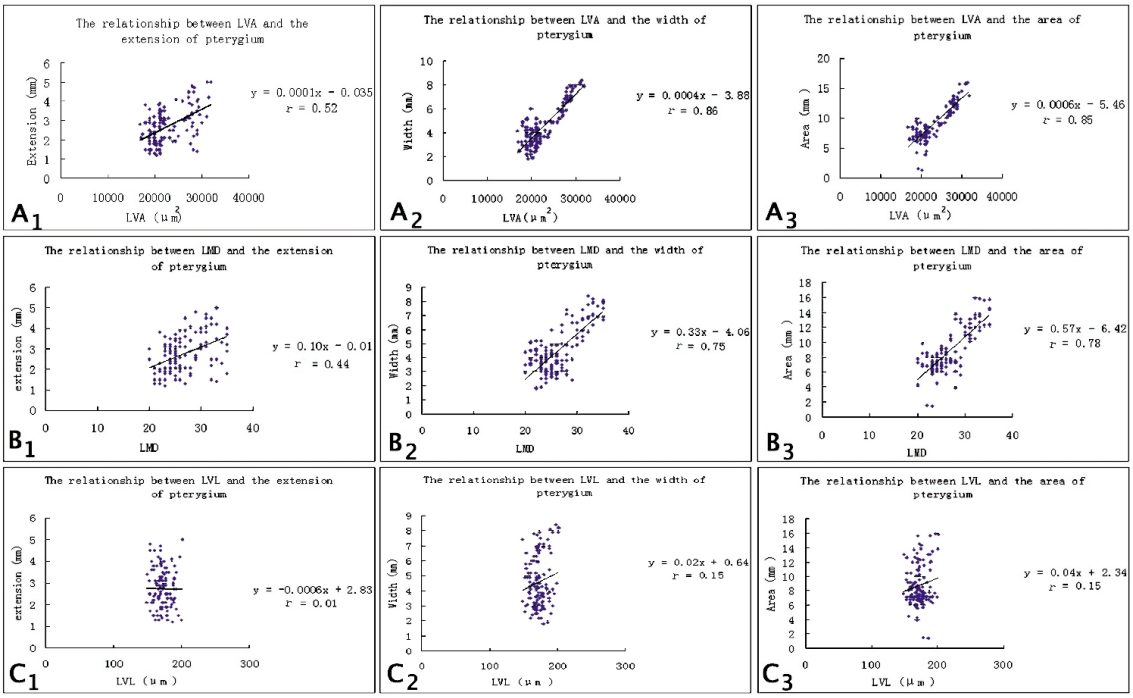

Figure 2 Relationship between the size of pterygium and lymphangiogenesis A:There were significant relationships between LVA and all three parameters of the size (extension, width and area) in pterygia; B:The relationship was significant between width, area and LMD, but the relationship between the extension of pterygia and LMD was not significant. However, the association of LVL with all three parameters (extension, width and area) was not significant.(all P>0.05).

Table 1 Comparative evaluation of LVA, LMD, and LVL in primary versus recurrent pterygia

Patients	Number	LVA(μm^2)	LMD (*n*)	LVL(μm)
Controls	7	12125±810.5	10.71±1.5	128.9±14.7
Primary pterygia	88	20601±1829.1[a]	24.49±2.10[a]	171.3±10.4[a]
Recurrent pterygia	34	28358±1456.9[a,c]	31.71±2.19[a,c]	174.5±13.9[a]

[a]P<0.05 *vs* Controls; [c]P<0.05 *vs* primary pterygia.

DISCUSSION

Among research focuses on pterygium, the development of blood vessels (angiogenesis) has been studied extensively, whereas that of lymphatic vessels (lymphangiogenesis), despite its critical relevance, has drawn little attention until recently. Besides maintenance of tissue fluid homeostasis,

the lymphatic system also plays an essential role in the immune response to infectious agents. While blood vessels provide routes of entry for immune effector cells (e.g., $CD4^+$ alloreactive T lymphocytes, memory T lymphocytes), afferent lymphatic vessels are the exit routes through which APCs migrate to the regional lymph nodes and lymphoid organs[11-13]. Recent researches on corneal lymphangiogenesis have provided evidence that, in corneal immunity, afferent corneal lymphatics may be as important as or even more important than efferent corneal blood vessels[14, 15]. It has also been reported that lymphangiogenesis occurs in conjunctiva, iris, ciliary body and intraocular tumor, and it is responsible for uveolympahtic pathway [16-19]. Therefore, anti-lymphangiogenesis therapies might inhibit ocular inflammation, allograft rejection and eye immunity.

Recently, Cimpean *et al* [8] have examined human pterygium by immunohistochemistry and discovered an increased LMD in pterygium compared with the normal conjunctiva. They also found that D2-40-positive lymphatic endothelial cells were actively proliferating in pterygium, consistent with Ki-67 immunostaining result, but not in normal conjunctiva. These data clearly indicate the presence of active proliferating lymphatic vessels in human pterygium, suggesting that lymphangiogenesis is active in this pathological condition. The presence of an increased LMD in primary pterygium proves that immunological mechanism is involved in the development of pterygium.

However, there were still some questions unanswered. Firstly, LMD can not completely represent the changes of lymphatic vessels. Besides LMD, there are some other parameters, such as LVA and LVL, for examining lymphangiogenesis. Secondly, although Cimpean *et al* [8] discovered higher LMD in pterygium than in normal control, they did not compare the severity of pterygium among patients. If lymphangiogenesis really contributes to pterygium growth, as was described by Cimpean, there would be some difference, say in quantity, in lymphatic vessels among patients with pterygium of different severity. Therefore, more evidence is needed to clarify whether the changes of lymphatic vessels is correlated with the development of pterygium.

To throw light upon them, we labeled lymphatic vessels through LYVE-1 staining to investigate lymphangiogenesis. Compared with angiogenesis, lymphangiogenesis is poorly understood, partly due to the lack of specific lymphatic endothelium markers. This situation has been improved by the identification of LYVE-1 [20]. As a hyaluronan receptor related to CD_{44} expression in lymph vessel endothelial cells, of both normal and neoplastic tissues and on both the luminal and abluminal surfaces of the lymphatic endothelial cells, LYVE-1 is a powerful marker of lymphatic structure and function [21,21]. Subsequently, we examined LMD, LVA and LVL by a computer software and then compared them between pterygium tissue and normal conjunctiva. Our data showed that all three parameters (LMD, LVA and LVL) significantly increased in pterygium, suggesting lymphangiogenesis was present. Lastly, with the methods described by Casey *et al* [23], we examined the size of pterygium by three parameters (width, extension and area), evaluated the degree of pterygium and then determined the correlations of LMD, LVA and LVL and the size of pterygium respectively. We found that both LMD and LVA were correlated with the width and area of pterygia. Together with the correlation between LVA and the extension of pterygia, they suggested that lymphatic vessels were responsible for the development of pterygia.

In conclusion, our study revealed that there was a significant increase in LMD, LVA and LVL in pterygium and lymphangiogenesis was correlated with the size of pterygium. The occurrence of lymphangiogenesis in pterygium provides additional evidence that immunological factors give rise to pterygia development, since lymphatic vessel is regarded as an exit "arm" in immunity. Anti-lymphangiogenic therapy can be designed to improve the prognosis of pterygium.

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