

Preliminary evidence of the presence of lymphatic vessels immunoreactive for D2-40 and Prox-1 in human pterygium

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Abstract. Human pterygium is a benign fibrovascular outgrowth of the corneo-conjunctival junction, characterized by tissue remodeling, cellular proliferation, angiogenesis and inflammation. No data are available concerning the presence of lymphatic vessels in this pathological condition. The aim of this study was to evaluate by immunohistochemistry, using antibodies against D2-40, Prox-1 and Ki-67, the presence and the proliferative activity of lymphatic vessels in human pterygium. An increased lymphatic microvessel density was observed in the human pterygium compared to the normal conjunctiva. Moreover, D2-40-positive lymphatic endothelial cells were also actively proliferating, as assessed by Ki-67 immunostaining, while in normal conjunctiva proliferating lymphatic endothelial cells were not detectable. Overall, these data clearly indicate the presence of active proliferating lymphatic vessels in human pterygium, suggesting that active lymphangiogenesis occurs in this pathological condition.

Introduction

Pterygium is a disease characterized by surface ocular lesions associated with chronic ultraviolet exposure in which the primary effect is a solar actinic elastosis within the stroma and all other changes are secondary to the primary effect (1-7). Pterygium is characterized by cellular proliferation, inflammatory infiltration, fibrosis, angiogenesis, and extracellular matrix breakdown; the lesion begins to grow from limbal epithelium and invades the cornea centripetally followed by conjunctival epithelium, exhibiting degenerative and hyperplastic changes (1-7).

Seifert and Sekundo identified intraepithelial capillaries in the optical half of 42.3% pterygia, providing the first evidence of neovascularization in this pathological condition (8). Other

reports followed (9,10) and several angiogenic cytokines, such as fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF) platelet-derived growth factor (PDGF), transforming growth factor β (TGF- β), tumor necrosis factor α (TNF- α), interleukin-6 and -8 (IL-6 and IL-8) and nerve growth factor (NGF) (11-14) have been implicated as mediators of angiogenesis. Furthermore, the presence of mast cells has been correlated with the degree of angiogenesis in pterygium (15,16).

Several antiangiogenic approaches have been proposed for the treatment of pterygium. One case report documented the successful treatment of an early recurrent pterygium using interferon $\alpha 2\beta$ (17) and more recently, bevacizumab, a human monoclonal antibody anti-VEGF, has been proposed in the treatment of pterygium (18-23).

Few data are available concerning lymphangiogenesis in the cornea and the presence of lymphatic vessels in pterygium (24). The aim of this study was to evaluate by immunohistochemistry, by using antibodies against D2-40 and Prox-1, the presence of lymphatic vessels in human pterygium.

Materials and methods

The study group included 20 bioptic specimens of surgical excised primary or recurrent pterygium. Ten nasal epibulbar conjunctival segments, excised during cataract surgery near the limbus, were used as control tissues. Specimens were fixed in buffered formalin for 48 h, embedded in paraffin, and 5 μ m sections were stained with hematoxylin-eosin for routine diagnosis.

Lymphatic vessels were immunostained with a monoclonal antibody against D2-40 as a cytoplasmatic marker (purchased from Dako, Carpinteria, USA) and a polyclonal anti-Prox-1 antibody as a nuclear marker (dilution 1:200; 2 h incubation at room temperature; code 11941 from Abcam, UK) by using an avidin-biotin-peroxidase method. Lymphatic endothelial cell proliferation was assessed using a Ki-67 antibody (clone MIB1; Dako) by incubating for 30 min at room temperature). The markers were detected by a simple immunostaining (the final product was visualized with 3,3'-diaminobenzidine hydrochloride) or were co-localized by applying a double-staining method using the Doublestain Envision kit (Dako). Counterstaining was performed with modified Lille's Hematoxylin. All immunohistochemical steps were performed in an

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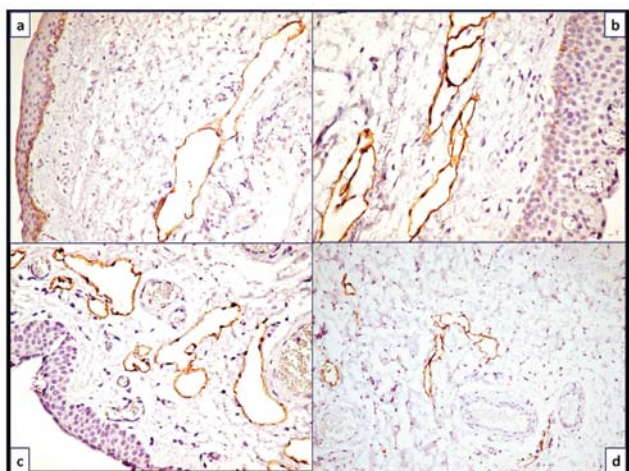


Figure 1. (a) In normal conjunctiva, basal cells are immunoreactive to D2-40 and scarce, large lymphatic vessels immunoreactive to D2-40 are observed deeply inside the stroma. Lymphatic vessels immunoreactive to D2-40 are located close to the epithelium (b and c) or deep inside the fibrous stroma (d) in human pterygium. Original magnification, x200.

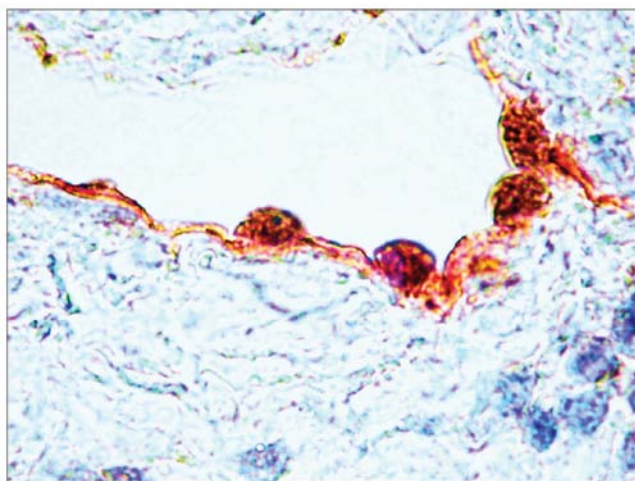


Figure 2. Intense proliferative activity assessed by Ki-67 (brown nuclear staining) along the D2-40 positive lymphatic endothelium (red, cytoplasmic reaction) in human pterygium. Original magnification, x400.

automated fashion with the use of PT Link for automated controlled antigen retrieval and the Dako Autostainer.

Lymphatic microvascular density (LMVD) was estimated at x200 magnification (covering an area of 0.74 mm²) by two independent observers (M.R. and A.M.C.), using the hot spot method. Microscopic images were captured in a JPEG format, and LMVD was calculated using the Nikon Lucia G program of analysis of the microscopic images (Nikon, Tokyo, Japan).

The local research ethics committee approved the protocol of the study, and informed consent was obtained from all subjects according to the World Medical Association (WMA) Declaration of Helsinki.

Results

D2-40 immunostaining specifically highlighted the lymphatic vessels in normal conjunctiva, whereas rare positive lymphatic vessels with normal morphology were observed deeply inside

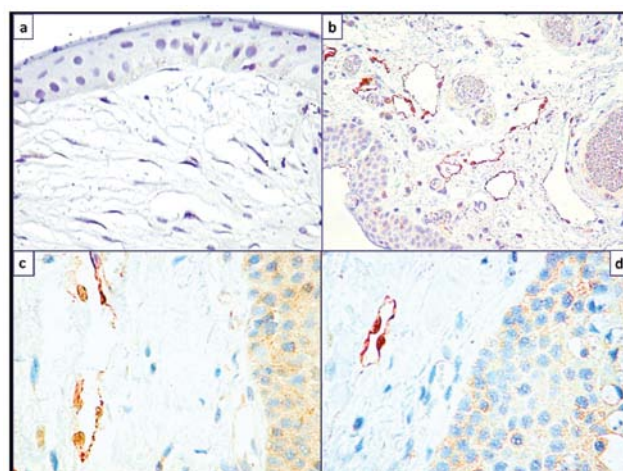


Figure 3. (a) Absence of immunoreactivity to Prox-1 in normal conjunctiva. (b) Strong immunoreactivity for both D2-40 and Prox-1 in human pterygium. D2-40 red cytoplasmic immunostaining may be (c) discontinuous or (d) continuous; Prox-1 staining in the nucleus is shown in red. Original magnification, x400 (a, c and d), x200 (b).

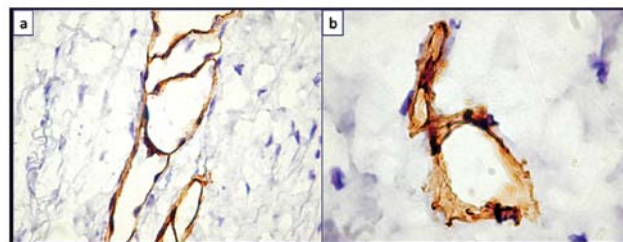


Figure 4. Lymphatic vessels in human pterygium immunoreactive to D2-40 suggestive of (a) intussusception or (b) a sprouting mechanism of new formation. Original magnification, x400.

the connective tissue (Fig. 1a). LMVD ranged between 1-3 vessels/field at x200 magnification.

In human pterygium the lymphatic vessels were distributed in close proximity of the epithelial layer, were tortuous, their lumen was highly splitted and the vascular wall was discontinuous (Fig. 1b). Most lymphatic vessels were distributed in a network-like fashion (Fig. 1c and d), while in normal conjunctiva lymphatic vessels were presented as distinctive structures, and were separated from each other by connective tissue stroma. The LMVD range was between 7-10 vessels/field at x200 magnification.

By means of a double immunostaining with antibodies anti D2-40 and anti-Ki-67, a high proliferative activity of lymphatic endothelial cells was detectable in human pterygium, because all D2-40-positive vessels were also positive for Ki-67 (Fig. 2), while in normal conjunctiva the proliferative activity of lymphatic endothelial cells was not detectable.

By using an antibody anti-Prox-1, a constitutive marker for lymphatic endothelium, numerous Prox-1-positive lymphatic endothelial cells or scattered fusiform stromal cells were found only in human pterygium, but not in normal conjunctiva. Many lymphatic vessels with Prox-1-positive cells showed a moderate reactivity for D2-40 and a discontinuous or continuous wall (Fig. 3c and d). Morphological pictures suggestive of an

intussusceptive (Fig. 4a) or sprouting (Fig. 4b) mechanism of lymphangiogenesis were also recognizable.

Discussion

In this study we have demonstrated the presence of lymphatic vessels immunoreactive to D2-40 and Prox-1 in human pterygium. We found a high number of lymphatics with a high proliferative activity in pterygium as compared with normal conjunctiva.

Few data are available concerning the presence of lymphatic vessels in human pterygium and lymphangiogenesis in the cornea. Seifert *et al* (24) by using conventional staining procedure (hematoxylin-eosin, alcian blue and toluidine blue) demonstrated the presence of many large-diameter lymphatic vessels in a markedly edematous human pterygium and more recently Pérez-Rico *et al* (6) showed the expression of fibulin-2 and fibulin-3 in the subepithelial connective tissue of human pterygium distributed along blood and lymphatic vessels.

In regards to lymphangiogenesis, in a mouse model of corneal induced angiogenesis and lymphangiogenesis, topical or systemic application of bevacizumab was shown to inhibit both inflammation-induced angiogenesis and lymphangiogenesis, suggesting an important role of VEGF-A in corneal lymphangiogenesis (22). Moreover, Lee *et al* (23) suggested that edema in conjunctivo-limbal autograft observed in patients treated with bevacizumab as adjuvant therapy might be due to a suppression of lymphangiogenesis induced by this drug.

In human pterygium, an increased number of proliferating lymphatic vessels could be the result of a compensatory mechanism to reduce edema occurring in this pathological condition. In fact, in the pathogenesis of pterygium, chronic irritation produces a chronic inflammatory cell infiltration with a consequent inflammatory edema (25). Moreover, active angiogenesis is responsible for the formation of immature, dilated and high permeable blood vessels.

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