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LYMPHATIC VESSELS IN HUMAN EYELIDS: AN IMMUNOHISTOLOGICAL STUDY IN DERMATOCHALASIS AND CHALAZION

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ABSTRACT

We investigated lymphatic morphology and expression of endothelin (ET-1) axis molecules in human eyelids affected by an inflammatory state (chalazion) and an age-related degenerative condition (dermatochalasis). Lymphatics were immunohistologically detected by D2-40/LYVE-1 staining. Absorbing lymphatic vessels were localized in papillary dermis and around skin appendages with distinctive morphology. In chalazion, D2-40 reactive flattened lymphatic profiles were compressed by inflammatory infiltrate; in dermatochalasis, large fully opened lymphatics were observed, with a significantly wider total area (lymphatic lumina/200x field; $p<0.05$). The lymphatic density (number/200x field) in the two groups was within the same range. Lymphatic dilation is possibly dependent on reduction and fragmentation of the dermal elastic network as well as of oxytalanic fibers in the papillary dermis of dermatochalasis, as shown by Weigert's reaction. Multifunctional peptide ET-1, involved in vasomotion, inflammation and connective proliferation, was faintly and discontinuously localized on lymphatics, as was its type A receptor. In contrast, the consistent expression of type B receptor indicates that lymphatic endothelium is a physiological target for ET-1, whose effects are modulated by multiple pathophysiological conditions. Thus, vasoactive factors play a role in the physiology of richly vascularized eyelids, and therefore, morphofunctional characterization of lymphatic vessels may be useful in suggesting treatment options.

Keywords: lymphatics, dermatochalasis, chalazion, endothelin-axis, eyelids

The main function of lymphatic vessels is to return lymph to the blood circulation through lymphatic-venous junctions; moreover, the lymphatic system contributes to the immune response, and it is involved in numerous pathological conditions such as lymphedema, inflammatory diseases, and tumor metastasis.

Whereas the detection of lymphatics previously relied exclusively on ultrastructural criteria (1), recent identification of several specific markers provides new insights into the lymphatic distribution and pathophysiology (2). These lymphatic markers include VEGFR-3, a tyrosine kinase receptor for vascular endothelial growth factor (VEGF)-C and VEGF-D (3); podoplanin, an O-linked transmembrane sialoglycoprotein, also recognized by D2-40 monoclonal antibody (4-6); transcription factor Prox-1, a homolog of the *Drosophila* homeobox gene *prospero* (7); and the

lymphatic vascular endothelial hyaluronan receptor-1 (LYVE-1) (8).

In the skin, lymphatics originate as a network of initial absorbing vessels in the superficial dermis which merge in precollectors draining into larger deep collectors. Little is known about lymphatic vessels in human eyelids. An histochemical analysis (9) on lymphatics of monkey and human eyelids, performed by 5'-Nase, demonstrated a superficial or pre-tarsal and a deep or post-tarsal lymphatic plexus. In our study, we examined lymphatics in two different conditions occurring in eyelids: dermatochalasis and chalazion. Dermatochalasis is an aging- related condition of upper or lower eyelids clinically characterized by loose redundant skin. Histological investigation shows disruption of elastic fibers and variable collagen degeneration (10). Chalazion is a chronic granulomatous inflammatory lesion, originating from meibomian glands (11). The inflammatory infiltrate consists of neutrophils, macrophages, T-cells and plasma cells. Chalazion is often associated with other meibomian gland dysfunctions, such as chronic blepharitis, or systemic pathology of sebaceous glands, e.g., acne rosacea and seborrhoea (12).

Different sets of vasoactive factors intervene in mechanisms of lymph propulsion. Endothelin-1 (ET-1) is a multifunctional peptide, first identified as a potent vasoconstrictor produced by endothelial cells (13). ET-1 activity is mediated by specific receptors, type A and type B (ETAR and ETBR) (14) with different levels of expression in various cell types. Physiological investigations have shown ET-1/ETAR mediated lymphatic vasomotion in guinea-pig mesenteric collectors (15). Among its varied biological effects, ET-1 is also active in inflammation as a proinflammatory cytokine (16) and in remodelling of extracellular matrix via interactions with fibrogenic cytokines and metalloproteases (17). Whereas the role of ET-1 has been widely investigated in fibrovascular, neural and epithelial structures of the human eye (18-20), little information is available on ET-1 localization in periocular structures (21).

The present study was carried out in order to investigate the distribution of human eyelid lymphatics and their ET-1, ETAR/ETBR expression in dermatochalasis and chalazion, disease models of degenerative and inflammatory changes of eyelids.

MATERIALS AND METHODS

Specimens of human upper eyelids were obtained from surgical treatment on seventeen patients (seven cases of dermatochalasis and ten cases of chalazion). All samples were provided by the Department of Ophthalmology and Neurosurgery (S. Maria alle Scotte, University Hospital, Siena, Italy). All research procedures involving human subjects were carried out according to the Helsinki declaration for the use of human tissue in research. Written informed consent was obtained from all patients. The patients age range was 56-75 years for dermatochalasis (1 man, 6 women) and 20-68 years for chalazion (4 men, 6 women). As controls, we used specimens obtained from human distal leg or thigh hairy skin. All the samples were routinely processed for light microscopy, fixed in Bouin's solution and paraffin embedded, or snap frozen in liquid nitrogen chilled isopenthane. Serial sections, 6-7 µm thick, were mounted on SuperFrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). For each sample a total length >250 µm was examined, with 35-45 serial sections. Each stain was performed on two slides and experiments were performed twice. For immunohistochemistry (IHC), paraffin-embedded sections were deparaffinized and rehydrated, whereas cryostat sections were dried and fixed for 10 min in cold acetone. All sections were then washed in phosphate buffer saline (PBS) and incubated for 30 min at room temperature (r.t.) in PBS containing 5% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) to block aspecific

TABLE 1
Specificities, Dilution, and Sources of Primary Antibodies Used in this Study

Antibody/antiserum	Host	Dilution	Source
D2-40	Mouse	1:50	DakoCytomation#
LYVE-1	Rabbit	1:100	Novus Biologicals*
CD-31	Mouse	1:20	DakoCytomation#
ET-1	Mouse	1:250	Sigma Aldrich^
ETAR	Rabbit	1:400	Sigma Aldrich^
ETBR	Rabbit	1:400	Sigma Aldrich^

*Novus Biologicals, Inc, Littleton, CO; #DakoCytomation, Carpinteria, CA; ^Sigma Aldrich, St. Louis, MO.

binding sites. They were then incubated overnight at 4°C with specific primary antibodies and antisera, diluted in PBS-1% BSA, as indicated in *Table 1*. For lymphatic immunostaining, D2-40 and LYVE-1 in serial sections were utilized. In order to inactivate endogenous peroxidases, sections were incubated with 6% H_2O_2 in methanol for 30 min at r.t. The reaction was detected by the EnVision peroxidase polymer (EnVision, + Labelled polymer, DakoCytomation, Carpinteria, CA) and visualized with 3,3'-Diaminobenzidine (DAB, Sigma Aldrich). All sections were counterstained with Mayer's hemalum (Sigma Aldrich). Negative controls were carried out by omission of primary or secondary antibodies. Images were acquired on a Carl Zeiss Axioplan 2 imaging microscope using an AxioCam HR CCD camera and AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany). For identification of elastic fibers Weigert stain was carried out.

We performed a quantitative morphometric study in D2-40 stained sections from dermatochalasis, chalazion and normal skin to evaluate the lymphatic density and area. In each sample, five random microscopic fields were photographed at 200x magnification and evaluated with AxioVision 4.6 software. Density of vessels was expressed as number of lymphatics/microscopic field ($1.436x10^5$ μm^2). Area was expressed as total surface of absorbing lymphatic vessels/ microscopic field. Counts and measurements were carried out by two independent operators in a blinded fashion, and repeated procedures ensured constant and reproducible results. The results of morphometric analysis are expressed as mean ± standard deviation (M±SD). Statistics were performed by one-way ANOVA followed by Dunnett's test for multiple comparisons; $p<0.05$ was considered significant.

RESULTS

Lymphatics were recognized by D2-40 and LYVE-1 antibodies that specifically and entirely immunostained lymphatic endothelium, as further confirmed by panendothelial marker CD-31 serial stain *(Fig. 1)*.

In dermatochalasis, eyelid lymphatic vessels were numerous and characterized by a wide lumen and a tortuous endothelial profile *(Fig. 2)*. All lymphatics, in spite of the large size and the occasional occurrence of

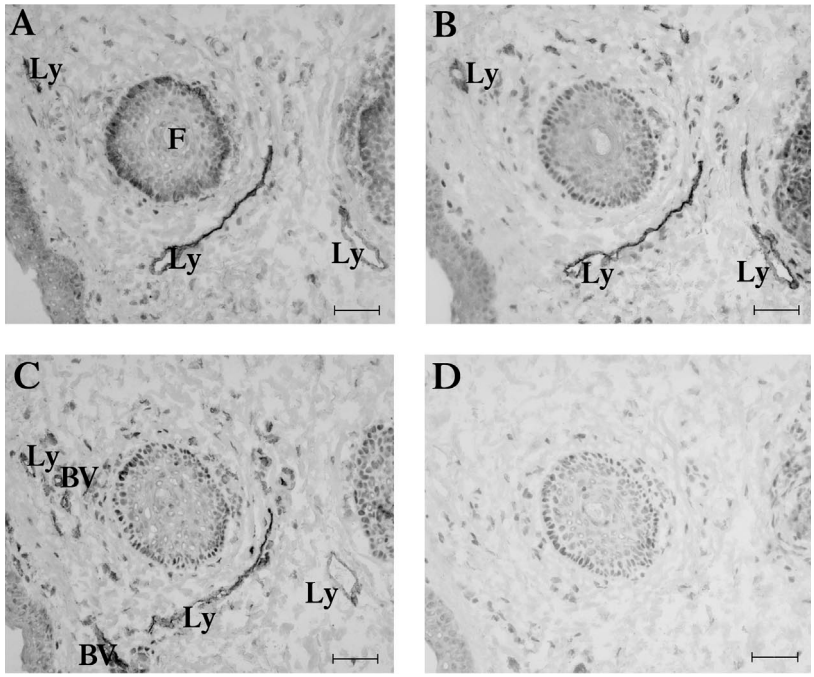

Fig. 1. Eyelid skin affected by dermatochalasis. Serial immunohistology stains. A: D2-40 immunoreactivity in lymphatic vessels (Ly) and hair follicles (F). B: Lyve-1 immunoreactivity in the same lymphatic vessels (Ly) and in some scattered cells. C: CD-31 immunoreactivity in lymphatic (Ly) and blood vessels (BV). D: negative control by omission of primary antibody. Immunoperoxidase cryostat sections. Bar=50 µm.

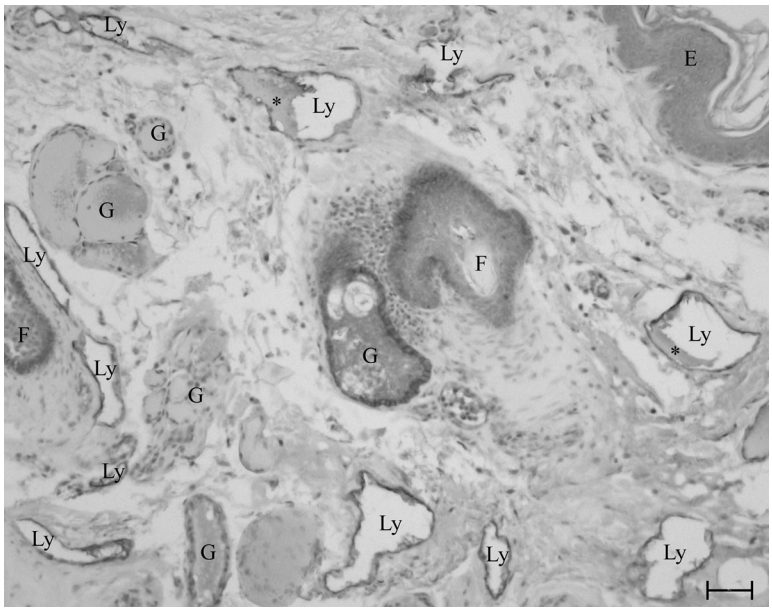

Fig. 2. Dermatochalasis. Numerous D2-40 positive lymphatic vessels (Ly) are dilated and may contain coagulated lymph (*). Epithelium (E), outer root sheath cells of hair follicles (F) and apocrine glands (G) are also immuno-stained by D2-40. Bar=50 µm.

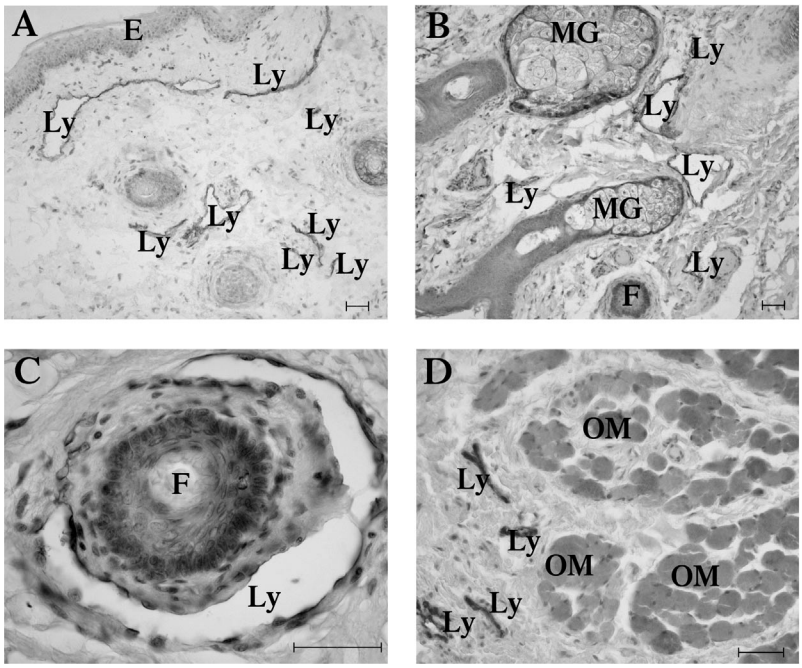

Fig. 3. Dermatochalasis. D2-40 positive lymphatic vessels (Ly). A: located beneath the epithelium (E), B: near meibomian glands (MG), C: surrounding a hair follicle (F) and D: near orbicularis muscle (OM). Bar=50 µm.

valves, were identified as absorbing vessels because no smooth muscle cells were detected in the wall (total length observed by serial sections >250 µm). Lymphatic vessels were localized beneath the epithelium in the papillary dermis, near meibomian glands, around hair follicles and orbicularis muscle bundles *(Fig. 3)*. No lymphatics crossing the muscle fascicles were detected. In chalazia, immunohistology demonstrated within areas of inflammatory infiltrate a network of flattened and collapsed lymphatic vessels *(Fig. 4)*. In some chalazion samples, lymphatics were few and small.

A similar lymphatic topography was observed in control skin samples. The area of lymphatic lumina ranged from 129.4 µm^2 to 22,655.48 µm^2 for dermatochalasis, from 46.13 µm^2 to 998.56 µm^2 for chalazion and from 132.06 µm^2 to 2,120.75 µm^2 for control skin. Statistical analysis of morphometric measurements *(Table 2)* showed significant differences in total lymphatic area between dermatochalasis/chalazion and controls ($p<0.05$). No variation among the three groups occurred for the global density of lymphatics ($p>0.05$). Elastic network surrounding dermal lymphatics was lessened. Dermal elastic fibers were regionally decreased with fragmentation and clumping, and focal loss of oxytalanic fibers was detected in the papillary dermis *(Fig. 5)*.

D2-40 immunoreactivity was present in the epithelium, in the outer root sheath of hair follicles, on apocrine gland adenomers and myoepithelial cells *(Fig. 2)*, and on a large part of orbicularis muscle fibers *(Fig. 3)*. LYVE-1 reaction was also detected in scattered non-endothelial cells, presumably macrophages, located in the connective tissue surrounding lymphatic vessels *(Fig. 1B)*. In both groups, eyelids presented with a high density of CD-31 positive, D2-40/LYVE-1 negative blood vessels *(Fig. 1)*.

Immunohistochemistry detected ET-1 in the blood vessel endothelium and smooth

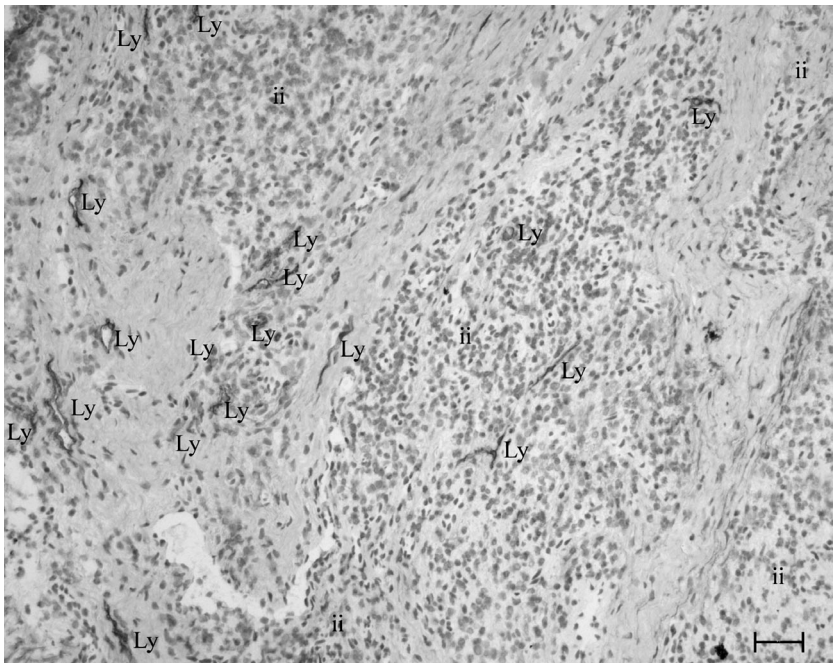

Fig. 4. Chalazion. Most D2-40 positive lymphatic vessels (Ly) within the inflammatory infiltrate (ii) are linear and collapsed. Inflammatory cells are also D2-40 reactive. Bar=50 µm.

TABLE 2
Morphometric Analysis

	Controls (n=3)*	Dermatochalasis (n=7)$^{\infty}$	Chalazion (n=10)^	Significance
Lymphatic density§ M±SD	1.7 ± 0.46	3.4 ± 3.02	1.3 ± 1.74	*vs$^{\infty}$ p>0.05 *vs^ p>0.05 $^{\infty}$vs^ p>0.05
Lymphatic area# M±SD	593.7 ± 509.47	2,581.5 ± 4,390.77	324.3 ± 235.68	*vs$^{\infty}$ p<0.01 *vs^ p<0.01 $^{\infty}$vs^ p<0.01
M±SD				

§ Number/200x microscopic field; # Area values are expressed in µm^2

muscle, in epithelium of epidermis, hair follicles and adenomers, as well as in scattered connective macrophages of dermatochalasis. In chalazia, most of the inflammatory cells such as macrophages, plasma cells and neutrophils, also were ET-1 reactive *(Fig. 6)*.

Both ET receptors were expressed in blood vessel endothelium, smooth muscle cells, orbicularis muscle, epithelial structures and inflammatory cells *(Fig. 6)*.

The pattern of expression of ET-1 and ETRs in absorbing lymphatic vessels was

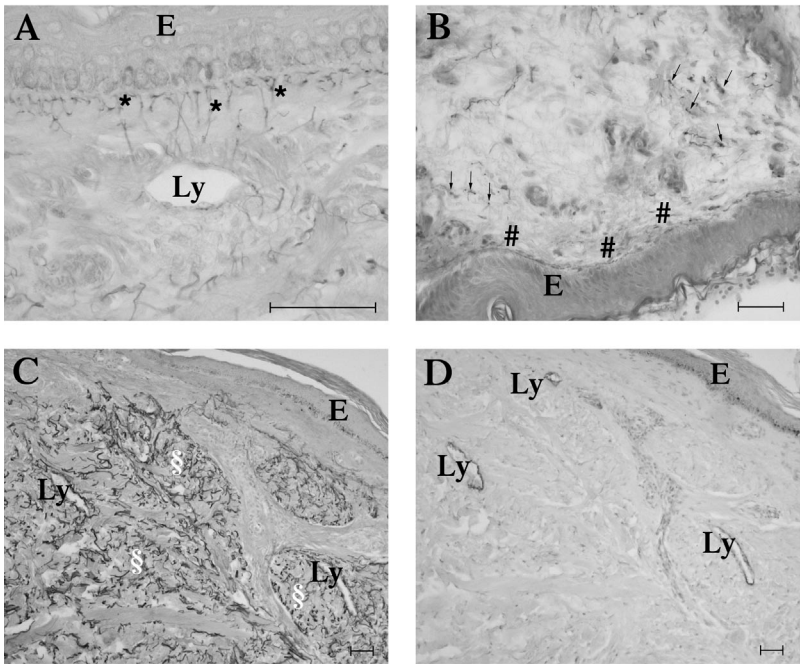

Fig. 5. Dermatochalasis (A-B). A: reduction of dermal elastic network around a lymphatic vessel (Ly) (E: epithelium). Normal, perpendicular distribution of oxytalanic fibers in the papillary dermis (*). B: loss of oxytalanic fibers (#), reduction, fragmentation, and clumping (?) of dermal elastic fibres. Normal skin serial sections (C-D). C: normal elastic dermal network (§) interspersed with collagen bundles and surrounding lymphatics (Ly). D: subepithelial and dermal D2-40 reactive lymphatics. A-C Weigert reaction. Bar=50 µm.

distinctive. ET-1 endothelial immunoreactivity was absent *(Fig. 7)* or faint, with punctate and patchy distribution *(Fig. 8)*. Also the expression of ETAR was similar to ET-1 (data not shown). On the other hand, the lymphatic endothelium was constantly strongly reactive for ETBR, with a diffuse cytoplasmic distribution *(Fig. 9)*.

DISCUSSION

The efficacy of lymph drainage plays a significant role in pathophysiological conditions of eyelids because of their extremely rich blood supply. To the best of our knowledge, however, few reported data are available on the distribution of lymphatics in this location (9). In our surgical specimens, we were able to examine the superficial lymphatic plexus. In accord with Cook's report, no link between the two plexuses, across the orbicularis muscle, was detected. The wide dilation of absorbing lymphatics that we observed in dermatochalasis is a distinctive feature as confirmed by statistical analysis.

Lymphatics in chalazia appeared linear and collapsed, in an environment of inflammatory deposits and fibrotic reaction. In our opinion, this condition shows a functional analogy with the morphology of intratumoral lymphatics compressed by tumor cell proliferation (22). Accordingly, Cook's study on normal eyelid lymphatics documents an intermediate morphological pattern with partially open lumina. Lymphatic vessels react to various pathophysiological stimuli from the extracellular matrix with changes in shape that could modulate their capacity for fluid filtration and reabsorption (23). Therefore, we suggest that the local enlargement of lymphatics is likely to be dependent on the structural tissue changes in dermato-

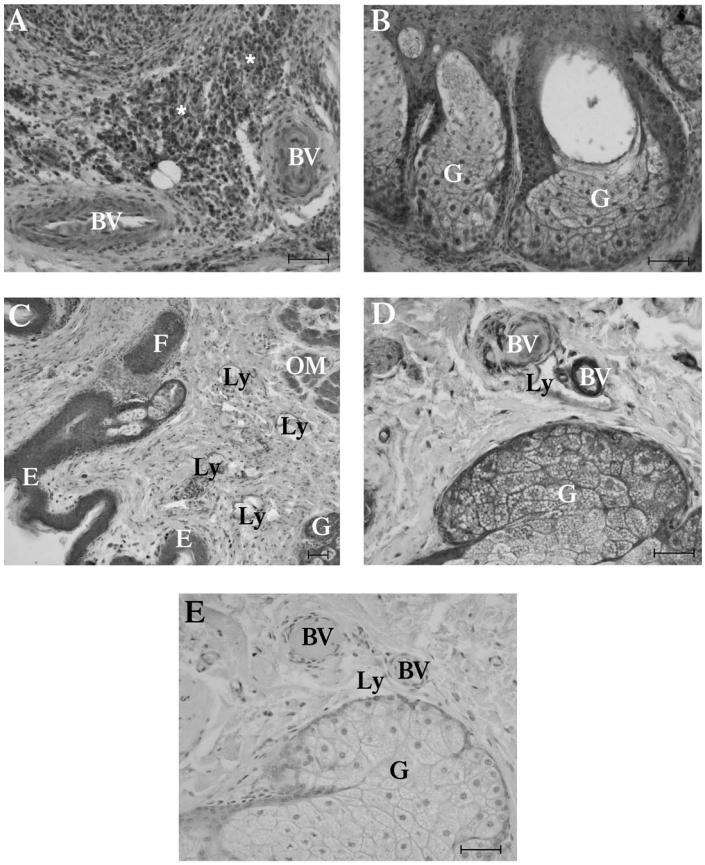

Fig. 6. ET-1/ETBR in non-lymphatic eyelids structures. ET-1 expression (A-B) in inflammatory cells (*), in the endothelium and smooth muscle cells of blood vessels (BV) and in gland adenomers (G). ETBR reactivity (C-D) in epidermial cells (E), eyelash follicles (F), glands (G), orbicularis muscle (OM) and in blood vessels (BV). Lymphatic endothelial is also reactive (Ly). E: negative control on serial section of D. Bar=50 µm.

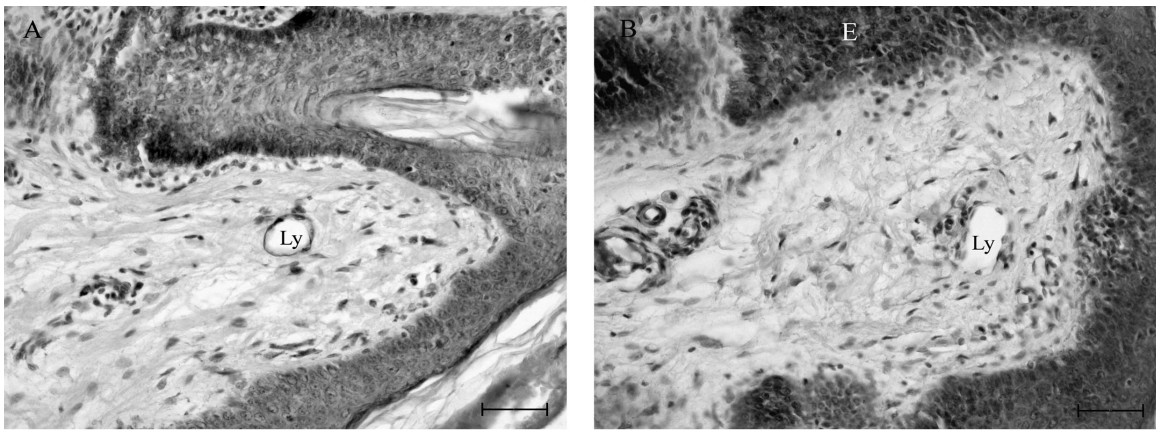

Fig. 7. Dermatochalasis. Serial non-adjacent sections of lymphatic vessel (Ly) immunostained by D2-40 (A) and ET-1 (B). ET-1 reactivity is strong in the epidermis (E) and absent in the lymphatic endothelium. Bar=50 µm.

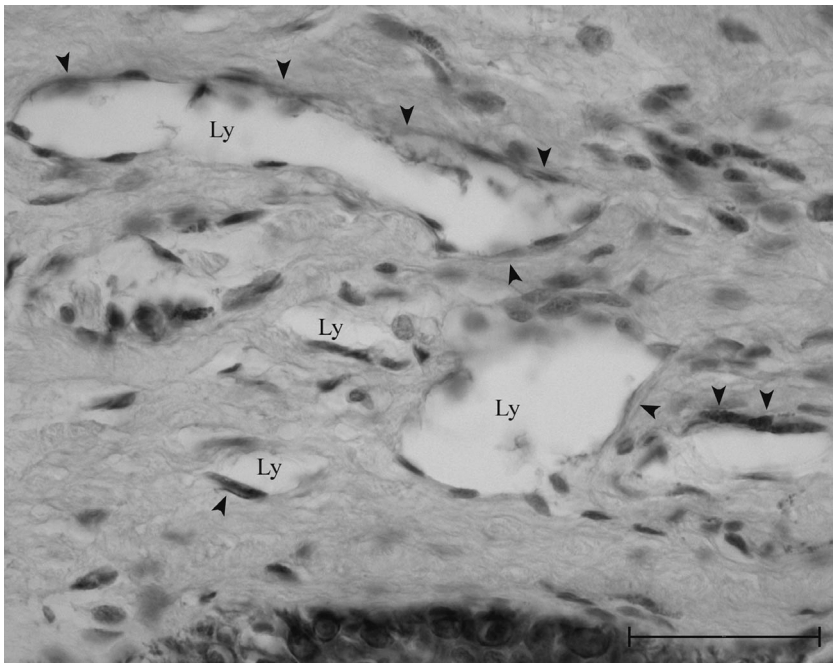

Fig. 8. Dermatochalasis. Faint and punctuate immunohistochemical localization of ET-1 (arrowheads) in lymphatic vessels (Ly) presents only in tracts of the endothelium. Bar=50 µm.

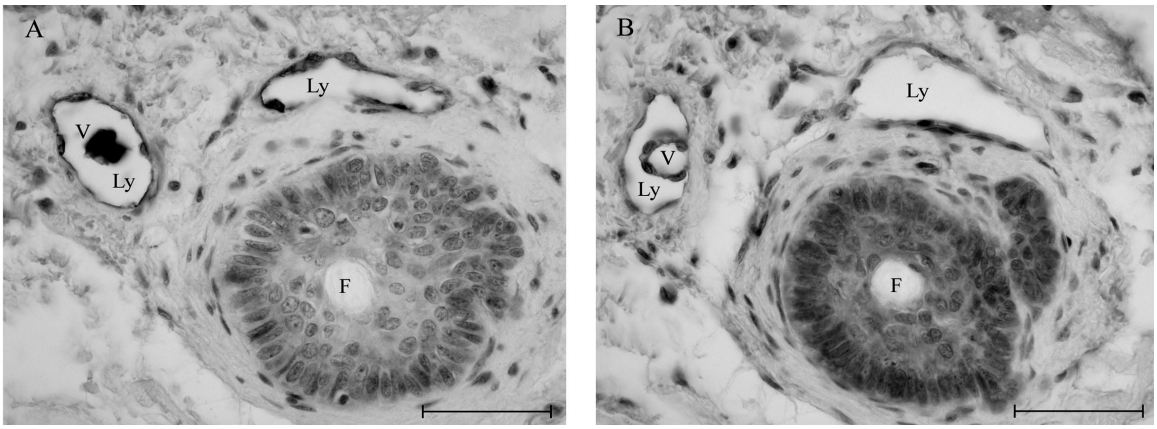

Fig. 9. Dermatochalasis. Serial sections. A: D2-40, lymphatic vessels (Ly) near a hair follicle (F) B: ETRB reactivity of lymphatic endothelium and epithelial structures. Valve (V). Bar=50 µm.

chalasis. Association of epidermal thinning and disruption of elastic fibers, both in the dermo-epidermal junction and the dermis, may lead to loss of mechanical support and disarray of elastic network with involvement of the lymphatic "fibrillar elastic apparatus" (24). This set of lesions, with no morphological changes of collagen, is also observed in acquired cutis laxa (25), a more generalized condition which often encompasses dermatochalasis .

The expression of ET-1 axis molecules by the lymphatic system is not yet fully elucidated, and multiple pathophysiological

mechanisms are probably going on at the same time.

Whereas in chalazion ETBR immunoreactivity adjacent to the inflammatory infiltrate is likely to be enhanced by a cytokine-induced upregulation, dermatochalasis presents with no inflammatory changes. Therefore, ETBR localization strongly suggests constitutive expression of this receptor and indicates lymphatic endothelium as a target site for ET-1. Although ETAR and ETBR on smooth muscle cells cause vasoconstriction, endothelial ETBR stimulation causes a regional vasodilation by releasing prostacyclin or nitric oxide (26). In this study, ET-1 immunoreactivity in absorbing lymphatic vessels was patchy or absent in accord with findings of Ohkuma (27) in human foreskin. In contrast, a strong continuous endothelial ET-1 reactivity has been detected in bovine (28) and porcine (29) lymphatic collectors, which is probably due to the different role and structure of the collecting vessels. As lymphatic smooth muscle cells utilize ET-1 pathway for vasomotion (15), a higher ET-1 endothelial synthesis may be required.

Age-related clinical pathological changes of eyelid skin are a rapidly expanding field of interest in medicine and corrective surgery, which takes into account the abundant blood supply and rich vascularization. Therefore, a detailed knowledge of lymphatic morphology and functional properties in this location may be useful in developing treatment strategies.

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