Lymphatic Vessels in Vascularized Human Corneas: Immunohistochemical Investigation Using LYVE-1 and Podoplanin

Claus Cursiefen,[1] Ursula Schlötzer-Schrehardt,[1] Michael Küchle,[1] Lydia Sorokin,[2] Silvana Breiteneder-Geleff,[3] Kari Alitalo,[4] and David Jackson[5]

Purpose. To determine whether lymphatic vessels exist in vascularized human corneas, by using immunohistochemistry with novel markers for lymphatic endothelium.

Methods. Human corneas exhibiting neovascularization secondary to keratitis, transplant rejection, trauma, and limbal insufficiency ($n = 21$) were assessed for lymphatic vessel content by conventional transmission electron microscopy and by immunostaining and immunoelectron microscopy with antibodies specific for the lymphatic endothelial markers, lymphatic vessel endothelial hyaluronan receptor (LYVE-1) and the 38-kDa integral membrane glycoprotein podoplanin. In addition, corneas were stained for the lymphangiogenic growth factor VEGF-C, and its receptor VEGFR3 by immunohistochemistry and in situ RNA hybridization, respectively.

Results. Thin-walled, erythrocyte-free vessels staining with lymphatic markers (LYVE-1 and podoplanin) were found to constitute 8% of all vessels, to be more common in the early course of neovascularization, to be always associated with blood vessels and stromal inflammatory cells, and to correlate significantly with the degree of corneal hemangiogenesis ($r = 0.6$; $P = 0.005$). VEGF-C, VEGFR3, podoplanin, and LYVE-1 colocalized on the endothelial lining of lymphatic vessels. With immunogold labeling, LYVE-1 and podoplanin antigen were found on endothelial cells lining vessels with ultrastructural features of lymph vessels.

Conclusions. Immunohistochemistry with novel lymph-endothelium markers and ultrastructural analyses indicate the existence of lymphatic vessels in vascularized human corneas. Human corneal lymphangiogenesis appears to be correlated with the degree of corneal hemangiogenesis and may at least partially be mediated by VEGF-C and its receptor VEGFR3. (*Invest Ophthalmol Vis Sci.* 2002;43:2127–2135)

From the [1]Department of Ophthalmology and [2]Nikolaus-Fiebiger-Zentrum for Molecular Medicine, University of Erlangen-Nürnberg, Erlangen, Germany; the [3]Department of Clinical Pathology, University of Vienna, Vienna, Austria; the [4]Haartman Institute, Molecular and Cancer Biology Laboratory, University of Helsinki, Helsinki, Finland; and the [5]Medical Research Council Human Immunology Unit, Institute of Molecular Medicine, Oxford, United Kingdom.

Supported by Federal Ministry of Education and Research (BMBF) Grant B13 to the Interdisciplinary Center for Clinical Research (IZKF), Erlangen, and German Research Council Grant Cu 47/1-1.

Presented in part at the annual meeting of the Association for Research in Vision in Ophthalmology, Fort Lauderdale, Florida, May 2001.

Submitted for publication January 7, 2002; accepted March 6, 2002.

Commercial relationships policy: N.

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Corresponding author: Claus Cursiefen, The Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, 20 Staniford Street, Boston, MA;
cursiefen@vision.eri.harvard.edu.

The normal human cornea has no blood or lymphatic vessels. Corneal avascularity contributes to the enhanced prognosis of corneal transplantation compared with other solid organ transplantation by suppressing both "arms" of a potential "immune reflex" that could lead to transplant rejection after keratoplasty.[1,2] However, secondary to a wide range of diseases, the cornea can become vascularized, a phenomenon that has been described both clinically and histopathologically.[3]

In recent years, it has become clear that—for example, in tumor growth and granulation tissue—lymphangiogenesis (i.e., the outgrowth of new lymphatic vessels) can occur together with hemangiogenesis (i.e., the outgrowth of new blood vessels).[4–6] Therefore, the question arises of whether lymphangiogenesis occurs in diseased vascularized human corneas. In the rabbit, both processes were indeed found to occur during experimental corneal neovascularization (CNV) in earlier studies in which lymphatic vessels were convincingly demonstrated both ultrastructurally and by drainage of ink, which was injected into vascularized corneas, into regional lymph nodes.[7–17] In humans, should lymphatic vessels occur in the pathologically vascularized cornea before keratoplasty, they could increase the risk of subsequent immunologic graft rejection, not only by accelerating antigen recognition, but also by eliminating anterior chamber associated immune deviation (ACAID).[1]

Because of the hitherto lack of specific markers for lymphatic endothelium, a detailed analysis of human corneal lymphangiogenesis (CL) has until now been unavailable. With the identification of VEGFR3, a receptor for VEGF-C, and more recently, podoplanin and lymphatic vessel endothelial hyaluronan (HA) receptor (LYVE-1), new specific markers of lymphatic endothelium have become available. VEGFR3 belongs to the family of endothelium-specific receptor tyrosine kinases and is expressed on venous and lymphatic endothelium in the fetal vasculature, but becomes restricted to lymphatic endothelium in the adult.[18–21] However, VEGFR3 is not solely expressed on lymphatic endothelium in the adult, but is also present on proliferating and some fenestrated endothelia.[22,23] Recently, in experimental rat CNV, expression of VEGF-C was reported in invading inflammatory cells, and VEGFR3 was detected in vessels with the ultrastructural features of lymphatics.[24] Podoplanin, a 38-kDa membrane glycoprotein originally identified on podocytes, is expressed on the endothelium of lymphatic capillaries, but not in quiescent or proliferating blood vascular endothelium.[25–28] LYVE-1, a new lymphatic-specific receptor for HA, is expressed on lymphatic but not blood vascular endothelium, with the possible exception of liver sinusoids (Prevo RK, Weigel PH, Jackson DG, unpublished observation, 2001).[29–31]

The purposes of this study were threefold: (1) To analyze neovascularized human corneas immunohistochemically and ultrastructurally for evidence of lymphatic vessels, by using new markers of lymphatic endothelium (LYVE-1, podoplanin), (2) to compare expression of the lymphangiogenic growth factor VEGF-C and its receptor VEGFR3 in normal and vascu-

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larized human corneas, and (3) to investigate the relationship between blood vessel density, duration of CNV and CL.

Material and Methods

Light Microscopy and Classification of Normal and Vascularized Human Corneas

Paraffin-embedded 4-μm sections of randomly retrieved vascularized corneas obtained by penetrating keratoplasty and sent to our Ophthalmic Pathology Laboratory for histopathologic evaluation were included in this study ($n = 21$; 11 male; mean age, 55 ± 18 years; range, 19–76). In 12 cases this was the first penetrating keratoplasty, in 9 it was a regrafting. CNV was secondary to trauma ($n = 2$); limbal insufficiency in aniridia ($n = 2$); transplant insufficiency ($n = 7$), two of which occurred after transplant rejection; and herpetic keratitis ($n = 10$). The duration of CNV was estimated retrospectively as the time interval between the first notion of CNV in patients' charts and subsequent penetrating keratoplasty (47 ± 55 months; range, 0.5–204). The degree of corneal hemangiogenesis was calculated as the number of all von Willebrand factor (vWF)$^+$/LYVE-1$^-$ vessel cross sections on a representative section of each vascularized human cornea analyzed ($n = 21$; degree of CL determined by the number of vWF$^+$/LYVE-1$^+$ vessel cross sections). Control samples included nonvascularized corneas obtained after transplant rejection[5] ($n = 5$), corneas with keratoconus and Fuchs endothelial dystrophy[3] ($n = 3$), and normal corneoscleral donor buttons not used for transplantation ($n = 3$).[3] For immunofluorescence microscopy, cryoconserved corneas vascularized secondarily to transplant rejection ($n = 2$) and herpetic keratitis ($n = 2$) were used with the above-mentioned control samples. The research was conducted according to the tenets of the Declaration of Helsinki.

Indirect Immunohistochemistry

Consecutive 4-μm sections from normal, diseased nonvascularized, and vascularized human corneas were stained with a monoclonal mouse anti-human antibody against VEGFR3 (1:100), and polyclonal rabbit anti-human antibodies against podoplanin (1:200), LYVE-1 (1:100), vWF (1:100; Dako, Hamburg, Germany) and VEGF-C (1:60; Zymed, South San Francisco, CA) at least twice, using the streptavidin-biotin-method, as described previously.[32] Briefly, for LYVE-1, vWF, and VEGFR3, tissue fixed in neutral buffered formalin was embedded in paraffin and 4-μm sections cut. After deparaffinization and rehydration, sections were digested with proteinase K (Dako) before incubation with peroxidase for 10 minutes. Sections were then incubated with primary antibody (30 minutes) and horseradish peroxidase (HRP)–conjugated secondary antibody before development with 3-amino-9-ethylcarbazole (AEC)$^+$ substrate (red reaction product) or 3,3′-diaminobenzidine (DAB; brown product). Finally, the sections were counterstained with Mayer hemalalm (Chroma, Münster, Germany) and mounted in an aqueous-based medium (Faramount; Dako). For VEGF-C, a kit was used (NBA, Zymed). For podoplanin, sections were pretreated by microwaving (600 W; 10 minutes)[25] and subsequently were incubated with podoplanin antibody (0.5 μg/mL) for 1 hour at 20°C, followed by biotinylated goat anti-rabbit IgG for 30 minutes and detection by a streptavidin-peroxidase complex (using DAB/AEC$^+$ as the chromogen substrate). Isotype-matched antibody and preimmune serum were included as the negative control and showed no staining of normal human tissue or tumor tissue (Human Tissue Checkerboard; Dako). Sections were photographed with a microscope (Axiophot; Carl Zeiss, Oberkochen, Germany) using color film (Ektachrome 64 T; Eastman Kodak, Rochester, NY).

Immunofluorescence Microscopy

Tissue specimens were embedded in optimal cutting temperature (OCT; Miles Diagnostics, Elkhart, IN) compound and snap frozen in isopentane-liquid nitrogen. Cryostat sections (6 μm) were fixed in cold acetone and incubated overnight at 4°C in a 10% solution of normal goat serum in PBS, containing primary antibody diluted in PBS (VEGFR3 and LYVE-1: 1:100; CD31 [Dako]: 1:40; podoplanin: 1:1000; VEGF-C: 1:200). Antibody binding was detected by incubating the sections with a mixture of fluorescent dye—conjugated (Alexa Fluor 488; Molecular Probes, Leiden, The Netherlands) and Cy3-conjugated secondary antibodies (1:100; Molecular Probes) in PBS for 30 minutes at room temperature.

Immunoelectron Microscopy

Immunoelectron microscopy was performed on a specimen confirmed to contain lymphatic vessels by immunochemical staining with affinity-purified LYVE-1 antibody (dilution: 1:2000) and podoplanin antibody (dilution: 1:8000). Briefly, ultrathin sections of resin embedded (LR-White; London Resin Co., Basingstoke, UK) corneal tissue were incubated successively in drops of Tris-buffered saline (TBS), 0.05 M glycine in TBS, 0.5% ovalbumin, and 0.5% gelatin in TBS, primary antibody diluted in TBS-ovalbumin overnight at 4°C, and 10 nm gold-conjugated secondary antibody (BioCell, Cardiff, Wales, UK) diluted in 1:30 in TBS-ovalbumin for 1 hour at room temperature. After they were rinsed, the sections were stained with uranyl acetate and examined with an electron microscope.

In Situ Hybridization

In situ hybridization was performed as described, using a VEGFR3 cDNA probe.[33] A 3564-bp plasmid (pBluescript II SK +/−) with a 595-bp *Eco*RI-*Sph*I fragment covering base pairs 1-595 from the coding sequence of human VEGFR3 was used. The construct was linearized with either *Eco*RI, to obtain the template for T3 antisense RNA, or with *Hind*III, to obtain the template for T7 sense RNA (negative control). Briefly, 4-μm paraffin-embedded vascularized corneal sections were fixed on silanized coverslips ($n = 15$). The RNA probe was obtained by in vitro transcription, DNase I digestion, and RNA hydrolysis. After prehybridization, hybridization for 12 hours with radioactively labeled uridine triphosphate (UTP), washing, and RNase digestion, sections were exposed (6 weeks), developed and mounted in rapid embedding medium (Entellan; EM Science, Darmstadt, Germany).

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed on segments of vascularized corneas when non–paraffin-embedded material was available for analysis ($n = 11$; model EM 906; Leo, Oberkochen, Germany). For the classification of corneal blood and lymphatic vessels at the TEM level, the definitions outlined in References 11, 34, and 35 were used.

Statistics

Correlation analyses between the number of lymph and blood vessel cross sections was performed with the Spearman test and group comparisons with the Mann-Whitney test (SPSS ver. 10.0; SPSS Science, Chicago, IL).

Results

Immunohistochemistry in Normal, Nonvascularized, and Vascularized Human Corneas

Podoplanin. In positive control tissue (e.g., normal conjunctiva), the endothelial lining of non–erythrocyte-filled, thin-walled (i.e., lymphatic) vessels stained positively, whereas erythrocyte-filled (i.e., blood) vessels were not stained. In normal corneas and corneas with keratoconus and Fuchs endothelial dystrophy, a positive reaction was seen in the basal layers of the corneal epithelium, and a weak reaction in some corneal endothelial cells and keratocytes. In vascularized human corneas, a positive reactivity was seen in non–erythrocyte-filled (i.e., lymphatic) vessels, whereas erythrocyte-filled, thick-walled (i.e., blood) vessels did not react with the antibody (Figs. 1, 2).

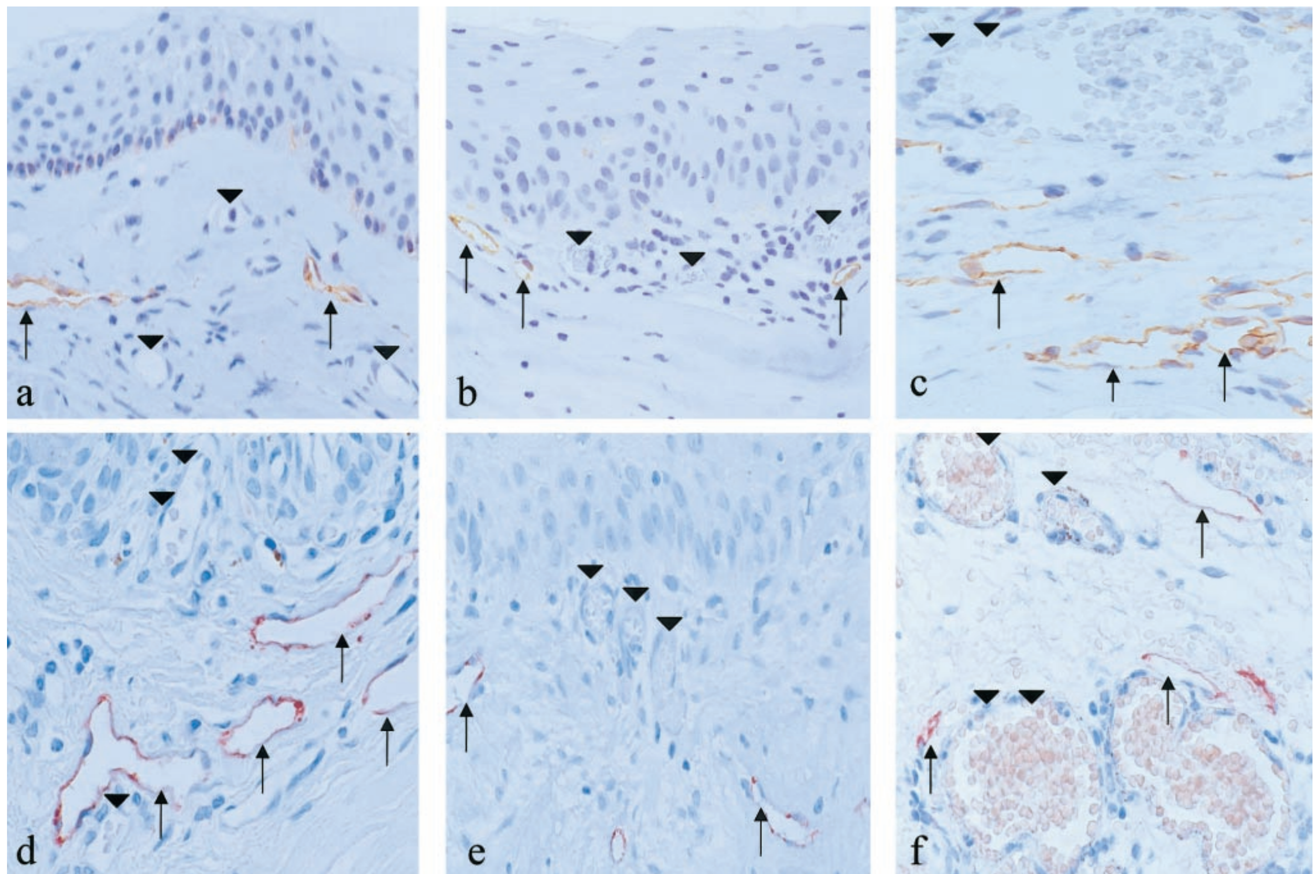

FIGURE 1. Immunohistochemical staining of lymphatic vessels in normal human limbus (**a**, **d**) and in a vascularized human cornea with neovascularization secondary to transplant insufficiency (**b**, **c**; **e**, **f**), detected by using two markers of lymphatic endothelium: podoplanin (**a–c**) and LYVE-1 (**d–f**). Note intense *brown* marking of endothelial lining of thin-walled, non–erythrocyte-filled lymphatic vessels with the antibody against podoplanin (**a**– **c**, *arrows*). In contrast, erythrocyte-filled, thick-walled blood vessels did not react with podoplanin (**a–c**; *arrowheads*). Lymph vessels were present both in the subepithelium and the stroma. Podoplanin in addition weakly stained basal corneal epithelial cells. Antibody against LYVE-1, a lymph-endothelium–specific HA receptor, marked thin-walled, non–erythrocyte-filled lymph vessels *red* (**d**– **f**, *arrows*), whereas thick walled, erythrocyte-filled blood vessels did not react (**d–f**; *arrowheads*). Magnification: (**a**, **b**) ×400; (**c**) ×800; (**d**) ×400; (**e**, **f**) ×800.

LYVE-1. In normal conjunctiva, LYVE-1 stained non–erythrocyte-filled, thin-walled (i.e., lymphatic) vessels, whereas erythrocyte-filled blood vessels were not stained. In normal corneas and in those with keratoconus and Fuchs endothelial dystrophy, no positive reactivity was seen (except for an occasional unspecific nuclear staining in corneal epithelium, keratocytes and endothelium). In vascularized human corneas, no reactivity was seen in erythrocyte-filled vessels, but a strong reactivity was found in non–erythrocyte-filled, thin-walled (i.e., lymphatic) vessels (Figs. 1, 2).

VEGF-C. In positive control tissue (e.g., pyogenic granuloma), a strong positive reaction was seen in inflammatory cells (not shown). In normal human corneas and in those affected by keratoconus and Fuchs endothelial dystrophy, a positive reaction was seen in the basal layers of the corneal epithelium and a weak reaction in some keratocytes and corneal endothelial cells. In vascularized human corneas, VEGF-C reactivity was also seen in inflammatory cells and on the endothelial cells lining some non–erythrocyte-filled, thin-walled (i.e., lymphatic) vessels (Fig. 3).

VEGFR 3. In normal conjunctiva, non–erythrocyte-filled, thin-walled vessels (i.e., lymphatic vessels) stained positively, whereas erythrocyte-filled vessels were not stained. When the VEGFR3 antibody was used in normal human corneas and in those with keratoconus and Fuchs endothelial dystrophy, a positive reaction was noted in the basal layers of the corneal epithelium and a weak reaction in some endothelial cells. In vascularized corneas in addition non–erythrocyte-filled, thin-walled (i.e., lymphatic) vessels and inflammatory cells were partly positive (Fig. 3).

Using in situ-hybridization with a VEGFR3-specific probe, a strong positive signal in dark- and bright-field analysis could be detected in the basal layers of the corneal epithelium of normal and vascularized corneas. Weak positive signals were seen in corneal endothelium of normal and vascularized corneas. In addition, in vascularized corneas a strong reaction was seen in association with pathologic corneal vessels and adjacent inflammatory cells. Tissue sections hybridized with the sense probes showed no signals above background.

Immunofluorescence Microscopy

The above findings were confirmed by single-antibody immunofluorescence microscopy for VEGFR3, VEGF-C, LYVE-1, and podoplanin in normal and vascularized corneas. In addition, double immunofluorescence microscopy revealed colocalization of all four markers in lymphatic vessels and confirmed that the podoplanin and LYVE-1–positive vessels also expressed the panendothelial marker CD31 (Fig. 3).

Clinicopathologic Correlation

According to immunohistochemical criteria, LYVE-1^+/podoplanin$^+$/vWF$^+$ lymphatic vessels were present in 10 (48%) of

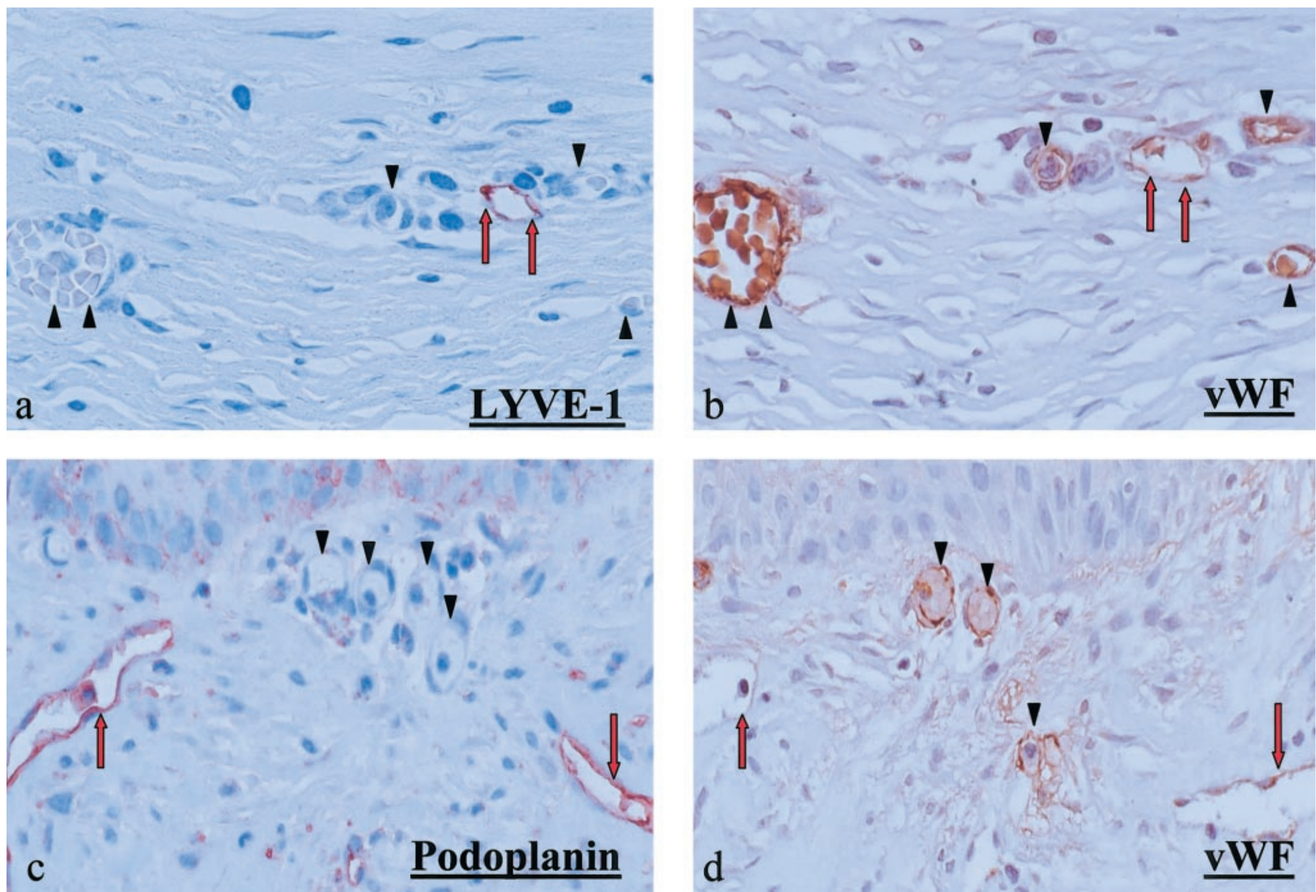

FIGURE 2. Comparative staining of lymphatics in serial sections of a vascularized human cornea with lymphatic endothelial markers (LYVE-1 [**a**] and podoplanin [**c**]) and the panendothelial marker vWF (**b**, **d**). Note that LYVE-1 stained a thin-walled, non–erythrocyte-filled lymphatic vessel *red* (**a**, *arrows*) and did not react with erythrocyte-filled blood vessels (*arrowheads*), whereas vWF stained both endothelium lined vessels. Staining intensity of vWF was weaker in lymph than in blood vessels. Podoplanin, besides basal epithelium, stained non–erythrocyte-filled lymphatic vessels bright *red* (**c**; *arrows*), whereas erythrocyte-filled blood vessels are not stained (*arrowheads*). vWF stained both vessel types but the staining intensity was weaker in lymph vessels than in blood vessels. Magnification: (**a**, **b**) ×800; (**c**, **d**) ×600.

21 vascularized corneas. From a total of 1124 vWF^+ vessel cross sections of the 21 vascularized human corneas, 93 (8.3%) were classified as lymphatic (vWF^+/LYVE-1^+) and 1031 as blood vessels (vWF^+/LYVE-1^-; Table 1). Lymphatic vessel cross sections constituted on average 8.5% ± 10.5% (0–35) of all vWF^+ vessels per section in vascularized corneas with lymphatic vessels present (mean number of lymph vessel cross sections of all corneas with lymphatics: 7.2 ± 4.9; range, 1–16). The duration of CNV was shorter in corneas with lymph vessels detectable by immunohistochemistry (mean, 39.9 ± 24.8 months; range, 0.5–60) compared with those without detectable lymph vessels (72.7 ± 80.5 months; range, 3–204; $P = 0.28$). All corneas with duration of CNV of less than 3 months had lymph vessels detectable by immunohistochemistry, but none was present in corneas with a duration of CNV of more than 60 months. There were significantly more blood vessel cross sections detectable in corneas with lymphatic vessels present (65.7 ± 66.6; range, 13–205) as in corneas without lymphatic vessels (22.1 ± 19.9; range, 3–56; $P = 0.037$), and there was a significant positive correlation between the number of blood (vWF^+/LYVE-1^-) and lymphatic vessel cross sections (vWF^+/LYVE-1^+; $r = 0.6$, $P = 0.005$; Fig. 4). No lymphatic vessels were detected in either the five nonvascularized corneas obtained after transplant rejection or in the nonvascularized control sections. Lymphatic vessels were located in the outer third of corneal buttons (toward the limbus), both within the stroma and beneath the epithelium and were always associated with inflammatory cells, blood vessels, and a disorganized stromal architecture in the vicinity (Fig. 5).

Ultrastructural Findings in Vascularized Human Corneas

All vascularized corneal specimens analyzed were scored as containing blood vessels (Fig. 6). However, only 1 of 11 specimens analyzed contained unequivocal lymphatic vessels (relative proportion: 17% [6 of 35 vessel cross sections]). Because only a small segment of each cornea was analyzed ultrastructurally, this datum cannot be compared with the immunohistochemical results in whole corneal sections. Furthermore, in disorganized and edematous vascularized corneas, ultrastructural identification of collapsed lymph vessel is difficult, and therefore only unequivocal lymph vessels with open lumens were counted. In inflamed and disorganized corneas, in addition, abundant lymphatic clefts (i.e., nonendothelium-lined clefts between collagen fiber bundles and keratocytes) were observed, partly filled with macrophages and lymphocytes.

Immunoelectron Microscopy with LYVE-1 and Podoplanin in Vascularized Human Corneas

TEM, with immunogold-labeled polyclonal antibodies to LYVE-1, revealed small clusters of gold particles along the luminal endothelial face of lymphatic vessel endothelial cells (Fig. 7). Gold particles were only rarely detectable within the

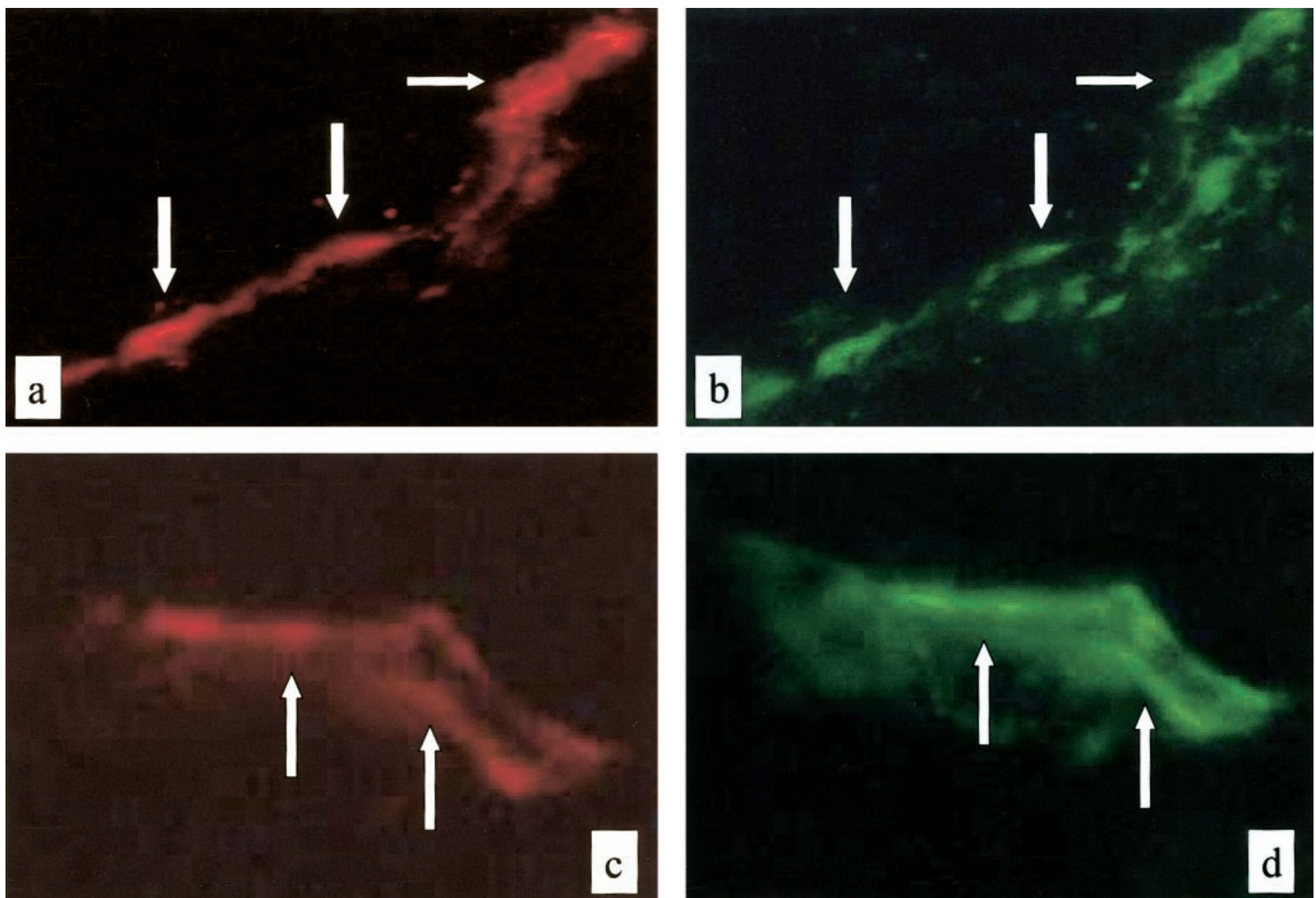

FIGURE 3. Double immunofluorescence microscopy demonstrating colocalization of VEGFR3 (**a**) and LYVE-1 (**b**), as well as CD31 (**c**) and VEGF-C (**d**) on the endothelial lining of a lymphatic vessel in a vascularized human cornea. Lymphatic vessels demonstrated reactivity with LYVE-1, podoplanin, VEGFR3 and VEGF-C. Magnification, ×800.

endothelial cells themselves. The polyclonal antibody against podoplanin yielded a similar staining pattern.

DISCUSSION

Evidence for human, in contrast to experimental, CL has until now been scarce. The concept of CL itself is well established, because Collin[8–13] and others[14–17] have provided evidence for the presence of lymphatic vessels in experimentally vascularized animal corneas, by using ultrastructural analysis and ink-injection experiments. In the case of vascularized human corneas, vessels with the ultrastructural features of lymphatics have been reported only in two corneas as the result of conjunctival transplantation secondary to chemical burns.[7] Immunohistochemical demonstration of CL has been hampered by the unavailability of specific markers until the recent discovery of VEGFR3, podoplanin, and LYVE-1. In this study, we present immunohistochemical evidence, by the use of each of these markers, together with ultrastructural evidence that lymphatic vessels exist in vascularized human corneas.

Whereas VEGFR3 may be expressed on both blood vascular and lymphatic endothelium, podoplanin and LYVE-1 are more specific markers of lymphatic endothelium.[4,25–31] VEGF-C and -D induce lymphangiogenesis through VEGFR3, which in the adult is expressed on lymphatic endothelium,[18,19] but can also be found on some proliferating[5,19,22] and fenestrated blood vascular endothelial cells.[23] According to our findings, VEGF-C and VEGFR3 may be involved in mediating human CL. Both

TABLE 1. Comparison of Vascularized Human Corneas, with and without Immunohistochemical Evidence of Lymphatic Vessels

	Lymph Vessels Present (n = 10)	No Lymph Vessels Present (n = 11)
Number of blood vessel cross sections (vWF$^+$/LYVE-1$^-$)	65.7 ± 66.6 (13–205)*	22.1 ± 19.9 (3–56)*
Number of lymph vessel cross sections (vWF$^+$/LYVE-1$^+$)	7.2 ± 4.9 (1–16)	0
Duration of corneal neovascularization (mo)	39.9 ± 24.8 (0.5–60)†	72.7 ± 80.5 (3–204)†

Data are expressed as the mean ± SD, with the range in parentheses.
* P = 0.037.
† NS.

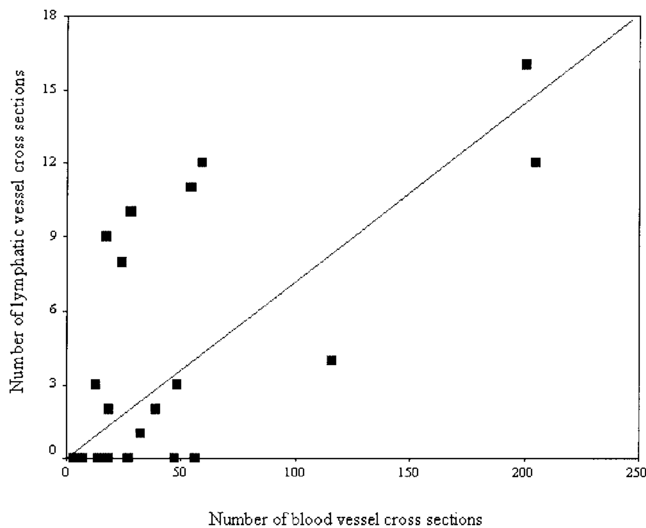

FIGURE 4. Positive correlation between the degree of human corneal hemangiogenesis and lymphangiogenesis. Corneas with more blood vessel cross sections (*x*-axis; $vWF^+/LYVE\text{-}1^-$ vessel) contained more lymph vessel cross sections (*y*-axis: $vWF^+/LYVE\text{-}1^+$; $r = 0.6$; $P = 0.005$; $n = 21$ vascularized corneas).

were detectable on the endothelial lining of corneal lymph vessels and VEGF-C, in addition, in infiltrating inflammatory cells. Podoplanin, a 38-kDa membrane glycoprotein, has been localized to lymphatic but not to blood vascular endothelium and colocalizes with VEGFR3.[25–28,36] LYVE-1, a major receptor for HA, is expressed on vessels exhibiting ultrastructural features of lymphatics, colocalizes with VEGFR3 and podoplanin, and is not expressed on blood vascular endothelium, apart from liver sinusoids (Prevo R., Weigel PH, and Jackson DG, unpublished observation, 2001).[4,29–31,37–39] HA, the ligand for LYVE-1 is only found in trace amounts in the normal corneal endothelium.[40] However, in the injured cornea, the synthesis of HA is upregulated in all layers,[40] most likely in response to cytokines released by infiltrating inflammatory leukocytes.[41] Consequently, it is tempting to speculate that LYVE-1 on the luminal face of lymphatic vessels could facilitate drainage of HA from the injured or inflamed cornea to regional lymph nodes. CL may therefore be beneficial for reestablishment of corneal transparency after injury or inflammation.

In relation to subsequent corneal transplantation, corneal lymphatics in vascularized human host beds adjacent to grafted tissue may enhance antigen presentation in regional lymph nodes and promote rejection.[8,12] This process may also be facilitated by the constitutive secretion of secondary lymphoid

FIGURE 5. Indirect immunohistochemistry for the lymphatic endothelial marker LYVE-1 demonstrates lymphatic vessels adjacent to donor tissue. (**b**) Higher magnification of *inset* in (**a**) demonstrates *red* LYVE-1 immunoreactivity in lymphatic vessels (*arrows*) associated with inflammatory cells and adjacent nonreactive erythrocyte-filled blood vessels (*arrowheads*). Magnification: (**a**) ×100; (**b**) ×800.

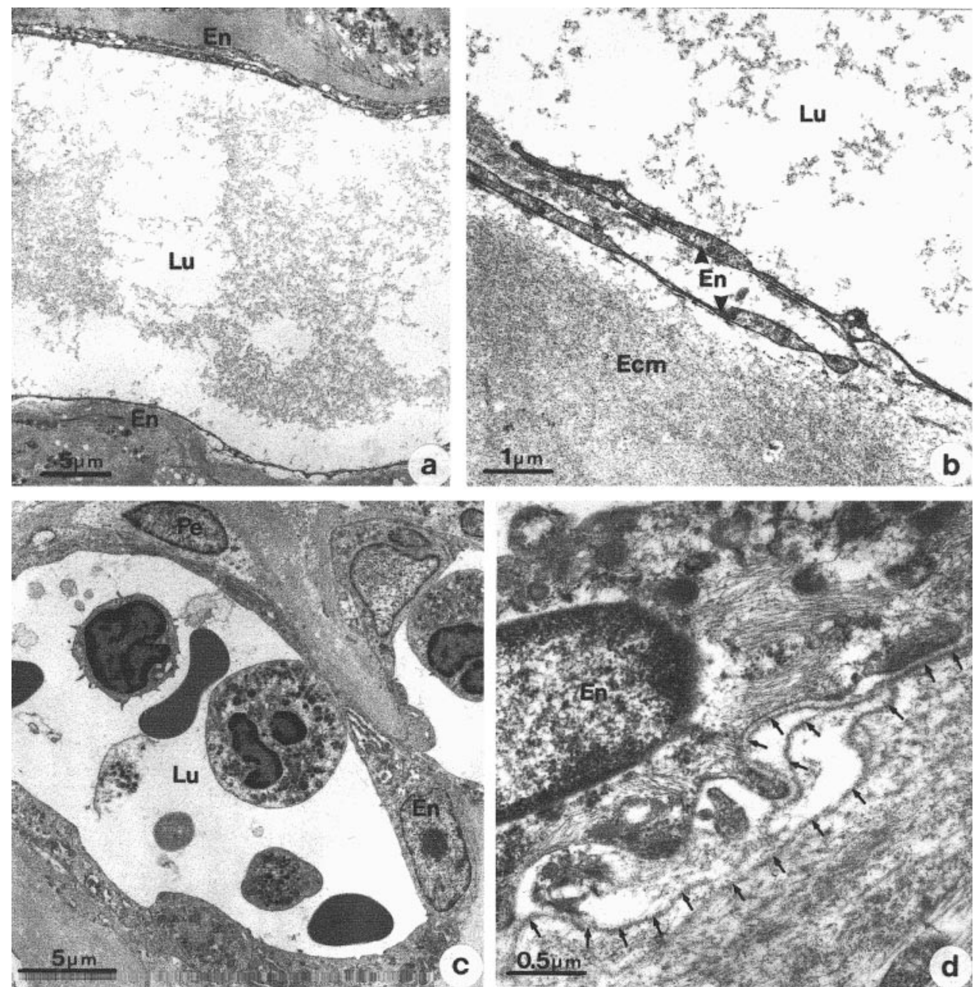

FIGURE 6. Ultrastructural features of a lymphatic (**a**, **b**) and a blood vessel (**c**, **d**) from a vascularized human cornea, 2 weeks after a penetrating injury, as assessed by transmission electron microscopy. Note partly overlapping endothelial cells (En, *arrowheads*) allowing material influx and thin endothelial cells, without pericytes (PE) or tight junctions, and absent continuous basement membrane (**c**, **d**), typical features of a lymphatic vessel. No erythrocytes were detectable within the lumen. A typical blood vessel from the same cornea displaying continuous multilayered basement membrane (**c**; *arrows* in **d**) erythrocytes in the lumen and pericytes covering the vessel. Lu, lumen; Ecm, extracellular matrix; En, endothelial cell.

tissue chemokine (SLC) by lymphatic endothelia which could lead to the recruitment of limbal and corneal dendritic cells to corneal lymphatics and draining lymph nodes.[38,42]

Because lymph vessels occurred only in vascularized corneas, correlated with the degree of corneal hemangiogenesis, were more common in corneas with a short duration of CNV, and were absent in corneas with a long history of CNV, it is suggested that CL follows CNV, is related to the degree of CNV, and regresses earlier than CNV. In experimental skin wounds, transient lymphangiogenesis occurred with a slight delay after hemangiogenesis, with lymphatic vessels being fewer and regressing earlier than blood vessels.[5] The delay of CL after CNV may be related to the fact that blood vascular endothelium secretes VEGF-C, so that blood vasculature has to be established before induction of CL by VEGF-C.[38] The mechanisms of adult lymphangiogenesis are not yet fully clear, but new corneal lymphatic vessels most likely develop from preexisting limbal lymphatic vessels, rather than from primary stem cells.[19,20]

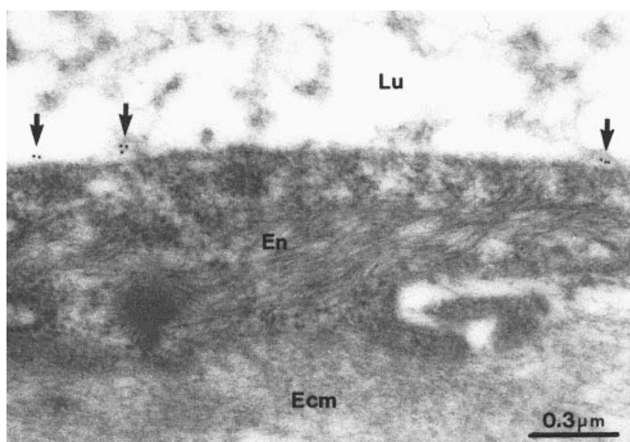

FIGURE 7. Electron microscopy of a vascularized human cornea with immunogold labeled antibody to LYVE-1. In the specimen shown, vascularization was secondary to a previous penetrating corneal injury. Note gold particles (*arrows*) along the luminal endothelial surface (En) of a vessel with the ultrastructural features of a lymphatic vessel. Similar results were found with the antibody against podoplanin. Lu, lumen; Ecm, extracellular matrix.

Human CNV potentially associated with CL can occur in two settings in relation to corneal grafting: CNV before and CNV subsequent to corneal transplantation.[43,44] Because most corneal diseases leading to CNV are inflammatory in nature[3] and VEGF-C is upregulated by proinflammatory cytokines, it is not surprising that CL here was found only in corneas with stromal inflammatory cells present.[20,45] In CNV occurring after keratoplasty,[43,44] VEGF-C may be released from inflammatory cells or by suture-induced damage of basal epithelium containing VEGF-C. Upregulation of proinflammatory cytokines was observed after corneal grafting,[46] suggesting release of lymphangiogenic growth factors by surgical trauma or wound healing.

In conclusion, immunohistochemical and ultrastructural findings support the existence of human CL. CL is less extensive than hemangiogenesis and seems to occur during the early

phase of CNV, to regress before blood vessels, and to be associated with inflammation. Demonstration of both VEGFR3 and its lymphangiogenic ligand VEGF-C may indicate involvement of these factors in mediating human CL. The existence of corneal lymphatic vessels could partly explain the increased rate of transplant rejections occurring in vascularized host corneas. Therapeutic inhibition of CL may thus enhance transplant survival both in the high- and low-risk setting.

Acknowledgments

The authors thank Carmen Hofmann-Rummelt, Ophthalmic Pathology Laboratory (immunohistochemistry); Elke Meyer, Electron Microscopy Laboratory, Department of Ophthalmology (electron microscopy); and Friederike Pausch, Nikolaus-Fiebiger-Zentrum for Molecular Medicine (in situ hybridization) for providing excellent technical assistance.

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