Cellular-Level Characterization of Lymph Vessels in Live, Unlabeled Corneas by In Vivo Confocal Microscopy

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PURPOSE. To determine whether in vivo confocal microscopy (IVCM) of the cornea can be used for the label-free detection and monitoring of lymph vessels in live corneas.

METHODS. Parallel corneal hemangiogenesis and lymphangiogenesis was induced by the placement of a single suture in one cornea of male Wistar rats. Fourteen days after suture placement and under general anesthesia, laser-scanning IVCM was performed in the vascularized region. Corneas were subsequently excised for flat-mount double immunofluorescence with a pan-endothelial marker (PECAM-1/CD31) and a lymphatic endothelial specific marker (LYVE-1). Using the suture area and prominent blood vessels as points of reference, the identical microscopic region was located in both fluorescent and archived in vivo images. Additionally, vessel diameter, lumen contrast, and cell diameter and velocity within vessels were quantified from in vivo images.

RESULTS. Comparison of identical corneal regions in fluorescence and in vivo revealed prominent CD31+/LYVE-1+ lymph vessels that were visible in vivo. In vivo, corneal lymph vessels were located in the vascularized area in the same focal plane as blood vessels but had a darker lumen (P < 0.001) sparsely populated by highly reflective cells with diameters similar to those of leukocytes in blood vessels (P = 0.61). Cell velocity in lymph vessels was significantly reduced compared with blood particle velocity (P < 0.001). Morphologic characteristics enabled subsequent identification of corneal lymphatics in live, vascularized rat corneas before immunofluorescence labeling.

CONCLUSIONS. IVCM enabled the nondestructive, label-free, in vivo detection of corneal lymphatics. IVCM provides the possibility of observing lymphatic activity in the same live corneas longitudinally and, as a clinical instrument, of monitoring corneal lymphatics in live human subjects. (Invest Ophthalmol Vis Sci. 2010;51:830–835) DOI:10.1167/iovs.09-4407

Under normal circumstances, the angiostatic nature of the cornea contributes to the maintenance of its immune privilege. Secondary to disease, trauma, or infection, however, this angiostasis can be compromised and can result in corneal neovascularization characterized by the parallel invasion of blood and lymphatic vessels (hemangiogenesis and lymphangiogenesis), with subsequent loss of corneal clarity and vision and a poorer prognosis for treatment.

Although immune cells enter the cornea through the afferent blood vasculature, it is the corneal lymph vessels that provide a direct efferent route for antigen-presenting cells to access regional lymph nodes. The critical role of lymph vessels is further underscored in cancer-induced lymphangiogenesis, by which the dissemination of tumor cells through lymph vessels is believed to initiate tumor metastasis. Despite the importance of lymphangiogenesis, however, relatively little is known about the dynamics of lymph vessel formation, growth, and regression or about cellular activity within lymphatics.

Unlike corneal blood vessels, which can be easily visualized and assessed by slit lamp biomicroscopy, corneal lymph vessels have long eluded in vivo detection because of the transparency of lymphatic endothelial cells and the lymph fluid. As a result, lymphatic research has advanced through the use of vascular endothelial markers, such as LYVE-1, Prox1, and Podoplanin, with the drawback that destructive ex vivo analysis precludes longitudinal observation of the same cornea or the study of cell transport within lymphatics. Moreover, current methods of visualizing lymph vessels limit lymphatic research to animal models or to a retrospective analysis of excised human corneal grafts in failed transplantations.

To date, there has been no report of nondestructive, label-free, in vivo observation of lymphatics or of any technique that can be directly applied to the observation of lymphatics in human subjects. Therefore, we undertook this study to determine whether in vivo confocal microscopy of the cornea, a nondestructive and label-free clinical technique, could be used to identify lymph vessels in their native state in a live cornea.

METHODS

Rat Model of Suture-Induced Inflammatory Corneal Neovascularization

Twelve- to 16-week-old male Wistar rats, each weighing 300 to 400 g (Scanbur AB, Sollentuna, Sweden), were used. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. With approval from the Linköping regional animal ethics review board, each rat was anesthetized by intraperitoneal injection of dexdomitor and xylazine (20 mg/kg body weight and 75 mg/kg body weight, respectively). Under an operating microscope, a single 10–0 nylon suture was placed through the corneal stroma approximately 1.5 to 2 mm from the temporal limbus at the 9 o’clock position in the right eye. Suture placement was chosen to limit neovascularization to a well-defined area and to facilitate access to the vessels by in vivo confocal microscopy. Two days before in vivo examination, chloramphenicol ointment (Chloromycetin; Pfizer AB, Sollentuna, Sweden) was applied in the sutured cornea (twice in 24 hours) to reduce the effect of any presumed secondary infection and thereby minimize the potential of corneal haze. Fourteen days after the...
In Vivo Confocal Microscopy

With rats under general anesthesia and in the prone position, corneas were examined with a laser-scanning in vivo confocal microscope with corneal imaging module (HRT3-RCM; Heidelberg Engineering, Heidelberg, Germany). The microscope was equipped with a 63×/0.95 NA water-immersion objective (Zeiss, Oberkochen, Germany), providing an en face view of a 400 × 400-μm corneal area at a selectable corneal depth. The instrument was prepared as for a routine clinical corneal examination according to the manufacturer’s instructions. Briefly, a large drop of transparent tear gel (Bausch & Lomb, Rochester, NY) was placed on the microscope objective lens, and a sterile disposable plastic cap (Tomocap; Heidelberg Engineering, Heidelberg, Germany) was affixed over the gel-coated lens. A second drop of tear gel was placed on the outer surface of the cap, and the gel-coated cap was brought into contact with the cornea in the suture region using the HRT3 manual alignment controls. The focus depth of the HRT3 was initially adjusted to image the corneal epithelial surface, and the lateral and transverse microscope alignments were adjusted to locate the suture in the real-time image display window. Once the suture was located, the focal plane was adjusted axially to locate blood vessels. Digital images were recorded at 5 frames/s, and the probed region was adjusted in lateral, transverse, and axial directions during image capture to locate and follow the path of vessels from the suture area to the limbus. A typical corneal examination consisted of 30 to 50 image sequences, with each sequence containing 100 successive digital image frames. Sequences could be analyzed frame-by-frame or in video mode, played at the acquisition rate.

Double Immunofluorescence of Corneal Hemangiogenesis and Lymphangiogenesis

After in vivo microscopy, animals were euthanatized by intraperitoneal injection of 100 mg/kg pentobarbital sodium (Apoteket AB, Stockholm, Sweden), and the entire cornea with scleral rim was excised and prepared for flat mounting with three short radial cuts. Corneas were immediately rinsed in PBS, fixed in acetone, rinsed in PBS, blocked in 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Bar Harbor, ME), and incubated with LYVE-1 overnight (goat polyclonal, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA). The next day, samples were washed and stained with Cy2 (donkey anti-mouse, 1:100; Jackson ImmunoResearch Laboratories), washed and stained with pan-endothelial marker (PECAM-1/CD31; mouse monoclonal, 1:100; Santa Cruz Biotechnology) over the following night, washed and stained with Cy3 (donkey anti-goat, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA). The Cy2 secondary antibody fluoresced green under blue laser (488 nm) excitation, and Cy3 fluoresced red under green laser (543 nm) excitation. Samples were scanned under dual laser excitation, and a digital camera was used to record images.

Quantification of In Vivo Vessel Morphology

Individual in vivo image frames of blood and lymph vessels within two rat corneas were analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/jij/index.html). Images with distinct vessels were selected that contained blood and lymph vessels at the same distance from the limbus and, where possible, at the same corneal depth. Blood and lymph vessel diameters were measured manually with a line tool by two independent observers; no significant differences were detected between observers for blood (P = 0.16, n = 38) or lymph (P = 0.24, n = 38) vessels. Lymph vessel diameter was measured in each vessel segment of differing diameter, whereas blood vessels had a constant diameter and were measured once per vessel. Lymph and blood particle diameters were measured once per cell and only for cells with distinct boundaries. The contrast ratio of the vessel lumen to the surrounding extracellular matrix (ECM) was determined by the ratio of the 8-bit grayscale pixel value at the center of the vessel lumen to the pixel value of the ECM at a distance of one vessel diameter from the center of the lumen in a direction perpendicular to the direction of flow. Cell velocity in lymph vessels represents the velocity of cells that were in motion when images were viewed in sequence (stationary cells were not included). Cell velocity in blood vessels was determined by identifying individual moving cells in the center of blood vessels and measuring their displacement in two successive image frames (200-ms interval) to limit the influence of motion artifacts.

Statistical Analysis

Statistical comparison of vessel parameters was performed with the independent t-test for values that were normally distributed and the nonparametric Mann-Whitney rank sum test for others. Normality was determined by the Kolmogorov-Smirnov test. All comparisons were performed using statistical software (SigmaStat; Systat Software Inc., Chicago, IL), and a two-tailed value of P < 0.05 was considered statistically significant.

RESULTS

In Vivo and Ex Vivo Correlation of Corneal Neovascularization

Fourteen days after suture placement in one eye of a rat cornea, aggressive corneal neovascularization was observed, concentrated in the area between the suture and the temporal limbus (Fig. 1a). With the rat under general anesthesia, the cornea was examined by laser scanning in vivo confocal microscopy with the clinical instrument shown in Figure 1b, which provides an en face view, parallel to the corneal surface. After in vivo examination, rats were anesthetized, and laser scanning in vivo confocal microscopy of the corneas was performed.

Sample images for In Vivo and Ex Vivo Correlation of Corneal Neovascularization

**FIGURE 1.** In vivo examination of a corneal rat model of angiogenesis. (a) Neovascularization observed 14 days after suture placement (white arrow). Blood vessels (black arrows) originating from the limbus extend into the cornea toward the suture region (note: underlying vessels are in the iris and not in the cornea). (b) A gel-coated confocal microscope objective lens is brought into contact with the rat cornea for in vivo examination.
examination, the rat was euthanatized, and the entire cornea with scleral rim was removed while the suture was kept in place. The full-thickness cornea was then prepared for flat mounting to preserve the en face view and was then processed for double immunofluorescence using the pan-endothelial marker PECAM-1/CD31 for blood vessels and the lymphatic endothelium-specific marker LYVE-1 for lymph vessels.

The suture area was located with a confocal laser-scanning fluorescence microscope, and the focal depth was adjusted to locate vascular structures. Blood (CD31+/LYVE-1−) and lymph (CD31+/LYVE-1+) vessels were observed in the immediate vicinity of the suture (Fig. 2). Concurrently, archived in vivo confocal microscope images were manually searched to locate the same microscopic region adjacent to the suture.

**Figure 2.** In vivo and ex vivo confocal image correlation. Retrospective comparison of the same corneal region with in vivo confocal microscopy (a) and ex vivo microscopy with immunofluorescent markers (b, c). Features of blood vessels (a, b, asterisks) aided in the location of the same tissue region. LYVE-1-positive lymph vessels (c, arrows) were also visible in vivo (a, arrows). Depth below corneal surface, 59 μm. Scale bar, 100 μm.

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**Figure 3.** In vivo confocal microscopy in a rat cornea. Presumed corneal lymphatics (a, b, arrows) observed in the rat cornea in vivo 14 days after suture placement. Note the presence of reflective cells (leukocytes) within the vessels and the presence of adjacent blood-filled vessels at the same corneal depth (b, asterisks). Depths below corneal surface: 43 μm (a), 42 μm (b). Images measure 400 × 400 μm.

**Figure 4.** Immunofluorescence confirmation of presumed lymphatics in the rat cornea. The presumed lymphatic observed in vivo (arrows) was confirmed as CD31+/LYVE-1−, with adjacent CD31+/LYVE-1− blood vessels (asterisks) serving as a point of reference. Depth below corneal surface, 43 μm. Scale bar, 100 μm.
Characteristic blood vessel branches and bends were used as points of reference. Once the identical region was found, a direct comparison of in vivo and ex vivo images was possible, revealing the in vivo morphology of blood-carrying vessels and lymph vessels containing cells (Fig. 2).

Prospective Morphologic Detection of Corneal Lymphatics In Vivo

Given that a retrospective, manual search of in vivo images was data intensive and did not permit observation of lymphatic activity in real time, we identified specific morphologic features of lymph vessels from Figure 2 that could aid in their prospective in vivo detection, before ex vivo analysis. In vivo, lymph vessels contained no discernible vessel walls, a dark lumen, and reflective cells (presumed leukocytes) of roughly uniform size that were larger than most particles observed in blood vessels.

To determine whether corneal lymphatics could be prospectively detected based solely on these morphologic features, the rat model of corneal angiogenesis was again used, followed by in vivo confocal microscopy after 14 days. During in vivo examination, several vessels with lumens substantially darker than the surrounding extracellular matrix were observed. These vessels (presumed lymphatics) carried reflective cells of approximately uniform diameter (Fig. 3). The vessels were superficially located at the same corneal depth as blood vessels, approximately 41 to 46 μm below the corneal epithelial surface. Video sequences depicting the motion of leukocytes within these vessels were also taken (Movies S1 and S2, http://www.iovs.org/cgi/content/full/51/2/830/DC1) and revealed a substantially slower movement of cells compared with the flow within blood vessels. After in vivo imaging, corneas were excised and prepared for immunofluorescence. Fluorescent images of the suture area were used to locate the suspected lymphatics, which were confirmed as lymph vessels by CD31+/LYVE-1+ expression (Fig. 4). Additionally, in a single rat cornea without neovascularization, we located presumed lymphatics normally present in the temporal bulbar conjunctiva that were similarly subsequently confirmed as conjunctival lymphatics by immunofluorescence (Fig. 5).

Quantitative In Vivo Analysis of Corneal Blood and Lymph Vessels

Longitudinal observation of corneal lymphatics in vivo in the same animal or in humans without the use of contrast agents requires a means to objectively distinguish lymph vessels from blood vessels. Therefore, we quantified from in vivo images the morphologic characteristics of blood and lymph vessels in the rat cornea (Table 1). Although blood and lymph vessel diameter did not significantly differ, lymph vessels appeared to be constructed of multiple adjoining vessel segments of variable diameter, whereas blood vessels appeared continuous and had constant diameters along their length. Blood vessel branches were abrupt, making well-defined angles relative to the vessel trunk, whereas lymph vessel junctions appeared rounded and more continuous. Additionally, lymphatic vessel walls could not be visualized in vivo. Lymph vessels had a lumen about half as reflective as the surrounding ECM, whereas blood vessels

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**Figure 5.** Immunofluorescence confirmation of lymphatics in the temporal perilimbal conjunctiva of a rat without corneal neovascularization. The same blood vessels seen in vivo in the limbal region (a, b, asterisks, plus signs) were located after immunofluorescent staining with CD31/LYVE-1 (c, asterisk, plus sign). Conjunctival lymph vessels in vivo had a wide, bulging dark lumen with only a few cells visible (a, b, arrows) compared with blood vessels exhibiting a smaller vessel diameter, thicker walls, and a high number of small reflecting cells. Lymphatics were revealed by immunofluorescence to be part of a three-dimensional conjunctival lymphatic network (c, CD31+/LYVE-1+, red). Note the vessels in the limbal region (asterisk, plus sign) appear with distortion in c because of the flat-mount artifact. Depths below corneal surface: 57 μm (a), 44 μm (b). Images measure 400 × 400 μm.

**Table 1. Quantitative Analysis of In Vivo Morphologic Parameters of Corneal Vessels**

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<tr>
<th>Parameter</th>
<th>Lymph Vessels</th>
<th>Blood Vessels</th>
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<tr>
<td>Vessel diameter, μm</td>
<td>19.9 ± 6.7</td>
<td>24.3 ± 11.7</td>
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<tr>
<td>Lumen/ECM contrast ratio</td>
<td>0.46 ± 0.16</td>
<td>1.85 ± 0.82</td>
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<tr>
<td>Leukocyte diameter, μm</td>
<td>7.6 ± 1.3</td>
<td>7.4 ± 1.4</td>
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<tr>
<td>Cell velocity, μm/s</td>
<td>53 ± 42</td>
<td>208 ± 73</td>
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<td>Value</td>
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* Bold values indicate significant parameter differences between blood and lymph vessels.
dense with reflective, flowing particles had a lumen almost twice as reflective as the ECM. Thus, the lumen of blood vessels was on average four times as reflective as the lymph vessel lumen in the same corneal region. Leukocytes within lymph vessels were of uniform diameter, were highly reflective, and did not appear to differ morphologically from leukocytes in blood vessels. Notably, however, cells with leukocyte morphology were the only cells observed in lymphatics, whereas blood vessels contained both smaller particles (presumed erythrocytes; most cells, 2–3 μm in diameter) and leukocytes (minority). Leukocyte velocity in lymph vessels was significantly lower than the central particle velocity in blood vessels, by a factor of 4 on average. Although leukocytes in blood vessels appeared to flow along with erythrocytes in the vessel center or to "roll" along the inner blood vessel walls, all leukocytes we observed in corneal lymphatics did not move along vessel walls, and no visual evidence of rolling could be detected. Additionally, within lymphatics, leukocytes sometimes appeared stationary within the duration of observation (tens of seconds), and sometimes changed their direction of motion.

**DISCUSSION**

Advances in noninvasive in vivo microscopy have enabled the repeated imaging of live human and animal tissue without the use of exogenous contrast agents but with the drawback that images contain purely morphologic information without molecular-level specificity. Although attempts have been made to correlate in vivo morphology with ex vivo staining of histologic sections or immunofluorescence from impression cytology, so far an exact correlation has not been possible because of the technical difficulty of imaging the identical tissue region in the same anatomic view and with similar resolution both in vivo and ex vivo.

We found that by providing the same en face, high-resolution confocal microscopic view of the same tissue region both in vivo and ex vivo, a direct correlation of tissue morphology with molecular expression was possible, leading to the identification of corneal lymph vessels in vivo. Moreover, the morphology of lymph vessels was highly specific and enabled subsequent monitoring of lymphatic activity—including the dynamics of leukocyte movement within lymphatics—prospectively and without the use of contrast agents. This result suggests that by repeatedly probing the same tissue region in vivo, the unique morphologic features of specific lymph vessels (such as their shape and relative location) can be used to longitudinally monitor the same lymph vessel in vivo.

In this study, corneal lymphatics in their native state appeared morphologically distinct from adjacent blood vessels. Lymph vessels had dark lumen in vivo, which is consistent with the transparency of the lymph fluid. Segmented, bulging lymph vessel walls resulted in a variable vessel diameter in vivo, consistent with the lymphatic vascular endothelial morphologic observed ex vivo. Although leukocyte morphology in blood and lymph vessels was similar, leukocyte velocity in lymphatics was on average four times as slow as blood velocity in the inflamed area. It was additionally observed that lymphatic leukocytes changed their direction of motion though others appeared stationary, behavior in accordance with known oscillations in the flow of lymph. Our simple method of calculating blood velocity by particle tracking, however, might have resulted in an underestimate of the true blood velocity by at least a factor of 5 because the cells we observed in the blood vessels were likely the slowest and most amenable to observation.

It is expected that the distinct differences in lymph and blood vessel morphology noted in the rat cornea can be applied to the direct detection of human corneal lymphatics given the broad morphologic similarity of the cornea across species and the ability to use the same clinical in vivo confocal microscope to examine animal and human corneas. Although corneal lymph vessels have yet to be observed in a live human subject, presumed conjunctival human lymph vessels have earlier been reported with in vivo confocal microscopy but without histologic proof. It would be of considerable clinical interest to detect lymph vessels in patients with ocular inflammatory conditions or in recipients of corneal transplants, in whom lymph vessels play a critical role in the acceptance or rejection of allograft tissue. In vivo screening for corneal lymphatics before transplantation could aid in the decision about when to perform surgery in patients at high risk. Additionally, in vivo detection of corneal lymph vessels after transplantation could provide an early indicator of the degree of risk for rejection and subsequent transplant failure.

Observation of cellular activity in lymph vessels may also contribute to a greater understanding of lymphatics in other areas of the body less amenable to noninvasive observation, such as cardiac, intestinal, renal, and lung tissue. Tumor-associated lymphangiogenesis and the metastasis of tumor cells through lymph vessels may also share cellular dynamics similar to those observed in corneal tissue. Additionally, studies examining the role of vascular endothelial growth factor-C in lymphangiogenesis and its therapeutic inhibition could benefit from the ability to study the same animals (and vessels) longitudinally.

Our technique of in vivo lymphatic visualization requires further refinement, specifically to determine the sensitivity and specificity of the technique in relation to ex vivo methods. In corneas in which lymphatics were observed prospectively, the path of the vessels was followed in an attempt to trace their entire course from the conjunctival lymphatics to the suture zone. In practice, however, this was difficult because of the three-dimensional course of the vessels and the presence of intervening blood vessels and nontransparent areas of ECM that served to obscure the lymphatics. Superficial lymphatics were the easiest to visualize. Further tests are required to determine the feasibility of in vivo quantification of lymph vessel density.

In conclusion, laser scanning in vivo confocal microscopy has been used to detect and monitor corneal lymph vessel activity in vivo. The technique provides a means to study cellular activity within lymphatics and to conduct longitudinal observations of a given vessel to complement ex vivo visualization and analysis methods. Finally, we suggest that IVCM could be used to observe lymph vessels in human corneas to be applied, for example, in assessing the risk for corneal transplant failure.

**References**


