Involvement of Platelet Coagulation and Inflammation in the Endothelium of Schlemm’s Canal

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PURPOSE. To investigate whether the endothelial cells of Schlemm’s canal (ECSCs) are connected to lymphatic vessels or are involved in platelet coagulation and inflammation, by comparing them to lung tissue cells.

METHODS. Three normal eyes, trabeculectomy specimens of 6 early-onset primary open-angle glaucoma (POAG), 15 late-onset POAG, and 6 normal-tension glaucoma (NTG), and lung tissues from 10 normal autopsy cases were used. The specimens were processed for light microscopy of immunohistochemical staining. The antibodies used in this study were von Willebrand factor (vWF) and thrombomodulin for evaluating the platelet coagulation system, CD 34 as a marker for blood vessels, platelet/endothelial cell adhesion molecule (PECAM-1) and E-selectin for evaluating the involvement of inflammation, and D2-40 as a marker for lymphatic vessels.

RESULTS. Thrombomodulin, CD 34, PECAM-1 and E-selectin were detected in ECSCs, the endothelial cells of Sondermann’s canal, and the endothelial cells of alveolar capillaries in the lung (EACL), whereas vWF was negative in those cells. Sondermann’s canal was often found in compact juxtacanalicular tissue (JCT) in eyes with early-onset POAG. D2-40 was positive in the endothelial cells of lymphatic vessels in the lung (ELVL) and trabecular cells. In early-onset POAG, trabecular cells in JCT were mostly negative for D2-40.

CONCLUSIONS. ECSCs and EACLs are suggested to have developed specifically for their functions from the aspects of the anti-platelet coagulation system and may be involved in inflammation for leukocyte infiltration. D2-40 and thrombomodulin seem to be useful for evaluating morphologic abnormality in POAG. (Invest Ophthalmol Vis Sci. 2010;51:277-283) DOI: 10.1167/iovs.08-3279

One of the most important functions of the endothelial cells of the inner wall of Schlemm’s canal is to control the flow of aqueous humor from the spaces of the juxtacanalicular tissue (JCT) into Schlemm’s canal. A review of the origin of the cells of Schlemm’s canal has been published.1 Because the aqueous flows toward the basolateral direction in the monolayer of endothelial cells, it is generally believed that Schlemm’s canal is a lymphatic vessel. However, endothelial cells have Weibel-Palade bodies2 that contain von Willebrand factor (vWF). Their containing vWF is relevant because it is involved in the platelet coagulation system, and thus, the endothelial cells of Schlemm’s canal also have components of blood vessels.

Breaks in the inner wall of Schlemm’s canal can be caused by a perfusion of ethylene diamine tetra-acetate (EDTA) through the anterior chamber or by raising the intraocular pressure (IOP) to an unphysiological level in monkey eyes. The breaks can be effectively repaired by platelet coagulation.3 However, if the platelet coagulation system is activated, the normal outflow through the giant invaginations or intercellular routes may be easily blocked by the coagulated platelets.

An elevation of the intraocular pressure in the absence of inflammation in the anterior chamber has been reported in patients with sarcoidosis or Posner-Schlossman syndrome. Occult keratic precipitates or trabeculitis can also be present in patients with elevated IOPs with an absence of inflammation in the anterior chamber.4 Histopathologic examination of the outflow channels of eyes with secondary open-angle glaucoma due to sarcoidosis showed granulomas in Schlemm’s canal and an infiltration of monocytes and macrophages around Schlemm’s canal and the collector channels.5 The inflammation caused by the infiltration of the inflammatory cells has led to a condition called Schlemm’s canalitis.6

Identifying and determining the properties of the cells in the JCT is important for the understanding the mechanisms that lead to primary open-angle glaucoma (POAG). There is some confusion about the role of the endothelial cells of Schlemm’s canal, because evidence has been published that they have properties of blood vessels and also properties of lymphatic vessels. Thus, the purpose of this study was to determine whether the endothelial cells of Schlemm’s canal (ECSCs) have properties of the endothelial cells of blood vessels or of lymphatic vessels. To accomplish this, we used histochemical markers (e.g., vWF, thrombomodulin, CD34, PECAM-1, E-selectin, and D2-40), which are standard markers for the endothelial cells of blood and lymphatic vessels.7 It would also be interesting to investigate whether ECSCs are involved in the transmigration of leukocytes through the intercellular junctions. We investigated by using antibodies to the ECSC adhesion molecule, PECAM-1, and E-selectin.

MATERIALS AND METHODS

Patient Selection, Tissue Samples, and Immunohistochemistry

Tissues from the anterior chamber (AC) angle were obtained from three normal eyes, and normal lung tissues were collected at 10 autopsies. Two of the normal eyes were obtained from two autopsies of persons with no known disease—a 58-year-old man and a 71-year-old woman—and one eye was enucleated from a 70-year-old woman because of a malignant melanoma of the choroid. Twenty-seven trabeculectomy specimens were examined. There were six specimens obtained from patients (mean age: 31.5 ± 5.8 years) with early-onset POAG, 15 specimens from 15 patients (age: 71.3 ± 5.0 years) with late-onset POAG, and 6 specimens from 6...
patients (age: 47.1 ± 1.9) with normal tension glaucoma (NTG). All the trabeculectomy specimens and the enucleated eyes were obtained at the Japanese Red Cross Medical Center after informed consent was obtained from the patient or a family member. The handling of the specimens conformed to the tenets of the Declaration of Helsinki. This study was approved by the Ethics Committee of the Japanese Red Cross Hospital.

Patients with early-onset POAG received the diagnosis before or in their 20s, and patients with late-onset POAG received it in their 50s. All the trabeculectomy specimens and the enucleated eyes were obtained at the Japanese Red Cross Medical Center after informed consent was obtained from the patient or a family member. The handling of the specimens conformed to the tenets of the Declaration of Helsinki. This study was approved by the Ethics Committee of the Japanese Red Cross Hospital.

All slides were processed for immunohistochemical staining and examined by light microscopy. The antibodies used were vWF and thrombomodulin, markers for the platelet coagulation system; CD34, a marker of blood vessels; PECAM-1 and E-selectin, markers for endothelial cells that are used to determine whether ECSCs are involved in inflammation similar to the endothelial cells of blood vessels; and D2-40, a marker for lymphatic vessels.

Paraffin sections of 2-μm thickness, adjacent to sections stained with hematoxylin-eosin, were mounted on silanized slides and dried overnight at 37°C followed by 1 hour at 60°C. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. The slides were pretreated with 0.1% trypsin for vWF and thrombomodulin for 30 minutes; at 121°C for CD34, E-selectin, and D2-40 for 15 minutes; and with a fuchsin substrate (Histofine kit [pH 9.0]; NichireiBioscience, Tokyo, Japan) for 15 minutes and cooled for 15 minutes. Immunostaining was performed with an autostainer (Ventana Medical System, Inc. Tucson, AZ).

After the slides were washed with phosphate-buffered saline (PBS; pH 7.2) and in Tween 20, endogenous peroxidase activity was neutralized by 1% H$_2$O$_2$ in methanol for 20 minutes and washed with PBS. The sections were incubated with antibodies for 60 minutes at the following dilutions: vWF, clone F8/86, dilution 1:50 (DAKO; Carpinteria, CA); thrombomodulin, dilution 1:50 (DAKO; Carpinteria, CA); CD34, clone QBEnd10, dilution 1:1 (Immunotech, Westbrook ME); PECAM-1, clone JC70A, dilution 1:20 (DAKO; Carpinteria, CA); E-selectin, dilution 1:1000 (R&D Systems, Minneapolis, MN); and D2-40, clone D2-40, monoclonal dilution 1:1 (Covance, Madison, WI). The sections for E-selectin were incubated with a paraffin section anti-goat immunostain (MAX-PO; NichireiBioscience) as a second antibody and those for the other antibodies were incubated with a multi-antibody immunostain (MAX-PO; NichireiBioscience). The immunocomplex was made visible by 150 mL of 0.05 M Tris HCl buffer solution with 30 μL of H$_2$O$_2$ and DAB for 30 minutes and washed with PBS. The sections were counterstained with hematoxylin for 1 minute, dehydrated, and mounted. These slides were examined and graded by one ophthalmologist and one pathologist who were masked to the source of the slides.

### Results

The results of staining with each antibody are summarized in Table 1. All the negative controls were negative for each antibody (Fig. 1 control, 18–26).

#### Immunohistochemical Staining with Antibodies to vWF

The ECSC were weakly positive or negative for antibodies to vWF (Fig. 1). The endothelial cells of the noncapillary blood vessels of the lung (ENCBLs), including the pulmonary artery/vein and bronchial artery/vein, stained positively for vWF. On the other hand, the endothelial cells of the alveolar capillaries in the lung (EACls) stained weakly positive or were negative for vWF (Fig. 2). Similar to the pattern in the ECSCs. The endothelial cells of the lymphatic vessels in the lung (ELVLs) were negative to vWF (Fig. 2).

#### Immunohistochemical Staining with Antibodies to Thrombomodulin

The ECSCs, trabecular cells (Fig. 3), and the EACls (Fig. 4) stained positive for thrombomodulin, and the ENCBLs stained weakly positive or negative for thrombomodulin (Fig. 4). The ELVLs were not stained by thrombomodulin (Fig. 4).

#### Immunohistochemical Staining with Antibodies to CD34, PECAM-1, and E-selectin

All types of endothelial cells except those of the ELVLs stained positive for CD34 and E-selectin (Figs. 5, 6, 9, 10), and all types of endothelial cells including ELVLs stained positive for PECAM-1 (Figs. 7, 8). The endothelial cells that line the collector channels showed the same staining pattern as the ECSCs with CD34, PECAM-1, and E-selectin (Figs. 5, 7, 9). The ECSCs in the normal eyes and trabeculectomy specimens fixed in formalin were stained positive for PECAM-1 but those fixed in a mixture of glutaraldehyde and formalin did not stain the ECSCs.

The other antibodies did not show different results with different fixatives.

### Immunohistochemical Staining with Antibodies to Thrombomodulin

The ECSC were negative for D2-40 (Fig. 11), but the endothelial cells of ELVLs were positive for D2-40 (Fig. 12). D2-40 stained the trabecular cells of normal eyes (Fig. 11).

The trabecular cells in the JCT had different staining patterns depending on the source of the specimen. In normal eyes (Fig. 11) and eyes with NTG (Fig. 13), the trabecular cells in the JCT stained strongly positive for D2-40, but they stained weaker when obtained from eyes with late-onset POAG (Fig. 14). In the eyes of early-onset POAG (Fig. 15), the trabecular

### Table 1. Immunohistochemical Staining Scores

<table>
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<tr>
<th></th>
<th>vWF</th>
<th>Thrombomodulin</th>
<th>CD34</th>
<th>PECAM-1</th>
<th>E-Selectin</th>
<th>D2-40</th>
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<tr>
<td>ECSC (n = 30)</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>EBVL (n = 10)</td>
<td>3</td>
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<td>3</td>
<td>3</td>
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<td>0</td>
</tr>
<tr>
<td>ENCBL</td>
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<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
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<tr>
<td>EACL</td>
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<td>0</td>
<td>0</td>
<td>3</td>
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Immunohistochemical scoring: no staining (−) → 0; weak and irregular staining (−, +) → 1; strong staining (+ +) → 3.
FIGURE 1. Immunohistochemical staining with antibody to vWF of the trabecular meshwork and Schlemm’s canal (SC) of a normal eye (left) and a control eye (right). Tissues were obtained from a 58-year-old man. vWF was weakly positive or negative (arrowhead) in the cells of the outer wall of Schlemm’s canal. Brown dots in both the vWF and control (arrows) sections are melanin granules.

FIGURE 2. Immunohistochemical staining with antibody to vWF of lung tissue obtained from a 71-year-old man. The endothelial cells of the noncapillary blood vessels (※) in the lung stained positive for vWF, but the endothelial cells of the alveolar capillaries (arrows) in the lung were weakly positive or negative for vWF (arrows). Endothelial cells of the lymphatic vessels ( chù ) in the lung were negative for vWF. See also positive staining of lymphatic vessels in an adjacent section for D2-40 (Fig. 12).

FIGURE 3. Immunohistochemical staining of trabecular meshwork and Schlemm’s canal with antibody to thrombomodulin. The tissues were obtained from a 46-year-old man. The endothelial cells of Schlemm’s canal (SC) were positive, but the trabecular cells were negative to thrombomodulin. AC, anterior chamber.

FIGURE 4. Immunohistochemical staining with antibody to thrombomodulin of lung tissue obtained from a 71-year-old man. The endothelial cells of Schlemm’s canal, trabecular cells, and the endothelial cells of alveolar capillaries (arrows) in the lung were thrombomodulin-positive. The endothelial cells of noncapillary blood vessels (※) in the lung were weakly positive or negative for thrombomodulin. Endothelial cells of the lymphatic vessels ( chù ) in the lung were negative for thrombomodulin.

FIGURE 5. Immunohistochemical staining of trabecular meshwork and Schlemm’s canal with antibody to CD34. Tissues were obtained from a 67-year-old man. The endothelial cells of Schlemm’s canal stained positive, but the trabecular cells were negative to CD34.

FIGURE 6. Immunohistochemical staining of lung tissue from a 71-year-old man with antibody to CD34. The endothelial cells of the alveolar capillaries (arrows) and noncapillary blood vessels (※) in the lung were positive, whereas the lymphatic vessels (ELVLs, chù ) in the lung were negative to CD34. Inset: higher magnification of the boxed area. ELVLs was negative for CD34, despite positive staining of the surrounding tissue.

FIGURE 7. Immunohistochemical staining of trabecular meshwork and Schlemm’s canal with antibody to PECAM-1. Tissues were obtained from a 68-year-old man. The endothelial cells of Schlemm’s canal were positive, but the trabecular cells were negative to PECAM-1.

FIGURE 8. Immunohistochemical staining of lung tissue from a 71-year-old man with antibody to PECAM-1. The endothelial cells of the alveolar capillaries (arrows), noncapillary blood vessels (※), and lymphatic vessels ( chù ) in the lung were positive to PECAM-1.
FIGURE 9. Immunohistochemical staining of trabecular meshwork and Schlemm’s canal with antibody to E-selectin. Tissues were obtained from an 85-year-old man. The endothelial cells of Schlemm’s canal were positive, but the trabecular cells were negative to E-selectin.

FIGURE 10. Immunohistochemical staining of lung tissue from a 71-year-old man with antibody to E-selectin. The endothelial cells of the alveolar capillaries (arrows) and noncapillary blood vessels (●) in the lung were positive, whereas the lymphatic vessels (☆) in the lung were negative for E-selectin.

FIGURE 11. Immunohistochemical staining of trabecular meshwork and Schlemm’s canal with antibody to D2-40. Tissues were obtained from a 70-year-old woman. The endothelial cells of Schlemm’s canal were negative, but the trabecular cells were positive for D2-40.

FIGURE 12. Immunohistochemical staining of lung tissue from a 71-year-old man with antibody to D2-40. The endothelial cells of the lymphatic vessels in the lung were positive (●), whereas the alveolar capillaries (arrows) and noncapillary blood vessels (☆) in the lung were negative to D2-40.

FIGURE 13. Immunohistochemical staining of trabecular meshwork and Schlemm’s canal with antibody to D2-40 in an eye of a 45-year-old woman with NTG. The trabecular cells in juxtacanalicular tissue (JCT) stained diffusely positive.

FIGURE 14. Immunohistochemical staining of trabecular meshwork and Schlemm’s canal with antibody to D2-40 in an eye with late-onset POAG of a 68-year-old man. The trabecular cells staining in JCT are more numerous than those in Figure 13.

FIGURE 15. Immunohistochemical staining with the antibody of D2-40 of trabecular meshwork and Schlemm’s canal in an eye with early-onset POAG (26-year-old man). In eyes with early-onset POAG, the JCT was thick and compact, and there were almost no D2-40-positive cells.
FIGURE 16. Immunohistochemical staining for thrombomodulin in an eye with early-onset POAG (37-year-old man). Thrombomodulin was detected in not only the collector channels (CC) and Schlemm's canal but also in Sondermann's canal (arrowheads).

FIGURE 17. Serial section stained with antibodies against D2-40. Note that the compact juxtacanalicular tissue did not contain D2-40-positive trabecular cells.

FIGURE 18–26. Negative controls of the outflow routes stained for thrombomodulin (Fig. 18), CD34 (Fig. 19), PECAM-1 (Fig. 20), E-selectin (Fig. 21), D2-40 (Fig. 22), D2-40 (Fig. 23), D2-40 (Fig. 24), D2-40 (Fig. 25), and thrombomodulin and D2-40 (Fig. 26).
cells in the JCT, where tightly packed and were all negative for D2-40.

**Sonnermann’s Canal**

Sonnermann’s canal in the JCT was made clearly visible by immunohistochemical staining by thrombomodulin (Fig. 16), CD34, PECAM-1, and E-selectin. Sonnermann’s canal was found in the compact JCT often in early-onset POAG (Fig. 16). The endothelial cells of Sonnermann’s canal were negative for D2-40 (Fig. 17).

**DISCUSSION**

Lung tissue was selected as the control because the blood and lymphatic vessels are well developed, and the lymphatic vessels are easy to recognize because they run parallel to the alveolar capillaries. Thrombomodulin has been shown to be widely distributed in the endothelial cells of blood vessels, and the endothelial cells of the nonvascular surface of body cavities (e.g., the mesothelia of pleura and the arachnoid membranes of the central nervous system). Thrombomodulin inactivates factors V and vWF by activating protein C, resulting in a suppression of platelet coagulation. The trabecular plexus of the rabbit eye and Schlemm’s canal of human eyes were immunopositive for thrombomodulin. The ECSCs of all our specimens stained positive with thrombomodulin, indicating that they had properties of blood vessels. On the other hand, the ECSCs were only weakly positive or negative to vWF, which was supported by the report that Weibel-Palade bodies are very rare in the inner-wall cells of Schlemm’s canal but are more often found in the outer walls. The weak positive staining of the outer wall of Schlemm’s canal with antibodies against vWF (Fig. 1) agrees with earlier findings.

Taken together, these findings indicate that the anti-platelet coagulation system may be prominent in ECSCs. Similar findings have been reported for the lung. The EACLs of normal lung tissues are positive for thrombomodulin and express vWF weakly or not at all. This staining pattern is the opposite of that for the ENCBLs, but they correspond well with our results (Figs. 2, 4). The difference in the staining by thrombomodulin and vWF in the ENCBLs, EACLs, and ECSCs may well reflect the functional differences of these tissues.

The prominent anti-platelet coagulation system in the ECSCs may also be important for the physiological outflow. It is also interesting that thrombomodulin staining could often detect Sonnermann’s canal in trabeculectomy specimens of eyes with early-onset POAG. Sonnermann’s canal is a blind in-pouching of Schlemm’s canal and is not usually recognized in light microscopic examination of hematoxylin and eosin (H&E)-stained sections. However, immunohistochemical staining with thrombomodulin and CD 54, which are markers for cells of mesodermal origin, clearly stained both the ECSCs and endothelial cells of Sonnermann’s canal. The function of Sonnermann’s canal is to drain aqueous fluid passing laterally along the corneoscleral trabecular meshwork. The frequent finding of Sonnermann’s canal in compact JCT of eyes with early-onset POAG specimens suggests that impairment of neural crest cells, which are future trabecular cells, may have caused a misreading of the ECSCs.

E-selectin is involved in the slow rolling and stabilization of leukocytes on the endothelium during inflammation. The E-selectin in the ECSCs may play a role in the infiltration of monocytes and macrophages around Schlemm’s canal in eyes with sarcoidosis and other types of anterior uveitis. E-selectin was also suggested to participate in angiogenesis by studies using antibodies against E-selectin in vitro and being upregulated in pathologic angiogenic tissues.

Fluorescein gonioangiography in patients with neovascular glaucoma due to diabetic retinopathy showed that the new blood vessels arose from the iris root and extended deep into the trabecular meshwork. These new vessels appeared to extend toward Schlemm’s canal. This suggests that E-selectin in the ECSCs may stimulate the formation of new vessels from the iris root.

PECAM-1 is enriched in the intercellular junctional domain of cultured human umbilical vein endothelial cells, and is crucial for leukocyte transmigration through the intercellular junctions of vascular endothelial cells. The PECAM-1 in the ECSCs may play a role in the transmigration of macrophages or monocytes through the JCT or the outer wall of Schlemm’s canal in patients with anterior uveitis. The macrophages migrating through the JCT may cause an elevation of IOP without inflammation in the anterior chamber in some types of anterior uveitis, such as Posner-Schlossman syndrome.

D2-40 is a commercially available antibody that reacts with O-linked sialoglycoprotein (MW: 40 kDa) and detects lymphatic vessels, testicular germ cell tumors, and squamous cell carcinomas. The lymphatic vessels in the lung were clearly positive for D2-40, but the ECSCs were completely negative. This supports our earlier findings that ECSCs had properties of blood vessels and not lymphatic vessels.

D2-40 is also known to be an antibody against podoplanin, which plays a role in stimulating platelet aggregation. It is reasonable that D2-40 was not detected in the endothelium of Schlemm’s canal, because these cells are prominent in the antiplatelet coagulation system.

It was somewhat surprising that trabecular cells of normal eyes were positive for D2-40, since trabecular cells are believed to originate from the neural crest. However, it seems reasonable that trabecular cells have some properties of lymphatic endothelial cells, since the aqueous humor resembles lymphatic fluid in the interstitial spaces. In addition, the aqueous drains into Schlemm’s canal which had immunohistochemical properties of blood vessels. It was very interesting that trabecular cells in compact JCT of early-onset POAG specimens were almost all negative for D2-40. This tendency toward more negative trabecular cells in JCT in eyes with early-onset POAG may be caused by additional developmental abnormalities.

It was recently reported that podoplanin is essential for the morphogenesis of lymphatic endothelial cells because of defects in the lymphatic vessel in homozygous podoplanin-knockout mice. It has also been reported that interfering RNA-mediated silence of the podoplanin gene blocks capillary tube formation by the primary human lung microvascular lymphatic endothelial cells. The lack of positive staining for D2-40 in JCT trabecular cells of patients with glaucoma suggested that a podoplanin deficiency in JCT trabecular cells compromises the formation of spaces in the JCT, resulting in higher IOPs. The immunohistochemical staining for D2-40 may be useful in evaluating the quantitative changes in the aqueous outflow routes in trabecular meshwork or developmental abnormalities in JCT of eyes with POAG.

Our results indicated that the ECSCs have similar properties to the EACLs and differ from those of the ENCBLs from the aspect of the blood coagulation system. It seemed very important for ECSCs to be prominent in the antiplatelet coagulation system for aqueous outflow by suppressing platelet coagulation in the intracellular (physiological pores of giant vacuole) or intercellular routes. Further investigations are needed regarding interaction between the development of Schlemm’s canal and trabecular cells in the embryonic stage, to understand the pathogenesis of POAG.
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References