Proliferative Diabetic Retinopathy with Lymphocyte-Rich Epiretinal Membrane Associated with Poor Visual Prognosis

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PURPOSE. The aim of this study was to analyze lymphocyte infiltration using immunohistochemistry in proliferative diabetic retinopathy (PDR) membranes.

METHODS. Sixteen patients, 13 with PDR and 3 without diabetes, underwent pars plana vitrectomy, and the epiretinal membrane was peeled. Formalin-fixed, paraffin-embedded epiretinal membrane tissues were processed for immunohistochemistry with anti-leukocyte common antigen (LCA), CD3, and CD20 antibodies. Lymphocyte density was determined by direct counting at a high magnification under a light microscope, which was compared with the patients' visual prognoses.

RESULTS. The lymphocyte density ranged from 1 to 52 (mean, 9.5) in high-power fields. Of 13 membranes, 5 showed a lymphocyte density of >5 cells, whereas the other 8 membranes showed a cell number of <2. The former type is defined as a lymphocyte-rich epiretinal membrane (LERM). Infiltated lymphocytes were immunohistochemically positive for CD3, a T-cell marker, but not for CD20, a B-cell marker. All patients with LERM had poor visual prognosis after vitrectomy. In contrast, the visual prognosis in 7 patients with non-LERM improved or remained unchanged. A significant association was observed between high-level lymphocyte infiltration in the epiretinal membrane and poor visual prognosis (P < 0.001). LCA− mononuclear cells were not observed in epiretinal membranes in the absence of diabetes.

CONCLUSIONS. These results suggest that the high-level infiltration of T lymphocytes into the PDR membrane is well correlated with poor visual prognosis. Histopathologic observation of the epiretinal membrane in patients with PDR may provide significant prognostic information. (Invest Ophthalmol Vis Sci. 2009;50:5909–5912) DOI:10.1167/iovs.09-3767

Diabetic retinopathy (DR) is a significant cause of severe vision loss and blindness in developed countries, in spite of recognized ocular treatments that are successful in reducing the rate of vision impairment.1 Proliferative DR (PDR) is characterized by fibrovascular proliferative changes, which may cause vitreous hemorrhage, or traction retinal detachment (TRD), which often requires surgical intervention. Pathologic analyses using the epiretinal membrane in PDR removed during vitrectomy have been widely performed to investigate the pathogenesis in membrane formation. Recently, Snead et al.2 proposed that the mechanisms underlying membrane formation in PDR are hypoxia and neovascular cytokine production on histopathologic examination. However, the correlation between epiretinal membrane pathology and subsequent clinical visual outcome is largely unknown.

It has been well documented that epiretinal membranes formed in PDR are mainly composed of neovascular stromal tissue.2,3 In contrast to neovascular endothelial cells, the number of lymphocytes infiltrating the epiretinal membrane is suggested to be variable in each case,4 indicating that the density of lymphocytes in the membrane may be of specific clinical importance. Leukocytes were found to contribute to angiogenic and fibrotic processes in the pathogenesis of diabetic retinopathy,5 whereas little is known about the role of lymphocytes in terms of visual outcome in patients with PDR.

We and other groups have documented an enhancement of intravitreal concentrations of several cytokines, underlining the importance of the inflammatory process in the development of PDR.6,7 The breakdown of the blood-retinal barrier causes the infiltration of immunologically competent cells into PDR membranes.4 Hence, Tang et al.7 demonstrated that immune-mediated processes or inflammatory reactions played a role in the formation of the epiretinal membrane in PDR. The aim of this study was to analyze lymphocyte infiltration using immunohistochemistry in PDR membranes.

MATERIALS AND METHODS

Thirteen membranes from patients with PDR were examined in this study. The ages of subjects with diabetes ranged from 36 to 73 (mean, 59.8 years). The clinical data of patients are summarized in Table 1. Peripheral blood was collected before surgery. All patients underwent pars plana vitrectomy from October 2004 to December 2008 at Hokkaido University Hospital. Follow-up periods ranged from 5 to 35 months (mean, 21.9 months) after vitrectomy. For subjects without diabetes, we collected two idiopathic epiretinal membranes (IEMs) and one inner limiting membrane (ILM) surgically removed from patients with macular hole retinal detachment. The membranes peeled and removed from the retina were fixed in 4% paraformaldehyde, and paraffin-embedded tissue sections were produced for hematoxylin and cosin staining, Masson-trichrome staining, and immunohistochemistry. Written informed consent was obtained from all patients before collection of the membranes. All studies conformed to the tenets of the Declaration of Helsinki.

Immunohistochemistry

Dewaxed paraffin sections were immunostained using the streptavidin-biotin peroxidase complex method. Formalin-fixed, paraffin-embedded serial tissue sections were cut to a thickness of 4 μm, and endogenous peroxidase activity was inhibited by immersing the slides in 0.3% hydrogen peroxide in methanol for 30 minutes. As a pretreatment,
microwave-based antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 10 minutes. Then, nonspecific binding of the primary antibody was blocked by incubating the slides in the blocking serum for 20 minutes. The slides were incubated with anti-lymphocyte common antigen (LCA) monoclonal (DakoCytomation, Glostrup, Denmark), anti-CD20 polyclonal (DakoCytomation), anti-CD20 monoclonal (Nichirei, Tokyo, Japan), and anti-CD34 monoclonal (DakoCytomation) antibodies overnight at 4°C, followed by the secondary antibody and biotin-streptavidin complex for 30 minutes each at room temperature. Immunoreactions were visualized with diamobenzidine, and the sections were counterstained with hematoxylin. To examine the specificity of immunostaining, the primary antibody was replaced with mouse normal IgG or Tris-buffered saline. All control slides were negative for immunostaining.

Evaluation of Tissue Specimens

Lymphocyte and microvessel densities, represented by the density of LCA+ cells and CD34+ vascular lumens in epiretinal membranes, respectively, were determined under a high-power field (objective lens, 40×). Masson-trichrome staining for detection of collagen was applied to evaluate the tissue fibrosis in PDR membranes. To evaluate the correlation with lymphocyte density, degree of fibrosis, and patient visual outcome, statistical analysis was performed using the χ² test for trend. Student’s t-test was applied to evaluate the correlation with the presence of TRD and lymphocyte density in the PDR membrane. The correlation between lymphocyte and microvessel densities was analyzed by Spearman correlation coefficient. The accepted level of significance for all tests was P < 0.05.

RESULTS

Histopathology and Lymphocyte Infiltration in PDR Membranes

All PDR membranes comprised a variety of vascular endothelial and stromal cells (Fig. 1a). Several mononuclear cells showing round or oval/spindle nuclei infiltrated the stroma in the membrane. Immunoreactivity for LCA was detected in the cytoplasm of several mononuclear cells in all PDR membranes examined (Fig. 1b). LCA+ lymphocytes were predominantly distributed in the stroma rather than vascular lumen. Lymphocyte densities ranged from 1 to 52 (mean, 9.5). Of 13 membranes, 5 membranes showed a lymphocyte density of >5 cells, whereas the other 8 membranes revealed a cell number of <2. The former is defined as lymphocyte-rich epiretinal membrane (LERM). Immunohistochemical study revealed that infiltrated lymphocytes were positive for CD3 (Fig. 1c), a T-cell marker, but not for CD20 (Fig. 1d), a B-cell marker.

Immunoreactivity for CD34 clearly detected microvessels in epiretinal membranes (Figs. 2a, b). In LERM, a variety of mononuclear cells infiltrated, whereas a few microvessels were noted (Fig. 2a). In non-LEM, several microvessels were present, whereas lymphoid cells were not observed (Fig. 2b). Dot graph discloses all data of lymphocyte and microvessel densities in PDR membranes (Fig. 2c). There was no statistically significant correlation between lymphocyte and microvessel densities (correlation coefficient, r = 0.02).

Masson-trichrome staining clearly represented collagen in all PDR membranes. PDR membrane with a dense collagen, strongly stained by blue, was evaluated as severe fibrosis (Fig. 2d). The fibrosis was classified into four grades: 0, absent; 1, mild; 2, moderate; 3, severe. Student’s t-test was applied to evaluate the correlation with the fibrosis grade and lymphocyte density.

TABLE 1. Clinicopathologic Profile of Patients With Proliferative Diabetic Retinopathy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Side</th>
<th>VH</th>
<th>TRD</th>
<th>Pre-VA</th>
<th>Post-VA</th>
<th>Follow-up (mo)</th>
<th>LD/HPF</th>
<th>Fibrosis</th>
<th>CRP</th>
<th>WBC (×/μL)</th>
<th>Lymph (%)</th>
<th>HbA1c (%)</th>
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<tr>
<td>1</td>
<td>58</td>
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<td>L</td>
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<td>+</td>
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<td>LP</td>
<td>35</td>
<td>52</td>
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<td>+</td>
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<td>LP</td>
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<td>52</td>
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<td>+</td>
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<td>36</td>
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<td>+</td>
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<td>CF</td>
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<td>0.04</td>
<td>31</td>
<td>5</td>
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<td>28.4</td>
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<tr>
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<td>0.9</td>
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<td>&gt;0.24</td>
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<td>0.5</td>
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<tr>
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<td>M</td>
<td>R</td>
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<td>+</td>
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<td>0.9</td>
<td>33</td>
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<td>&gt;0.24</td>
<td>6.1</td>
<td>33.9</td>
<td>6.9</td>
</tr>
</tbody>
</table>

M, male; F, female; L, left; R, right; VH, vitreous hemorrhage; TRD, traction retinal detachment; Pre-VA, preoperative visual acuity; Post-VA, postoperative visual acuity; HM, hand motion; CF, counting fingers; LP, light perception; LD/HPF, lymphocyte density/high-power field; 3+, severe; 2+, moderate; 1+, mild, CRP, C-reactive protein; WBC, white blood cell; Lymph, lymphocyte; ND, not done.

FIGURE 1. Hematoxylin and eosin staining (a, c), and immunohistochemistry (b–d, f) in LERM and non-LEM in PDR. In LERM, mononuclear cells are infiltrating the membrane, where vascular endothelial cells are intermingled (a). Cytoplasmic immunoreactivity for leukocyte common antigen (LCA) (b) and CD3 (c) is detected in mononuclear cells. In contrast, CD20+ cells are barely detectable in LERM (d). In non-LEM, vascular endothelial cells with vascular lumens are present in the membrane, whereas mononuclear cell infiltration is absent (e). LCA+ cells are barely observed in non-LEM (f).
Figure 2. Microvessel density in LERM and non-LERM in PDR. Immunoreactivity for CD34 clearly detects microvessels in epiretinal membranes (a, b). In LERM, a variety of mononuclear cells infiltrate, and a few microvessels are noted (a). In non-LERM, several microvessels are present, but lymphoid cells are not observed (b). Dot graph reveals all data of lymphocyte and microvessel densities in PDR membranes (c). There is no statistical significant correlation between the lymphocyte and microvessel densities ($r = 0.02$). HPF, high-power field.

3a). In contrast, collagen, which was weakly stained in the PDR membrane, was evaluated as mild fibrosis (Fig. 3b). Moderate fibrosis was shown by an intermediate staining pattern. As shown in Table 1, 4 of 5 LERM showed severe fibrosis (Fig. 3a), whereas mild to moderate fibrosis was predominantly observed in 7 non-LERM (Fig. 3b). Fibrosis was significantly more severe in LERM than non-LERM ($P < 0.01$).

Clinical Significance in LERM

In fundus examination, both LERM and non-LERM revealed fibrous proliferative membranes. Intraoperative findings demonstrated that epiretinal membrane was firmly attached to the retina in LERM compared with non-LERM. TRD was confirmed in 7 patients with PDR before or during vitrectomy. All patients with LERM had TRD, whereas 2 of 8 non-LERM accompanied TRD. Significant association was observed between the presence of TRD and LREM ($P < 0.01$). There was no significant difference between the follow-up periods of patients with LERM and non-LREM ($P = 0.9$). As shown in Table 1, all patients with LERM developed severe vision loss caused by the marked reproliferation of the epiretinal membrane with retinal redetachment after vitrectomy. In contrast, the final visual outcome in the 7 patients with non-LERM after vitrectomy was improvement in 6 patients; the condition remained unchanged in 1 patient. Significant association was observed between high-level lymphocyte infiltration in the epiretinal membrane and a poor visual prognosis ($P < 0.001$, Table 2). Blood tests including C-reactive protein, number of white blood cells (WBCs), lymphocytes (%), and hemoglobin A1C (%) were not different between LREM and non-LREM.

Lymphocyte Infiltration in IERM and ILM

All membranes from IERM comprised several flattened cells with oval nuclei and pale-staining indistinct cytoplasm. ILM revealed abundant collagenous tissue in which a few mononuclear cells were intermingled. Immunohistopathologically, LCA + mononuclear cells were not observed in IERM or ILM.

Discussion

We found that the high-level infiltration of T lymphocytes in the PDR membrane was well correlated with the presence of TRD and a poor irreversible visual outcome because of the subsequent reproliferation of the epiretinal membrane. The mechanism underlying membrane reproliferation by lymphocyte infiltration after vitrectomy remains unknown. It has been shown that ischemia-reperfusion leads to the long-term infiltration of activated T lymphocytes and to the production of inflammatory factors, which consequently leads to tissue damage and fibrosis.8,9 These results also indicate that changes in ocular blood flow may lead to the activation of T lymphocytes and fibrosis. Indeed, vitrectomy induces a significant reduction in ocular blood flow in patients with DR.10 Taken together, this suggests that lymphocytes markedly infiltrating the retina may be activated after vitrectomy through the alteration of retinal blood flow, which may contribute to membrane reproliferation and fibrosis.

We showed that lymphocyte infiltration was not observed in IERM or ILM from patients without diabetes. In contrast, lymphocyte infiltration was observed in all PDR membranes, although the degree of cellular density varied in each membrane. Canton et al.11 confirmed the presence of T lympho-

Table 2. Correlation with Lymphocyte Density in Epiretinal Membrane and Postoperative Visual Outcome in Patients with Proliferative Diabetic Retinopathy

<table>
<thead>
<tr>
<th>Lymphocyte Density</th>
<th>Improved</th>
<th>Worse/Stable</th>
<th>Total</th>
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<td>High (≤ 5/HPF)</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Low (&gt; 5/HPF)</td>
<td>7</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>6</td>
<td>13</td>
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</table>
cytes infiltrating the vitreous fluid of all patients with PDR using flow cytometry, which seems to be consistent with the results obtained in this study. Indeed, it is presumed that intravitreal cellularity reflects peripheral blood entering the intraocular cavity because of disruption of the blood-retinal barrier in PDR. In this study, we showed that the high number of T lymphocytes infiltrating the vitreous were related to improved prognosis in patients with PDR. In this study, we showed that the high number of T lymphocytes infiltrating the epiretinal membrane, determined by histopathology, was correlated with a poor visual prognosis. These results may not be consistent with those observed in the previous report. This study also showed that the number of WBCs and the population of lymphocytes in peripheral blood did not reflect lymphocyte infiltration in the epiretinal membrane. Moreover, the histopathology of PDR membranes showed that lymphocytes predominantly infiltrated the stroma and were rarely distributed in the vascular lumen. These results suggest that the population and distribution of lymphocytes in the vitreous may be different from those in the epiretinal membrane. Further studies are needed to clarify the direct association between lymphocyte infiltration in the vitreous and epiretinal membrane.

It is indisputable that pathologic analyses of epiretinal membranes contribute to the investigation of pathogenesis in membrane formation. However, as far as we know, there have been no reports presenting a clear correlation of pathologic findings in the epiretinal membrane with patients’ visual prognosis. In this study, we demonstrated that patients with PDR with LREM subsequently had poor visual prognoses, likely caused by the marked reproliferation of the epiretinal membrane. Thus, observation of the epiretinal membrane pathology may provide significant information not only for research on the pathogenesis but also for the clinical application of adjunctive treatments after vitrectomy. It is known that triamcinolone acetonide is a long-acting corticosteroid that has been shown to be effective against diabetic macular edema and PDR when administered by intravitreal injection. Importantly, the anti-inflammatory effects of glucocorticoids are mediated by the redirection of T lymphocyte responses from proinflammatory to an anti-inflammatory. In addition, it is known that glucocorticoids suppress collagen synthesis and fibrosis. Our study may indicate topical administration of glucocorticoids to specific PDR eyes with LREM after vitrectomy to suppress fibrosis and subsequent severe vision loss. Knowing the status of lymphocyte infiltration in the PDR membrane may aid ophthalmologists in making visual prognoses.

References