A Significant Role of Stromal Fibroblasts in Rapidly Progressive Dry Eye in Patients with Chronic GVHD

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**PURPOSE.** To elucidate histopathologic features of the lacrimal gland in chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation.

**METHODS.** Lacrimal gland specimens from five patients who had dry eye as part of the symptoms of chronic GVHD were examined by immunohistochemistry and transmission electron microscopy. Lacrimal gland specimens from five patients with Sjögren’s syndrome (SS) were used as control samples.

**RESULTS.** Lymphocytes, predominantly T cells, were found primarily in the periductal areas of the lacrimal gland from patients with chronic GVHD, whereas B cells were the dominant infiltrating cells in the acinar areas of the lacrimal gland from patients with SS. Notable findings in the lacrimal gland from patients with chronic GVHD were marked fibrosis of the glandular interstitium and an increase in the number of CD34+ stromal fibroblasts. These findings were more prominent in patients with severe dry eye than in those with mild dry eye. Electron microscopic observations of the lacrimal gland from patients with chronic GVHD revealed that stromal fibroblasts were attached to various inflammatory cells, especially T cells, through primitive or rudimentary contacts. In addition, the presence of a well-developed rough endoplasmic reticulum in the fibroblasts and newly synthesized collagen fibrils in the extracellular matrix indicated an active production of extracellular matrix components. Electron micrographs revealed multilayered and thickened basal laminae of blood vessels, ducts, and lobules in the lacrimal gland of patients with chronic GVHD; however, these observations were infrequently observed in the lacrimal glands of patients with SS.

**CONCLUSIONS.** The results suggest substantial differences in the lacrimal gland histopathology of patients with chronic GVHD and SS. In addition, it is likely that stromal fibroblasts are actively involved in the pathogenic process of chronic GVHD in the lacrimal gland by producing excessive extracellular matrix components. (Invest Ophthalmol Vis Sci. 2001;42:111–119)

**Materials and Methods**

**Patients**
We examined lacrimal gland specimens from five patients who had received allogeneic SCT and in whom dry eye developed later (cases 1–5). Lacrimal gland specimens from five patients with Sjögren’s syndrome (SS) were examined as control samples (cases 6–10). All subjects were recruited from patients attending the dry eye outpatient clinic at Keio University Hospital. Dry eye was diagnosed as a disorder of the tear film caused by tear deficiency or excessive tear evaporation, which causes damage to the ocular surface, with or without symptoms of ocular discomfort. Severe dry eye was defined as reduced reflex tearing (Schirmer test with nasal stimulation ≤10 mm). The diagnosis and classification of chronic GVHD and SS were based on previously reported criteria.

**Lacrimal Gland Biopsy**
Written informed consent was obtained in advance from all patients in accordance with the tenets of the Declaration of Helsinki. After a lid retractor was positioned, a local anesthetic was injected into the superior temporal portion of the palpebral lacrimal tissue. A 1-cm incision was then made in the anesthetized region of the conjunctiva and a 5-mm-diameter specimen of lacrimal gland tissue was removed and placed in fixative. No sutures were required, and digital compression was applied for 10 minutes after surgery.

**Light Microscopic Examination**
All lacrimal gland specimens were immediately fixed in 10% neutralized buffered formalin, embedded in paraffin wax, and processed according to conventional histologic techniques, including hematoxylin-eosin and Mallory stainings.
For immunohistochemistry, deparaaffinized sections were examined, as previously described. 15 Briefly, sections were incubated with 2% H2O2 for 5 minutes at room temperature to remove any endogenous peroxidase and were rinsed with Tris-buffered saline. The respective primary antibodies listed in Table 1 were added to the sections and incubated for 60 minutes at room temperature with a subsequent incubation of the corresponding peroxidase-conjugated secondary antibodies (En Vision; Dakopatts, Glostrup, Denmark). The bound antibody was visualized with 3,3′-diaminobenzidine tetrahydrochloride, and cell nuclei were counterstained with hematoxylin for 1 minute, with subsequently rinsing in water. All incubation steps were performed in a moist chamber.

Antigenic epitopes were unmasked using the antigen retrieval method described in Table 1. Briefly, for CD45RO, CD20, or CD34 staining, the slides were treated in a microwave oven for 10 minutes. 16 For laminin staining, the tissue sections were digested with 0.4% pepsin in 0.01 M HCl (pH 2.0) at 37°C for 180 minutes. 17

Transmission Electron Microscopic Examination

Transmission electron microscopic analysis was performed according to standard protocols. 18 Specimens were immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 hours at 4°C and washed three times with 0.1 M phosphate buffer. The samples were then postfixed in 2% osmium tetroxide, dehydrated in a series of ethanol and propylene oxide, and embedded in epoxy resin. One-micrometer sections were stained with methylene blue and the lacrimal gland portions exhibiting findings of interest were then sectioned on an ultratome (LKB; Gaithersburg, MD) with a diamond knife. Sections in the range of gray to silver were collected on 150-mesh grids, stained with uranyl acetate and lead citrate, and examined under an electron microscope (model 1200 EXII; JEOL, Tokyo, Japan).

Semiquantitative Microscopic Analysis

The degree of infiltrating T and B cells was scored on a scale of 0 to 4 according to the grading scale of Greenspan et al. 19 with some modifications (grade 0, none; grade 1, 1–9 cells; grade 2, 10–49 cells; grade 3, 50–99 cells; and grade 4, ≥100 cells per 4-mm² section). Other pathologic findings were evaluated at the same magnification and categorized into four groups (−, +, +, and ++). In electron microscopic analysis, at least 50 micrographs were taken and assessed for each subject.

RESULTS

Clinical Data

The clinical characteristics of patients with chronic GVHD enrolled in this study are listed in Table 2. They had undergone allogeneic SCT for various hematologic malignancies. At the time of biopsy, two patients (cases 1 and 2) had mild dry eye, whereas the remaining three (cases 3, 4, and 5) had severe dry eye. Case 1, the patient experienced dry eye soon after discontinuing cyclosporin A, but the severity of dry eye did not progress during the following 18 months. In case 2, a lacrimal gland biopsy was performed soon after the onset of dry eye. Because this patient exhibited bilateral severe dry eye within 3 months after the onset of dry eye, the tissue sample from this patient was particularly useful for assessing early pathologic changes. In cases 3 and 5, the patients had systemic chronic GVHD with severe dry eye that developed rapidly within 3 months after the initial symptoms. In contrast, severe dry eye was the only symptom related to chronic GVHD in case 4.

In five patients with SS (cases 6–10), four were diagnosed as having primary SS, whereas one patient (case 7) had secondary SS accompanied by rheumatoid arthritis. All except one (case 6) had severe dry eye. The mean age at the time of biopsy of the patients with SS was significantly more than the mean age of patients with chronic GVHD (54.0 ± 12.4 years versus 34.0 ± 8.3 years, P = 0.02 by Student’s t test).

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age at Biopsy (mo)</th>
<th>Degree of Dry Eye</th>
<th>Underlying Disease</th>
<th>Donor</th>
<th>TBI</th>
<th>Prophylaxis for GVHD</th>
<th>GVHD in Other Organs</th>
<th>Time of Biopsy after SCT (mo)</th>
<th>Interval Between Onset of Dry Eye and Biopsy (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>32</td>
<td>Mild</td>
<td>APL</td>
<td>Related</td>
<td>+</td>
<td>CyA</td>
<td>Skin (grade II)</td>
<td>Liver</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>47</td>
<td>Mild</td>
<td>AML</td>
<td>Unrelated</td>
<td>+</td>
<td>CyA</td>
<td>Skin (grade II)</td>
<td>Liver</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>35</td>
<td>Severe</td>
<td>CML</td>
<td>Unrelated</td>
<td>+</td>
<td>FK506</td>
<td>Skin, intestine (grade IV)</td>
<td>Skin, mouth, lung</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>26</td>
<td>Severe</td>
<td>CML</td>
<td>Related</td>
<td>–</td>
<td>CyA</td>
<td>None</td>
<td>None</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>28</td>
<td>Severe</td>
<td>ALL</td>
<td>Related</td>
<td>+</td>
<td>FK506</td>
<td>Skin, intestine, mouth, lung</td>
<td>None</td>
<td>9</td>
</tr>
</tbody>
</table>

TBI, total body irradiation; GVHD, graft-versus-host disease; APL, acute promyelocytic leukemia; CyA, cyclosporin A; AML, acute myeloblastic leukemia; CML, chronic myeloblastic leukemia; FK506, tacrolimus; ALL, acute lymphoblastic leukemia.

* Degree of dry eye was determined based on reflex tearing.

Table 2. Clinical Characteristics of Patients with Chronic GVHD Who Have Dry Eye
**Light Microscopic Findings**

In patients with chronic GVHD, mononuclear cells were mainly found around medium-sized ducts and almost normal acinar sites in a patient with mild dry eye (case 1). Immunostaining with CD45RO (C) and CD20 (D) in the case 3 specimen shows that lymphocytes infiltrating the periductal areas are mostly T cells with only a few B cells. In the fibrotic areas, proliferation and dilatation of small ducts are seen. (E, F) Hematoxylin and eosin staining in the same specimen from a patient with severe dry eye shows a markedly fibrotic interstitium and irregular loss of the periductal margins of the acini. (G) Mallory staining in a specimen of severe dry eye (case 5) shows a severely fibrotic interstitium that is more intense in the periductal areas than in the acinar areas. (H) CD34 staining in the case 3 specimen shows intense CD34 expression in the periductal areas, as well as at the periductal margins of the acinar areas. (I) A high magnification view of (H) shows that CD34^+^ cells have a *spindlelike shape*, consistent with the shape of fibroblasts. (J) Laminin staining in the case 3 specimen shows multilayered and attenuated basal laminae of acini and ducts. Magnification, (A) ×155; (B) ×100; (C through H) ×70; (I) ×900; (J) ×340. A, acini; D, duct.

In patients with chronic GVHD, mononuclear cells were mainly found around medium-sized ducts in the interlobular areas, whereas there were only a small number of mononuclear cells in the acinar area (Figs. 1A, 1B). Infiltrating lymphocytes in the periductal areas were predominantly T cells, regardless of the severity of dry eye (Figs. 1C, 1D). In patients with severe dry eye, the intralobular interstitium was fibrotic, and partially myxedematous (Figs. 1E, 1F). The margins of the lobules were irregularly replaced by fibrotic tissue, and the small ducts of fibrotic areas appeared to dilate and proliferate (Figs. 1E, 1F). In contrast, fibrosis in the intralobular interstitium was not apparent, and the acini had an almost normal structure in one patient with mild dry eye (case 1, Figs. 1A, 1B). Mallory staining confirmed dense and severe fibrosis of the interstitium, which was more intense in the periductal areas than in the acinar areas (Fig. 1G). CD34 was expressed in the periductal areas and at the margins of the acinar areas, where the fibrosis was apparent (Fig. 1H). The majority of CD34^+^ cells had a spindle-like shape consistent with the morphology of fibroblasts (Fig.
An increase in the number of CD34+ fibroblasts was more prominent in patients with severe dry eye than in those with mild dry eye. There were CD34+ cells in the walls of small blood vessels, and those cells appeared to be endothelial cells. Laminin staining revealed multilayered and thickened basal lamina around ducts (Fig. 1J). The basal laminae of blood vessels were also multilayered in all patients with chronic GVHD.

Electron Microscopic Findings

On initial overview of lacrimal gland specimens from patients with chronic GVHD by electron microscopy, we noted an increased number of fibroblasts in a collagenous matrix of the interlobular stroma. In addition, we found a number of lymphocytes around ducts and sometimes in ductal epithelia (Figs. 2A, 2B). Therefore, we focused on stromal fibroblasts and lymphocytes located in the periductal areas and the changes in the basal laminae of ducts and blood vessels.

Stromal Fibroblasts

Stromal fibroblasts in the lacrimal gland had abundant, well-developed, rough endoplasmic reticula and Golgi apparatus (Figs. 2A, 2B, 2C). Subplasmalemmal linear densities (i.e., cell-to-matrix junctions having a lamina-like structure,20,21) were observed on the surface of fibroblasts in two patients (Fig. 2C, arrowhead and inset). Fibroblasts were attached to various types of inflammatory cells, including lymphocytes (Figs. 2A through 2F), macrophages (Fig. 2E), plasma cells, and myoepithelial cells (Fig. 2G). Primitive or rudimentary contacts between fibroblasts and inflammatory cells were frequently observed around ducts and vessels (Figs. 2C through 2F, 2F inset)—i.e., closely apposed plasma membranes that were separated by a narrow interspace and an electron-dense amorphous material was visible near the membrane surface of the apposed membranes.22 In the high-magnification view of the area within the square is shown in (G). (C) Subplasmalemmal linear densities are observed on the surface of fibroblasts (arrowhead and inset; case 1). T cells with clustered dense bodies (star) have infiltrated the periductal areas and attached to fibroblasts. (D) T cells with clustered dense bodies (star) in the periductal areas are attached to another (arrowheads) and to fibroblasts (arrow) through primitive contacts (case 1). (E) Lymphocyte and macrophages attached to fibroblasts in the perivascular area (case 1). (F) A primitive contact between a fibroblast and a lymphocyte (case 4). Inset: Typical primitive contact (arrow).
presence of newly synthesized collagen fibrils was independent of dry eye severity.

**Lymphocytes Infiltrated around Ducts**

The lymphocytes in the periductal areas were mostly T cells with clustered dense bodies and attached to one another by primitive contacts (Figs. 2C, 2D). Lymphocytes, in conjunction with macrophages, were often observed in the perivascular areas (Fig. 2I). Figure 2J shows the disrupted basal lamina of a large duct (arrows) and a number of lymphocytes infiltrated into the ductal epithelia in a patient with mild dry eye (case 1). The lymphocytes are attached one another and also make contact with myoepithelial cells. A dead epithelial cell is found in the duct’s lumen. (K) Multilayered and thickened basal lamina of a capillary (case 1).

**Changes in the Basal Laminae of Ducts and Blood Vessels**

Multilayered basal laminae of capillaries were frequently observed in all patients with chronic GVHD (Fig. 2K). The basal laminae of ducts and lobules were also multilayered and thickened (Figs. 2L, 2M, 2N). Thickening of the basal laminae of lobules and ducts was more prominent in patients with severe dry eye (Figs. 2L, 2M) than in those with mild dry eye (Fig. 2J). In patients with severe dry eye, the basal laminae were markedly thickened and the cytoplasmic processes of the myoepithelial cells were elongated (Fig. 2M). In contrast, the thickening of the blood vessel basal laminae was similar between patients with severe dry eye and those with mild dry eye.
Pathologic Features in Chronic GVHD in Comparison with SS

Table 3 summarizes the pathologic features of the lacrimal glands in five patients with chronic GVHD and five patients with SS. Lymphocytes were the predominant infiltrating cells in the periductal areas in four of the five patients with chronic GVHD, whereas lymphocyte infiltration of the acinar areas was found in three patients with SS. The degree of infiltrating T cells was similar between chronic GVHD and SS, but the number of infiltrating B cells was significantly lower in chronic GVHD than in SS. Moreover, clusters of plasma cells (Fig. 2O) were frequently observed in the interlobular areas of patients with SS, but these were not seen in patients with chronic GVHD. Excessive fibrosis in the extracellular matrix was more prominent in chronic GVHD than in SS. An increase in the number of CD34⁺ fibroblasts was found in all patients with chronic GVHD, but in only one with SS. These findings, associated with chronic GVHD, were more prominent in patients with severe dry eye compared with those with mild dry eye.

Using ultrastructural techniques, we observed primitive contacts between fibroblasts and lymphocytes, newly synthesized collagen fibrils, thickening of the basal laminae in lobules and ducts, and multilayered basal laminae in blood vessels of all lacrimal gland samples collected from patients with chronic GVHD.

<table>
<thead>
<tr>
<th>Table 3. Histopathologic Features of Lacrimal Glands in Patients with Chronic GVHD and in Patients with SS</th>
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<tbody>
<tr>
<td><strong>Histopathologic Findings</strong></td>
</tr>
<tr>
<td>Severity of dry eye</td>
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<tr>
<td><strong>Light microscopic findings</strong></td>
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<tr>
<td>The predominant site of lymphocyte infiltration*</td>
</tr>
<tr>
<td>Degree of infiltrating T and B cells (grade 1–4)†</td>
</tr>
<tr>
<td>T cells</td>
</tr>
<tr>
<td>B cells</td>
</tr>
<tr>
<td>Excessive fibrosis of extracellular matrix‡</td>
</tr>
<tr>
<td>Increase in CD34-positive fibroblasts‡</td>
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<tr>
<td><strong>Ultrastructural microscopic findings</strong></td>
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<tr>
<td>Primitive contacts between fibroblasts and lymphocytes‡</td>
</tr>
<tr>
<td>Subplasmalemmal linear densities‡</td>
</tr>
<tr>
<td>Newly synthesized collagen‡</td>
</tr>
<tr>
<td>Thickening of basal lamina of lobules and ducts‡</td>
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<tr>
<td>Multilayered basal lamina of vessels‡</td>
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</table>

* D. periductal area; DA. periductal and acinar area; A. acinar area.
† Scored according to the grading scale of Greenspan et al. with modifications.
‡ None (−), slight (±), frequent (+), and marked (++).
GVHD, but infrequently in the samples from patients with SS. Moreover, we detected thickening of the basal laminae of lobules and ducts in samples from all the patients with chronic GVHD, but in only one sample from a patient with SS. This thickening was apparent even though the mean age of the patients with chronic GVHD was less than that of the patients with SS. One patient with SS who had rheumatoid arthritis (case 7) had multilayered basal laminae of blood vessels that was comparable with those seen in lacrimal gland samples from patients with chronic GVHD.

**DISCUSSION**

This is the first comprehensive study examining detailed histopathologic characteristics of the lacrimal gland in patients with chronic GVHD. Although clinical presentation is similar between chronic GVHD and SS, there are significant differences in pathologic findings between these two diseases. The histopathologic findings in chronic GVHD that were different from SS can be summarized as follows: predominant infiltration of T cells into the periductal area, an increased number of stromal fibroblasts, excessive fibrosis in the extracellular matrix, and multilayered basal laminae of ducts, lobules, and vessels. These results indicate substantial differences in the pathogenic process between chronic GVHD and SS.

In chronic GVHD, lymphocytes, mainly T cells, appeared to infiltrate the periductal areas and the ductal epithelia. These pathologic observations are consistent with those seen in the lacrimal glands in an animal model of GVHD. In a published study of patients with chronic GVHD, in whom cell invasion of ductal epithelium led to the destruction of the epithelium and ductal ectasia. Multilayered basal laminae of blood vessels are believed to be the result of repeated damage and repair of the vessel walls. Multilayered basal laminae of capillaries may be one of the initial processes involved in the pathogenesis of chronic GVHD. Fibrosis in the lacrimal glands of patients with chronic GVHD are stromal fibroblasts residing in the intra- and interlobular areas. Another possibility is that fibroblasts in the lacrimal gland originate from donor fibrocytes, a novel population of blood-borne cells with a spindlelike morphology and a distinct cell-surface phenotype (collagen/CD13/CD45). Multilayered basal laminae of blood vessels are believed to be the result of repeated damage and repair of the vessel walls. Multilayered basal laminae of capillaries are frequently observed in patients with systemic vasculopathy, such as diabetes mellitus and rheumatoid arthritis. In fact, the blood vessels of a patient with SS who had rheumatoid arthritis in this study (case 7) contained multilayered basal laminae. The breakdown of the basal lamina in patients who received SCT may be associated with vascular injury due to the use of antitumor drugs or immunosuppressants or to a cytomegalovirus infection. Because multilayering of the blood vessel basal laminae was observed in all patients with chronic GVHD, including a patient in the early stage of the disease, the breakdown of capillaries may be one of the initial processes involved in the pathogenesis of dry eye.

We found multilayered and thickened basal laminae of lobules and ducts to be characteristic of lacrimal glands in patients with chronic GVHD. The degree of thickening of the basal lamina was correlated with the severity of dry eye. Similar findings were previously reported in the skin of patients with chronic GVHD but not in the lacrimal gland. Based on the findings of a breakdown of the basal laminae of the larger ducts and the infiltration of T cells in a patient with mild dry eye (case 1), multilayering of the basal laminae may be due to the accelerated production of extracellular matrix by fibroblasts and myoepithelial cells through contacts with the infiltrating T cells.

Because four of five patients with chronic GVHD had received total body irradiation (TBI) as a part of the conditioning regimen for SCT, the effects of TBI on the pathologic findings in these patients should be considered when interpreting our results. Therefore, the possibility that marked fibrosis and alteration of the basal laminae observed in patients with chronic GVHD may be caused by TBI cannot be excluded. However,
Sale et al.\textsuperscript{5} reported no significant correlation between TBI and histopathologic findings, which included ductal epithelial changes and the invasion of duct walls by lymphocytes. Moreover, excessive fibrosis and thickening of the basal laminae of vessels, lobules, and ducts were also found in the one patient (case 4) who did not receive TBI (Table 3). To further clarify this possible discrepancy, a large-scale clinical study comparing patients with GVHD with and without having undergone TBI would have to be undertaken.

It is well known that some aspects of the clinical and histopathologic features in chronic GVHD resemble those in scleroderma,\textsuperscript{39} an autoimmune disease exhibiting excessive fibrosis in the skin and internal organs such as the lung and the gastrointestinal tract. A recent report has shown that both scleroderma and chronic GVHD are T helper cell type 2 (Th2)-dominant diseases, based on high levels of serum Th2 cytokines and high CD30 expression on T cells infiltrating the skin.\textsuperscript{40} In addition, pathologic features, such as fibroblast and T cell interaction, observed in the lacrimal gland of patients with chronic GVHD, have also been documented in the skin of patients with scleroderma.\textsuperscript{41,42} Therefore, it is likely that there are similarities between the pathogenesis of scleroderma and chronic GVHD. In scleroderma, the interaction between fibroblasts and T cells is mediated in part by CD54 and LFA-1.\textsuperscript{43,44} Moreover, transforming growth factor (TGF)-β, interleukin (IL)-4, and IL-6, which are produced by fibroblasts alone in an autocrine fashion or by activated T cells, have been shown to promote the activation of fibroblasts.\textsuperscript{45} These observations suggest that the activation of fibroblasts in the lacrimal gland of patients with chronic GVHD is mediated through a cognate contact with T cells through receptor-ligand interactions, as well as by cytokines produced by activated T cells.

Our findings suggest an hypothesis for the pathogenesis of dry eye in patients with chronic GVHD. Specifically, a breakdown of the blood vessel basal laminae induces the migration of donor T cells into the lacrimal gland tissues. These donor T cells are then activated, migrate into the periductal areas, and contribute to the destruction of the ductal epithelia. At the same time, CD34\textsuperscript{+} stromal fibroblasts are activated by cytokines released by inflammatory cells. These activated fibroblasts synthesize an excessive amount of extracellular matrix, resulting in rapid interstitial fibrosis. In this scenario, the suppression of activated donor T cells and fibroblasts early in the course of the disease could lead to the prevention of progressive dry eye. Further investigation to identify the target antigens recognized by the infiltrating donor T cells and the functional properties of stromal fibroblasts are needed to further elucidate the pathogenesis of dry eye in patients with chronic GVHD.

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\section*{References}


