Role of Heat Shock Protein 47, a Collagen-Binding Chaperone, in Lacrimal Gland Pathology in Patients with cGVHD

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PURPOSE. Uncontrolled fibrosis due to excessive accumulation of extracellular matrix proteins in the lacrimal glands of patients with chronic graft-versus-host disease (cGVHD) is well documented. Heat-shock protein 47 (HSP47) is involved in the molecular maturation of collagen and has been shown to have a fibrogenic role in various fibrotic diseases. In this study, the role of HSP47 in the pathogenesis of lacrimal gland of patients with cGVHD was investigated.

METHODS. The expression of HSP47, Ki67 (a proliferation marker), types I and III collagen, and α-smooth muscle actin (α-SMA) was examined in tissue sections and in primary cultures of fibroblasts obtained from the lacrimal glands of patients with cGVHD (n = 8) and Sjögren’s syndrome (SS; n = 7).

RESULTS. Tissue sections of the lacrimal glands of patients with cGVHD showed markedly increased expression of HSP47 in fibroblasts around the mediumsized ducts than did those from patients with SS. The elevated expression of HSP47 in patients with cGVHD was mostly detected in Ki67-positive fibroblasts and was associated with increased accumulation of types I and III collagen in and around the fibrotic areas. Primary fibroblast cultures generated from cGVHD lacrimal gland showed higher HSP47 mRNA expression than did fibroblasts isolated from SS biopsy tissue, as determined by RT-PCR (P < 0.05). In contrast, α-SMA was higher in the SS than cGVHD fibroblasts at both mRNA and protein levels, and more lacrimal gland fibroblasts in the SS were positive for α-SMA than cGVHD (P < 0.01).

CONCLUSIONS. In cGVHD, increased expression of HSP47 may promote excessive collagen assembly in and around the periductal areas where fibroblasts are mostly in an active state. The less α-SMA in the cGVHD lacrimal gland fibroblasts suggests a relative lack of myofibroblastic transformation. It is likely that fibroblasts incapable of myofibroblastic transformation are the main source of HSP47 and collagen production, and the resultant effect is the periductal fibrotic changes seen in lacrimal glands of patients with cGVHD. (Invest Ophthalmol Vis Sci. 2007;48:1079–1086) DOI:10.1167/iovs.06-0601

Chronic graft versus host disease (cGVHD) is one of the late complications after allogeneic hematopoietic stem cell transplantation (HSCT).1–3 Dry eye is a major complication of cGVHD that affects the quality of life of patients who undergo HSCT.3–4 We have shown that an increased number of CD34+ fibroblasts are associated with the excessive fibrosis in and around the periductal areas in the lacrimal glands in cGVHD, resulting in rapidly progressing dry eye.5 Furthermore, we reported that the periductal area is the primary site for T-cell activation and is where the activated T cells interact with the activated fibroblasts.6 These findings indicate that fibroblasts play a central role in the pathologic condition in lacrimal glands of patients with cGVHD.

Heat shock protein 47 (HSP47) is a 47-kDa stress glycoprotein that resides in the endoplasmic reticulum of collagen-secreting cells. It functions as a collagen-specific molecular chaperone in the folding, and assemblies of procollagen molecules are well-documented. It interacts with various collagens, including types I to V and is required for the formation of correct triple-helical structure.7,8 Recently, several studies have shown an association between increased expression of HSP47 by the matrix-producing cells, and the excessive accumulation of collagens in human and experimental fibrotic diseases, including ocular cicatricial pemphigoid,8 pulmonary fibrosis,9 renal scarring,10 and neointima in atherosclerosis.11 However, little is known about the role of HSP47 in the fibrosis of the lacrimal gland disease in systemic cGVHD.

We studied the expression of HSP47 and several other molecules involved in fibrosis, including collagen types I and III and α-smooth muscle actin (α-SMA), in lacrimal gland biopsy specimens and in fibroblasts isolated from patients with cGVHD. Earlier studies have shown that the expression of HSP47 in various organs is closely associated with increased accumulation of various types of collagens.12–14 Furthermore, the proliferation status of the fibroblasts in the lacrimal glands was evaluated by assessing the expression of Ki67, a marker of proliferating cells.15

MATERIALS AND METHODS

Study Design

Biopsy specimens of lacrimal glands from eight patients with cGVHD, including tissue sections from five patients (cases 1, 3, 4, 5, and 13) and cultured fibroblasts from three patients (cases 15–17), were examined, and compared with samples from seven control patients with Sjögren’s syndrome (SS) including tissue sections from three patients (cases 7–9) and cultured fibroblasts from four patients with SS (cases 18–21). The clinical characteristics of the eight patients with cGVHD are summar-
ized in Table 1. Written informed consent was obtained in advance from all the patients in accordance with the guidelines of the Institutional Review Board and the Declaration of Helsinki for research involving human subjects. Lacrimal gland tissues obtained by biopsy were used for histologic analysis and primary fibroblast cultures, as described previously. Proliferating adherent cells were passaged and expanded, and at the third to fifth passages the cells were used for further study.

Clinicopathological diagnosis of cGVHD was based on previously reported criteria. Patients with localized skin and/or liver dysfunction were diagnosed with limited cGVHD. When patients had localized skin involvement or liver dysfunction plus chronic aggressive hepatitis, eye involvement (results of Schirmer’s test or liver dysfunction in addition to chronic aggressive hepatitis), or myoepithelial involvement, or involvement of other target organs, we diagnosed extensive cGVHD. SS was diagnosed in the patients, as proposed by Fox et al., when all four of the following criteria were met: (1) objective evidence of keratoconjunctivitis sicca, as documented by rose bengal or fluorescein dye staining; (2) objective evidence of diminished salivary or lacrimal gland flow; (3) minor salivary gland or lacrimal gland biopsy, obtained through normal mucosa, with the specimen containing at least four evaluable salivary or lacrimal gland lobules, and having an average of at least two foci/4 mm²; and (4) evidence of a systemic autoimmune process, as manifested by the presence of autoantibodies, such as rheumatoid factor and/or antinuclear antibody and/or antibodies to SSA or SSB.

Dry eye was diagnosed according to published criteria. Severe dry eye was defined as reduced reflex tearing (Schirmer test with nasal stimulation ≥10 mm) and ocular surface abnormality (rose bengal score ≥3 and/or fluorescein score ≥1).

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections, as described previously. Briefly, deparaffinized sections were soaked in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature to inactivate endogenous peroxidase activity. The sections were then blocked with 10% goat serum for 30 minutes at room temperature to inactivate endogenous peroxidase. The sections were then washed with PBS and incubated further with a mixture of 3,3′-diaminobenzene-4 HCl (DAB) and H₂O₂. The blocking solution, secondary antibody, and streptavidin-peroxidase solution were from a histology kit (Histostain kit; Nichirei, Tokyo, Japan). The staining pattern was graded semiquantitatively according to the intensity and distribution of the staining, as described in our earlier reports. Positive staining for HSP47, collagens and α-SMA was determined by comparison with smooth muscle cells of the vessels or myoepithelium in the same section as a positive reference. We regarded the expression as positive when more than one cell showed a staining intensity similar to that of the reference cells. The staining intensity of HSP47, collagen types I and III, and α-SMA was graded semiquantitatively according to the following scale: (−) no staining, (+) moderate staining, and (++) strong staining.

To double-stain for HSP47 and Ki67 in paraffin-embedded tissue sections, we performed antigen unmasking by autoclaving the sections at 120°C for 20 minutes in 10 mM sodium citrate buffer (pH 6.0). For the immune reaction, we used a mouse anti-HSP47 antibody (Stressgen Biotechnologies Corp.) followed by an Alexa 568-conjugated rabbit anti-mouse secondary antibody (Invitrogen-Molecular Probes, Eugene, OR) and a FITC-conjugated mouse anti-Ki67 antibody (Dako). For double-staining of cultured fibroblasts, the endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide for 5 minutes. A mouse anti-human HSP47 (Stress Gen Biotechnologies Corp., Victoria, BC, Canada), mouse anti-human type I collagen, mouse anti-human type III collagen (Daiichi Fine Chemicals, Takaoka City, Japan), or mouse anti-human α-SMA (Dako, Glostrup, Denmark). Normal mouse serum was used as a negative control. After being washed with PBS, the sections were treated with a biotinylated mouse secondary antibody for 15 minutes, washed with PBS, and incubated further with streptavidin-peroxidase. The reaction products were developed with a mixture of 3,3′-diaminobenzene-4 HCl (DAB) and H₂O₂. The blocking solution, secondary antibody, and streptavidin-peroxidase solution were from a histology kit (Histostain kit; Nichirei, Tokyo, Japan). The staining pattern was graded semiquantitatively according to the intensity and distribution of the staining, as described in our earlier reports. Positive staining for HSP47, collagens and α-SMA was determined by comparison with smooth muscle cells of the vessels or myoepithelium in the same section as a positive reference. We regarded the expression as positive when more than one cell showed a staining intensity similar to that of the reference cells. The staining intensity of HSP47, collagen types I and III, and α-SMA was graded semiquantitatively according to the following scale: (−) no staining, (+) moderate staining, and (++) strong staining.

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**Table 1. Clinical Characteristics of Patients with cGVHD**

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age at Biopsy</th>
<th>Degree of Dry Eye</th>
<th>Underlying Disease</th>
<th>Donor</th>
<th>TBI</th>
<th>Clinically Affected Chronic GVHD Organs</th>
<th>Time of Biopsy after HSCT (mo)</th>
<th>Interval Between Onset of Dry Eye and Biopsy (mo)</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>32</td>
<td>Mild</td>
<td>APL</td>
<td>Related</td>
<td>+</td>
<td>Eye, liver</td>
<td>36</td>
<td>18</td>
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<tr>
<td>3</td>
<td>F</td>
<td>35</td>
<td>Severe</td>
<td>CML</td>
<td>Unrelated</td>
<td>+</td>
<td>Eye, skin, mouth, lung, liver</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>26</td>
<td>Severe</td>
<td>CML</td>
<td>Related</td>
<td>−</td>
<td>Eye</td>
<td>26</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>28</td>
<td>Severe</td>
<td>CML</td>
<td>Related</td>
<td>+</td>
<td>Eye, skin, intestine, mouth, lung</td>
<td>9</td>
<td>4</td>
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<tr>
<td>13</td>
<td>F</td>
<td>44</td>
<td>Severe</td>
<td>MDS</td>
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<td>Eye, skin, intestine, mouth</td>
<td>11</td>
<td>5</td>
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<tr>
<td>15</td>
<td>M</td>
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<td>Mild</td>
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<td>16</td>
<td>F</td>
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<td>Eye, mouth, liver</td>
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<tr>
<td>17</td>
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<td>36</td>
<td>Severe</td>
<td>MM</td>
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<td>−</td>
<td>Eye, mouth, liver</td>
<td>11</td>
<td>9</td>
</tr>
</tbody>
</table>

TBI, total body irradiation; APL, acute promyelocytic leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma.

**Table 2. Primer Sequences Used in RT-PCR Analysis**

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Analyzed</th>
<th>Primer Sequences</th>
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<tr>
<td>HSP47</td>
<td>Sense</td>
<td>5′-GAGGTGACGACGCAACGAGCTAGTGGATGACGAGCAT-3′</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Sense</td>
<td>5′-ACC CCC ACG TGTGACGAGCAT-3′</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>Sense</td>
<td>5′-ACGGTACGTGCGCGCAACGCAGCAT-3′</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Sense</td>
<td>5′-GAGGTGACGACGCAACGAGCTAGTGGATGACGAGCAT-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-AAAACACATTAACCGTGCAG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-TATGCTCGCTGCGCGCAACGAGCAT-3′</td>
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</table>

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of the primary antibody. At least 300 cells per culture were evaluated, to enumerate the proportion of cells staining positive for each protein.24

For fluorescent staining, the coexpression in cultured fibroblasts of HSP47, collagen types I and III, and α-SMA was examined by double-staining with a rabbit anti-HSP47 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) used with an Alexa 488-conjugated goat anti-rabbit secondary antibody (Invitrogen-Molecular Probes), or with a mouse anti-collagen type I, anti-collagen type III, or anti-α-SMA (Dako) antibody used with an Alexa 568-conjugated rabbit anti-mouse secondary antibody (Invitrogen-Molecular Probes). Nuclei were counterstained with TO-PRO-3 (Invitrogen-Molecular Probes). Isotype-matched mouse antibodies were used as control subjects. These tissue sections and the cultured fibroblasts used for fluorescent staining were mounted on glass slides and examined with a confocal microscope (LSM5 Pascal; Carl Zeiss Meditec, GmbH, Göttingen, Germany).

Transmission Electron Microscopy
A portion of lacrimal gland tissue was immediately fixed with 2.5% glutaraldehyde and subjected to electron microscopic examination, as described previously.9 One-micrometer-thick sections were stained with methylene blue, and the portions of interest were thin sectioned and examined with an electron microscope (1200 EXII, JEOL, Tokyo, Japan).

Reverse Transcription–Polymerase Chain Reaction
A standard RT-PCR procedure was used to measure transcript levels. The gene-specific primers for collagen types I and III, HSP47, α-SMA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control) are listed in Table 2. Briefly, total RNA was extracted from cultured fibroblasts that were isolated from the lacrimal glands of patients with cGVHD or SS by using an RNA isolation kit (Qiagen, Valencia, CA). The PCR reactions were as follows: denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds (except for α-SMA, which was at 60°C for 30 seconds), and extension at 72°C for 30 seconds. Twenty-four cycles were performed for HSP47 and α-SMA, and 30 cycles for collagen types I and III (Bio-Rad, Hercules, CA). Control RT-PCRs of GAPDH were conducted for 30 cycles with the GAPDH-specific primers. The reaction products were subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. PCR was performed at least twice to confirm the reproducibility of the results. The semiquantitative analysis was performed using NIH Image (available by ftp at zippy.nimh.nih.gov or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).25

Immunoblot Analysis
Cellular protein of cultured lacrimal gland fibroblasts from patients with cGVHD or SS was prepared by homogenizing the cells using potter homogenizer (10 strokes), and protein concentration was estimated by protein assay (DC Protein Assay Kit; Bio-Rad). Five micrograms of the samples were electrophoresed on polyacrylamide gel under a reducing condition, and resolved proteins were electrophoretically transferred to nitrocellulose membrane. The membrane was incubated for 1 hour in a blocking solution (5% skim milk in TBS containing 0.05% Tween-20) and probed with mAbs (1 μg/ml in TBS containing 1% skim milk and 0.05% Tween-20) for 1 hour. Rabbit anti-human Erk1 antibody (Santa Cruz Biotechnology, Inc.) was used for confirmation of equal protein loading. After the membrane was extensively washed with TBS containing 0.05% Tween-20, it was further incubated with a 1:2000 diluted horseradish peroxidase–conjugated goat anti-mouse IgG antibody for detecting HSP47 and α-SMA, or anti-rabbit IgG antibody for Erk. For detection of type I or III collagen, the membrane was then incubated with 1:2000 diluted biotin-conjugated goat anti-mouse IgG antibody, followed by 1:1000 diluted horseradish peroxidase–conjugated goat anti-biotin antibody. The bands of antigens were detected by chemiluminescence of the product of peroxidase reaction (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology, Inc., Rockford, IL).

Statistical Analysis
Statistical analysis was performed with the Mann-Whitney test.

RESULTS
Fibroblast Activation in the Periductal Area of Lacrimal Glands in cGVHD
First, we examined the distribution of HSP47, α-SMA, collagen, and Ki67 on tissue sections of lacrimal gland specimens obtained from patients with cGVHD and SS. All the biopsied

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932941/ on 07/22/2018)

**Figure 1.** Expression of HSP47, α-SMA, and type III collagen in lacrimal gland tissue sections prepared from patients with cGVHD or SS. (A) Hematoxylin and eosin staining of a section exhibiting typical histologic findings of cGVHD (case 13). Immunostaining of HSP47 for lacrimal gland tissue sections prepared from patients with cGVHD (cases 1, 3, and 5). (C-E) Predominant expression on spindle-shaped fibroblasts (arrows) around the medium-sized ducts, endothelium of capillaries (*asterisks*), and myoepithelium of the ducts (arrowheads). (B) HSP47 immunostaining in an SS specimen, showing a scarcity of HSP47+ fibroblasts (case 9). In serial sections of the cGVHD lacrimal gland biopsy tissue (case 3) that were consecutive to the ones (F) used for HSP47 immunohistochemistry (E), almost no α-SMA-positive interstitial cells are detected in and around the periductal areas. α-SMA is expressed on endothelium of vessels (F, *asterisks*) and myoepithelium of the acini and ducts (F, *arrowheads*). Immunostaining for collagen type III (G, H) shows predominant expression around the periductal and perilacinar areas (case 13) (G), in comparison with a lacrimal gland section from a patient with SS (case 8) (H). Brown: positive immunoreactivity. Dc, duct; Aci, Acinus. Original magnification: (A, B, E, F, G, H) ×100; (C, D) ×200.
lacrimal glands from the patients with cGVHD exhibited a common histologic appearance: the margins of the lobules were irregularly replaced by fibrotic tissue compared with SS control tissue. In the cGVHD lacrimal gland, excessive fibrosis was observed around medium-sized ducts (Fig. 1A), and HSP47 was expressed on the stromal fibroblasts around these ducts, the endothelium of the surrounding vessels, and the myoepithelium of ducts and acini (Figs. 1C–E). The HSP47-expressing stromal fibroblasts were located at the margins of the lobules and around medium-sized ducts (Figs. 1C–E), where an increased accumulation of CD34+/H11001 fibroblasts has been reported. In contrast, the samples from the patients with SS showed only diffuse, faint staining for HSP47 (Fig. 1B).

In serial sections of the cGVHD lacrimal gland biopsies (Figs. 1E, 1F) that were consecutive to the ones used for HSP47 immunohistochemistry (Fig. 1E), almost no α-SMA-positive interstitial cells were detected in and around the periductal areas, except in the myoepithelium of the acini and capillary walls (Figs. 1F). In contrast, increased type III collagen accumulation was detected in the fibrotic areas, especially in the periacinar and periductal areas (Fig. 1G) of the cGVHD samples, compared with the SS control samples (Fig. 1H).

To confirm that the fibroblasts expressing HSP47 in the cGVHD samples were activated and proliferating, we studied the coexpression of HSP47 and Ki67 on tissue sections. We found that a subpopulation of the fibroblasts located around medium-sized ducts in the SS lacrimal gland tissue (Fig. 2C).

Table 3 summarizes the pathologic features of lacrimal gland tissue sections from five patients with cGVHD and three patients with SS. HSP47-expressing fibroblasts accumulated in the periductal areas in all the patients with cGVHD, whereas the staining was diffuse, and few fibroblasts were labeled in these areas in the samples from one of the three patients with SS. The degree of HSP47-expressing endothelium was similar between the cGVHD and SS samples, but the number of myoepithelial cells expressing HSP47 was significantly higher in the cGVHD than in the SS samples. The accumulation of collagens types I and III in the extracellular matrix around the medium-sized ducts was more prominent in the cGVHD than in the SS lacrimal glands.

Table 3. Immunohistochemistry of Tissue Sections from Patients with Lacrimal Gland cGVHD and SS

<table>
<thead>
<tr>
<th>Antibodies of Interest</th>
<th>GVHD1</th>
<th>GVHD3</th>
<th>GVHD4</th>
<th>GVHD5</th>
<th>GVHD13</th>
<th>SS7</th>
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<tbody>
<tr>
<td>HSP47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Endothelium</td>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Epithelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Type I collagen</td>
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<td>+</td>
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<td>Type III collagen</td>
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<td>+</td>
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<td>–</td>
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<tr>
<td>α-SMA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

m, myoepithelium.
* Periductal area.

FIGURE 2. (A–C) Coexpression of Ki67 and HSP47 on stromal fibroblasts in a lacrimal gland section from a patient with cGVHD (case 17) (A, B) and a patient with SS (case 8) (C), by immunofluorescence double staining. Arrows: stromal fibroblasts expressing both Ki67 and HSP47 around medium-sized duct. (D) Electron microscopic findings on lacrimal gland specimens from patients with cGVHD. Note the presence of collagen bundles from the acinar myoepithelium (arrows). Ac, acini; Dc, duct; ME, myoepithelium. Original magnification, (A–C) ✕630; (D) ✕2000.
Transmission electron microscopic observation showed collagen fibers apparently secreted from myoepithelia, suggesting that collagens are secreted from myoepithelia that express HSP47 (Fig. 2D). Together, these findings suggest that the subpopulation of fibroblasts that accumulates at the restricted areas around medium-sized ducts in the cGVHD lacrimal gland is active and produces HSP47. It, therefore, appears that it is not the overall increase in HSP47; rather, it is the increased expression in a selected area in cGVHD lacrimal gland that may be responsible for regulating fibrogenic responses.

**Bone Marrow-Derived Fibroblasts and the Lacrimal Gland Fibrosis of cGVHD**

Bone marrow-derived fibroblasts are shown to be active cells in the synthesis of various matrix proteins, and they do not express α-SMA, a marker of differentiated myofibroblasts, whereas a subset of residential fibroblasts express α-SMA. α-SMA is usually expressed by fibroblasts that have completed their differentiation into myofibroblasts, which generally produce relatively little extracellular matrix proteins. There is evidence also that donor-derived fibroblasts in the pathogenic fibrotic lesion may contribute to the excessive production of extracellular matrix in lacrimal gland cGVHD.

To determine the similar pattern of molecular expression on fibroblasts of the lacrimal gland of patients with cGVHD, we established primary cultures of fibroblasts from the lacrimal gland tissue of patients with cGVHD and SS. We first examined the expression of HSP47, collagens, and α-SMA mRNAs in cultured lacrimal gland fibroblasts from patients with cGVHD versus SS by RT-PCR. The corresponding semiquantitative data, normalized to the internal control, GAPDH, are shown in Table 4. The mRNA expression of HSP47 was higher in fibroblasts isolated from the biopsy tissues of patients with cGVHD than in those from patients with SS. (1.12 ± 0.24 vs. 0.62 ± 0.23, P < 0.05). The expression of α-SMA mRNA trended higher in SS than in cGVHD (0.70 ± 0.94 vs. 0.04 ± 0.02; Table 4). Consistent with the results of our RT-PCR observation, the protein expression of α-SMA was also higher in the fibroblasts isolated from patients with SS than in cGVHD (Fig. 3). However, despite increased mRNA expression of HSP47 in the fibroblasts isolated from the biopsy tissues of patients with cGVHD, we were not able to detect any significant changes in the expression of HSP47 in the protein level through immunoblot analysis.

To further verify the characteristics of cultured lacrimal gland fibroblasts from patients with cGVHD, we used triple immunofluorescence staining to determine the expression of HSP47, collagen types I or III, and α-SMA. The nuclei were labeled with the nuclear stain TO-PRO-3, and the cells were examined by confocal microscopy. All the cultured cGVHD fibroblasts expressed HSP47 and coexpressed collagen types I or III (Figs. 4A, 4B). DAB and immunofluorescence staining for anti-α-SMA, showed that very few cultured lacrimal gland fibroblasts from patients with cGVHD expressed α-SMA (Figs. 4C, 4E). In contrast, the percentage of α-SMA-positive fibroblasts isolated from patients with SS was significantly higher than that of cGVHD (62.2 ± 13.5% vs. 3.1 ± 2.9%, P < 0.01; Figs. 4F, 4G).

These findings suggest that the fibroblasts from the cGVHD lacrimal gland are largely incapable of myofibroblastic transformation, and bone marrow-derived fibroblasts may be actively involved in the fibrotic processes in lacrimal gland cGVHD.

**DISCUSSION**

In this study, the periductal area was identified as the primary site for fibroblast activation and subsequent lacrimal gland fibrosis in patients with cGVHD, as demonstrated by coexpression of HSP47 and Ki67 in the matrix-producing fibroblasts. In addition, there were relatively fewer α-SMA-positive fibroblasts in the lacrimal glands of patients with cGVHD than in those of patients with SS. This finding suggests that in patients with cGVHD, periductal fibroblasts are largely incapable of myofibroblastic transformation and raises the possibility of the contribution of donor-derived fibroblasts to the pathogenic process of lacrimal gland cGVHD.

Our recent histopathologic study of the lacrimal glands of patients with cGVHD demonstrated prominent fibrosis and an increase in stromal fibroblasts in the glandular interstitium around medium-sized ducts, signifying a role for stromal fibroblasts in rapidly progressive dry eye. Moreover, the accumulation of CD4+ and CD8+ T cells, CD154+ activated T cells, and mononuclear cells and stromal fibroblasts expressing HLA-DR, CD54, and costimulatory molecules, were found predominantly around the glandular ducts in lacrimal gland biopsy tissue from patients with cGVHD. Of interest, the periductal area is also the site of increased stromal donor-derived fibroblast accumulation and excessive fibrosis in patients with cGVHD. It is well known that the expression of HSP47 mRNA is markedly induced during the progression of fibrosis, in parallel with the expression of collagen types I and III. Consistent with these earlier studies, the expression of HSP47 was found to be substantially higher in the cGVHD lacrimal gland than in the SS lacrimal gland and was accompanied by an increased deposition of types I and III collagen, mostly in and around the periductal areas. We cultured fibroblasts from cGVHD lacrimal glands to examine their ability to synthesize HSP47 and collagens. As expected, all the cultured fibroblasts isolated from cGVHD lacrimal glands to examine their ability to synthesize HSP47 and collagens. As expected, all the cultured fibroblasts isolated from lacrimal gland cGVHD expressed HSP47. In tissue sections, the activated and proliferating cGVHD lacrimal gland fibroblasts (i.e., those expressing both HSP47 and Ki67) were located mostly in areas...
around medium-sized ducts at the restricted sites of T-cell activation, where fibrogenic responses are initiated. Fibrosis usually consists of an initiation phase, followed by inflammatory and fibrogenic events. Our results indicate that the periductal area was not only the primary site of fibroblast activation and T-cell accumulation, but also the site where fibrotic processes began.

We found that the myoepithelium of ducts and acini in cGVHD lacrimal glands expressed HSP47. Studies reported that adult fibroblasts may be derived from epithelial cells by epithelial–mesenchymal transition, and HSP47 expression in the epithelium could be an early sign of epithelial-mesenchymal transition. Our findings suggest that the myoepithelium may contribute to the production of excessive extracellular matrix. HSP47 expression by myoepithelial cells and the observation of collagen fibrils adjacent to the myoepithelia by electron microscopy suggested that myoepithelial cells indeed secrete collagens. Recently, it was reported that epithelial and mesenchymal interactions play a pivotal role in organogenesis, and fibroblasts regulate the proliferation and differentiation of epithelial tissues. Activated fibroblasts around the ducts and acini in lacrimal gland cGVHD may respond to the epithelia injured by acute GVHD, the conditioning regimen, or infection.

We also found the expression of HSP47 in both fibroblasts and endothelium in the cGVHD lacrimal gland. There are published reports of HSP47 expression in the endothelial cells of blood vessels; in this study, we found an intensity of HSP47 staining similar to that in endothelial cells in different components of the lacrimal gland specimens obtained from patients with cGVHD, validating the specificity of HSP47 staining in our sections.

HSP47, synthesized by cGVHD lacrimal gland fibroblasts, was upregulated in tissue sections and cultures from patients with cGVHD. The number of α-SMA-expressing cultured fibroblasts isolated from the lacrimal gland appeared to be higher in SS than in cGVHD. The expression of α-SMA was confirmed to be higher in SS than in cGVHD at both the mRNA and protein levels. The HSP47-expressing stromal fibroblasts were located at the margins of the lobules and around medium-sized ducts in the tissue sections. In contrast, the HSP47-expressing stromal fibroblasts from the patients with SS distributed diffusely. The almost equal expression of HSP47 and collagen types I and III by immunoblot analysis reemphasizes that it is not the total amount but the distribution and site of expression of HSP47 in the lacrimal gland that determines the focal fibrogenic responses in lacrimal glands of patients with cGVHD. Our results suggest that the HSP47α-SMA fibroblasts were activated locally around medium-sized ducts in lacrimal gland in patients with cGVHD.

Terminally differentiated fibroblasts are usually less active cells in terms of its ability to produce various matrix protein...
synthesis and expresses α-SMA when fibroblasts are transformed into myofibroblasts. Recently, it has been reported that bone-marrow-derived fibroblasts are resistant to TGF-β-induced myofibroblast differentiation and do not express α-SMA. In these observations, most of the collagen-producing fibroblasts were also found to be of bone marrow rather than of local origin. Those bone marrow-derived fibroblasts could not be induced by TGF-β to express α-SMA. Therefore, the pathologic collagen-producing fibroblasts not only were bone marrow-derived but were incapable of transforming into myofibroblasts.

We have reported that nearly half of the CD34+ fibroblasts at the site of pathogenic fibrosis in lacrimal gland cGVHD are of donor origin. In the present study, lacrimal gland fibroblasts of patients with cGVHD were active and proliferating around medium-sized ducts and may have contributed to the production of extracellular matrix. A lower expression of α-SMA in the cultured lacrimal gland fibroblasts of patients with cGVHD is suggestive of a relative lack of myofibroblast transformation, an observation that is very similar to those in earlier reports. Nontransformed fibroblasts in the lacrimal glands of patients with cGVHD may be involved in the excessive synthesis of HSP47 and collagens. A recent study suggested that a substantial percentage of telomerase reverse transcriptase (TERT)-positive fibroblasts with a low expression of α-SMA are derived from bone marrow precursor cells and that TERT may be related to its ability to enhance fibroblast proliferation and prolonged survival. Thus, cGVHD lacrimal gland fibroblasts may have a prolonged lifespan that could contribute to the excessive extracellular matrix production seen in this disorder. Therefore, fibroblasts rather than α-SMA-positive myofibroblasts are the main matrix-regulating cells in patients with cGVHD. Further studies are needed to settle these issues.

Together, these findings suggest that the lacrimal gland cGVHD fibroblast population expresses HSP47 and Ki67 but little α-SMA. It has been reported that bone marrow-derived fibroblasts express several fibroblastic markers in vivo and can be efficiently recruited into fibrotic lesions in response to injurious stimuli, but the degree of recruitment frequency may depend on the tissue microenvironment. Because coexpression of HSP47 and α-SMA has been reported in several fibrotic diseases such as allograft renal tissues and lung, the finding of low α-SMA expression in lacrimal gland cGVHD is dependent on the higher proportion of bone marrow-derived fibroblasts in cGVHD lacrimal gland and seems to be unique to lacrimal gland cGVHD. These findings suggest that bone marrow-derived fibroblasts may contribute to the fibrotic processes in lacrimal gland cGVHD.

Based on our present study and our previous report, we propose a hypothesis for the pathogenetic process of lacrimal gland cGVHD fibrosis (Fig. 5). It is likely that the suppression of activated fibroblasts early in the course of the disease can lead to the prevention of progressive dry eye. Studies have shown that inhibition of the expression of HSP47 reduces collagen synthesis both in vivo and in vitro. It has been documented that phosphorothioate antisense oligodeoxynucleotides to HSP47 inhibit the synthesis of HSP47 and subsequently attenuates the synthesis of type I procollagen. The reduction of the number of HSP47+ cells and protein contents was inhibited by injection of HSP47 antisense oligodeoxynucleotides resulted in the suppression of collagen type I production and the attenuation of skin scar formation in a rat model. Because there is no effective treatment available for cGVHD fibrosis, further studies investigating the fibrotic process in the periductal area in patients with cGVHD would be useful for clarifying the pathogenesis of cGVHD, as well as for the development of focused therapeutic intervention.

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References


