Epithelial Mesenchymal Transition in Human Ocular Chronic Graft-Versus-Host Disease

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Chronic graft-versus-host disease (cGVHD) of the ocular surface and lacrimal gland is a vision-threatening condition that occurs after allogeneic bone marrow transplantation. In this study, we used immunohistochemistry and electron microscopy to investigate whether epithelial mesenchymal transition (EMT) contributed to the pathogenesis of ocular cGVHD. We detected down-regulation of E-cadherin and translocation of β -catenin from the intercellular junction to the cytoplasm and nucleus of cGVHD conjunctival basal epithelia and lacrimal gland myoepithelia. Notable findings included expression of Snail, an inducer of EMT, in the nucleus of ocular cGVHD epithelia. The fibrosis markers heat shock protein 47, α -smooth muscle actin, and fibroblast specific protein-1 were overexpressed in ocular cGVHD epithelia. In addition, p63, a marker of conjunctival basal epithelia, was observed in the nuclei of subconjunctival cells beneath disrupted basal lamina. Disrupted basal lamina and the presence of altered collagen bundles were observed in the cytoplasm and beneath cGVHD epithelia. In contrast, these observations were rarely observed in the normal conjunctiva and in Sjögren's syndrome lacrimal gland epithelia. These findings together indicate that ocular cGVHD epithelia gain the mesenchymal phenotype and the capacity to migrate into the subepithelial stroma. Our findings suggest that EMT may be partially responsible for the conjunctival and lacrimal gland fibrosis found in patients with cGVHD. (*Am J Pathol* 2009, 175:2372–2381; DOI: 10.2353/ajpath.2009.090318)

However, chronic graft-versus-host disease (cGVHD) is a major complication after allogeneic hematopoietic stem cell transplantation (HSCT), which has hampered the success of HSCT.¹ Although numerous advances have been made for treating acute GVHD, the pathogenesis of cGVHD remains largely unknown and effective therapy has not been established. We previously studied the pathogenesis of cGVHD by focusing on the ocular surface and lacrimal gland and found that excessive fibrosis and a subset of fibroblasts contribute to the pathogenesis of ocular cGVHD.^{2,3}

Recently, several studies have reported that epithelial-mesenchymal transition (EMT) contributes to various fibrotic diseases of the kidney,⁴ lung,⁵ and liver.⁶ For example, 40% of fibroblasts in kidney fibrosis arise from epithelial cells via local EMT triggered by inflammatory stress.⁷ EMT is involved in embryonic development, organ fibrosis, and also cancer metastasis.⁸ EMT is characterized by the loss of apical/basal cell polarity and loss of cell-to-cell adhesions, followed by the acquisition of a mesenchymal phenotype, ie, migration and invasion ability and expression of mesenchymal markers.

EMT is triggered by various stimuli including irradiation,⁹ hypoxia,¹⁰ reactive oxygen species,¹¹ inflammatory cytokines such as transforming growth factor- β and fibroblast growth factor,⁸ disruption of basal lamina, and exposure of cytoplasm to extracellular matrix.¹² These triggers of EMT also participate in conjunction with the pathogenesis of cGVHD after HSCT. In a clinical setting, total body irradiation before HSCT and migrating inflammatory cells after HSCT generate substantial proinflammatory cytokines.¹ This “cytokine storm” then acts on T cells in the graft, prompting them to attack host antigens.¹³ In addition, reactive oxygen species-mediated organ injury was reported in bone marrow transplant recipients.¹⁴ The purpose of this study was to elucidate

Supported by the Japanese Ministry of the Education, Science, Sports and Culture (20592058).

Accepted for publication August 17, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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Allogeneic hematopoietic stem cell transplantation is a potentially curative treatment for hematological malignancies.

Table 1. Demographics and Medication of Patients with Conjunctival GVHD

Case	Sex	Age at biopsy (yr)	Degree of dry eye	Underlying disease	Type of donor	Clinically affected cGVHD organ	Time of after HSCT (mo)	Interval between onset of dry eye and biopsy (mo)	Systemic therapy for GVHD	Topical therapy for GVHD
1	F	32	Mild	APL	Related	Eye, liver	36	18	PSL, 3 mg*	AT, HA, VA, AS
2	F	35	Severe	CML	Unrelated	Eye, skin, mouth, lung, liver	14	7	PSL, 35 mg*	AT, S, T
3	F	37	Mild	CML	Related	Eye	13	2	Cys A, 50 mg*	AT, VA
4	F	42	Severe	ALL	Related	Eye, mouth, lung, liver	26	12	Off	AT, HA, VA, S
5	F	36	Severe	MM	Related	Eye, mouth, liver	11	9	Cys A, 150 mg/100 mg [†]	AT, HA,
6	F	47	Mild	AML	Unrelated	Eye, liver	5	8	Cys A, 50 mg [†]	AT, HA, VA
7	M	28	Severe	ALL	Related	Eye, skin, mouth, lung, intestine	9	4	PSL, 70 mg/8 mg [†]	AT, VA, S, B
8	M	38	Mild	MDS	Unrelated	Eye	9	2	FK506, 1 mg [†]	AT, HA
9	M	50	Severe	AML	Related	Eye, mouth	11	7	Off	AT, HA, VA
10	M	50	Severe	CML	Related	Eye, skin, mouth, lung, liver	72	69	PSL, 40 mg*	HA, S, B
11	M	53	Mild	ALL	Unrelated	Eye, skin, mouth, intestine	3	7	PSL, 5 mg*	AT, HA, VA

F, female; M, male; APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia; ALL, acute lymphocytic leukemia; MM, multiple myeloma; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; PSL, prednisolone; Cys A, cyclosporine A; FK506, tacrolimus; AT, artificial tears frequently/day; HA, topical hyaluronic acid 5 times/day; VA, topical vitamin A 5 times/day; AS, autologous serum 5 times/day; S, topical steroid 4 times/day; B, topical antibiotics 3 times/day.

*Per day.

[†]Alternative day.

whether EMT is involved in mucosal and exocrine gland cGVHD.

Materials and Methods

Patients

We studied 13 allogeneic HSCT recipients who had clinically significant dry eye¹⁵ and were diagnosed with cGVHD.¹⁶ Conjunctive biopsies from 11 patients with cGVHD (Table 1) and lacrimal gland biopsies from 9 patients with cGVHD (Table 2) were taken for diagnostic purposes in all patients. Biopsy specimens were compared with controls consisting of 5 normal conjunctival tissue samples, one non-GVHD conjunctival tissue sam-

ple from a HSCT patient suspected of a malignant tumor of the conjunctiva, and 5 lacrimal gland samples from patients with Sjögren's syndrome serving as controls. Written informed consent was obtained in advance from all patients in accordance with the principles expressed in the Declaration of Helsinki. This study was approved by the Keio University Institutional Review Boards.

Histology and Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed paraffin-embedded tissue sections as described previously. In brief, antigen unmasking for p63 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), heat shock protein (HSP) 47 (Stress Gen Biotechnologies Corp, Vic-

Table 2. Demographics and Medication of Patients with Lacrimal Gland cGVHD

Case	Sex	Age at biopsy (yr)	Degree of dry eye	Underlying disease	Donor	TBI	Clinically affected chronic GVHD organs	Time of biopsy after HSCT (mo)	Interval between onset of dry eye and biopsy (mo)	Systemic therapy for GVHD	Topical therapy for GVHD
1	F	32	Mild	APL	Related	+	Eye, liver	36	18	PSL, 3 mg*	AT, HA, VA, AS
2	F	35	Severe	CML	Unrelated	+	Eye, skin, mouth, lung, liver	14	7	PSL, 35 mg*	AT, S, T
3	F	37	Mild	CML	Related	-	Eye	13	2	Cys A, 50 mg*	AT, VA
4	F	42	Severe	ALL	Related	+	Eye, mouth, lung, liver	23	12	Off	AT, HA, VA, S
5	F	36	Severe	MM	Related	-	Eye, mouth, liver	11	9	Cys A, 150 mg/100 mg [†]	AT, HA,
12	F	44	Severe	MDS	Unrelated	+	Eye, skin, intestine, mouth	11	5	PSL/FK506, 35/1.5 mg*	AT, HA, VA
13	M	26	Severe	CML	Related	-	Eye	26	11	PSL, 3 mg*	AT, VA, S
7	M	28	Severe	ALL	Related	+	Eye, skin, intestine, mouth, lung	9	4	PSL, 70/8 mg [†]	AT, VA, S, B
8	M	38	Mild	MDS	Unrelated	+	Eye	9	2	FK506, 1 mg*	AT, HA

TBI, total body irradiation; F, female; M, male; APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; MDS, myelodysplastic syndrome; PSL, prednisolone; Cys A, cyclosporine A; FK506, tacrolimus; AT, artificial tears frequently/day; HA, topical hyaluronic acid 5 times/day; VA, topical vitamin A 5 times/day; AS, autologous serum 5 times/day; S, topical steroid 4 times/day; B, topical antibiotics 3 times/day.

*Per day.

[†]Alternative day.

toria, BC, Canada), and Snail (Abgent, San Diego, CA) was performed by autoclaving the sections at 120°C for 20 minutes in 10 mmol/L sodium citrate buffer, pH 6.0, to detect intranuclear and intracytoplasmic antigen. Deparaffinized sections were soaked in 0.3% hydrogen peroxidase in methanol for 30 minutes at room temperature to inactivate endogenous peroxidase activity. The sections were blocked with 10% goat serum for 1 hour and then were incubated overnight at 4°C with the following primary antibodies: mouse anti-human HSP47, mouse anti-human Snail, and mouse anti- β -catenin (Dako, Glostrup, Denmark). After washing with PBS, the sections were treated with peroxidase-conjugated secondary antibody (EnVision+, Dako) in combination with nuclear staining with hematoxylin. The negative controls were tissue sections incubated with normal mouse IgG instead of the primary antibody. In some experiments, frozen sections were used to stain for E-cadherin (Santa Cruz Biotechnology, Inc), β -catenin (Chemicon, Temecula, CA), α -smooth muscle actin (α -SMA) (Dako), type IV collagen (Chemicon), and matrix metalloproteinase (MMP)-9 (Santa Cruz Biotechnology, Inc.).

The DAB and fluorescein staining pattern was graded semiquantitatively according to the intensity and distribution of the staining as described in our earlier reports.^{2,3} Positive staining for HSP47 and α -SMA was determined by comparison with blood vessels and smooth muscle cells or myoepithelium in the same section as the positive reference. We regarded the expression as positive when more than one cell showed a staining intensity similar to that of reference cells. Images were photographed with a microscope (COOLSCOPE II, Nikon Corporation, Tokyo, Japan). All of the slides were reviewed twice in a blind manner by three independent ophthalmologists and a pathologist and the histological architecture and staining were assessed. The number of p63-positive cells in the conjunctival stroma in the subepithelial lesion was counted at $\times 200$ images of each photograph as described previously.¹⁷ To investigate the amount of cells undergoing EMT, the number of cells expressing both the epithelial marker p63 and the mesenchymal marker HSP47 were counted in conjunctival samples. We analyzed at least five separated fields of subepithelial stroma including conjunctiva and lacrimal gland in each sample. The degraded basement membrane area was assessed and graded as none (score of 0), one area of disrupted lamina (score of 1), or more than two areas of disrupted basal lamina (score of 2).

To double stain for p63 and type IV collagen, we used a rabbit anti-type IV antibody followed by an Alexa 568-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Carlsbad, CA) and a mouse p63 antibody followed by an Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes). Double staining for p63 and HSP47 was done using a mouse p63 antibody visualized by diaminobenzidine (DAB) staining, and then a mouse anti-HSP47 antibody followed by an Alexa 488-conjugated goat anti-mouse secondary antibody (Molecular Probes). Tissue sections for fluorescent staining were mounted on glass slides and examined with an LSM5 PASCAL confocal microscope (Carl Zeiss, Göttingen,

Germany) or an Axio Imager ZI fluorescein microscope (Carl Zeiss).

Transmission Electron Microscopy

A portion of lacrimal gland and conjunctival tissue was immediately fixed with 2.5% glutaraldehyde and subjected to electron microscopic examination as described previously.² One-micrometer-thick sections were stained with methylene blue, and the portions of interest were thin-sectioned and examined under an electron microscope (1200 EXII or 1230; JOEL, Tokyo, Japan).

Results

cGVHD Affects Ocular Surface Mucosa, Leading to Excessive Fibrosis

To examine whether EMT is involved in mucosal cGVHD, we selected the conjunctiva as a target organ of mucosal cGVHD. Typical histopathological findings in the conjunctiva from patients with cGVHD were a fibrotic subconjunctival stroma with mild lymphocytic infiltration. The conjunctival epithelium was thin, and the basal lamina was attenuated (Figures 1A and 2A) compared with normal conjunctiva (Figure 1B). Mallory staining revealed excessive fibrosis of submucosal stroma (Figure 1C), which was not observed in normal conjunctiva (Figure 1D). Electron microscopic findings of conjunctival epithelia revealed separation of conjunctival basal epithelia, which exhibited a flattened and spindle-shaped morphology resembling a fibroblast (Figure 2A). cGVHD conjunctival basal lamina showed attenuated and elongated processes extended into the subconjunctival stroma through disrupted basal lamina (Figure 2B, arrows). Fibroblasts in the subconjunctival stroma from patients with cGVHD showed an activated form containing a large amount of protein in the rough endoplasmic reticulum (high magnification of boxed area in Figure 2B, arrowheads).

To examine the expression of epithelial markers, we examined the expression of E-cadherin (Figure 1, E and F), a key epithelial cell adhesion molecule. In cGVHD, intercellular staining of E-cadherin was diminished in the conjunctival basal epithelia, suggesting the loss of cell-to-cell junctional adhesion (Figure 1E). In contrast, normal conjunctiva showed characteristic staining of E-cadherin at the intercellular junctions of basal and suprabasal epithelial cells (Figure 1F). In cGVHD samples, β -catenin was expressed in the cytoplasm and nucleus of the conjunctival basal epithelia but not in suprabasal epithelia (Figure 1G). In contrast, β -catenin was colocalized with E-cadherin in normal conjunctiva (Figure 1H). Snail, a transcriptional repressor of E-cadherin and an inducer of EMT,¹⁸ was also expressed in the nucleus of basal epithelial cells (Figure 1I), but not in the suprabasal epithelial layers in samples from patients with cGVHD (Figure 1I) and normal conjunctival epithelia (Figure 1J). This observation is consistent with the expression of E-cadherin being repressed by Snail, suggesting the involvement of EMT in cGVHD-related fibrosis.

Figure 1. Chronic GVHD affects ocular surface mucosa, leading to excessive fibrosis. **A** and **B**: H&E staining of the cGVHD conjunctiva showed mild lymphocytic infiltration in the stroma. The conjunctival epithelium from patients with cGVHD was thin, and the basal lamina was attenuated (**A**, from patient 2) compared with a normal control (**B**). **C** and **D**: Mallory staining in a patient with cGVHD showed a severely fibrotic subconjunctival stroma (**C**, from patient 10), which was not observed in normal conjunctiva (**D**). **E** and **F**: Intercellular staining of E-cadherin was diminished in the conjunctival basal epithelia of patients with cGVHD (**E**). In contrast, a characteristic pattern of E-cadherin staining was seen at the intercellular junctions of basal and suprabasal epithelial cell layers in the normal conjunctiva (**F**). **G** and **H**: In cGVHD samples, β -catenin was expressed in the cytoplasm and nuclei of the conjunctival basal epithelia but not in suprabasal epithelia (**G**, arrows, from patient 5). β -Catenin showed its characteristic expression pattern in normal conjunctiva, which is essentially the same as E-cadherin (**H**). **I** and **J**: Snail staining was found in the nucleus of basal epithelial cells (**I**, arrows, from patient 11), but not in the suprabasal epithelial layers in samples from patients with cGVHD and normal conjunctiva (**I** and **J**). Original magnification: $\times 200$ (**A–D**); $\times 400$ (**E–J**).

Figure 2. Electron microscopy of disrupted basal lamina and epithelial cells in cGVHD. **A**: Conjunctival epithelia in cGVHD consisted of only two to three cell layers, and cell-to-cell adhesion in basal cells were dissociated. A magnified view of the boxed area shows that dissociated basal epithelia had a spindle-shaped morphology with cytoplasmic extensions containing extracellular matrix (**star**) in the cytoplasm. Abnormal collagen bundles with unusual periodicity were present in the subepithelial stroma (**A**, arrows). **B**: cGVHD conjunctiva showed attenuated and elongated processes extended into the subconjunctival stroma through a disrupted basal lamina (**B**, arrows). Fibroblasts in the subconjunctival stroma showed an activated form containing a large amount of protein in the rough endoplasmic reticulum (**B**, arrowheads).

Epithelial Mesenchymal Transition as a Manifestation of Basal Epithelial Plasticity in Mucosal cGVHD

To examine the epithelial plasticity in conjunctival cGVHD, we examined whether mesenchymal markers were expressed in epithelial cells of cGVHD conjunctiva. When we immunostained for α -SMA, localized staining was observed in the basal epithelium (Figure 3A, arrows), which was not observed in normal conjunctiva (Figure 3B).

Electron microscopic findings confirmed the presence of collagen bundles within the cytoplasm of cGVHD basal epithelial cells, with elongated processes extending into the subepithelial stroma (Figures 2B and 3C). Abnormal collagen bundles with unusual periodicity were present in the subepithelial stroma (Figure 2A, arrows). These findings were not observed in normal conjunctiva (Figure 3D). The EMT and mesenchymal marker HSP47 was expressed in the basal epithelia of cGVHD conjunctiva (Figure 3E) but not in normal controls (Figure 3F). Electron microscopy again revealed abnormal collagen bundles in these basal cells (Figure 3, G and H) but not in normal conjunctiva (Figure 3I). These results suggest that basal epithelial cells adopt a mesenchymal phenotype in

Figure 3. Epithelial mesenchymal transition as a manifestation of basal epithelial plasticity in conjunctival cGVHD. **A** and **B**: α -SMA expression was restricted to the basal epithelium (**A**, arrows), but not in the normal conjunctiva (**B**). **C** and **D**: Electron microscopic findings confirmed the presence of collagen bundles (black arrow-head) within the cytoplasm of basal epithelial cells (red arrow), as well as the presence of their elongated processes, extending into the subepithelial stroma (**C**). Normal conjunctiva (**D**). **E** and **F**: HSP47 was expressed in the basal epithelia (**E**, from patient 2), but not in normal controls (**F**). Electron microscopy again revealed abnormal collagen bundles in the basal cells in cGVHD samples (**G** and **H**, from patient 3) but not in normal control (**I**). Original magnification: $\times 200$ (**A**, **E**, and **F**); $\times 100$ (**B**); $\times 5000$ (**C** and **D**); $\times 15,000$ (**G-I**).

cGVHD and secrete collagen bundles that may extend into the stroma through the disrupted lamina.

Basal Epithelial Cell Invasion through Disrupted Basal Lamina in Conjunctival cGVHD

We next examined the expression of p63, an epithelia-specific transcription factor expressed predominantly in the basal cells of stratified epithelia as an epithelial marker.¹⁷ In cGVHD samples, p63 was also found in the nucleus of cells in the conjunctival stroma (Figure 4, A and B), whereas p63 was limited to the basal cells in normal conjunctiva (Figure 4C). Recently, it was reported that the interaction between several extracellular matrices such as collagen bundles and the cytoplasm through disrupted lamina could trigger EMT.¹² To examine the relationship between the basal lamina and the location of

p63-positive cells, we stained for type IV collagen and p63. Type IV collagen staining revealed that the basal lamina was disrupted in cGVHD conjunctiva (Figure 4D). In contrast, a continuous basal lamina was observed in normal conjunctiva (Figure 4E). The areas of disrupted basal lamina per field in the cGVHD conjunctiva were larger than normal conjunctiva ($P = 0.006$, Student *t*-test) (Figure 4F).

p63-positive stromal cells were preferentially located below the type IV collagen layer in degenerated regions (Figure 4G). Under the electron microscope, we observed conjunctival basal epithelial cells attached to lymphocytes, with elongated processes extending from the basal cells into the subconjunctival space (Figure 4H). Therefore, these basal epithelial cells seem to be activated by binding to lymphocytes. Cells expressing both the epithelial marker p63 and mesenchymal marker

Figure 4. Basal epithelial cell invasion through disrupted basal lamina in conjunctival cGVHD. **A, B,** and **C:** In the cGVHD samples, p63 was also found in the nucleus of some cells in the conjunctival stroma (**A** and **B**, from patient 9). In contrast, p63 was found in the nuclei of basal cells in the normal conjunctiva (**C**). **D** and **E:** Type IV collagen staining revealed the disrupted basal lamina in cGVHD conjunctiva (**D**, patient 2), whereas a continuous basal lamina is observed in normal conjunctiva (**E**). **F:** The average area of disrupted basal lamina was statistically larger in the GVHD lacrimal gland compared with a normal control ($P = 0.006$, Student's *t*-test) (**F**). **G** and **H:** Double staining of p63 (green) and type IV collagen (red). p63-positive stromal cells were preferentially located below the type IV collagen layer in regions where this layer showed degradation (**G**, arrows, from patient 2). Cap, capillary; Bc, Basal epithelial cell; Ly, Lymphocyte. **I–L:** Double staining revealed that cells expressing both nuclear p63 (DAB) and cytoplasmic HSP47 (green) were found in basal cells and subconjunctival cells from patients with cGVHD (**I** and **J**, Axio imager ZI image, **K**, confocal microscopic image of the same sample, from patient 11). In contrast, double-positive cells were not detected in the basal or stromal cells from normal conjunctiva (**L**). **M** and **N:** MMP9 was expressed in the conjunctival epithelia (**M**, patient 3), but not in normal conjunctiva (**N**). **O:** The number of p63/HSP47 double-positive invading cells in the cGVHD conjunctival samples was significantly higher than that in normal samples ($P = 0.0008$, Student's *t*-test). Original magnification: $\times 400$ (**A, B, G, I–L**); $\times 200$ (**C** and **D**); $\times 15,000$ (**H**).

HSP47 were found in the basal conjunctiva, as well as in the subconjunctival areas in patients with cGVHD (Figure 4, I, J, and K). In contrast, double-positive cells were hardly detected in the basal epithelium or stroma in normal conjunctiva (Figure 4L).

MMP9, a 92-kDa type IV collagenase known to degrade type IV collagen, was expressed in cGVHD conjunctival epithelia (Figure 4M) but not in normal conjunctiva (Figure 4N). These findings suggest that some of the basal epithelia had migrated into the conjunctival stroma through the disrupted basal lamina after digestion by MMP9. The number of p63/HSP47 double-positive invading cells in the cGVHD conjunctival samples was significantly higher than that in normal samples ($P = 0.0008$, Student's *t*-test) (Figure 4O). These EMT-associated changes were not observed in a conjunctival biopsy from a HSCT patient without cGVHD, suggesting that the pathological findings were not due to the stress of HSCT alone (Supplemental Figure S1, see <http://ajp.amjpathol.org>).

Lacrimal Gland Is Also a Target of Exocrine Gland cGVHD and EMT

We next examined the lacrimal gland as a target of exocrine gland cGVHD and examined whether lacrimal gland epithelia underwent EMT during the process. For the lacrimal gland study, normal lacrimal gland controls were not available for ethical reasons. Thus, we used Sjögren's syndrome lacrimal glands from biopsy samples that were obtained for diagnostic purposes. H&E staining of cGVHD lacrimal gland showed mild lymphocytic infiltration in the stroma (Figure 5A) compared with Sjögren's syndrome (SS) lacrimal gland, in which extensive lymphocytic infiltration was observed (Figure 5B). Mallory staining in a patient with cGVHD showed a severely fibrotic stroma (Figure 5C) that was not prominent in the SS lacrimal gland (Figure 5D). Similar to the conjunctiva, intercellular staining of E-cadherin was diminished in the lacrimal gland epithelia of patients

Figure 5. Excessive fibrosis and Wnt/ β -catenin pathway activation in cGVHD lacrimal gland. **A** and **B**: H&E staining of the cGVHD lacrimal gland showed mild lymphocytic infiltration in the stroma (**A**, from patient 3) compared with SS lacrimal gland (**B**). **C** and **D**: Mallory staining in a patient with cGVHD showed a severely fibrotic stroma (**C**, from patient 8) that was not observed in the SS lacrimal gland (**D**). **E** and **F**: Intercellular staining of E-cadherin was diminished in the lacrimal gland epithelia of patients with cGVHD (**E**, from patient 5). In contrast, a characteristic pattern of E-cadherin staining was observed at the intercellular junctions in SS lacrimal gland epithelia (**F**). **G** and **H**: In cGVHD samples, β -catenin was expressed in the cytoplasm and nuclei of the ductal epithelia but not in SS ductal epithelia (**G**, arrow). β -Catenin showed characteristic expression in SS lacrimal gland, which is essentially the same as E-cadherin staining (**H**). **I** and **J**: Snail was expressed in the nucleus of acinar epithelial cells (**I**, from patient 5), but not in the nucleus of epithelial layers and weakly expressed intercellular junction of lacrimal gland epithelia in samples from lacrimal gland of patients with SS (**J**). Original magnification: $\times 200$ (**A–D**); $\times 630$ (**E** and **F**); $\times 400$ (**G** and **H**).

with cGVHD (Figure 5E) compared with the intercellular junctional localization in SS lacrimal gland epithelia (Figure 5F).

Distribution of β -catenin was localized in the cytoplasm and nucleus of the acinar and ductal epithelia of cGVHD samples (Figure 5G) but not in SS epithelia, in which only weak expression was observed in the intercellular junction (Figure 5H). Snail staining was found in the nucleus of acinar and ductal epithelial cells of cGVHD (Figure 5I) but not in SS lacrimal glands (Figure 5J). These findings indicate that lacrimal gland epithelia may undergo EMT

during cGVHD after the down-regulation of E-cadherin, translocation of β -catenin, and expression of Snail.

EMT as a Manifestation of Myoepithelial Cell Activation in Exocrine Gland cGVHD

In exocrine organs, stellate myoepithelial cells are observed between the basal lamina and acinar or ductal cells. These cells have structural features of both epithelium and smooth muscle cells. Their functions include contraction when the gland is stimulated to secrete, compressing or reinforcing the underlying parenchymal cells and preventing damage to surrounding cells.¹⁹ To examine whether the lacrimal gland epithelia are involved in EMT, we examined the expression of mesenchymal markers in lacrimal gland epithelia. Fibroblast-specific protein-1 (FSP1) expression was found on the basal side of cGVHD lacrimal gland epithelia (Figure 6A) but not in SS lacrimal gland epithelia (Figure 6B). Some of the spindle-shaped cells adjacent to the myoepithelia expressed FSP1, suggesting that these cells in the stroma were derived from myoepithelia (Figure 6A). α -SMA expression was restricted to the myoepithelia in samples from SS controls (Figure 6D), whereas the GVHD lacrimal gland myoepithelium had altered morphology, suggesting cell activation (Figure 6C). Type IV collagen staining revealed a disrupted basal lamina in cGVHD lacrimal gland epithelia (Figure 6E), whereas a continuous basal lamina was observed in the SS lacrimal gland epithelia (Figure 6F). The area of disrupted basal lamina per field in the cGVHD lacrimal gland was statistically larger than that in the SS lacrimal gland ($P = 0.002$, Student's t -test) (Figure 6G). MMP9 was expressed on the basal side of the cGVHD lacrimal gland epithelia (Figure 6H), but not in the SS lacrimal gland control (Figure 6I). These findings suggest that lacrimal gland myoepithelia in cGVHD may acquire the mesenchymal phenotype or activation of myoepithelia.

Myoepithelial Cells Invade the Lacrimal Gland Stroma through Disrupted Basal Lamina in Patients with cGVHD

Electron microscopy revealed a remarkable change in microfilament cytoskeleton conformation (Figure 7, A and B) and elongation of cytoplasmic processes along the apicobasal axis into the subepithelial stroma (Figure 7B). Abundant collagen bundles were observed adjacent to the myoepithelium (Figure 7C) with elongated processes rich in actin filaments extending into the subepithelial stroma (Figure 7D). Residual myoepithelium in cGVHD lacrimal glands had a spindle-shaped morphology with excessive extracellular matrix accumulation and seemed to interact with lymphocytes (Figure 7E). These findings suggest that the myoepithelia convert to an activated phenotype to become fibroblasts.

Figure 6. Epithelial mesenchymal transition as a manifestation of myoepithelial activation in cGVHD exocrine gland. **A** and **B**: FSP1 expression was found on the basal side of cGVHD lacrimal gland epithelia (**A**, from patient 4) but not on that of SS lacrimal gland epithelia (**B**). **C** and **D**: α -SMA expression was found on the altered myoepithelia (**C**), but not in the SS lacrimal gland myoepithelia (**D**). **E** and **F**: Type IV collagen staining revealed the disrupted basal lamina in cGVHD lacrimal gland epithelia (**E**, from patient 8) but the continuous basal lamina in the SS lacrimal gland epithelia (**F**). The average area of disrupted basal lamina was statistically larger in the GVHD lacrimal gland compared with SS control (* $P = 0.002$, Student's t -test) (**G**). **H** and **I**: MMP9 was expressed on the basal side of the cGVHD lacrimal gland epithelia (**H**, from patient 8), but not on the SS lacrimal gland epithelia (**I**). Aci, Acinus. Original magnification: $\times 630$ (**A–D**, **H**, and **I**); $\times 400$ (**E** and **F**).

Discussion

cGVHD is a major complication of allogeneic hematopoietic stem cell transplantation and has features resembling scleroderma, exhibiting prominent fibrosis in skin lesions, pulmonary fibrosis, and chronic immunodeficiency. Clinical features of ocular cGVHD include onset

of dry, gritty, or painful eyes, cicatricial conjunctivitis including subconjunctival fibrovascular tissue formation, and scleral shortening, which is characteristic feature of conjunctival fibrosis. In addition to sclerotic features in skin lesions, mucosal atrophy in the mouth, strictures or stenosis in the upper to mid third of the esophagus, joint stiffness or contracture due to sclerosis, and bronchitis obliterans in lung together indicate the characteristic features of systemic GVHD-mediated fibrosis.¹⁶

Recently, several studies have shown that EMT also plays a role in the genesis of fibroblasts during organ fibrosis.⁷ Examples include alveolar epithelial cells during pulmonary fibrosis,⁵ tubular epithelial cells during renal fibrosis,⁷ and functional involvement of adult hepatocytes in the accumulation of activated fibroblasts in the fibrotic liver.⁶ In the field of ophthalmology, EMT contributes to subepithelial fibrosis of lens, limbal stem cells, and pterygium.^{17,20,21} Typical features of EMT involve a series of events during which epithelial cells lose many of their epithelial characteristics such as loss of intercellular adhesion, destruction of basal lamina, and loss of basolateral polarity. Cells undergoing EMT show a mesenchymal phenotype and acquire the ability to migrate and invade stromal tissue.¹⁸ MMPs can also break down molecules that mediate cell-cell contractions and contribute to tissue fibrosis via EMT and can lead to increased levels of cellular reactive oxygen species. In addition, irradiation,⁹ hypoxia,^{22,23} and reactive oxygen species²⁴ orchestrate the disassembly of junctional complexes and lead to the changes in cytoskeletal organization that occur during EMT.

In this study, we observed several findings suggesting that EMT may be involved in mucosal membrane and exocrine gland cGVHD. First, down-regulation of E-cadherin and the loss of adhesion molecules were seen in conjunctival and lacrimal gland epithelia. Up-regulation of Snail, which plays a central role in EMT as a master regulator and transcriptional repressor of E-cadherin,⁸ was seen at the site of conjunctival basal epithelia and lacrimal gland epithelia. Therefore, both mucosal and exocrine cGVHD epithelia may undergo EMT in patients with cGVHD.

Second, HSP47, a collagen-specific molecular chaperon and an early marker of EMT,²⁵ and α -SMA, a marker of mesenchymal cells, were exclusively expressed in the conjunctival basal epithelia. In cGVHD lacrimal glands, HSP47-expressing cells were observed adjacent to HSP47-expressing myoepithelia in the subepithelial stroma. Moreover, FSP1-expressing spindle-shaped cells were also found in around FSP1⁺ myoepithelia, suggesting that these cells were derived from myoepithelia. These findings indicate that cGVHD epithelia gain the mesenchymal phenotype.

Third, altered collagen bundles were observed in the cytoplasm of conjunctival basal epithelia and adjacent to the myoepithelia of lacrimal gland. One possibility is that type I collagen bundles migrate into the site of vacuolar degeneration of conjunctival epithelia after being targeted by T cells. It has been shown that interaction between type I collagen and epithelial cytoplasm after degradation of type IV collagen facilitate EMT *in vitro*.²⁶

Figure 7. Myoepithelial cell invasion in lacrimal gland cGVHD through disrupted basal lamina. Electron microscopic observation revealed the remarkable change in microfilament cytoskeleton (**A** and **B**) and elongation of cytoplasmic processes along its apicobasal axis into subepithelial stroma (**B**). **C**: An abundant collagen bundle adjacent to the myoepithelium. **D**: Elongated processes of myoepithelia, extending into the subepithelial stroma. **E**: Residual myoepithelium in cGVHD lacrimal gland. **A–E** from patient 13. ME, myoepithelia; BL, basal lamina; Ly, Lymphocyte. Original magnification: $\times 2000$ (**A** and **D**); $\times 15,000$ (**B** and **C**); $\times 5000$ (**E**).

Extracellular matrix may also provide the cue for migration of conjunctival basal cells and lacrimal gland myoepithelial cells. It has been reported that type I collagen induces disruption of E-cadherin-mediated cell-cell contacts,²⁷ and interaction of type I collagen down-regulates E-cadherin adhesion, resulting in EMT.¹² Moreover, type I collagen contributes to facilitate the translocation of nucleus from intercellular location to nucleus.²⁸ Our findings suggest that partially degraded basal lamina by MMP9 and type IV collagenase may facilitate EMT²⁹ by interacting with extracellular matrix. Another possibility is that conjunctival basal epithelia and lacrimal gland myoepithelia secrete collagen bundles after acquiring the activated mesenchymal phenotype as shown by HSP47 expression.

The basal lamina regulates epithelial polarity, and this polarity is lost by the disrupted basal lamina, which may lead to facilitation of EMT.^{30,31} We also have shown actin rearrangement-like findings in the epithelial cytoplasm under electron microscopy. Rearrangement of the cytoskeleton such as depolymerization and homogeneous distribution of filamentous actin in the cytoplasm was observed, which is also an important initiator of EMT.³⁰ One study using an animal model has shown that the basement membrane is a key component for apicolateral F actin accumulation and that the disruption of bone marrow promotes homogeneous distribution of F actin in the cytoplasm.³⁰ Our electron microscopic findings of conjunctival basal epithelia and myoepithelia demonstrated that these processes were due to altered distribution of actin cytoskeleton, ie, microfilament. Microfilaments in cGVHD epithelia prominently increased in the cytoplasm at the site of disrupted lamina. These findings suggest that the degradation of basal lamina and interaction between extracellular matrix and epithelial cyto-

plasm play an important role during EMT related to cGVHD.

On the basis of our findings, we propose the following model of the pathogenic process of mucosal and exocrine cGVHD. Donor lymphocytes and cytokines released by migrated inflammatory cells target the conjunctival basal epithelia and lacrimal gland myoepithelia. The conjunctival basal epithelia and lacrimal gland myoepithelia are activated by the interaction between the lymphocytes and encounter cytokine storm released by migrated inflammatory cells and ocular cGVHD epithelia, promoting the transition from the epithelial to the mesenchymal phenotype. The activated basal epithelia secrete abnormal collagen bundles, leading to conjunctival and lacrimal gland EMT and fibrosis in patients with cGVHD.

Serial specimens for evaluating the potential association between epithelial-derived fibroblasts and disease progression will help to elucidate the mechanism of cGVHD-mediated fibrosis as well as that of other fibrotic diseases using animal models. A full understanding of the contribution of different extracellular triggers in the conjunctival and lacrimal gland cGVHD fibrosis will elucidate the mechanism of the dynamic process of EMT in cGVHD and improve the quality of life of patients with cGVHD.

Acknowledgments

We thank Toshihiro Nagai and Mai Tadaki, Keio University School of Medicine, for expert technical assistance on transmission electron microscopy and immunohistochemistry and Hideyuki Miyashita for helpful discussion on the article.

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