Conflict of interest

The authors declare no conflict of interest.

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Morphologic evaluation of meibomian glands in chronic graft-versus-host disease using in vivo laser confocal microscopy

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Purpose: To evaluate the morphological changes of the meibomian glands (MGs) using in vivo laser confocal microscopy (CM) in dry eye (DE) patients with chronic graft-versus-host disease (cGVHD).

Methods: Seventeen eyes from 9 patients with a diagnosis of DE associated with cGVHD (DE/cGVHD group; 6 males, 3 females; median 50.5 years) and 16 eyes of 8 hematopoietic stem cell transplantation (HSCT) recipients without DE (non-DE/non-cGVHD group; 5 males, 3 females; median 47.0 years) were enrolled. CM was used to investigate the MG and MG acinar unit density (MGAUD), MG acinar longest diameter (MGALD), MG acinar shortest diameter (MGASD), and the fibrosis grade. Clinical findings of the lid margin were obtained. Tear dynamics, ocular surface vital staining, meibography, and MG expressibility were also examined. Data were compared between the 2 groups using the unpaired t and Mann–Whitney tests.

Results: The mean MGAUD value was significantly lower in the DE/cGVHD group than in the non-DE/non-cGVHD group ($p=0.01$, 57.8±38.3 glands/mm^2, 88.8±26.6 glands/mm^2, respectively), and the mean MGALD and MGASD were significantly shorter in the DE/cGVHD group than in the non-DE/non-cGVHD group ($p=0.0018$, 37.3±24.4 μm and 60.4±11.8 μm, $p=0.0106$, 17.7±11.8 μm and 26.6±6.03 μm, respectively). The mean fibrosis grade was significantly higher in the DE/cGVHD group than the non-DE/non-cGVHD group ($p<0.0001$, 1.39±0.71 grade, 0.06±0.25 grade, respectively). Clinical findings in the lid margin, tear dynamics, and ocular surface findings were significantly worse in the DE/cGVHD group than in the non-DE/non-cGVHD group.

Conclusions: CM clearly depicted the morphological changes of the MG in the DE/cGVHD group, and revealed the severity of the meibomian gland dysfunction. Patients with severe DE after HSCT showed atrophic MG and excessive fibrosis.

Meibomian glands (MGs) are sebaceous glands embedded within the tarsal plates. Each MG comprises multiple acini connected by a long common central duct running throughout the entire length of the gland. Meibomian glands secrete lipids into the preocular tear film. The meibomian secretions form the outer layer of the tear film to suppress evaporation and function as lubricants for the eyelids during blinking [1]. Meibomian gland dysfunction (MGD) is a chronic, diffuse abnormality of the meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative and quantitative changes in the glandular secretion. This may result in alteration of the tear film, symptoms of eye irritation, clinically apparent inflammation, and ocular surface disease [2].

Chronic graft-versus-host disease (cGVHD) is a major cause of morbidity and mortality in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) for hematologic malignancies [3], and MGD is a second most frequent complication of ocular cGVHD. The reported prevalence of MGD in ocular cGVHD is 47.8% [4]. However, to our knowledge, no report on the morphology of MG in cases with cGVHD related dry eye (DE) has been published to date.

Existing methods for assessing MG status and function include slit-lamp examination of the lid margins and ocular surface epithelium, meibometry [5,6] (the assessment of the volume and properties of the meibum), meibography (evaluation of MG morphology, including "drop-outs") [7-10], and in vivo laser confocal microscopy (CM) [11-13]. The last of these, CM is an emerging new imaging technique that can noninvasively detect structural changes in many ocular surface diseases and anterior segment disorders. In this study, we used CM to investigate the MG of patients with cGVHD-associated DE and compared the results with those from patients who did not develop DE after HSCT.

METHODS

Subjects and examinations: We had prospectively followed up the HSCT patients at DE outpatient clinic and worked up ocular findings before and after HSCT by communicating with the KEIO BMT program transplant internist since 1996.

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Table 1. Demographic data for patients with chronic GVHD related dry eye (DE/cGVHD group).

Case	Age	Gender	HSCT source	Underlying disease	Interval between HSCT and CM analysis (mo)	Interval between DE onset and CM analysis (mo)	Interval between HSCT and DE onset (mo)	DE severity level	CC	Clinically affected cGVHD organs
1	57	Male	PBSC	ML	17	1	16	3	-	Eye, Skin
2	47	Male	PBSC	AML	19	6	13	4	+	Eye, Skin, Mouth, Liver
3	50	Male	PBSC	MM	12	1	11	4	+	Eye, Mouth
4	47	Female	BM	MM	43	1	42	4	+	Eye, Mouth
5	38	Female	BM	AML	12	0	12	2	-	Eye
6	57	Male	BM	MDS	14	N.A.	N.A.	3	+	Eye
7	51	Male	BM	CML	120	48	72	2	-	Eye
8	46	Female	PBSC	MM	116	108	8	2	-	Eye
9	55	Male	PBSC	AML	25	17	8	4	+	Eye, Skin

HSCT=hematopoietic stem cell transplantation; CM=confocal microscopy; DE=dry eye; CC=cicatricial change; PBSC=peripheral blood stem cell; BM=bone marrow; ML=malignant lymphoma; AML=acute myeloid leukemia; MM=multiple myeloma; MDS=myelodysplastic syndrome; CML=chronic myeloid leukemia; N.A.=not applicable.

Table 2. Demographic data for HSCT recipients without DE (non-DE/non-cGVHD group).

Case	Age	Gender	HSCT source	Underlying disease	Interval between HSCT and CM analysis (mo)
1	55	Male	BM	MM	20
2	28	Male	BM	ALL	168
3	52	Male	PBSC	CML	24
4	62	Male	BM	AML	72
5	26	Male	BM	MDS	26
6	61	Female	BM	AML	39
7	44	Female	BM	CML	199
8	48	Female	BM	AML	177

HSCT=hematopoietic stem cell transplantation; CM=confocal microscopy; DE=dry eye; PBSC=peripheral blood stem cell; BM=bone marrow; MM=multiple myeloma; ALL=acute lymphocytic leukemia; CML=chronic myeloid leukemia; AML=acute myeloid leukemia; MDS=myelodysplastic syndrome.

Confocal microscopy was introduced to our outpatient clinic in 2006. We started this case controlled study since 2010. Between February 2010 and January 2011, we examined 17 patients for this study using CM. Among them, 9 patients had newly developed DE after HSCT. The median period between DE development and HSCT was 22.8 months (range; 8–72 months). Seventeen eyes of 9 patients who had cGVHD related dry eye (DE/cGVHD group; 6 males and 3 females; median age: 50.5 years; range: 38–57 years) and 16 eyes of 8 age- and gender-matched patients who did not develop DE after HSCT (non-DE/non-cGVHD group; 5 males and 3 females; median age: 47.0 years; range: 26–62 years) were analyzed. The demographic and clinical characteristics of the DE/cGVHD group and non-DE/non-cGVHD group are shown in Table 1 and Table 2. There were no statistically significant differences in interval between HSCT and CM analysis between two groups. In addition, we divided the DE/cGVHD group in this study into two groups with or without systemic cGVHD (10 eyes of 5 cases, 7 eyes of 4 cases, respectively) to examine the relationship with systemic cGVHD. The reason why we subdivide the DE/cGVHD group into two groups with or without systemic cGVHD is follows. Conjunctival involvement in GVHD has been reported as a marker for severe systemic GVHD [14]. In our previous study, cGVHD patients with conjunctival fibrosis had systemic complications and a poor prognosis following HSCT [15]. Therefore, we compared between DE/cGVHD group with and without systemic involvement regarding as the meibomian gland acinar unit density (MGAUD), the meibomian gland acinar longest diameter (MGALD) and the meibomian gland acinar shortest diameter (MGASD), and the fibrosis severity.

The definition of systemic GVHD is based on accompanied with or without clinically affected organs other than the eyes as mentioned in the Table 1 and is according to

a previous report [16]. We excluded one eye from a patient (Case 5; Table 1) in the DE/cGVHD group because of phthisis from all of the analysis of this study. Topical eye drops, including artificial tears, vitamin A, and autologous serum drops, were instilled five times a day, immediately after the diagnosis of DE following HSCT.

We used the global diagnostic criteria for DE, which is based on the recommendation of the 2007 International Dry Eye Workshop Report [17]. Patients who had a history of surgical lacrimal punctal occlusion, allergies, contact lens use, glaucoma eye drop use, or other ocular surgery, including refractive surgery or radiation to the eyes, were excluded, as were patients with infectious blepharitis, blink disorders, disorders of the lid aperture or lid/globe congruity, or other ocular surface disorders. In addition, patients with trachoma and ocular cicatricial pemphigoid were excluded. The research procedures in this study followed the tenets of the Declaration of Helsinki Principles, and informed consent was obtained from all subjects. IRB/Ethics Committee approval at Keio University was obtained for this study.

Ocular surface vital staining: The fluorescein and Rose-Bengal staining scores for the ocular surface were obtained using the double vital staining method [18]. Both stains were scored on a scale of 0–9 [18,19]. The van Bijsterveld scoring system was used for the Rose-Bengal staining. Briefly, the ocular surface was divided into three zones: nasal conjunctival, corneal, and temporal conjunctival areas. A score of 0–3 points was used for each zone, with a minimum possible score of 0 and a maximum of 9 points. Scarce punctuate staining was given 1 point, denser staining that did not cover the entire zone was given 2 points, and Rose-Bengal staining over the entire zone was given 3 points. For the fluorescein staining, the cornea was divided into three equal zones (upper, middle, and lower). A score of 0 to 3 points was used for each zone, as with the Rose-Bengal stain. The presence of scarce staining in a zone was scored as 1 point; frequent puncta not covering the entire zone as 2 points, and punctuate staining covering the entire zone as 3 points.

Meibomian gland secretions: The expression of meibomian secretion (meibum) was graded as described by Shimazaki et al. [10]. Briefly, to assess obstruction of the meibomian gland orifices, digital pressure was applied on the upper tarsus, and the expression of meibomian secretion (meibum) was scored semiquantitatively as follows: grade 0, clear meibum, easily expressed; grade 1, cloudy meibum, expressed with mild pressure; grade 2, cloudy meibum, expressed with more than moderate pressure; and grade 3, no meibum expressed even under hard pressure. Two independent investigators (Y.B. or Y.O.) pressed gently on the upper eyelids to express the meibomian lipids.

Meibomian gland dropout grades (meibography): The meibography apparatus was composed of a slit lamp (RO 5000; Rodenstock, Munich, Germany) and an infrared charge-coupled device video camera (XC-EI50; Sony, Tokyo, Japan) [7-9].The MG structure was observed on the under side of the upper and lower eyelids, by everting the lids manually. The degree of MG drop out was scored as reported previously (Shimazaki grading): 0, no gland drop out; 1, drop out with loss of less than half of the glandular structures; and 2, drop out with loss of more than half of the glandular structures [20]. We obtained the values as the average of the upper and lower grades.

Lid margin abnormalities: The presence or absence of four parameters in lid margin abnormalities (i.e., irregular lid margin; absence=0 or presence=1, vascular engorgement; absence=0 or presence=1, plugged MG orifices; absence=0 or presence=1 and anterior or posterior replacement of the mucocutaneous junction, absence=0 or presence=1) were scored from 0 through 4, according to the number of these abnormalities using slit lamp examination [6,7,21-23]. Therefore, all four parameters for lid margin abnormality were present, the maximum score was given as 4.

Tear function test: Tear film break up time (TBUT) was measured three times, and the median value was calculated [18]. The Schirmer's test was performed using standard strips (Showa Yakuhin Kako Co. Ltd., Tokyo, Japan) placed in the lower conjunctival sac for 5 min without anesthesia.

In vivo laser confocal microscopy: In vivo laser CM was performed on all subjects, using a newly developed confocal microscope, the Heidelberg Retina Tomograph II-Rostock Cornea Module (Heidelberg Engineering GmbH, Dossenheim, Germany), as described previously [11-13]. Briefly, after the lower eyelid was everted, the center of the Tomo-Cap was placed onto the palpebral conjunctiva, and the MGs were scanned while moving the applanating lens. To evaluate the morphologic changes in the MG, we measured the acinar unit density, the longest and the shortest acinar unit diameters, as described previously [11-13]. In addition, we devised one new parameter, the fibrosis severity. The degree of fibrosis severity was scored as follows: grade 0, no fibrosis; grade 1, fibrosis in less than half of the lower eyelid; grade 2, fibrosis in more than half of the lower eyelid. Clearly visible acinar units in a 400×400-μm frame were all counted, and the acinar density was described as the number of units per square millimeter. We calculated the longest and shortest diameters in μm using ImageJ software (Java software program developed by the National Institutes of Health, Bethesda, MD). Three randomized nonoverlapping high-quality digital images of the lower eyelid were used for the CM-based assessments. We calculated the mean MGAUD, MGALD, MGASD, and the fibrosis grade from three randomized nonoverlapping images.

Diagnosis of dry eye (DE): The diagnosis and classification of DE disease based on its severity was performed as recommended by the 2007 International Dry Eye Workshop Report. Dry eye is a multifactorial disease of the tears and

Table 3. Comparison of ocular surface/tear dynamics between the DE/cGVHD group and non-DE/non-cGVHD group.

Examinations	DE/cGVHD group	Non-DE/non-cGVHD group	p-value
F (points)	4.88±3.06	0.38±0.62	<0.0001‡
RB (points)	5.06±2.41	0.13±0.34	<0.0001‡
MG expressibility (points)	2.47±0.92	0.75±0.93	<0.0001‡
Meibography (grade)	1.41±0.80	0.50±0.52	0.0014‡
Lid margin (grade)	2.94±1.35	0.38±0.72	<0.0001‡
TBUT (seconds)	2.35±1.90	6.94±3.04	<0.0001*
Schirmer (mm)	1.64±1.21	5.80±2.30	<0.0001*

DE=dry eye; cGVHD=chronic graft-versus-host disease; F=fluorescein score; RB=Rose-Bengal score; MG=meibomian gland; TBUT=tear film break up time; *=Unpaired *t*-test, ‡=Mann–Whitney test.

Table 4. Comparison of meibomian glands between the DE/cGVHD group and non-DE/non-cGVHD group using in vivo laser confocal microscopy.

Examinations	DE/cGVHD group	Non-DE/non-cGVHD group	p-value
MGAUD (glands/mm^2)	57.8±38.3	88.8±26.6	0.01*
MGALD (μm)	37.3±24.4	60.4±11.8	0.0018*
MGASD (μm)	17.7±11.8	26.6±6.03	0.0106*
Fibrosis (grade)	1.39±0.71	0.06±0.25	<0.0001‡

DE=dry eye; cGVHD=chronic graft-versus-host disease; MGAUD=meibomian gland acinar unit density; MGALD=meibomian gland acinar longest diameter; MGASD=meibomian gland acinar shortest diameter; *=Unpaired *t*-test; ‡=Mann–Whitney test.

Table 5. Comparison of meibomian glands between the DE/cGVHD group with systemic cGVHD and the DE/cGVHD group without systemic cGVHD using in vivo confocal microscopy.

Examinations	DE/cGVHD group with systemic cGVHD (10 eyes, 5 cases)	DE/cGVHD group without systemic cGVHD (7 eyes, 4 cases)	p-value
MGAUD (glands/mm^2)	38.4±30.0	85.5±32.3	0.0097*
MGALD (μm)	19.3±7.31	63.0±14.2	< 0.0001*
MGASD (μm)	10.7±4.33	27.6±12.0	0.0009*
Fibrosis (grade)	1.87±0.23	0.71±0.59	0.0007‡

DE=dry eye; cGVHD=chronic graft-versus-host disease; MGAUD=meibomian gland acinar unit density; MGALD=meibomian gland acinar longest diameter; MGASD=meibomian gland acinar shortest diameter; *=Unpaired *t*-test; ‡=Mann–Whitney test.

ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface [17].

Diagnosis of cGVHD: All the patients in our study fulfilled the revised consensus criteria for cGVHD [24]. Briefly, a diagnosis of cGVHD requires the following: (1) a distinction from acute GVHD, (2) the presence of at least one distinctive manifestation (e.g., keratoconjunctivitis sicca) confirmed by pertinent biopsy or other relevant tests (e.g., Schirmer's test) in the same or other organs, and (3) the exclusion of other possible diagnoses.

Conjunctival fibrosis: We diagnosed conjunctival fibrosis in patients who had subconjunctival fibrosis, fornix shortening, symblepharon, and/or ankyloblepharon [14,25]. We evaluated these findings by using slit-lamp microscopy during a routine examination.

Statistical analyses: The unpaired *t*-test for TBUT, Schirmer test value, MGAUD, MGALD, and MGASD and nonparametric Mann–Whitney test for fluoresecein score, Rose-Bengal score, MG expressibility, meibography, lid margin abnormality and fibrosis grade were used to compare between two groups (the DE/cGVHD group versus the non-DE/non-cGVHD group; Table 3 and Table 4). We also compared between the DE/cGVHD group with and without systemic cGVHD using this test (Table 5). In addition, we compared between the DE/cGVHD group without systemic cGVHD and the non-DE/non-cGVHD group (Table 6). A p value of 0.05 was considered statistically significant. GraphPad Instat (GraphPad Software, San Diego, CA) was used for the statistical analyses.

RESULTS

Data on tear function and ocular surface evaluation: The mean value of tear function test including TBUT and Schirmer

TABLE 6. COMPARISON OF MEIBOMIAN GLANDS ASSESSED BY IN VIVO LASER CONFOCAL MICROSCOPY, MG EXPRESSIBILITY, MEIBOGRAPHY AND LID MARGIN ABNORMALITY BETWEEN THE DE/CGVHD GROUP WITHOUT SYSTEMIC CGVHD AND NON-DE/NON-CGVHD GROUP.

Examinations	DE/cGVHD group without systemic cGVHD (7 eyes, 4 cases)	non-DE/non-cGVHD group (16 eyes, 8 cases)	p-value
MGAUD (glands/mm²)	85.3±32.3	88.8±26.6	0.8021
MGALD (μm)	63.0±14.2	60.4±11.8	0.65
MGASD (μm)	27.6±12.0	26.6±6.03	0.7888
Fibrosis (grade)	0.71±0.59	0.06±0.25	0.0016‡
MG expressibility (points)	2.20±1.10	0.75±0.93	0.0189‡
Meibography (grade)	1.14±0.69	0.50±0.52	0.0399‡
Lid margin (grade)	2.0±1.41	0.38±0.72	0.0019‡

DE=dry eye; cGVHD=chronic graft-versus-host disease; MGAUD=meibomian gland acinar unit density; MGALD=meibomian gland acinar longest diameter; MGASD=meibomian gland acinar shortest diameter; MG=meibomian gland; ‡=Mann–Whitney test.

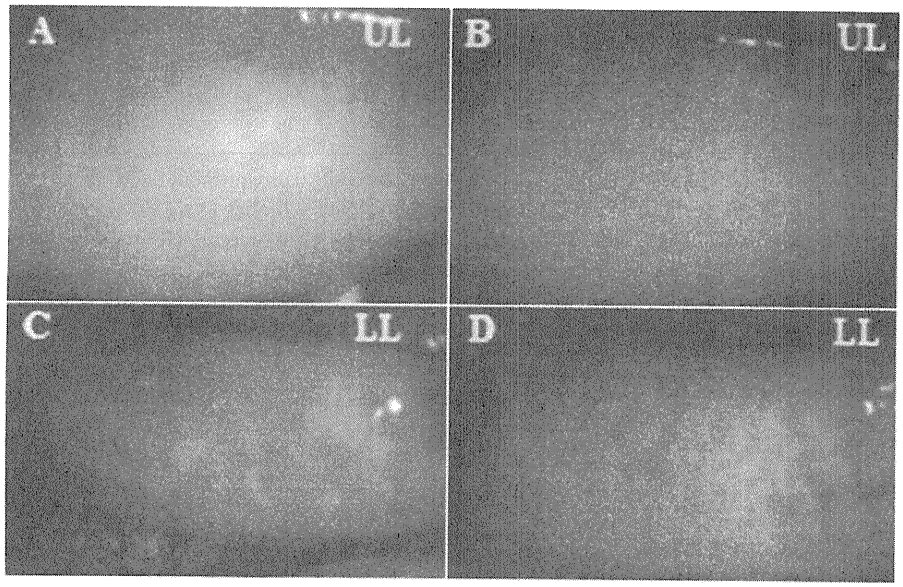

Figure 1. Meibomian gland images observed by noncontact infrared meibography. **A**, **C**: DE/cGVHD group, 57 year-old male (Case 1; Table1). Note the numerous meibomian gland dropouts. **B**, **D**: Non-DE/Non-cGVHD group, 28-year-old male (Case 2; Table2) No loss of meibomian glands was observed in the patient who did not develop DE after HSCT. UL=Upper, Left, LL=Lower, Left.

test and ocular surface staining scores including Rose-Bengal and fluoresecein were significantly worse in the DE/cGVHD group than in the non-DE/non-cGVHD group ($p<0.0001$; Table 3).

Meibomian gland secretions: The mean MG expressibility score was significantly higher (grade 2.47±0.92 grade) in the DE/cGVHD group than in the non-DE/non-cGVHD group (grade 0.75±0.93 grade; $p<0.0001$; Table 3).

Meibography: The numerous meibomian gland dropouts were observed by using noncontact infrared meibography from a 57-year-old male (Case 1; Table 1) in the DE/cGVHD group (Figure 1A,C). In contrast, there was no loss of MG in a 28-year old male (Case 2; Table 2) who did not develop DE after HSCT in the non-DE/non-cGVHD group (Figure 1B,D). The mean meibomian gland dropout grade was significantly higher (1.41±0.80 grade) in the DE/cGVHD group than in the non-DE/non-cGVHD group (0.50±0.52 grade; $p=0.0014$; Table 3).

Lid margin abnormalities: Slit lamp examination revealed irregular lid margin, vascular engorgement, plugging of MG orifices and posterior replacement of the mucocutaneous junction from a representative 50-year-old male (Case 3; Table 1) in the DE/cGVHD group (Figure 2).

The mean lid margin score was significantly higher (2.94±1.35 grade) in the DE/cGVHD group than in the non-DE/non-cGVHD group (0.38±0.72 grade; $p<0.0001$; Table 3).

In vivo laser confocal microscopy: CM clearly depicted the glandular acinar units in the non-DE/non-cGVHD group. The CM images also revealed morphologic alterations in the DE/cGVHD group, including atrophic MG and extensive fibrosis. The confocal microscopic images observed at the onset of DE related to cGVHD from a representative 47-year-old male

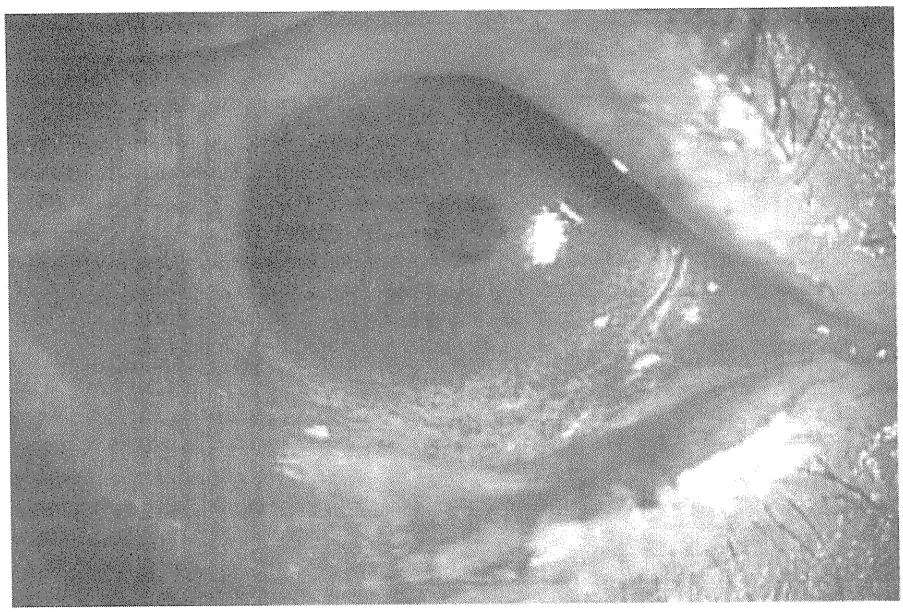

Figure 2. Anterior slit lamp photograph; DE/cGVHD group, 50-year-old male (Case 3; Table 1). The anterior slit lamp photograph showing irregular lid margin, vascular engorgement, plugging of MG orifices, and posterior replacement of the mucocutaneous junction.

(Case 2; Table 1) in the DE/cGVHD group showed the excessive fibrosis around the atrophic glands and the moderate infiltration of inflammatory cells (Figure 3B,D). Seventeen months after the onset of DE related to cGVHD showed the excessive fibrosis around the atrophic glands and the mild infiltration of inflammatory cells from a representative 55-year-old male patient (Case 9; Table 1) in the DE/cGVHD group (Figure 4). In contrast, the confocal microscopic images from a representative 61-year-old female (Case 6; Table 2) in non-DE/non-cGVHD group showed the presence of numerous and compact acinar units (Figure 5).

The mean MGAUD was 57.8±38.3 gland/mm^2 in the DE/cGVHD group, which was significantly lower than in the non-DE/non-cGVHD group (88.8±26.6 gland/mm^2, p=0.01). The MGALD was significantly shorter in the DE/cGVHD group than in the non-DE/non-cGVHD group (37.3±24.4 μm versus 60.4±11.8 μm, p<0.0018). In addition, the MGASD was also significantly shorter in the DE-cGVHD group than in the non-DE/non-cGVHD group (17.7±11.8 μm versus 26.6±6.03 μm, p=0.0106). The fibrosis grade was significantly higher (1.39±0.71 grade) in the DE/cGVHD group than in the non-DE/non-cGVHD group (0.06±0.25 grade, p<0.0001, Table 4).

When the DE/cGVHD group was subdivided into patients with systemic cGVHD and patients with only ocular cGVHD, the mean MGAUD value was significantly lower in the patients with systemic cGVHD (38.4±30.0 gland/mm^2) than in those without it (85.5±32.3 gland/mm^2, p=0.0097). The MGALD was significantly shorter in the patients with systemic cGVHD than in those without systemic GVHD (19.3±7.31 μm versus 63.0±14.2μm, p<0.0001). In addition, the MGASD was also significantly shorter in the patients with systemic cGVHD than in those without systemic GVHD (10.7±4.33 μm versus 27.6±12.0 μm, p=0.0009; Table 5). The fibrosis grade was significantly higher (1.87±0.23 grade) in the DE/cGVHD group with systemic cGVHD than in those with non-systemic cGVHD (0.71±0.59 grade, p=0.0007; Table 5).

The value of the MGAUD, the MGALD and the MGASD in the DE/cGVHD group without systemic cGVHD was similar to the value of those in the non-DE/non-cGVHD group (Table 6).

DISCUSSION

To the authors' knowledge, this is the first comprehensive study to conduct a morphologic assessment of the MG in DE patients with cGVHD after HSCT using in vivo CM. We found that in patients with cGVHD related DE, the MG showed severe abnormalities, as assessed by in vivo CM. The characteristics of MGD in cGVHD are that atrophic MG and excessive fibrosis assessed by CM accumulated the almost entire MG including acini, ductules, main ducts, and orifices. In addition, MGD in cGVHD progressed rapidly shortly after the onset of DE [4].

MGs are specialized sebaceous glands. It has been reported that the bulge of hair follicles located below the opening of the sebaceous duct is destroyed by donor T cells in cGVHD skin lesions [26]. Blisters formation of the mucous membrane due to pseudomembrane or donor lymphocyte and/or donor macrophages attack to the recipient epithelia can occur in ocular cGVHD [27]. Similar pathogenic process might contribute recipient MG epithelia of orifices, ducts, ductule or acini, leading to secondary fibrotic spontaneous occlusion and the adhesion of various structures of meibomian glands.

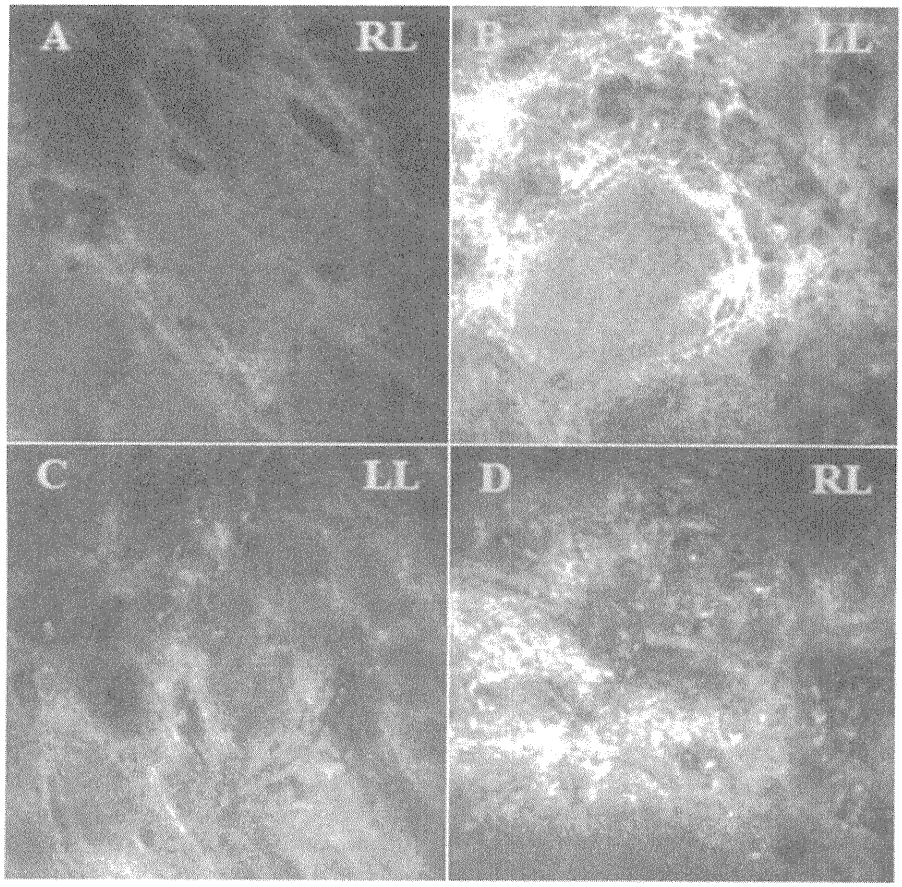

Figure 3. Meibomian gland of DE/cGVHD patients images observed by in vivo laser confocal microscopy. **A**, **C**: DE/cGVHD group, 47-year-old female (Case 4; Table1). **B**, **D**: DE/cGVHD group, 47-year-old male (Case 2; Table 1). The images observed at the onset of DE related to cGVHD. Note the excessive fibrosis around the atrophic glands and the moderate infiltration of inflammatory cells in the dry eye patients with cGVHD. RL=Right, Lower, LL=Left, Lower.

Previous studies showed that tissue atrophy and excessive fibrosis are prominent histological features of salivary gland and lacrimal gland in cGVHD [28-30]. We have reported T cells and fibroblasts in lacrimal gland cGVHD patients primarily are activated in the periductal area, and contribute to the pathogenic fibrosis [30]. In addition, activated fibroblasts derived from epithelia via local epithelial mesenchymal transition [31] or arise from bone marrow under inflammatory stress [29] might contribute to excessive fibrosis around the MG ducts. Therefore, we proposed that extensive MG fibrosis were exacerbated by inflammation under inflammatory cells and fibroblasts interaction and subsequent activation leading to excessive accumulation of extracellar matrix and pathogenic fibrosis in meibomian gland microenvironment. These factors are considered to be characteristic in cGVHD in comparison with other MGD.

Aging may also exacerbate the periglandular inflammation and fibrosis. Because it is reported that MGD is facilitated by aging [7,22], median age is 50.5 years in our cases may have a potential to develop MGD by aging. However, MGD parameter of age-matched non-GVHD patients was statistically better, the possibility influenced by aging is unlikely. Therefore, we propose that our results obtained this study is associated with cGVHD.

Because we prospectively followed the HSCT recipients since 1996, we could observe the MG at the onset of DE related to cGVHD. Excessive fibrosis was detectable by in vivo CM shortly after the onset of cGVHD-related DE. Previous reports have found that the MGALD (86.3±18.9 μm) and MGASD (34.8±9.2 μm) were both significantly larger in MGD patients than in controls (56.3±10.4 μm, $p<0.0001$, and 17.4±4.2 μm, $p<0.0001$, respectively) [11,12]. In age related MGD, the mechanism of morphologic changes in MG was suggested that hyperkeratinization of the ductal epithelium, shedding of keratinized material into the glandular ducts leading to orificial obstructions, and eventual cystic dilatation and atrophy [32].

Recently, morphological changes of MG in Sjogren's syndrome (SS) have been reported using CM. In this study, MGALD has been reported 53±31μm in primary SS and 70±42 μm in secondary SS. These values are longer than that of cGVHD obtained in this study [33]. In cGVHD, tissue atrophy and excessive fibrosis were prominent histological

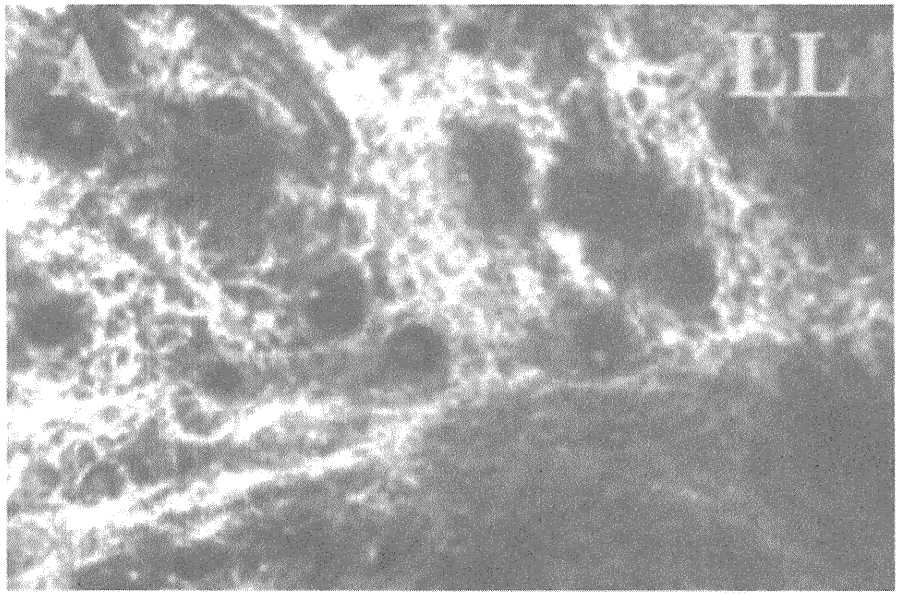

Figure 4. Meibomian gland of DE/cGVHD patient images observed by in vivo laser confocal microscopy. DE/cGVHD group, 55-year-old male (Case 9; Table 1). The images observed after 17 months on the onset of DE related to cGVHD. Note the excessive fibrosis around the atrophic glands and the mild infiltration of inflammatory cells in the dry eye patients with cGVHD. LL=Lower, Left.

features, may explain the disaccordance of our results with those of previous reports.

Interestingly, we also found that the mean MGAUD in the DE/cGVHD group with systemic cGVHD was lower than in the DE/cGVHD group without systemic cGVHD, and the mean MGALD and MGASD were shorter. These patients had severe DE, and 5 out of 9 HSCT recipients (55.6%) showed excessive fibrosis of the MG. The MG of patients with DE but without systemic cGVHD resembled those of the non-DE group. A previous report showed that cGVHD patients with conjunctival fibrosis had systemic complications and a poor prognosis following HSCT [14]. Our results suggested that the MG of DE/non-systemic cGVHD resembled those of the non-DE group. In other words, these findings suggested that dry eye related to cGVHD did not affected meibomian gland alterations. Although we analyzed a small number of patients, there is a statistically significant difference for fibrosis grades between the MG of DE/non-systemic cGVHD and those of the non-DE group. There is a possibility that fibrosis rather than dry eye itself may play a central role for the development of the MGD in cGVHD, similar to cGVHD lacrimal gland pathology. Our previous study have insisted that fibrosis is a leading cause of dry eye related to cGVHD and even in the early course of the diseases [34]. In cGVHD, there is a possibility that fibrosis is a primary event and consequently leads to MGD and dry eye separately.

Taken together, we believe that the cause of MGD in cGVHD is multifactorial and multistep. Destruction of the ductal epithelia due to lymphocyte infiltration, slouging of epithelial cells due to lymphocyte attack or pseudomembrane formation, and subsequent excessive fibrosis around the orifice, ducts, ductules, and acini of the MG, may explain for the development of MGD after HSCT.

Although further studies are necessary, our study suggests that CM findings on the MG in HSCT recipients may be useful for evaluating their likely progression to MGD. In this study, we conducted CM for the lower lid. It is difficult for us to evaluate the upper eyelid margin, whereas the lower one is easily positioned and examined. A confocal examination require a prolonged contact for several minutes between instrument and examined inverted upper eye lid tarsal conjunctiva, which can be uncomfortable for the patient during examination as reported previously [33]. Moreover the instrument has a space fix orientation, so it requires the polymethacrylate sterile cap (Tomo-cap) face, leads to more difficult to evaluate the upper lid meibomian glands. It is necessary for us to develop the methods as for examining upper tarsal conjunctiva more comfortable for further evaluating the entire MG morphology.

We used topical immunosuppressants when the patients were tapering off their systemic immunosuppressive therapy for cGVHD related dry eye [35]. Furthermore, our group reported that topical tranilast for the treatment of early fibrosis may effectively retard cGVHD related DE progression when given in the early stages [36]. Therefore, early use of topical immunosuppressant and anti-fibrotic intervention might be useful strategy for preventing or retarding the MGD associated fibrosis in cGVHD.

In summary, we conducted a morphometric assessment of the MG in DE patients with cGVHD using in vivo laser CM. CM clearly demonstrated morphological changes including inflammatory cell infiltration and excessive fibrosis, even in early stage, in the MG of DE patients with

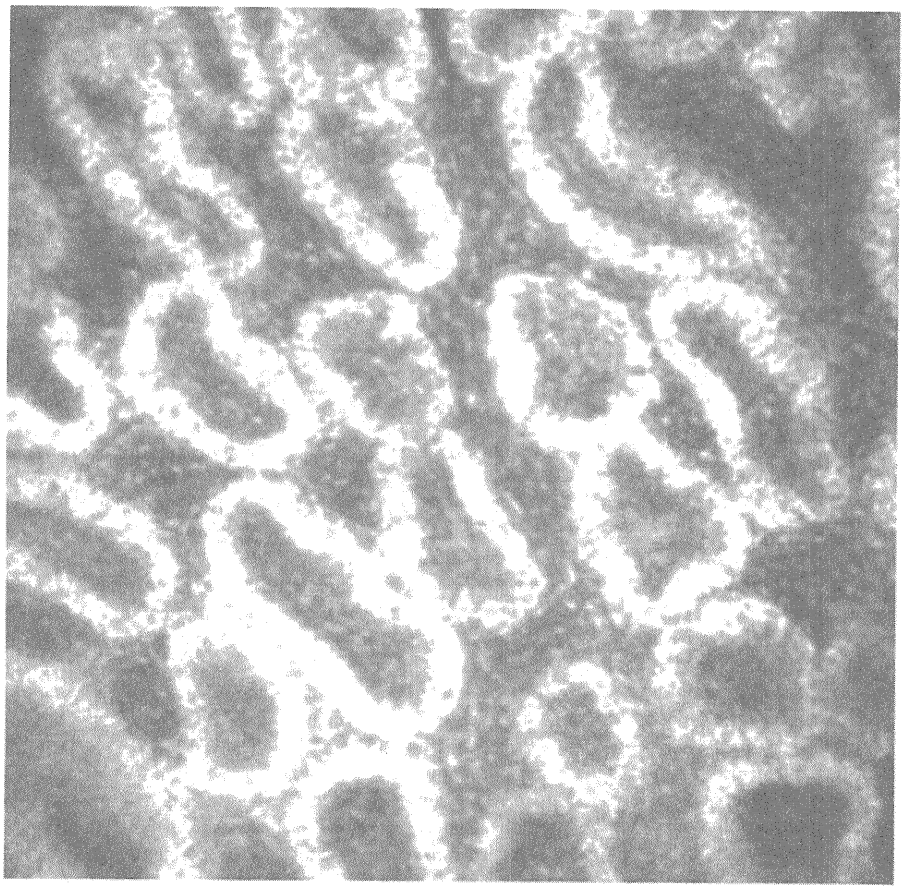

Figure 5. Meibomian gland of non-DE/ non-cGVHD recipient images observed by in vivo laser confocal microscopy. Non-DE/Non-cGVHD group, 61-year-old female (Case 6; Table 2). Note the presence of numerous and compact acinar units in patients without dry eye after HSCT.

cGVHD. Our results also suggested that MGD may allow us to diagnose severe DE and cGVHD early in the course of the disease. Early detection of MGD could be important, because it could signal a progression to severe DE, and extensive cGVHD, which can lead to blindness and become life-threatening.

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Comparison of stem cell sources in the severity of dry eye after allogeneic haematopoietic stem cell transplantation

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Comparison of stem cell sources in the severity of dry eye after allogeneic haematopoietic stem cell transplantation

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ABSTRACT

Aims To compare the incidence and severity of dry eye (DE) after allogeneic haematopoietic stem cell transplantation (HSCT) according to the stem cell source. The authors specifically focused on patients who received bone marrow transplantation (BMT), peripheral blood stem cell transplantation (PBSCT) and cord blood transplantation (CBT).

Methods Ninety-nine HSCT recipients who were prospectively followed-up for at least 100 days at Keio University Hospital were recruited. Ophthalmological examinations included evaluation of ocular surface findings and tear dynamics. The data on systemic graft-versus-host disease were collected by chart review.

Results Of the 99 patients (BMT, 67; PBSCT, 18; CBT, 14), 42 developed DE or showed worsened pre-existing DE after HSCT; 31 (46.3%) BMT group; 8 (44.0%) PBSCT group; and 3 (21.4%) CBT group (p=0.78). The median onset time of DE tended to be later in the PBSCT group (474 days, range 95—1559) than in the BMT (287 days, range 67—1216) or CBT (168 days, range 33—481) group, but the difference was not significant (p=0.23). However, the proportion of patients with severe DE was significantly higher in the PBSCT group (N=7, 87.5%) than in the BMT (N=12, 38.7%) or CBT (N=1, 33.3%) group (p=0.04) and CBT showed the lowest among all three stem cell sources.

Conclusion The data in this study suggested that the severity and onset time of DE were affected by the stem cell source. Close attention must be paid to the development of late-onset severe DE in PBSCT recipients.

INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) has become an established treatment modality for the management of haematological malignancies.[1] In addition to bone marrow transplantation (BMT), both umbilical cord blood transplantation (CBT) and peripheral blood stem cell transplantation (PBSCT) have been increasingly used over the last 15 years,[2] and several recent studies have suggested that CBT and PBSCT lead to outcomes similar to those observed with BMT in the setting of unrelated volunteer donor HSCT in adults.[3 4]

Despite improvements in post-transplantation immunosuppressive therapy, graft-versus-host disease (GVHD) remains a major complication that impedes the success of allogeneic HSCT.[5] In the clinical setting, GVHD is divided into acute and chronic forms. Acute GVHD (aGVHD) usually occurs during the first 3 months following HSCT.[5] T cells present in the donor's bone marrow at the time of transplant identify the HSCT patient as 'non-self' and attack the patient's skin, liver, stomach and/or intestines. Chronic GVHD (cGVHD) usually develops after the third month post-transplant[5] and has features resembling scleroderma, exhibiting prominent fibrosis in skin lesions, pulmonary fibrosis and chronic immunodeficiency. The pathophysiology of cGVHD was originally considered to be a later phase of aGVHD, but several recent studies suggest that an autoimmune-like process induced by dysfunctional immunological recovery also plays some role. However, the exact mechanism of cGVHD, which is distinct from aGVHD, is still largely unknown and considered more complex, multistep and needs to be explored.

With the steady and significant increase in the number of long-term survivors after HSCT, the management of cGVHD has become increasingly important for improving their quality of life. The eye is one of the major target organs of cGVHD and dry eye (DE) is the major late ocular complication associated with this disease.[6 7] Although there are other ocular complications as well and DE is not fatal, corneal epithelial defects can sometimes lead to blindness.[8] Therefore, it is important to manage DE after HSCT properly for the recipient's quality of life and as a prophylaxis for blindness. In this prospective study, we compared the incidence and severity of DE after HSCT according to the stem cell source with the ultimate goal of improving the prophylaxis and treatment of this ophthalmic complication.

MATERIALS AND METHODS

Patients

One hundred and forty-eight patients who underwent allogeneic HSCT (98 received BMT, 32 PBSCT and 18 CBT) from January 2000 to June 2004 at Keio University Hospital were prospectively followed by ophthalmologists before and after the transplant. We limited the study to those who underwent myeloablative HSCT and excluded reduced-intensity or mini-transplant recipients. Thirty-four patients who died and 15 who relapsed and needed to reduce or discontinue immunosuppressive drugs early in their course of treatment were also excluded from this study. Thus, 99 patients who survived at least 100 days with sustained engraftment were included in this study.

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Of these 99 patients, 67 received BMT, 18 PBSCT and 14 CBT. All patients underwent standard clinical and ophthalmological examinations just before and 3, 6, 9, 12, 18 and 24 months after the transplantation and whenever indicated. cGVHD was diagnosed according to standard criteria.[9] The research followed the tenets of the Declaration of Helsinki and informed consent was obtained from all patients.

Ophthalmological evaluation

Tear function tests and ocular surface evaluations

Ophthalmic examinations included the best corrected visual acuity for distance, assessment of conjunctival and corneal vital staining with rose bengal and fluorescein, tear film break up time (TBUT), Schirmer's test-I and lens and fundus examinations. The condition of the ocular surface was evaluated as reported previously.[8] Briefly, the cornea was evaluated by the double vital staining method. Two microlitres of a preservative-free combination of 1% rose bengal and 1% fluorescein dye was instilled into the conjunctival sac by micropipette. For rose bengal score, the ocular surface was divided into three zones: nasal conjunctival, corneal and temporal. A score of 0–3 points was used for each zone with a minimum possible score of 0 and a maximum possible score of 9. Scarce punctate staining was given 1 point. Denser staining not covering the entire zone was given 2 points. Denser staining over the entire zone was given 3 points. For fluorescein staining, the cornea was divided into three equal zones—upper, middle and lower. Each zone has a staining score ranging from 0 to 3 points, as with the rose bengal stain and the minimum and maximum scores were 0 and 9, respectively. The presence of scarce staining in a zone was scored 1, frequent punctate staining not covering the entire zone was scored as 2 points and punctate staining covering the entire zone was scored as 3 points.

Tear dynamics were assessed by three different methods[10]: the TBUT, Schirmer's test-I, and Schirmer's test with nasal stimulation. To determine the TBUT, double vital staining was performed and the patients were requested to blink three times to ensure adequate mixing of the fluorescein dye in the tears. The time interval between the last complete blink and the appearance of the first corneal black spot was measured by a stopwatch and the mean of the three measurements was regarded as the TBUT in this study.

The Schirmer's test-I was performed without topical anaesthesia, after all the other examinations. Strips (Whatman No 41, Showa, Tokyo) were placed at the outer one-third of the temporal lower conjunctival fornix for 5 min. The strips were then removed and the length of wet filter paper (in mm) was recorded. Schirmer's test with nasal stimulation,[10] which is considered to evoke maximal reflex tearing, was performed by applying a cotton swab to the nasal cavity.

Diagnostic criteria

DE was diagnosed as described previously.[11] Briefly, any sign of tear film instability (TBUT <5 s, Schirmer's test-I <5 mm) with any abnormality of the ocular surface (rose bengal score >3 points and/or fluorescein score >1 point) was diagnosed as DE. We divided the cases of DE into two groups according to the degree of severity as severe dry eye (S-DE) and mild dry eye (M-DE). S-DE was defined as reduced reflex tearing in the Schirmer's test with nasal stimulation to less than 10 mm and abnormality of the ocular surface (rose bengal score ≥3 and/or fluorescein score ≥3). M-DE was defined as abnormality of the ocular surface (rose bengal score ≥3, fluorescein score ≥1) without reduced reflex tearing (Schirmer's test with nasal stimulation <10 mm).[10] We also divided the DE cases into two groups according to the time of onset: within 100 days and after 100 days of HSCT.

Statistical analysis

The major endpoint of this study was to compare the incidence and severity of DE in patients who received HSCT with BMT, PBSCT or CBT. The analyses were performed with the StatView software (SAS Institute Inc, USA) for Windows 98/2000. The data are presented as medians with ranges. One-way analysis of variance was used to compare groups with respect to normally distributed continuous variables. The χ^2 test was used to compare nominally scaled variables. Two-tailed p values of less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

The clinical profiles of the 99 patients are shown in table 1. There were no statistically significant differences in the median age (p=0.78) or sex (p=0.37) among the three groups.

Forty-two patients (42.4%) developed DE or experienced a worsening of pre-existing DE after HSCT (table 2). DE was diagnosed in 31 patients (46.3%) in the BMT group, 8 (44.0%) in the PBSCT group and 3 (21.4%) in the CBT group (p=0.57). The median onset time of the DE was 287 days (range 67–1216) in the BMT group, 474 days (range 95–1559) in the PBSCT group, and 168 days (range 33–481) in the CBT group. Although it was not statistically significant, the CBT group showed an earlier onset of DE compared to the other groups (p=0.23). However, the incidence of DE onset within 100 days was significantly higher in patients who received CBT (2 patients, 66.7%) than BMT (4, 12.9%) or PBSCT (1, 12.5%) (p=0.05 for both; table 2). The incidence of DE did not correlate significantly with the recipients' age, sex or stem cell source.

Considering the severity of the DE, S-DE was observed in 12 patients (38.7%) in the BMT group, 7 (87.5%) in the PBSCT group and 1 (33.3%) in the CBT group. The incidence of S-DE was significantly higher in the PBSCT group than BMT or CBT group (p=0.04 for both; table 2). Following the onset of DE, the conjunctival and corneal findings of the S-DE group rapidly worsened despite treatment. For the M-DE group, the development of DE without reduced reflex tearing was observed in 19 patients (61.3%) in the BMT group, 1 patient (12.5%) in the PBSCT group and 2 patients (66.7%) in the CBT group. These patients' ocular surface findings were well controlled by applying commercially available artificial tears. None of the patients in this study experienced DE that led to corneal ulcer or blindness.

The relationship between the development of systemic GVHD and DE after HSCT is shown in table 3. We found that

Table 1 Characteristics of bone marrow, peripheral blood stem cell and cord blood transplants recipients

Characteristics	BMT group (N=67)	PBSCT group (N=18)	CBT group (N=14)	p Value
Patient-related				
Age (years)				
Median	40.5	42.4	39.2	0.78
Range	15–57	21–58	3–66	
Sex				
Male	34 (50.7%)	12 (66.6%)	6 (42.9%)	0.37
Female	33 (49.3%)	6 (33.3%)	8 (57.1%)	

BMT, bone marrow transplantation; CBT, cord blood transplantation; PBSCT, peripheral blood stem cell transplantation.

Table 2 Characteristics of patients who developed dry eye after haematopoietic stem cell transplantation

Characteristics	BMT group (N=31)	PBSCT group (N=8)	CBT group (N=3)	p Value
Age (years)				
Median	42	40	32	0.36
Range	15–56	21–58	3–60	
Sex				
Male	14 (45.1%)	4 (50%)	1 (33.3%)	0.69
Female	17 (54.9%)	4 (50%)	2 (66.6%)	
Median onset of DE (days)	287	474	168	0.23
Range (days)	67–1216	95–1559	33–481	
Type of DE				
DE <100 days	4 (12.9%)	1 (12.5%)	2 (66.7%)	0.05*
DE >100 days	27 (87.1%)	7 (87.5%)	1 (33.3%)	
Severe DE/Total	17.9% (12/67)	38.9% (7/18)	7.1% (1/14)	0.06
Severe DE	12 (38.7%)	7 (87.5%)	1 (33.3%)	0.04*
Mild DE	19 (61.3%)	1 (12.5%)	2 (66.7%)	

*statistically significant.
DE, Dry eye; BMT, bone marrow transplantation; CBT, cord blood transplantation; PBSCT, Peripheral blood stem cell transplantation.

the patients with GVHD had a higher OR for DE in the BMT (OR 12.28, 95% CI 2.48 to 60.5) and CBT (OR 13.8, 95% CI 0.4 to 448.6) groups compared with the PBSCT group (OR 3, 95% CI 0.4 to 22.7).

DISCUSSION

This is the first report to evaluate the incidence of GVHD-related DE according to the stem cell source, including bone marrow, peripheral blood and cord blood using detailed diagnostic techniques for DE. Unrelated CBT has been increasingly used as an alternative stem cell source for HSCT[1]; however, there have been no reports so far on the incidence and the severity of DE related to cGVHD after CBT in comparison with BMT or PBSCT. In this study, we found that the incidence of DE was comparable among the three stem cell recipient groups but the incidence of S-DE was significantly higher in the PBSCT recipients, while that in the BMT or CBT recipients was comparable. This finding is consistent with previous systemic cGVHD research examining the relationship between stem cell sources and the severity of systemic cGVHD.[12] Our present results suggest that DE reflects the condition of systemic cGVHD well, and indicate that the careful evaluation and diagnosis of DE after HSCT can help predict the systemic condition.

Table 3 Relationship between dry eye and systemic graft-versus-host disease

	Number of patients	Number with DE (%)	OR (95% CI)
BMT			
Systemic GVHD (−)	52	18 (34.6%)	1
Systemic GVHD (+)	15	13 (87%)	12.28 (2.48 to 60.5)
PBSCT			
Systemic GVHD (−)	7	2 (28.6%)	1
Systemic GVHD (+)	11	6 (54.5%)	3 (0.4 to 22.7)
CBT			
Systemic GVHD (−)	13	2 (15.4%)	1
Systemic GVHD (+)	1	1 (100%)	13.8 (0.4 to 448.6)

BMT, bone-marrow transplantation; CBT, cord blood transplantation; DE, dry eye; GVHD, graft-versus-host disease; PBSCT, peripheral blood stem cell transplantation.

The pathogenic process of DE associated with cGVHD is thought to involve T cells interacting with stromal fibroblasts in the cGVHD lacrimal gland. Because unmodified blood stem cells contain one log more T cells than unmodified bone marrow grafts,[13] the number of donor T cells and fibrocytes and the timing of the infiltration may lead to the increased severity of DE in PBSCT recipients.

Studies comparing CBT, PBSCT or BMT published to date have not examined the histological events of ocular cGVHD with regard to the stem cell source and further studies will be required to elucidate them. Detailed studies assessing the time course and extent of the immune reconstitution after PBSCT compared with BMT or CBT are also needed.[14]

Our observation that DE was diagnosed later in PBSCT than in BMT and CBT recipients remains to be elucidated. One possible explanation is that the early development of DE may have been prevented by the earlier use of immunosuppressants in the PBSCT recipients. Systemic immune suppressants are often applied early in PBSCT because PBSCT can result in more severe systemic GVHD than CBT or BMT.[15 16] Studies have shown the duration of systemic immunosuppressant treatment was longer in peripheral blood stem cell recipients than in the bone marrow recipients, suggesting that cGVHD after PBSCT is more protracted than BMT.[16 17] In peripheral blood stem cell recipients, cGVHD continue to occur over 6 months after transplantation. This type of cGVHD is defined as late onset of cGVHD.[15 17] Our results suggest that, when the immunosuppressants were being tapered off in the PBSCT recipients, late-onset DE related to cGVHD appear and rapidly progress, leading to S-DE. We also found that the patients with systemic GVHD had a higher OR for DE in the BMT and CBT groups than in the PBSCT group. These results also suggested that the prolonged use of systemic immunosuppressants in PBSCT patients masked their DE when systemic GVHD occurred. In contrast, once systemic GVHD occurred in the BMT and CBT recipients, mild DE, but not severe DE might have developed due to earlier taper of systemic immunosuppressants than PBSCT. These findings suggest that we may be able to start using topical immunosuppressants such as ciclosporin or FK506 before systemic immunosuppressants are tapered off to prevent or slow down the onset or progression of DE related to cGVHD. Close communication between ophthalmologists and internists would be important to determine the timing of commencement or cessation and the dose of systemic and topical immunosuppressants.

The patients with haematological malignancies receiving CBT from unrelated donors with partially mismatched human leucocyte antigen (HLA) had a lower risk of S-DE and earlier onset of DE than the PBSCT or BMT recipients. The earlier onset of DE after CBT compared with BMT or PBSCT validated the specificity of our results, because cord blood cells proliferate more rapidly and generate a larger number of progeny compared with bone marrow cells.[18] In CBT, the incidence of aGVHD is lower than that expected based on the degree of HLA mismatch and the time for haematopoietic recovery is consistently longer than that for other haematopoietic stem cell sources; furthermore, the dosage of both nucleated cells and CD34 cells influences the success of HCST.[19 20] The main differences between CBT and BMT are reported to be the number of nucleated cells in the graft and HLA compatibility.[21]

Cord blood has unique immunological features.[18 22] The reason for the low immunological reaction in cord blood cells has been attributed to the significant decrease in transforming growth factor β-1 and macrophage chemoattractant protein-1[22] and the increase in anti-inflammatory cytokine interleukin-10,

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which probably results in downmodulation of GVHD.[23] It has been reported that cord blood contains a high proportion of 'naive' phenotype T cells expressing the CD45RA/CD45RO, CD62L.[24] These properties may influence and reduce the ocular surface and lacrimal gland immune response, leading to the low incidence of S-DE after CBT.

In particular, in terms of the DE associated with cGVHD, the clinical results reported in this study suggest that cord blood from unrelated donors could be a safe and effective stem cell source. However, there is a report of a rapidly worsening case of severe DE with pseudomembrane after CBT.[25]

Taken together, our study suggests that the following series of events occur in ocular cGVHD depending on the stem cell sources. First, breakdown of the blood vessel results in the invasion of donor T cells and fibroblasts or fibrocytes into the ocular tissues, perhaps due to homing signals released as part of an alloimmune response.[26–28] The donor cells from various stem cell sources interact with recipient residual inflammatory cells, then activate and migrate to the ocular surface and lacrimal gland microenvionment. These activated fibroblasts exacerbate the production of excessive extracellular matrix with the end effect being impaired ocular surface and lacrimal gland function, resulting in S-DE. Peripheral blood stem cells contain a large number of mature T cells, and some donor fibrocytes[29] may have the potential to activate these inflammatory cascades, leading to S-DE than BMT or CBT. Therefore, PBSCT recipients require more dose of immune suppressive treatment. In contrast, cord blood has various anti-inflammatory properties[23] facilitating the inhibition of developing S-DE. Further studies investigating the immune process depending on the stem cell sources in the ocular cGVHD would be useful for clarifying the complex pathogenesis of cGVHD as well as for the development of therapeutic strategies for this disease.

In conclusion, we found that the incidence and severity of DE differ with the stem cell source used. Improvements in the early diagnosis of DE and therapeutic strategies for treating it may lead to a substantial decrease in the morbidity associated with DE. Studies aimed at improving the diagnosis and treatment of cGVHD after HSCT should be continued, with a focus on the effects of stem cell sources and the importance of DE.

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Competing interests None.

Ethics approval The examination procedure in the outpatient clinic and the examination of documents are within the routine work for patients in the outpatient clinic. The examination procedure for the patients and examination of the patients' documents were approved for dry eye patients by the Institutional Review Board of Keio University. However, no specific approval was obtained for this study.

Contributors YO and MU developed the original concept for this study, recruited patients, collected and analysed data, and wrote the manuscript. YO received funds and supervised the study. MU and YU statistically analysed the data and wrote the manuscript. TM and SO recruited patients, performed haematopoietic stem cell transplantation and were involved in critical review in terms of internal medicine and revised the manuscript. KT recruited patients, received funds, coordinated between ophthalmology and internal medicine and revised the manuscript.

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Comparison of telomere length and association with progenitor cell markers in lacrimal gland between Sjögren syndrome and non-Sjögren syndrome dry eye patients

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Purpose: Indicators of aging such as disruption of telomeric function due to shortening may be more frequent in dysfunctional lacrimal gland. The aims of this study were to 1) determine the viability of quantitative fluorescence in situ hybridization of telomeres (telo-FISH) for the assessment of telomere length in lacrimal gland in Sjögren and non-Sjögren syndrome patients; and 2) investigate the relationship between progenitor cell markers and telomere length in both groups.

Methods: Quantitative fluorescence in situ hybridization with a peptide nucleic acid probe complementary to the telomere repeat sequence was performed on frozen sections from human lacrimal gland tissues. The mean fluorescence intensity of telomere spots was automatically quantified by image analysis as relative telomere length in lacrimal gland epithelial cells. Immunostaining for p63, nucleostemin, ATP-binding cassette, sub-family G, member 2 (ABCG2), and nestin was also performed.

Results: Telomere intensity in the Sjögren syndrome group (6,785.0±455) was significantly lower than that in the non-Sjögren syndrome group (7,494.7±477; p=0.02). Among the samples from the non-Sjögren syndrome group, immunostaining revealed that p63 was expressed in 1–3 acinar cells in each acinar unit and continuously in the basal layer of duct cells. In contrast, in the Sjögren syndrome group, p63 and nucleostemin showed a lower level of expression. ABCG2 was expressed in acinar cells in both sjogren and non-Sjogren syndrome.

Conclusions: The results of this study indicate that 1) telo-FISH is a viable method of assessing telomere length in lacrimal gland, and 2) telomere length in Sjögren syndrome is shorter and associated with lower levels of expression of p63 and nucleostemin than in non-Sjögren syndrome.

Telomeres are specialized DNA sequences located at the ends of chromosomes which shorten with each successive round of cell division. Accumulating evidence indicates that telomere length in human somatic cells shortens with chronological aging [1,2]. The maximum number of possible cell divisions in a given cell population is fixed. It has been suggested that this replicative life span, also known as the "Hayflick limit," is determined by the telomere having a "critical" length [3]. In human, telomere length has been measured extensively in leukocytes in relation to chronological aging [4-6]. Recently, telomere length was reported to decline with age in mature endothelial cells and to contribute to endothelial dysfunction and atherogenesis [7-9].

Alteration in telomere length may play a role in the development of several diseases in human, including cancer and benign inflammatory diseases such as idiopathic pulmonary fibrosis and type 2 diabetes [10-15]. On the other hand, some studies have indicated that pathological stresses themselves may affect telomere shortening, with inflammation, for example, reported as one possible cause [10-12,14], perhaps due to the concomitant increase in turnover of cells. Sjögren syndrome is a chronic inflammatory disease affecting the lacrimal glands [16,17]. We hypothesized that telomere length shortening in lacrimal gland was related to inflammation of lacrimal gland.

Flores et al. [18] reported that telomeres shorten with age in mouse stem cells from various tissues, suggesting that telomere loss contributes to stem cell dysfunction with aging. In addition, they also reported that the longest telomeres were a general feature of the adult stem cell compartment. Although no reports have demonstrated the presence of stem cells in lacrimal gland, tissue-committed progenitor cells are believed to be present. A recent report showed that injured lacrimal gland can undergo repair after acinar cells are lost through apoptosis or autophagy, which is followed by an increase in the number of stem/progenitor cells, stimulation of proliferation and upregulation of the bone morphogenetic

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protein 3 (BMP7) pathway [19]. We hypothesized that progenitor cell markers reported in the corneal and conjunctival epithelia, which are of the same origin as the lacrimal gland during development, were related to telomere shortening. Therefore we selected p63, nucleostemin, ATP-binding cassette, sub-family G, member 2 (ABCG2), and nestin as progenitor cell markers for this study. p63 has been recognized as markers for epithelial cells which have potential to proliferate and stratified [20]. Nucleostemin has been reported to be related to small cell size and similar expression pattern as p63 in corneal epithelium [21,22]. ABCG2 has been identified as a molecular determinant for bone marrow stem cells and proposed as a universal marker for stem cells including corneal limbal epithelial stem cells [23,24]. Nestin has been used as progenitor marker in the study of lacrimal gland tissue repair after injury [19,25].

To the authors' knowledge, no studies have been published on telomere shortening in lacrimal gland. Therefore, the aims of this study were to 1) determine the viability of quantitative fluorescence in situ hybridization (FISH) of telomeres (telo-FISH) in the assessment of telomere length in lacrimal gland in Sjögren and non-Sjögren syndrome patients; and 2) investigate the relationship between progenitor cell markers and telomere length in both groups.

METHODS

Tissue samples: Human tissue samples were obtained with written informed consent from patients treated at the Department of Ophthalmology, Keio University Hospital, Tokyo, Japan. These were in accordance with the principles expressed in the Declaration of Helsinki. The approval of the Keio University Ethics Committee was obtained for the use of human materials for this research. Lacrimal gland biopsy specimens were collected from 11 patients with dry eye to determine the presence or absence of Sjögren syndrome. Sjögren syndrome diagnosis was based on the revised American-European consensus criteria [26,27]. For the lacrimal gland study, normal lacrimal gland controls were not available for ethical reasons. Thus, we used lacrimal glands biopsy samples that were obtained for diagnostic purposes.

All tissue samples were routinely embedded in Optimal Cutting Temperature (OCT) compound and stored at −80 °C. Some of the samples were processed for electron microscopy. Frozen blocks were sectioned to a thickness of 5 μm and used for telo-FISH, immunohistochemical analyses and hematoxylin and eosin (H&E) staining.

Telomere-FISH analysis: Telo-FISH was performed using a 5′-Cy3-labeled, telomere-specific peptide nucleic acid (PNA) probe (5′-CCC TAA CCC TAA CCC TAA-3′; Fasmac, Kanagawa, Japan) as described previously with some modifications [28]. Sections were fixed with 4% paraformaldehyde (PFA). Slides were incubated with 10 mM sodium citrate at 80 °C for 30 min. After washing with 1× PBS, slides were dehydrated using an ethanol series (25%, 50%, and 100%) and air-dried. Twenty microliters Cy3-labeled telomere-specific PNA probe (2 μg/ml PNA in 70% formamide buffer with blocking reagent and 10 mM $MgSO_4$) was added to the sample and denaturation then performed by incubation for 15 min at 95 °C. Slides were incubated overnight at 37 °C for hybridization. Slides were then washed in 70% formamide buffer 4× for 15 min each time, followed by washing in PBS–0.025% Tween-20 four times for 5 min each. Slides were then dehydrated using an ethanol series and air-dried. The nuclei were stained with DAPI (Molecular Probes, Eugene, OR) for 30 min. The slides were then washed in PBS for 1 min, briefly dehydrated using the ethanol series and air-dried. The slides were then mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Image capturing and analysis: Tissue structure was identified on H&E-stained adjacent tissue sections before fluorescent microscopy. For telo-FISH, fluorescence images of DAPI, and Cy3 were recorded with an Olympus IX-81 inverted fluorescence microscope with spinning disk confocal unit (IX81-DSU; Olympus, Tokyo, Japan) equipped with a cooled CCD ORCA-AG camera (HAMAMATSU, Shizuoka, Japan) using a UplanSApo 100× /NA.1.40 (Olympus) oil immersion objective lens. All quantitative image analysis were analyzed in MetaMorph 7.6.3.0 software (MDS Analytical Technologies, Sunnyvale,CA). Exposure times were optimized with respect to the intensities of telomere and nuclear signals to prevent overexposure/saturation and kept constant for all slides to ensure consistency in intensity measurement. To avoid differences due to variation in section thickness, we used slices of the same thickness (5 μm) in all tissues analyzed.

The DAPI image was used to define the nuclear area and the Cy3 image was used to quantify telomere fluorescence. The nuclei of lacrimal gland cells were outlined based on the DAPI signal, followed by outlining of the telomere signals in individual nuclei. The intensities of all outlined pixels of both telomere signals and DAPI were summed respectively on a per-cell basis and tabulated. Inflammatory cells were excluded from the analysis. Telomere signals were standardized with DAPI signals and telomere intensity was calculated for each nucleus as follows: telomere intensity (TI)=(sum of all telomere signal intensities)/(intensity of DAPI signal). Calculation at each step in the analysis was performed automatically. Ten fields were examined for each sample, and average telomere intensity in each sample calculated.

Statistical analysis: We used the Student *t* -test to analyze the results of the clinical findings and the Mann–Whitney *U*-test to analyze the results of telo-FISH. A probability value of $p<0.05$ was considered to indicate statistical significance.

Immunohistochemistry: Lacrimal gland tissue slides were fixed with 2% paraformaldehyde (PFA; Wako, Osaka, Japan)

Table 1. Patient's demographic data and telomere intensity.

Number	Diagnosis	Age	Fluorescein score	Rose bengal score	Schirmer value	Schirmer value with nasal stimulation
1	SS	24	6	8	2	1
2	SS	49	1	7	5	NA
3	SS	54	2	NA	2	NA
4	SS	66	9	9	5	4
mean±SD		**48.3±17.6**	**4.5±3.6**	**8.0±1.0**	**3.5±1.7**	**2.5±2.1**
5	Non-SS	42	1	4	2	35
6	Non-SS	54	6	2	8	NA
7	Non-SS	54	0	2	2	16
8	Non-SS	59	3	0	5	7
9	Non-SS	66	0	0	4	24
10	Non-SS	71	2	NA	4	2
11	Non-SS	74	0	2	4	4
mean±SD		**60.0±11.1**	**1.7±2.2**	**1.7±1.5**	**4.1±2.0**	**14.7±12.9**

SS, Sjögren syndrome; Non-SS, Non-Sjögren syndrome; NA, Not applicable; SD, standard deviation.

for immunostaining for p63 and nucleostemin. After background staining was blocked with 10% normal donkey serum, samples were treated with the following monoclonal primary antibodies: anti-p63 (4A4; Calbiochem, A Brand of EMD Biosciences, Inc., San Diego, CA), anti-nucleostemin (R&D Systems, Minneapolis, MN), anti-ABCG2 (Chemicon, Millipore, Billerica, MA), and anti-nestin (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were then treated with Cy3 (Jackson ImmunoResearch, West Grove, PA)-conjugated secondary antibodies. Nuclei were counterstained with 4',6'-diamino-2-phenylindole (1 mg/ml, DAPI; Dojindo Laboratories, Tokyo, Japan).

Transmission electron microscopy: To investigate the structural change of cells in both groups, we performed transmission electron microscopy analysis. A portion of lacrimal gland tissue was immediately fixed with 2.5% glutaraldehyde and subjected to electron microscopic examination as described previously [29]. One-micrometer-thick sections were stained with methylene blue and portions exhibiting lacrimal gland structure thin-sectioned and examined with an electron microscope (1200 EXII; JEOL, Tokyo, Japan).

RESULTS

Demographic data: Profiles of the patients are shown in Table 1. A diagnosis of Sjögren syndrome was made in 4 of 11 patients and non-Sjögren syndrome in 7 patients. Patients ranged in age from 24 to 74 years (mean±SD age, 55.7±14.3 years). No significant difference was found in age of Sjögren (48.3±17.6 years) and non-Sjögren syndrome (60±11.1 years) patients. Sjögren syndrome had significantly higher Rose Bengal score than non-Sjögren syndrome ($p<0.001$), which was the only significant difference among the clinical parameters between the two groups.

Telomere shortening in Sjögren syndrome: Large numbers of inflammatory cells invade lacrimal gland tissue in Sjögren syndrome. Therefore, we selected only locations where acinar unit structure was well preserved for telo-FISH. In this study, telo-FISH was successfully performed on fixed frozen tissue sections. Representative photos are shown in Figure 1A,B. High levels of Cy3 expression were observed in the nuclei in lacrimal gland in the non-Sjögren syndrome group, whereas expression was weak in the Sjögren syndrome group (Figure 1A,B). Telomere intensity in lacrimal gland epithelial cells in the Sjögren syndrome group (6,785.0±455) was significantly lower than that in the non-Sjögren syndrome group (7,494.7±477; $p=0.02$, Figure 1C,D).

Progenitor cell markers showed relationship with telomere shortening: To investigate the relationship between telomere length and progenitor cell markers, we performed immunostaining for p63 and nucleostemin. In the non-Sjögren syndrome group, p63 was expressed in 2–4 acinar cells in each acinar unit (Figure 2A) and in the basal layer of duct basal cells continuously (Figure 2B). In contrast, p63 showed a lower level of expression and was only sparsely expressed in the basal layer of duct cells in the Sjögren syndrome group (Figure 2F,G). Nucleostemin showed a similar pattern of expression as p63, being higher and more regularly expressed in the non-Sjögren syndrome group than in the Sjögren syndrome group (Figure 2C,H). ABCG2 was expressed in intercellular junction and cytoplasm of most acinar unit regularly in the non-Sjögren syndrome group (Figure 2D). Whereas acinar unit was decreased in the Sjögren syndrome group, and acinar unit with ABCG2 expression was also decreased (Figure 2I). Nestin was expressed outside the acinar unit in both group, Elongated cells with nestin expression were observed more frequently in the SS group, compared with the non-SS group. Those nestin-positive cells were not observed uniformly in all locations, but formed cell clusters at tissue damaged areas in the SS group (Figure 2F,J). Telomere length was shorter and expression of progenitor