We conclude that the drug interaction between voriconazole and calcineurin inhibitors varies significantly among patients; thus, the dose adjustment of calcineurin inhibitors on initiating or discontinuing voriconazole should not be decided uniformly. Rather, close monitoring of the concentration in each individual is necessary to guide dosage adjustments with the goal of minimizing dose-related toxicity and maximizing efficacy of calcineurin inhibitors. The relationship between the blood concentration of voriconazole and its drug interaction with calcineurin inhibitors should be examined in a future study.

Conflict of interest

The author(s) declare no financial conflict of interest.

Acknowledgements

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ORIGINAL ARTICLE

Baseline profiles of ocular surface and tear dynamics after allogeneic hematopoietic stem cell transplantation in patients with or without chronic GVHD-related dry eye

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We evaluated ocular surface alterations in allogeneic hematopoietic stem cell transplantation (HSCT) recipients with or without chronic GVHD-related dry eye in a prospective study. Fifty eyes of 25 post-HSCT patients and 28 eyes of 14 age-matched healthy controls were included. Meibomian gland (MG) obstruction, tear evaporation rate, corneal sensitivity (CS), Schirmer test-I, tear break-up time (BUT) and ocular surface vital staining were examined. Conjunctival impression and brush cytology specimens were collected to evaluate the goblet cell density (GCD) and the inflammatory cell numbers. Obvious MG obstruction, decreased CS and enhanced tear evaporation rate were found in post-HSCT patients compared with normal controls. In addition, decreased conjunctival GCD, increased conjunctival squamous metaplasia and inflammatory cells were noted in cGVHD-related dry eyes compared with normal controls and post-HSCT without dry eye subjects. Furthermore, the conjunctival inflammatory cells were significantly higher in severe dry eyes compared with mild dry eyes ($P=0.03$). We found comprehensive ocular surface alteration in post-HSCT patients, regardless of whether they had cGVHD-related dry eye or not. The results suggest that the extent of inflammatory process seems to have a pivotal role in the outcome of the cGVHD-related dry eye.
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Introduction

Chronic GVHD is a major complication of allogeneic hematopoietic stem cell transplantation (HSCT).[1] Ocular surface is one of the target tissues of cGVHD. About 50% of patients develop dry eye or experience a worsening of the pre-existing dry eye after HSCT.[2] Dry eye is a distinctive sign and symptom for the diagnosis of cGVHD.[1] However, the pathogenesis of dry eye associated with cGVHD is still unclear, and effective treatments have not yet been established.[3] Pathogenic studies of dry eye associated with cGVHD depend on the lacrimal gland and conjunctival biopsy.[4–6] It is impossible to follow the alterations of the ocular surface pathologic process after HSCT by repeated biopsy. On the other hand, impression cytology and brush cytology are widely used methods to evaluate the ocular surface pathologic changes.[7] They are noninvasive, repeatable, and useful in following the changes in the ocular surface.[8,9] However, there are few reports on impression cytology changes and brush cytology characteristics in patients with cGVHD-related dry eye.[10] On the other hand, the conditioning regimen including total body irradiation and high incidence of meibomian gland dysfunction (MGD) in post-GVHD patients contributes to the ocular surface and tear function changes.

However, there is no report comparing the tear functions and ocular surface alterations between post-HSCT patients with or without dry eye. In a previous study,[2] we noticed there were two types of dry eye after HSCT. One had severe ocular surface and tear function damage with decreased reflex tearing that occurred soon after the onset of dry eye, whereas the other was mild with normal reflex tearing.

There are no data comparing the ocular surface and tear function differences between these two types of dry eye.

Patients and methods

Patients

Fifty eyes of 25 patients who underwent HSCT were enrolled at the dry eye clinic at Keio University from January 2006 to December 2006. Included were 20 eyes of 10 patients (5 males and 5 females; range, 30–66; median, 50 years) with cGVHD-related severe dry eye, 20 eyes of 10 patients (6 males and 4 females; range, 37–62; median, 51 years) with cGVHD-related mild dry eye and 10 eyes of 5 patients (3 males and 2 females; range, 39–50; median, 45 years) without dry eye. All the patients had no previous conjunctival or corneal disease or infections or other ocular disease at clinical examination. Twenty-eight eyes of 14 healthy subjects (10 males and 4 females; range, 20–70; median, 39 years) were also recruited as normal controls. The control subjects did not have any history of ocular or systemic disease or a history of topical eye drops or contact lens use that would alter the ocular surface as well. According to the global diagnostic criteria of dry eye, and the severity grading of the Dry Eye Workshop Report 2007,[11,12] we diagnosed the patients as having dry eye when patients had any sign of tear film instability (tear break-up time (BUT) $\leqslant$5 s, Schirmer test $\leqslant$5 mm), any abnormality of the ocular surface (Rose Bengal score $\geqslant$3, Fluorescein score $\geqslant$1) and/or symptoms of ocular irritation. Severe dry eye was defined as previously described[2,13] In brief, patients were diagnosed as having severe dry eye if the Schirmer test with nasal stimulation (reflex tearing) was $\leqslant$10 mm, and the FS and RB scores were $\geqslant$3 points and/or grade 3 and 4 according to the DEWS report 2007. The study was carried out in accordance with the principles of the Declaration of Helsinki. Informed consents and ethics board reviews for the examination procedure were obtained.

Clinical examinations

The ocular surface was examined by the double vital staining method. Two microliters of a preservative-free combination of 1% Rose Bengal and 1% fluorescein was instilled in the conjunctival sac by a micropipette.[14] The staining of Rose Bengal was scored for the temporal and nasal conjunctiva and the cornea, on a scale of 0–3 points. Fluorescein staining score also ranged between 0 and 9 points, but only for the cornea.[15] The BUT value was measured three times at the time of double staining, and the mean value was used for calculation. Schirmer 1 test was performed with standardized strips of filter paper (Alcon Inc., Fort Worth, TX, USA). To evaluate the obstruction of the MG orifice, digital pressure was applied on the tarsus. The expression of meibomian secretion (meibum) was scored as follows:[16] grade 0, clear meibum is easily expressed; grade 1, cloudy meibum is expressed with mild pressure; grade 2, cloudy meibum is expressed with more than moderate pressure; and grade 3, meibum cannot be expressed even with hard pressure.

Tear evaporimetry

The tear evaporation was measured with the evaporimeter (KAO Corporation, Tokyo, Japan).[17] Briefly, the eyecup of the evaporimeter tightly covered the subject's eye, and then the device measured the tear evaporation rate in both eyes-closed and eyes-open conditions. In this way, we can eliminate the evaporation from the eyelid. The computer system calculated the difference between these two conditions and gave the tear evaporation rate. The unit of tear evaporation rate is 10^{-7} g/cm^2 s.

Corneal sensitivity

Measurement of corneal sensitivity (CS) was performed using a Cochet-Bonnet aesthesiometer. The measurements were begun with the nylon filament fully extended. The tip of the nylon filament was applied perpendicularly to the surface of the cornea making certain not to touch the eyelashes and was pushed until the fiber's first visible bending. The length of the fiber was gradually decreased until a blink reflex was observed. The length was recorded in units of millimeters. Measurements were taken from the central cornea and the mean of the measurements was recorded as the CS reading of that eye.[18,19]

Conjunctival impression cytology

The impression cytology samples were obtained under topical anesthesia with 0.4% oxybuprocaine. A piece of cellulose acetate filter paper (Millipore HAWP 304, Bedford, MA, USA) was put on the temporal bulbar conjunctiva and gently pressed by forceps for several seconds. The specimens were fixed with 10% formalin neutral buffer solution and stained with Periodic Acid-Schiff (PAS). Five nonoverlapping areas of $\times$400 magnification were randomly selected and photographed. The goblet cell density (GCD) was reported as cells per square millimeter. The conjunctival epithelial squamous metaplasia was evaluated according to Nelson's grading scheme.[20]

Conjunctival brush cytology

The brush cytology samples were collected after administration of topical anesthesia with 0.4% oxybuprocaine. The central upper palpebral conjunctiva was gently brushed seven times with a disposable dental brush 1.5 mm in diameter (Dentalpro, Jacks. Co., Osaka, Japan). After sampling, the brush was immediately put in 1 ml of Hank's solution and shaken several times to detach the cells from the brush. The suspended cells were centrifuged with cytocentrifuge at 700 r.p.m. for 10 min to make the monolayer cell smears. The slides were stained by diff-quick staining. We counted up to 500 cells including inflammatory cells and epithelial cells in nonoverlapping fields under microscopic observation (magnification, $\times$400). The inflammation was reported as the number of inflammatory cells in the total number of 500 brush cells.[10]

Statistical analysis

The data were analyzed by Instat (GraphPad Software, San Diego, CA, USA). Mann–Whitney *U*-test was used to compare the onset duration of dry eye. Kruskal–Wallis *H*-test

was used for the comparisons of clinical examination parameters, tear evaporation rates, GCD, conjunctival squamous metaplasia, and inflammatory cell amount. The probability level of 5% was chosen as the statistical significance.

Results

Demographic characteristics

Patients' demographic characteristics were summarized in Table 1. The onset of dry eye in cGVHD-related severe and mild dry eye was 6.8 ± 2.5 and 13.2 ± 9.1 months, respectively, after HSCT. The onset of dry eye in the severe dry eye group was significantly earlier than the onset in the mild dry eye group ($P = 0.02$). Nine out of 10 severe dry eye patients had systemic cGVHD, but only 3 in 10 mild dry eye patients had systemic cGVHD.

Clinical examination parameters

The baseline scores of CS, ocular surface vital staining and tear function were summarized in Table 2. Obviously decreased CS was found in post-HSCT patients either with or without dry eye, but statistically significant decrease was found only in the severe dry eye group. Although the mean CS in the severe dry eye group was considerably lower than those with mild dry eye and post-HSCT without the dry eye groups, there was no statistically significant difference among the three groups. Obvious MG orifice obstruction (grade >1) was noted in 40 of 50 eyes of the post-HSCT patients as shown in Table 3. MG orifice obstruction degree in post-HSCT patients was statistically higher than normal controls, but there was no significant difference between the three post-HSCT groups. The tear evaporation rate in normal control, post-HSCT without dry eye, mild dry eye, and severe dry eye group was $2.2 \pm 1.53 \times 10^{-7}$ g/cm^{2}s, $4.42 \pm 2.13 \times 10^{-7}$, $3.6 \pm 1.66 \times 10^{-7}$,

Table 1 Demographic characteristics

Case no	*Age (years)*	*Gender*	*Diagnosis*	*Systemic cGVHD*	*Dry eye*	*Onset (month from HSCT to dry eye)*	*Month since HSCT*
1	52	M	MDS	Lung, skin, mouth	Severe	7	29
2	63	M	MDS	Liver, skin, mouth	Severe	6	65
3	64	M	MM	Skin, mouth	Severe	7	30
4	35	F	CML	Mouth	Severe	7	61
5	30	F	AML	Mouth	Severe	7	93
6	50	M	AML	Mouth, skin, liver	Severe	3	69
7	36	M	CML	Mouth	Severe	7	88
8	66	F	MM	Mouth, skin	Severe	11	58
9	57	F	MDS	Mouth, skin	Severe	10	34
10	34	F	ALL	(–)	Severe	3	144
11	49	M	ALL	Lung, liver, skin	Mild	9	24
12	54	M	ALL	Mouth, skin, intestinal	Mild	12	66
13	59	F	ALL	(–)	Mild	11	96
14	56	M	MDS	Liver	Mild	36	156
15	62	F	ALL	(–)	Mild	2.5	19
16	37	M	MDS	(–)	Mild	5	28
17	45	M	MDS	(–)	Mild	12	12
18	61	F	ALL	(–)	Mild	16	36
19	51	M	AML	(–)	Mild	15	36
20	58	F	NHL	(–)	Mild	13	30
21	45	M	AA	(–)	(–)	(–)	60
22	44	F	AML	(–)	(–)	(–)	120
23	39	F	CML	(–)	(–)	(–)	144
24	50	M	AML	(–)	(–)	(–)	30
25	48	M	AML	(–)	(–)	(–)	3

Abbreviations: AA = aplastic anemia; cGVHD = chronic GVHD; F = female; HSCT = hematopoietic stem cell transplantation; M = male; MDS = myelodysplastic syndrome; MM = multiple myeloma; NHL = non-Hodgkin lymphoma.

Table 2 The scores of tear functions, corneal sensitivity and vital stainings

	Tear evaporation rate ($\times 10^{-7}$g/cm^{2}s)	*CS (mm)*	*Schirmer test (mm)*	*BUT (s)*	*FS (points)*	*RB (points)*
Normal controls	2.2 ± 1.53	60	16.35 ± 11.82	8.92 ± 3.17	0.54 ± 0.66	0.13 ± 0.34
Post-HSCT without dry eye	4.42 ± 2.13	57.5 ± 4.63	14.7 ± 10.34	10	0.3 ± 0.95	0.3 ± 0.95
cGVHD-related mild dry eye	3.6 ± 1.66	57.25 ± 4.16	13.06 ± 11.05	$4.85 \pm 2.18^{a,b}$	$2.4 \pm 1.93^{a,b}$	1.8 ± 1.85^{a}
cGVHD-related severe dry eye	5.98 ± 3.61^{a}	54.98 ± 7.75^{a}	$2.45 \pm 2.28^{a,b,c}$	$2.68 \pm 1.4^{a,b}$	$5.6 \pm 2.56^{a,b,c}$	$5.55 \pm 2.06^{a,b,c}$

Abbreviations: BUT = tear break-up time; cGVHD = chronic GVHD; FS = fluorescein score; HSCT = hematopoietic stem cell transplantation; RB = Rose Bengal score.
[a]$P < 0.05$, compared with normal controls, Kruskal–Wallis test.
[b]$P < 0.05$, compared with post-HSCT without dry eye patients, Kruskal–Wallis test.
[c]$P < 0.05$, compared with cGVHD-related mild dry eye patients, Kruskal–Wallis test.

Table 3 Comparison of orifice obstruction grade of meibomian gland

Orifice obstruction	*Normal controls*	*Post-HSCT without dry eye*	*cGVHD-related mild dry eye*	*cGVHD-related severe dry eye*
Grade 0	26 (92.86%)	2 (20%)	0	2 (10%)
Grade 1	2 (7.14%)	2 (20%)	4 (20%)	0
Grade 2	0	2 (20%)	7 (35%)	5 (25%)
Grade 3	0	4 (40%)	9 (45%)	13 (65%)

Abbreviations: cGVHD = chronic GVHD; HSCT = hematopoietic stem cell transplantation.

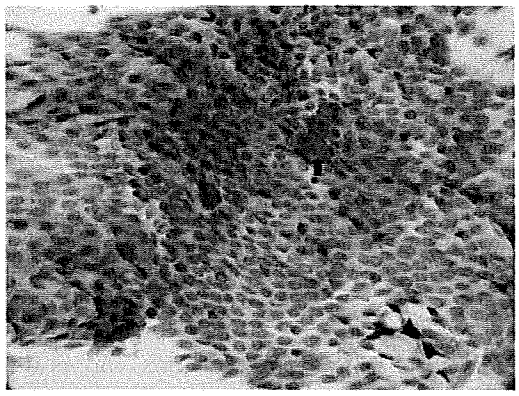

Figure 1 Representative conjunctival impression cytology specimens from a 50-year-old male, post-HSCT without dry eye subject. Note plenty goblet cells (black arrow) and mucin pick up (yellow arrows). Periodic acid-Schiff (PAS) staining, magnification, ×400.

and $5.98 \pm 3.61 \times 10^{-7}$ g/cm^2 s, respectively. Although the mean tear evaporation rate in mild dry eye and post-HSCT without dry eye patients was higher than in normal controls, statistically increased tear evaporation was found only in cGVHD-related severe dry eye patients ($P<0.001$).

Conjunctival impression cytology

Conjunctival specimens from normal controls and post-HSCT without dry eye subjects showed plenty of goblet cells and mucin pick up (Figure 1). The goblet cell densities in these two groups were 1313.13 ± 733.82 and 1030 ± 433.14 cells/mm^2. The mean GCD in the cGVHD-related mild and severe dry eye groups was 706.49 ± 583.52 and 396.36 ± 381.00 cells/mm^2. Both were obviously lower than the former two groups without dry eye (Table 4). Moreover, significant conjunctival epithelial squamous metaplasia was noted in severe dry eye patients. The mean grades of squamous metaplasia in normal control, post-HSCT without dry eye, and mild dry eye groups were 0.70 ± 0.46, 0.71 ± 0.52, and 0.72 ± 0.56, respectively. There was no statistical difference among the three groups. However, the average grade of squamous metaplasia in severe dry eye subjects was 1.61 ± 0.72, which was significantly higher than that in the other three groups (Table 4). Except decreased GCD, the PAS staining also showed inflammation in some impression cytology specimens from the cGVHD-related severe dry eye and mild dry eye patients (Figures 2 and 3). In addition, the PAS staining also indicated the intense inflammatory cell infiltration that frequently appeared with the abnormal mucin conglomeration.

Brush cytology

There was no inflammatory cell in the brush cytology specimens from normal controls. In contrast, a different extent of inflammatory cell infiltration was found in the specimens from post-HSCT patients (Figure 4). The mean number of inflammatory cells in 500 brush cells in post-HSCT without dry eye, mild dry eye, and severe dry eye specimens were 5.44 ± 6.04 cells, 14.64 ± 9.75 cells, and 22.64 ± 11.69 cells, respectively. The mean inflammatory cell numbers in both cGVHD-related mild and severe dry eye specimens were significantly higher than in normal controls and post-HSCT without the dry eye group ($P<0.001$). Moreover, the inflammatory cell number in the severe dry eye group was statistically higher than in the mild dry eye group ($P=0.03$).

Discussion

In this study, we evaluated the detailed baseline profiles of ocular surface and tear function alterations in post-HSCT patients with or without dry eye disease. We found obviously decreased CS in post-HSCT subjects either with or without dry eye disease. Although the reduction of CS in severe dry eye patients seemed to be more prominent, there were no statistical differences compared with post-HSCT without dry eye and mild dry eye patients. A reduction of CS has been reported in dry eye patients.[18,19] We also noted decreased CS in cGVHD-related dry eye patients in our previous study.[3] Considering the conditioning regimens before HSCT, such as total body irradiation, which includes orbital irradiation, we thought decreased CS in cGVHD-related dry eye patients may not be because of the dry eye pathologic process. Therefore, we recruited post-HSCT without dry eye subjects in this study. According to the present results, decreased CS was obvious even in post-HSCT without dry eye patients. Our study suggested that the conditioning regimens before HSCT may be more responsible for the decreased CS in cGVHD-related dry eye disease.

Moreover, increased MG obstruction grade was found in post-HSCT both with and without dry eye patients. Consistent with this, an increased tendency in the tear evaporation rate was noted in post-HSCT patients. However, the statistical increase was found only in the severe dry eye patients. MGs produce lipid material that spread and cover the ocular surface during the blink to keep the tear film stable and to reduce the tear evaporation. The dysfunction of the MG can induce evaporative dry eye.[21] On the other hand, decreased tear production

Table 4 Comparison of conjunctival GCD and epithelium squamous metaplasia

	Normal controls	*Post-HSCT without dry eye*	*cGVHD-related mild dry eye*	*cGVHD-related severe dry eye*
GCD (cells/mm^2)	1313.13 ± 733.82	1030 ± 433.14	706.49 ± 583.52[a]	396.36 ± 381.00[a,b]
Squamous metaplasia (Nelson's)	0.70 ± 0.46	0.71 ± 0.52	0.72 ± 0.56	1.61 ± 0.72[a,b,c]

Abbreviations: cGVHD = chronic GVHD; GCD = goblet cell density; HSCT = hematopoietic stem cell transplantation.
[a]$P<0.05$, compared with normal controls, Kruskal–Wallis test.
[b]$P<0.05$, compared with post-HCT without dry eye patients, Kruskal–Wallis test.
[c]$P<0.05$, compared with cGVHD-related mild dry eye patients, Kruskal–Wallis test.

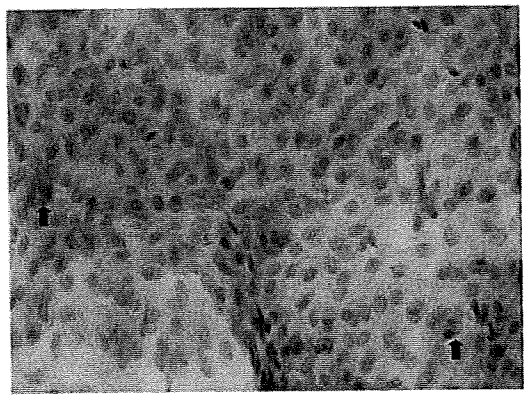

Figure 2 Representative conjunctival impression cytology specimens from a 37-year-old male, cGVHD-related mild dry eye patient. Note decreased goblet cell number (black arrow), and the conjunctival epithelium has no obvious keratinization. Periodic acid-Schiff (PAS) staining, magnification, ×400.

can also cause an increase in the tear evaporation rate.[22] Taken together, these findings suggest that increased MGD in post-HSCT patients cause enhanced tear evaporation in both groups of patients with or without dry eye, and enhanced tear evaporation acting together with the decreased tear production, induced the enhancement of ocular surface changes and tear function changes in cGVHD-related severe dry eye patients.

In our previous study,[2] we noticed the fact that there were two types of dry eye after HSCT. One had severe ocular surface and tear function alterations with decreased reflex tearing, which occurred soon after the onset of dry eye. Another was mild with normal reflex tearing. In this study, we performed a further comparison about the difference between these two types of dry eye. We found that the onset of cGVHD-related severe dry eye was obviously earlier than that of mild dry eye. In our patients, severe dry eye occurred around 6.8 ± 2.5 months after HSCT, but the onset of mild dry eye was around 13.2 ± 9.1 months after HSCT. There was one patient in whom the mild dry eye occurred 3 years after the HSCT. Moreover, most severe dry eye patients had systemic cGVHD, whereas only a few patients in the mild dry group had systemic cGVHD. Those findings indicated the different pathologic processes in cGVHD-related severe and mild dry eye disease.

For further comparison, we performed conjunctival impression cytology to evaluate the alterations and differences in GCD and squamous metaplasia in these two types of dry eye disease. GCD and squamous metaplasia are two parameters that were widely used to evaluate the ocular surface epithelial condition in dry eye and other ocular surface disease.[18,23,24] In addition, goblet cell content has been reported to be a sensitive indicator of primary ocular surface disease.[25,26] However, the report concerning the conjunctival impression cytology characteristics in cGVHD-related dry eye disease was still rare.[8] In this study, we found that both cGVHD-related mild and severe dry eye specimens showed significantly decreased GCD compared with normal controls and post-HSCT without dry eye specimens. Moreover, the mean GCD in severe dry eye patients was only about half of the density in mild dry eye patients with decrease in goblet cell numbers along with increased squamous metaplasia and keratinization of the ocular surface.[27] In cGVHD-related mild dry eye, although the GCD decreased, there was no obvious squamous metaplasia. However, high grades of squamous metaplasia with a further decrease in goblet cell numbers were found in severe dry eye patients. On the basis of these findings, we confirmed GCD to be a sensitive indicator for evaluating the extent of cGVHD-related dry eye disease. Except for decreased GCD and squamous metaplasia, some impression cytology specimens from cGVHD-related dry eye patients showed inflammatory cell infiltration in the conjunctival epithelium. These intense inflammatory areas often appeared in the area with clustered abnormal mucin. It indicates that the inflammation process involves the pathologic changes of cGVHD-related dry eye, which may influence the secretion and physiological characteristics of the ocular surface mucin.

For revealing the inflammation status in cGVHD-related dry eye disease and comparing the inflammation extent between cGVHD-related severe and mild dry eye disease, we collected the conjunctival brush cytology specimens and calculated the amount of inflammatory cells. We found considerably increased inflammatory cell numbers in both cGVHD-related severe dry eye and mild dry eye patients compared with normal controls and post-HSCT without dry eye subjects. Moreover, the number of inflammatory cells in severe dry eye specimens was significantly higher than in mild dry eye specimens. Recently, increased evidence suggests that dry eye is an inflammation-related disease.[28] Our previous study also found many inflammatory markers expressed in biopsy samples of the conjunctiva and lacrimal gland from cGVHD-related dry eye patients.[4–6] The present findings confirm that inflammation is involved in the pathogenesis of cGVHD-related dry eye. Moreover, our results suggest that the extent of

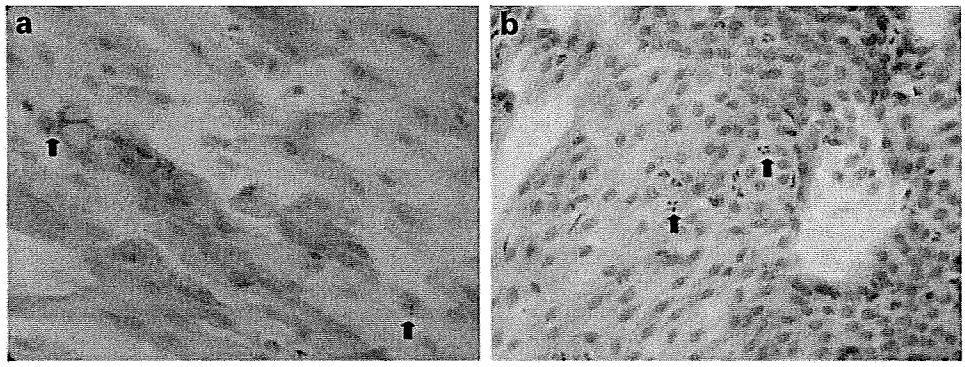

Figure 3 Representative conjunctival impression cytology specimens from a 52-year-old male cGVHD-related severe dry eye patient. (**a**) Note obvious squamous meterplasia and decreased GCD. Black arrows indicate the small goblet cells with decreased cytoplasmic mucin. (**b**) Note inflammatory infiltration (black arrows) in the conjunctival epithelium. Periodic acid-Schiff (PAS) staining, magnification, ×400.

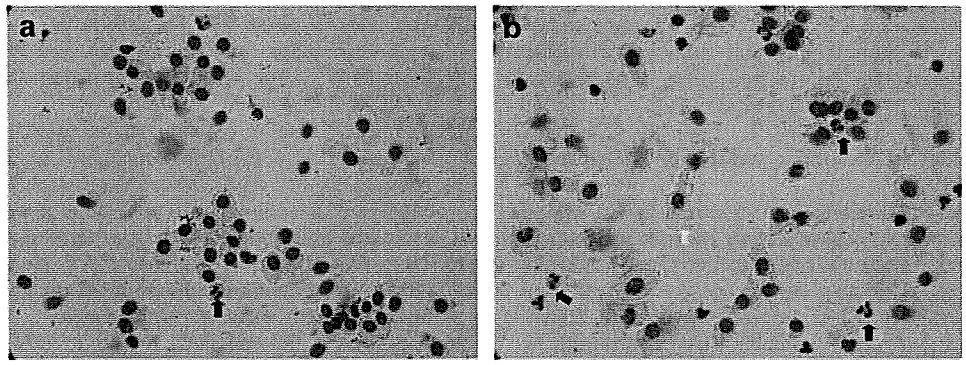

Figure 4 Representative brush cytology (BC) specimens. (**a**) Specimen from a 37-year-old male, cGVHD-related mild dry eye patient. Note inflammatory cells (black arrow). (**b**) Specimen from a 66-year-old female cGVHD-related severe dry eye patient. Note obviously inflammatory cells (black arrows) and keratinized conjunctival epithelial cells (yellow arrow). Diff-quick staining, magnification, ×400.

inflammation may be responsible for the different extent of pathologic damage in cGVHD-related mild and severe dry eye disease.

As the time we collected the brush cytology and impression cytology samples was relatively far from the onset of the dry eye, it is hard to distinguish whether the inflammation is the consequence or a cause of cGVHD-related dry eye. However, these two techniques are relatively noninvasive and repeatable examinations. They are very suitable to monitor the dynamic changes of the ocular surface epithelium and inflammation after HSCT. Moreover, the impression cytology and brush cytology samples can also be used to perform immunohistochemical staining, enzyme-linked immunosorbent assay, flow cytometry, and mRNA expression analysis.[9,10,29,30] Therefore, they are also useful to monitor the pathologic progress in cGVHD-related dry eye disease and helpful for investigating the etiology of cGVHD-related dry eye disease.

In this study, we used the tear evaporimetry, MG expression examination, impression cytology, and brush cytology to give a comprehensive evaluation of the changes of ocular surface and tear functions in patients with cGVHD-related mild and severe dry eye disease, and compared the results with healthy controls and post-HSCT patients without dry eye disease. According to the findings in this study, we speculated that the extent of the inflammatory process seems to have a pivotal role in the outcome of cGVHD-related dry eye disease with changes in tear evaporation, CS and GCD acting as determinants of the differences of the ocular surface healthy status.

In conclusion, our present data provide the baseline data of each type of dry eye disease associated with cGVHD using these methods. These data are also useful for future therapeutic evaluation.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Rituximab for the treatment of corticosteroid-refractory chronic graft-versus-host disease

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Abstract We prospectively evaluated the safety and efficacy of the anti-CD20 chimeric monoclonal antibody rituximab for the treatment of corticosteroid-refractory chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation. Seven patients were treated with 375 mg/m^2 rituximab weekly for 4 consecutive weeks. Rituximab was well tolerated with no severe toxicity observed during treatment. At 1 year, 3 patients showed a partial response to rituximab therapy, 3 had stable disease, and 1 had progressive disease. Rituximab allowed a reduction in the dose of steroids in 4 patients. Responsive manifestations included mild to moderate skin and oral lesions, and immune hemolytic anemia, and thrombocytopenia. Severe manifestations involving the skin, fascia, and eye did not respond to treatment. These observations suggest that rituximab therapy may be effective for select patients with corticosteroid-refractory chronic GVHD that is not advanced.

Keywords Rituximab · Chronic GVHD · Corticosteroids · Allogeneic transplantation

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1 Introduction

Chronic graft-versus-host disease (GVHD) remains to be the major cause of late morbidity and mortality, and has a significant effect on the functional status and quality of life in long-term survivors after allogeneic hematopoietic cell transplantation (HSCT). Chronic GVHD is a pleiomorphic syndrome with highly variable clinical manifestations, involving the skin, liver, eyes, mouth, esophagus, lung, serosal surfaces, lower gastrointestinal tract, female genitalia, and fascia [1, 2]. Corticosteroids in addition to the continuous administration of a calcineurin inhibitor are the standard treatment for chronic GVHD. The prognosis of patients with corticosteroid-refractory chronic GVHD is extremely poor, and there is no standard treatment for these patients [1, 3].

Although the biological mechanisms leading to chronic GVHD are not well understood compared with those leading to acute GVHD, multiple cellular and humoral mechanisms are likely to be involved in chronic GVHD [4, 5]. Much evidence suggest that B cells and humoral immunity are likely to play a role in the pathogenesis of chronic GVHD; the B cell compartment paradoxically shows simultaneous B lymphocytopenia and B cell hyperreactivity manifested by the production of autoantibodies.

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CD86 expression is highly upregulated in B cells upon stimulation with toll-like receptor 9 in patients with chronic GVHD, as compared to that in controls [6]. Alloantibodies specific for recipient minor histocompatibility antigens have been detected in patients with chronic GVHD, usually 4–6 months after transplantation [7, 8]. Patients with antibodies to recipient minor histocompatibility antigens also have T cells specific for the same antigens [9]. A more direct role of B cells has been suggested by experiments showing that the depletion of donor B cells can protect mice from chronic GVHD [10].

Rituximab is a chimeric mouse/human anti-CD20 monoclonal antibody. It binds with high affinity to $CD20^+$ cells and specifically depletes B cells in vivo. Several phase II studies and case series studies have suggested that rituximab may be effective in the treatment of chronic GVHD [11–17]. Such beneficial effects of B cell depletion by rituximab further emphasize a potential pathogenic role of B cells in the development of chronic GVHD. However, the organ-specific responses observed between studies are substantially different, possible, in part, because previous retrospective studies involved patients who were heavily treated with different types of immunosuppressive therapy.

Ethnicity is associated with the incidence and severity of GVHD [18]. Japanese that have remained geographically isolated for significant periods of time are likely to have less genetic diversity than other ethnic populations experiencing recent and multiple immigrations. Japanese patients receiving allogeneic HSCT have a lower incidence of acute and chronic GVHD compared with patients in Western countries [19–22]. Furthermore, immunosuppressants other than calcineurin inhibitors and corticosteroids are rarely used to prevent and treat GVHD in Japan because they have not been approved for use. Thus, Japanese patients with chronic GVHD might represent a more homogeneous population in terms of genetic background and prior therapies. Here, we prospectively evaluated the safety and efficacy of rituximab in the treatment of corticosteroid-refractory chronic GVHD in Japanese patients undergoing allogeneic HSCT.

2 Patients and methods

2.1 Patients

An open-labeled and early phase II study of rituximab therapy for corticosteroid-refractory chronic GVHD was conducted. The primary objective was to determine the safety, toxicity, and efficacy of 4 courses of rituximab therapy. Eligible subjects had extensive chronic GVHD, which had shown resistance to prednisolone (PSL) at doses greater than 0.5 mg/kg for 30 days within the previous 12 months, who were receiving a stable dose of cyclosporine (CSP) or tacrolimus (TAC). The patients excluded from the study had a previous history of HSCT, an uncontrolled infection, were carriers of hepatitis B or C viruses, and younger than 18 years. This study was approved by the Institutional Review Board of each participating institute, according to the Declaration of Helsinki, and written informed consent was obtained from each participating patient.

2.2 Rituximab therapy

The patients were premedicated with acetaminophen and diphenhydramine, and then 375 mg/m^2 rituximab was intravenously administered weekly for 4 weeks. The initial rate of infusion was 25 mg/h, which was increased to 100 mg/h if there was no reaction to the infusion. During 4 courses of treatment, all patients were required to receive a stable dose of immunosuppressive agents. Following 4 courses of rituximab therapy, decisions regarding the tapering of the dose of immunosuppressive medications were prepared by the transplant physician. The recommended sequence was the withdrawal of corticosteroids and then the withdrawal of the calcineurin inhibitors based on the resolution of chronic GVHD.

2.3 Study evaluation

The diagnosis of chronic GVHD required the presence of at least one diagnostic clinical sign of chronic GVHD or diagnostic manifestation confirmed histologically or by other relevant tests in the absence of acute characteristics of GVHD [2]. The disease was classified as limited or extensive and as de novo, quiescent, or progressive GVHD [1, 23]. Chronic GVHD was staged and graded according to National Institute of Health consensus criteria [2]. The global assessment of the severity of chronic GVHD was derived by combining organ- and site-specific scores. Each organ or site was scored according to a 4-point scale (0–3), with 0 representing no involvement and 3 representing severe impairment. In addition, performance status (PS) was evaluated on this 4-point scale. For thrombocytopenia, a score of 0 was defined as platelets $\geq 140 \times 10^9$/l, 1 as platelets $\geq 100 \times 10^9$/l, 2 as platelets $\geq 50 \times 10^9$/l, and 3 as platelets $< 50 \times 10^9$/l. For autoimmune hemolytic anemia (AIHA), a score of 0 was defined as hemoglobin ≥ 12 g/dl and a negative Coombs test result. Scores of 1, 2, and 3 were defined as hemoglobin ≥ 10, ≥ 7, and < 7 g/dl, respectively. A post-treatment evaluation was performed every week until 6 weeks and then 2, 3, 4, 6, and 12 months thereafter, which included an assessment of the severity of chronic GVHD in each organ or tissue and a safety analysis. The analysis included the monitoring of

blood counts and liver and renal function test results and documenting unexpected side effects. The severity of adverse events attributable to rituximab was evaluated on the basis of the Common Terminology Criteria for Adverse Events, version 3.0. The therapeutic response was assessed 1 year after the initiation of the study, and was defined as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). CR was defined as the resolution of all symptoms and signs of chronic GVHD. PR was defined as a partial improvement in scores of ≥ 2 for at least one organ with no progression in any other organs and no requirement of additional systemic immunosuppressive therapy for chronic GVHD. SD was defined as no change in score and no requirement of additional systemic therapy. PD was defined as the objective worsening of the disease or the need for dose escalation of immunosuppressive agents or additional systemic treatment. Statistical analysis was performed using an unpaired 2-tailed *t* test.

3 Results

3.1 Patient characteristics

Seven patients (5 men and 2 women; median age 48 years, age range 24–55 years) were enrolled in this study between April 2006 and March 2007. The patients' characteristics are summarized in Table 1. All patients had extensive and corticosteroid-refractory chronic GVHD after allogeneic HSCT. The diseases for which transplantation was performed were as follows: acute myelogenous leukemia (AML, $n = 3$), chronic myelogenous leukemia (CML, $n = 2$), acute lymphoblastic leukemia (ALL, $n = 1$), and myelodysplastic syndrome (MDS, $n = 1$). Four patients underwent bone marrow transplantation (BMT) from a human leukocyte antigen (HLA)-matched or HLA-DR-mismatched unrelated donor, and 3 underwent peripheral blood stem cell transplantation (PBSCT) from an HLA-matched sibling donor. Myeloablative conditioning regimens were used in 5 patients, whereas fludarabine-based reduced-intensity conditioning regimens were used in 2. GVHD prophylaxis consisted of CSP and short-term methotrexate (MTX) ($n = 4$), TAC and short-term MTX ($n = 2$), or TAC alone ($n = 1$). All patients developed acute GVHD (grade II in 6 patients and grade I in 1 patient), which was successfully treated with 1–2 mg/kg of methylprednisolone (mPSL) or PSL and subsequently developed into quiescent and extensive chronic GVHD. On the basis of the global staging system [2], 4 patients had "severe" chronic GVHD, and 3 had "moderate" disease. The median time from transplantation to study enrollment was 42 months (range 19–112 months). The median time

Table 1 Patients' characteristics

UPN	Age/sex	Diagnosis	Donors	HLA	Stem cell source	GVHD prophylaxis	Type of onset	Prior therapy	Interval from transplantation to rituximab (months)	Interval from onset of chronic GVHD to rituximab (months)
1	24/F	CML	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, CSP	19	8
2	39/M	MDS	Unrelated	Identical	BM	TAC+MTX	Quiescent	PSL, pulse mPSL, CSP, TAC	42	39
3	48/M	AML	Unrelated	Identical	BM	TAC	Quiescent	PSL, TAC	46	43
4	51/M	CML	Unrelated	DR mismatch	BM	TAC+MTX	Quiescent	PSL, CSP, TAC	112	109
5	55/F	AML	Unrelated	Identical	BM	CSP+MTX	Quiescent	PSL, CSP	34	30
6	55/M	AML	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, CSP	47	37
7	29/M	ALL	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, mPSL, CSP	27	25

from the onset of chronic GVHD to study enrollment was 37 months (range 8–109 months). In all patients, prior therapy for chronic GVHD was a combination of corticosteroid and CSP or TAC. None of the patients received other immunosuppressive medications. The intervals between dose escalations of corticosteroids and rituximab administration were at a minimum of 1 month. All subjects were followed for 1 year after the initiation of rituximab therapy.

3.2 Toxicity

All patients completed a 4-week course of rituximab treatment. Only one patient developed grade 2 allergic toxicity, i.e., an infusion reaction after the first dose of rituximab. None of the patients developed grade 3 or 4 adverse events attributable to rituximab during the 4-week treatment. Later adverse events, occurring within 1 year of the initiation of therapy, included the following: grade 3 bacterial infection that required intravenous administration of cephepim in 1 patient at 2 months, grade 2 herpes simplex virus infection that required treatment with valaciclovir in 1 patient at 4 months, grade 1 hepatic injury in 1, and grade 2 renal damage in 1. These adverse events were likely related to other drugs that were used or to pronounced immune suppression related to transplantation and chronic GVHD.

3.3 Efficacy

All patients were evaluable for their response to rituximab therapy at 1 year after the study initiation (Table 2). Unique patient number (UPN) 1 developed skin sclerosis, which was initially treated with 0.5 mg/kg of PSL. Six month later, her chronic GVHD progressed to "severe" skin sclerosis and contracture. Chronic GVHD initially responded to rituximab with an improvement of symptoms, leading to successful tapering of PSL by 67% over 6 weeks. However, sclerosis progressed thereafter, and the PSL dose was increased. The PSL dose was subsequently reduced again by 67% of the initial dose at 1 year, at which time the global staging and organ-specific scores were unchanged as compared to those before rituximab therapy. The overall response at 1 year was classified with PD because of the need for an escalation in the dose of PSL. UPN 2 developed chronic GVHD in the skin and mouth, which was initially responded to 250 mg of mPSL. Skin and oral lesions were exacerbated 10 months before enrollment to this study. CSP was replaced with TAC and PSL dose was increased to 0.5 mg/kg, but chronic GVHD progressed to "moderate" cutaneous and oral disease. Rituximab therapy was started, but was not effective. However, the disease was stable during the study period without the need for an escalation in dose of CSP and PSL.

UPN 3 developed extensive chronic GVHD, including cutaneous, oral, and hepatic lesions, and autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia. This patient had steroid-induced diabetes mellitus and a history of tuberculosis. The patient was initially treated with 1 mg/kg of PSL. Three months before study enrollment, PSL was increased to 0.8 mg/kg, which was maintained until study entry according to the past history of exacerbation with less doses of PSL. Rituximab therapy improved "severe" GVHD to "moderate" GVHD, and allowed an 82% reduction in the dose of PSL within 1 year of the study.

UPN 4 had a 9-year history of chronic GVHD. The most severe manifestation was slowly progressive sclerodermatous lesions in the cervical and lower facial skin and fascia, which resulted in severe flexion and rotation contracture and difficulty in mouth opening and swallowing. Rituximab therapy failed to improve these manifestations, but the disease did not progress during the study period with stable doses of CSP and PSL. However, the patient required additional immunosuppressive therapy with high-dose cyclophosphamide 17 months after rituximab therapy and died of bacterial pneumonia, which developed during cyclophosphamide-induced neutropenia.

UPN 5 had "severe" sclerodermatous skin lesions in both the upper and lower extremities. The patient also had recurrent pleural effusion and ascites and a motility disorder of the intestine. The patient was initially treated with 0.5 mg/kg of PSL. Nine months before study enrollment, the disease was deteriorated and PSL dose was increased to 0.5 mg/kg, which was discontinued before rituximab therapy because of a lack of improvement and steroid intolerance. Rituximab therapy temporally improved serositis and diarrhea, but global staging and organ-specific scores were unchanged at 1 year. The patient died of bacterial pneumonia 19 months after the initiation of rituximab therapy.

UPN 6 developed corticosteroid-refractory chronic GVHD in the skin, mouth, eyes, and muscles. Rituximab improved these symptoms, and the patient was able to discontinue PSL by 1 year. Interestingly, the patient developed conductive hearing loss due to inflammation in the bilateral middle ear at the onset of chronic GVHD. The patient recovered dramatically from deafness after the fourth dose of rituximab therapy. UPN 7 developed cutaneous chronic GVHD and treated with PSL. The disease was progressed to sclerodermatous skin disease and the patient was started on 2 mg/kg of mPSL, which was reduced due to a lack of improvement and the patient entered to this study. Sclerodermatous skin lesion improved slowly after rituximab therapy and disappeared

Table 2 Response to rituximab therapy

UPN	Pretreatment			2 months			1 year				
	Global staging	Organ/ manifestation	Score	Global staging	Score	% PSL reduction	Global staging	Score	% PSL reduction	Global response	Follow-up
1	Severe	PS	1	Severe	1	67	Severe	1	67	PD	Alive at 36 months
		Skin	2		2			2			
		Mouth	1		1			1			
		Joints and fascia	3		3			3			
2	Moderate	PS	1	Moderate	1	0	Moderate	1	0	SD	Alive at 35 months
		Skin	2		2			2			
		Mouth	2		2			2			
3	Severe	PS	1	Moderate	1	40	Moderate	1	72	PR	Alive at 34 months
		Skin	1		1			1			
		Mouth	1		1			1			
		Liver	3		2			2			
		Thrombocytopenia	2		1			1			
		AIHA	1		0			0			
4	Severe	PS	1	Severe	1	0	Severe	1	0	SD	Died of infection at 20 months
		Skin	3		3			3			
		Eye	1		1			1			
		Joints and fascia	3		3			3			
5	Severe	PS	2	Severe	2	–	Severe	2	–	SD	Died of infection at 19 months
		Skin	3		3			3			
		Eye	1		1			1			
		Intestine	1		1			1			
		Joints and fascia	1		1			1			
		Serositis	2		2			2			
		Thrombocytopenia	2		2			1			
6	Moderate	PS	2	Moderate	1	0	Moderate	1	100	PR	Alive at 30 months
		Skin	2		1			1			
		Mouth	2		1			1			
		Eye	2		1			1			
		Muscle	1		0			0			
7	Moderate	PS	1	Moderate	1	0	Moderate	1	25	PR	Alive at 23 months
		Skin	2		2			0			
		Mouth	1		1			1			
		Eye	2		2			2			
		Joints and fascia	1		1			0			

at 1 year, although dry eye and oral mucositis did not improve.

Overall, none of the patients achieved a CR, whereas a PR was noted in 3 patients. SD was noted in 3 patients and PD in 1. One year after rituximab therapy began, PSL was discontinued or reduced in 4 of 6 patients; the median reduction rate was 67% (range 0–100%). None of the 7 patients required additional immunosuppressive therapy within 1 year after the initiation of the study. At a median follow-up of 30 months, 5 patients were alive with active and continuing chronic GVHD, and 2 had died of infection after the study period.

On the basis of global staging, only 1 patient with "severe" disease improved to "moderate" disease at 1 year, whereas 3 others with "severe" disease experienced no change. Patients with severe (score 3) skin sclerosis and joint contracture related to sclerodermatous skin GVHD and fascitis did not respond to rituximab therapy. One patient with severe (score 3) hepatic GVHD responded partially to rituximab therapy. Clinical responses were

observed primarily in patients with moderate (score 2) to mild (score 1) manifestations. It is noteworthy that 6 of 11 manifestations with a score 2 responded to rituximab therapy. Improvement in the skin, mouth, eye, liver, joints and fascia, intestine, and serous membrane was observed in 2 of 7, 1 of 5, 1 of 4, 1 of 1, 1 of 4, 0 of 1, and 0 of 1 cases, respectively. Notably, all cases of immune thrombocytopenia and anemia were responded well to rituximab. However, PS improved only in 1 patient who achieved a PR.

3.4 Immunological monitoring

B cell numbers were monitored after rituximab therapy using a flowcytometric analysis. $CD19^+$ B cells were quickly eliminated within 2 weeks after the first treatment and did not repopulate at least by 12 weeks (Fig. 1). Serum levels of IgG and IgA were unchanged by 6 weeks, but gradually declined thereafter. Serum IgM levels decreased much earlier and more profound compared with those of IgG and IgA.

4 Discussion

The prognosis of corticosteroid-refractory chronic GVHD is poor, and no standard therapy for corticosteroid-refractory chronic GVHD is available [1, 3]. In the present study, we evaluated the efficacy and safety of rituximab therapy in patients with quiescent-type chronic GVHD. This condition may have been related to the ethnicity of the transplant patients. The incidence of progressive-type chronic GVHD is high, reportedly 10–70% in Western countries [24, 25]. In contrast, progressive-type GVHD is rare and quiescent-type GVHD is common in Japanese patients [22]. Rituximab therapy was well tolerated, and no severe adverse events were attributed to rituximab therapy. A 4-week course of rituximab treatment produced an overall response rate of 43% at 1 year, which is slightly lower than

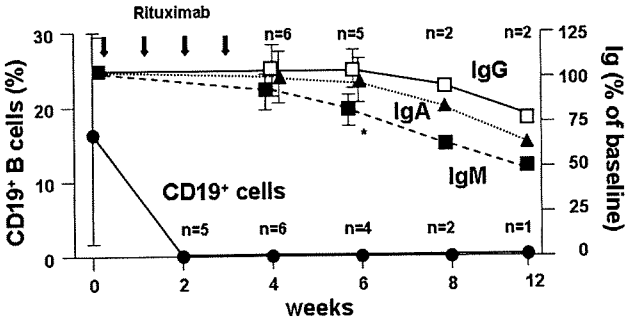

Fig. 1 Laboratory parameters over time after rituximab therapy. IgG, IgA and IgM levels are shown as percentage of baseline levels.*$P < 0.01$ compared with IgG or IgA

the overall response rate of 50–83% reported in previous studies testing the efficacy of rituximab [11–13, 15–17]. CR rates ranged from 0 to 20% in previous studies [12, 13, 16, 17]. In the present study, none of the patients achieved CR. The steroid-sparing effect is an important indicator of efficacy assessments of GVHD [26]. Rituximab therapy resulted in a median reduction in the dose of corticosteroids of 67%, which was slightly lower than the 75–86% reduction in dose observed in 3 previous studies that addressed the steroid-sparing effect of rituximab in this setting [13, 15, 16]. These results were surprising because we initially hypothesized that rituximab would be more effective in Japanese patients who tend to develop less severe chronic GVHD than Caucasians [22].

Previous studies of the efficacy of rituximab therapy for steroid-resistant chronic GVHD highlight the potential activity of rituximab against skin involvement, including scleroderma, whereas the responses to rituximab appear to be less pronounced in other organs or tissues [11–17]. These studies also suggested that the steroid-sparing effect might be more pronounced in the skin and oral lesions than others in chronic GVHD [15, 16]. In addition, hematologic abnormalities associated with chronic GVHD also respond well to rituximab therapy [11, 15, 27]. In our study, rituximab was most effective against immune thrombocytopenia and AIHA, and less effective against skin sclerosis and joint contracture related to sclerodermatous skin lesions and fascitis. This discrepancy between the current study and previous studies might have resulted because more patients with advanced sclerodermatous chronic GVHD were enrolled in our study than in the previous studies. The interval between the time of the onset of chronic GVHD and the time of study enrollment was longer in the present study (median duration 37 months) than in most of the previous studies (median duration 14–37 months) [11–13, 15, 17]. Nonetheless, our patients had undergone less immunosuppressive therapy before study enrollment than did the patients in the previous studies, most of whom had received multiple courses of immunosuppressive therapy [11, 12, 15]. Thus, the long-term duration of disease without sufficient intervention might have resulted in the development of irreversible damage in our patients.

Many advanced manifestations in chronic GVHD are potentially irreversible, including skin and joint contracture, chronic dry eye, esophageal and vaginal stricture, and bronchiolitis obliterans in the lung. The enrollment of patients with advanced chronic GVHD may not be appropriate when the endpoint of the study is the response to treatment. Alternatively, irreversible lesions could be excluded from consideration in the assessment of response [28, 29]. Such considerations were not specified in our protocol. The results of our study suggest that rituximab

may be more effective against mild to moderate manifestations than against severe manifestations of chronic GVHD. Thus, earlier treatment with rituximab or with other investigational agents for corticosteroid-refractory chronic GVHD may increase the chances of a good response. Another possible explanation for the poorer response to rituximab in our study than in previous studies, although unlikely, is that dominant immunological mechanisms associated with chronic GVHD and treatment outcomes may differ by ethnicity, because the prognostic scoring system [25], which was developed on the basis of clinical findings in Western patients, is not prognostic in Japanese patients [22].

We confirmed complete depletion of B cells after rituximab therapy. B cells were still absent 2 months after the last infusion of rituximab. In the initial multi-institutional trial evaluating a single four dose course of rituximab in patients with follicular lymphoma, the median B cell count did decline to almost undetectable levels after the first dose in the majority of patients, with recovery beginning from 6 to 9 months post-treatment, and return to normal levels between 9 and 12 months [30]. Similarly, B cells were undetectable in patients with chronic GVHD until 1 year after rituximab therapy [13]. Such a profound and prolonged B cell depletion may explain why rituximab treatment is effective in several antibody-mediated autoimmune diseases with some responses ongoing for more than 1–2 years [31]. On the other hand, rituximab therapy could results in impaired humoral immune responsiveness [32]. We also found that serum immunoglobulin levels decrease after rituximab therapy. Of note, IgM fell much more than IgG and IgA. This phenomenon was observed in patients with rheumatoid arthritis and chronic GVHD [13, 33]. This may be due to higher sensitivity of IgD^+ memory B cell subset, which produces natural mutated IgM antibodies as a first-line of defense against blood-borne antigens [33, 34], to rituximab than plasma cells.

In conclusion, the current study suggests that rituximab therapy may be effective for selective patients with corticosteroid-refractory chronic GVHD that is not advanced. A recent study indicated that that low-dose rituximab therapy is also effective [17]. However, the optimal schedule and dosing regimens for rituximab need to be determined. Furthermore, a well-designed, large-scale, prospective study is needed to conclusively address the efficacy of rituximab in the treatment of corticosteroid-refractory chronic GVHD.

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CASE REPORT

Second unrelated cord blood transplantation using a reduced-intensity conditioning regimen combined with gemtuzumab ozogamicin in patients with relapsed acute myelogenous leukemia

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Abstract Gemtuzumab ozogamicin (GO) is an effective molecular-targeted agent for CD33-positive acute myelogenous leukemia (AML) patients who are resistant to conventional chemotherapy. Recent prospective trials have revealed the safety and efficacy of GO as part of conditioning following allogeneic bone marrow or peripheral blood stem cell transplantation (SCT). We report here for the first time three AML cases that relapsed after allogeneic SCT and underwent unrelated cord blood transplantation (UCBT) following reduced-intensity conditioning (RIC) comprising fludarabine, melphalan, and low-dose total body irradiation combined with GO. Primary neutrophil engraftment occurred in all cases, while recovery of platelet count was delayed. Only one case of reversible hepatic sinusoidal obstruction syndrome was documented. Non-relapse mortality at day 100 was not documented. Notably, one patient who responded to GO survived for 6 months after UCBT in remission with excellent performance status, while the remaining cases relapsed early. These data suggest that GO may be safely combined with RIC for UCBT after previous allogeneic SCT.

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1 Introduction

The prognosis of relapsed acute myelogenous leukemia (AML) is generally poor. In particular, outcomes of patients who relapse after allogeneic hematopoietic stem cell transplantation (HSCT) are dismal [1, 2]. A response to the reduction of immunosuppressive agents or donor lymphocyte infusion is seen in only a few cases [3], and most cases require cytoreduction with a subsequent second transplantation of hematopoietic stem cells to achieve durable remission. Recently, reduced-intensity preparative conditioning (RIC) regimens have been developed to obtain primary engraftment with tolerable toxicity and an immune-mediated graft-versus-leukemia effect. This procedure has allowed successful extension of a second HSCT to patients who relapse after HSCT [4]. To improve the outcome of relapsed or refractory AML cases, the most important requirement is reduction of the pre-transplant tumor burden to reduce the risk of relapse [5].

Gemtuzumab ozogamicin (GO) is an immunoconjugate that targets the CD33 antigen expressed in approximately 90% of AML patients [6]. The humanized IgG4 monoclonal antibody is linked to the cytotoxin calicheamicin, which causes double-strand breaks in DNA and ultimately apoptosis. A response was observed in up to approximately 20% of AML patients with first relapse when GO was used as monotherapy [7]. Because of its limited extramedullary toxicity, GO might become an attractive agent for preparative treatment before allogeneic HSCT. However, a previous report [8] indicating that prior exposure to GO

within 3 months before HSCT increased the risk of hepatic sinusoidal obstruction syndrome (SOS) led us to hesitate before using GO as part of a conditioning regimen. Recently, two prospective studies revealed the safety and efficacy of concurrent administration of GO with a fludarabine-based RIC protocol and allogeneic HSCT [9, 10]. In these reports, the mobilized peripheral blood stem cells (PBSC) or bone marrow (BM) were used as the source of stem cells, and the safety and benefit of including GO in reduced-intensity unrelated cord blood stem cell transplantation (UCBT) remained unclear.

We report for the first time three AML cases that relapsed early after conventional allogeneic HSCT and underwent UCBT with RIC combined with GO. Primary neutrophil engraftment was obtained in all three cases, and non-relapse mortality at day 100 was not documented. Despite the limited number of cases, we consider that GO may be safely combined with RIC for a second UCBT in the treatment of AML that relapses after HSCT.

2 Case presentation

2.1 Case 1

A 31-year-old man was diagnosed with AML (M4) in April 2007. The leukocyte count was 155 × 10^9/L with 69% myeloblasts, and the immunophenotype of the blast cells was positive for CD13, CD33, CD34, CD38, and HLA-DR. Cytogenetic analysis revealed a normal karyotype, but FMS-like tyrosine kinase 3 (FLT3)-internal tandem duplication (ITD) and *NUP98-HOXA9* fusion was detected by polymerase chain reaction (PCR) assay. He achieved remission after induction chemotherapy and high-dose chemotherapy with autologous stem cell rescue. However, the patient relapsed in March 2008 with an additional acquisition of t(1;21)(p32;q22). He achieved complete remission (CR) after re-induction chemotherapy. After two cycles of consolidation chemotherapy, he underwent allogeneic bone marrow transplantation (BMT) from an unrelated donor with an RIC regimen consisting of fludarabine, busulfan, and low-dose total body irradiation (TBI). He relapsed again 5 months after allogeneic BMT. Leukemia progressed rapidly and was resistant to chemotherapy; UCBT was therefore scheduled. He received 6 and 3 mg/m^2 of GO 21 and 14 days before UCBT, respectively, as part of conditioning. Blast cells disappeared from his peripheral blood soon after first GO administration, and he received subsequent RIC regimen consisting of 25 mg/m^2 of fludarabine (days −8 to −4), 40 mg/m^2 of melphalan (days −3 to −2), and 4 Gy of TBI on day −1. Cord blood cells were transplanted on 21 November 2008, 175 days after the previous BMT (Table 1). Prophylaxis for graft-versus-host disease (GVHD) consisted of cyclosporine (CsA) and mycophenolate mofetil (MMF). Furthermore, to prevent hepatic SOS, the patient received 75 IU/kg/day of low-molecular-weight heparin (LMWH) from the first day of conditioning.

On day 10, he developed water retention, hyperbilirubinemia (2.9 mg/dL), and platelet transfusion-refractory thrombocytopenia. No ascites or hepatomegaly was detected by abdominal ultrasonography, but the level of serum bilirubin was elevated to 4.9 mg/dL. The patient was therefore treated with prostaglandin E1 (PGE1) and anti-thrombin III (AT-III) against the development of hepatic SOS. Subsequently, the serum level of bilirubin gradually decreased and the patient did not develop hepatic SOS. Engraftment of neutrophils (>0.5 × 10^9/L) was achieved on day 19 and that of platelets (>20 × 10^9/L) on day 63. The patient obtained hematological and molecular CR, and chimerism analysis showed complete donor type. He was discharged with fair performance status on day 67 with stage 2 skin GVHD (Grade I), which required no systemic corticosteroid treatment. He has maintained CR for 6 months after the second UCBT.

2.2 Case 2

A 31-year-old man was diagnosed with de novo AML-M2 with a normal karyotype in February 2008. The leukocyte count was 20.3 × 10^9/L with 31% myeloblasts. FLT3-ITD was detected by PCR assay. Blast cells were positive for CD13, CD33, CD34, CD38, and HLA-DR. The patient received two courses of chemotherapy, and thereafter remained in CR during consolidation therapy. Allogeneic HSCT was performed, but relapse of leukemia was documented in July 2008 immediately before allogeneic BMT from an HLA-matched unrelated donor. The myeloablative conditioning consisted of 12 Gy of TBI and 60 mg/kg of cyclophosphamide. He attained a transient second CR, but relapse of leukemia was observed again 50 days after transplantation. The immunophenotypic analysis of blasts was identical to that at the initial diagnosis. Because of the progression of leukemia refractory to re-induction therapy, a second UCBT was scheduled. RIC consisted of 6 mg/m^2 (day −21) and 3 mg/m^2 (day −12) of GO following 25 mg/m^2 of fludarabine (days −8 to −4) and 40 mg/m^2 of melphalan (days −3 to −2) and 2 Gy of TBI on day 0. Besides this RIC regimen, Ara-C (3 g) was given (days −6 to −5) against a rapid increase in leukemia blasts. He underwent UCBT on 25 November 2008, 125 days after the previous transplantation (Table 1). Administration of LMWH for prophylaxis against hepatic SOS was initiated on the first day of conditioning therapy. GVHD prophylaxis consisted of CsA and MMF.

Table 1 Summary of reduced-intensity UCBT combined with GO

	Case		
	1	2	3
Age/sex	31/M	31/M	55/F
Diagnosis	M4, Rel2	M2, Rel2	MDS/AML, Rel1
Cytogenetics/genetic anomalies	FLT3-ITD, NUP98/HOXA9	FLT3-ITD	Normal
Prior stem cell source	HLA-matched unrelated BM	HLA-matched unrelated BM	HLA-matched unrelated BM
Prior conditioning	Flu + BU + TBI	TBI + CY	TBI + CY
Second transplantation procedures time to prior transplant (days)	175	125	174
Graft source	UCB	UCB	UCB
Cell number ($\times 10^7$/kg)	1.91	3.27	3.13
$CD34^+$ cells ($\times 10^5$/kg)	0.82	1.17	1.17
HLA match	4/6	4/6	4/6
Conditioning	GO (9 mg/m^2) + Flu/L-PAM/TBI (4 Gy)	GO (9 mg/m^2) + Flu/L-PAM/TBI (4 Gy)	GO (6 mg/m^2) + Flu/L-PAM/TBI (2 Gy)
GVHD prophylaxis	CsA + MMF	CsA + MMF	CsA + MMF
Engraftment			
ANC > 0.5 × 10^9/L	Day 19	Day 21	Day 22
Plt > 20 × 10^9/L	Day 63	Not achieved	Not achieved
RRT			
Bilirubin elevation	Grade 3	Grade 3	Grade 3
Transaminase elevation	Grade 1	Grade 1	Grade 3
Hepatic SOS	–	+	–
aGVHD	Grade I	0	Grade I
Outcome	CR (6 months)	Dead (71 days)	Dead (61 days)

Rel relapse, *MDS* myelodysplastic syndrome, *AML* acute myelogenous leukemia, *BM* bone marrow, *Flu* fludarabine, *BU* busulfan, *TBI* total body irradiation, *CY* cyclophosphamide, *UCB* unrelated cord blood, *GO* gemtuzumab ozogamicin, *L-PAM* melphalan, *CsA* cyclosporine, *MMF* mycophenolate mofetil, *ANC* absolute neutrophils count, *RRT* regimen-related toxicity, *SOS* sinusoidal obstruction syndrome, *aGVHD* acute graft-versus-host disease, *CR* complete remission

On day 7, he presented right upper quadrant abdominal pain, hyperbilirubinemia (1.7 mg/dL), and coagulation abnormalities from day 13. Abdominal ultrasonography showed slight hepatomegaly, moderate ascites, and irregular reverse flow in the portal vein, which was compatible with hepatic SOS. After treatment with PGE1 and AT-III, his symptoms and hyperbilirubinemia (maximum 6.9 mg/dL) improved gradually and portal vein flow was normalized on day 24. Neutrophils exceeded to 0.5 × 10^9/L on day 21, but platelet recovery was delayed. No GVHD was documented, and mixed donor/recipient chimerism was confirmed. On day 41, leukemia cells were documented in the peripheral blood, indicating failure of the second UCBT combined with GO. The patient died of disease progression on day 71.

2.3 Case 3

A 55-year-old woman was diagnosed with AML developed from myelodysplastic syndrome with a normal karyotype in March 2008. Blast cells were positive for CD13, CD33, CD34, CD38, and HLA-DR. The patient achieved CR after conventional induction chemotherapy and subsequently received maintenance therapy. In the first CR, she underwent myeloablative conditioning (busulfan 12.8 mg/kg plus cyclophosphamide 120 mg/kg) and BMT from an HLA-matched unrelated donor in June 2008. However, she suffered from relapse of leukemia 3 months after BMT and did not gain remission despite re-induction chemotherapy. A second transplantation was therefore conducted to perform with UCB for relapsed AML. The following preparative conditioning was originally scheduled: Doses of GO 6 mg/m^2 (day −21) and 3 mg/m^2 (day −14), 25 mg/m^2 of fludarabine (days −6 to −2), 40 mg/m^2 of melphalan (days −3 to −2), and 2 Gy TBI on day −1. However, leukemia blasts reappeared rapidly after administration of GO on day −21. Therefore, GO on day −14 was avoided and instead high-dose Ara-C (2 g, days −8 and −7) was added to the conditioning regimen. The patient received UCBT on 11 December 2008, day 174 after the first

transplantation (Table 1). CsA and MMF were administered as GVHD prophylaxis, and LMWH was given to prevent hepatic SOS, as in Cases 1 and 2. On day 8, she showed high fever with unexplained weight gain. The serum bilirubin level increased to 5.4 mg/dL, but there were no obvious findings of hepatosplenomegaly, ascites, or reversed portal vein flow by abdominal ultrasonography, indicating that the patient did not develop hepatic SOS. Primary neutrophil engraftment ($>0.5 \times 10^9$/L) was achieved on day 22, but platelet count recovery was delayed. Complete donor chimerism was found in a bone marrow specimen on day 28. However, blast cells reappeared on day 61. Rapid tapering of the immunosuppressive agent was not effective in inducing the graft-versus-leukemia effect. She died on day 116 as a result of disease progression.

3 Discussion

A second HSCT may be the only way to provide a survival benefit in some AML patients who relapse after a first HSCT, despite the high mortality rate associated with transplant-related toxicity and the high relapse rate. To overcome this dilemma by reducing the toxicity of the treatment and burden of leukemia before HSCT, GO-combining RIC regimens have been developed for patients with CD33-positive AML as a means of conditioning for their second HSCT. However, higher doses of GO (9 mg/m^2 in two doses separated by 2 weeks) were associated with hepatic SOS as well as severe myelosuppression. Two groups tried to determine the safety and optimal dose of GO as a preconditioning treatment: Bornhauser et al. assigned 6 and 3 mg/m^2 of GO on days -21 and -14, and Lima et al. assigned 2 or 4 mg/m^2 of GO days -12 before HSCT, in order to clear immunotoxins and hence minimize possible interference with engraftment. They observed successful primary engraftment in all cases except one, and reversible hepatic SOS was documented in only two cases out of a total of 83 patients, and non-relapse mortality at day 100 was reported in approximately 20% of patients [9, 10]. In our cases, for prophylaxis against the development of hepatic SOS, we excluded busulfan and methotrexate from the conditioning and prophylaxis for GVHD in order to reduce liver damage [11], and standard dose heparin prophylaxis was initiated at the start of conditioning. Despite their advanced disease status, two patients did not develop hepatic SOS; the third patient developed reversible SOS, which was successfully treated with PGE1 and AT-III. Non-relapse mortality at day 100 was not observed in our cases. Based on these results, GO may not affect engraftment and may be safely combined with an RIC regimen for the second UCBT.

An impediment to a second HSCT is the limited availability of graft sources. In general, relapsed disease usually progresses rapidly, so there is little time to find an adequate donor source for the second HSCT. Recently, UCBT has become available after an RIC regimen for the second allogeneic HSCT to treat relapsed leukemia or engraftment failure because of the rapid availability of stored transplantable units. On the other hand, UCBT is associated with delayed neutrophil and platelet recovery and a higher incidence of engraftment failure compared with the use of BM or PBSC. In two prospective studies, allogeneic BM or PBSC were used in preference to CB, as the graft source for the second HSCT with GO-combining conditioning. CB may therefore be unsuitable for GO-combining RIC regimens for the second HSCT in terms of engraftment. Our three patients, who had no HLA-identical siblings, underwent allogeneic BMT from unrelated donors but leukemia relapsed early and progressed rapidly. Their leukemia cells expressed CD33 antigens, and therefore GO-combining reduced-intensity UCBT was conducted. As shown in Table 1, an adequate number of total CB cells and CD34-positive cells were infused: doses of 6 and 3 mg/m^2 of GO, days -21 and -14 before UCBT, did not entail any adverse consequences for neutrophil engraftment, but platelet engraftment was delayed (in Case 1) or not achieved (the remaining two cases); this is comparable to a previous report on a Japanese population that underwent UCBT [12].

In summary, previous reports and our experience suggest that GO may be safely combined with an RIC regimen for a second allogeneic HSCT, including UCBT. However, the probability of disease-free survival was up to 30% in two previous reports, and relapse of leukemia was the major reason for treatment failure, which was also observed in our cases. Extensive studies with large numbers of patients are required to evaluate and improve this treatment.

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