Table 1 Laboratory and viral PCR data of the CSF

Post CBT day	38	42	49	61	70	84	98
Cell number (/3 μl)	3464	1136	1336	861	376	277	217
PMN/MNC (%)	83/17	2/98	4/96	0/100	1/99	0/100	1/99
Protein (mg/dl)	261	268	289	118	107	100	96
CMV in CP CSF (copy/μg DNA)	2.8×10^6	7.5×10^5	2.0×10^4	1.6×10^2	NT	–	–
CMV in whole CSF (copy/ml)	2.8×10^6	4.9×10^7	9.1×10^5	6.7×10^2	NT	–	–
HHV6 in CP CSF (copying DNA)	–	–	–	–	NT	–	–
HHV6 in whole CSF (copy/ml)	–	–	–	–	NT	–	–
ADV in whole CSF (copy/ml)	–	–	–	–	NT	–	–
BKV in whole CSF (copy/ml)	–	–	–	–	NT	–	–

The amount of DNA of viruses, including CMV, HHV6, ADV, and BKV, in the whole or centrifuged pellet (CP) samples of the CSF was measured using real-time PCR. There was no PCR data on day 70 because most of the CSF sample was used for the flow cytometry and dextramer assay

PMN polymorphonuclear cells, *MNC* mononuclear cells, *CMV* cytomegalovirus, *HHV6* human herpes virus 6, *ADV* adenovirus, *BKV* BK virus, *NT* not tested

dextrameric-HLA A*24:02-restricted QYDPVAALF peptide complex (Immudex, Copenhagen, Denmark). After lysing red blood cells and washing twice with bovine serum albumin containing phosphate-buffered saline, cells were examined on a flow cytometer (Cytomics FC 500, Beckman Coulter, Inc., USA). More than 100,000 cells were acquired in the lymphocyte gate and analyzed using CXP software. The percentage of CMV-specific dextramer-positive cells in the $CD3^+$ $CD8^+$ fraction is shown in Table 2. The dextramer-negative control value in the CSF was a little high; however, these data suggest that the percentage of CMV-specific T cells is higher in the CSF than in the PB at least for A*02:01 dextramer. CMV-specific CD8 T cells seemed to be dominantly HLA A*02:01-restricted, but direct comparison was limited due to the difference in the efficacy of the two dextramers. Of note, CSF cell numbers were maintained still at high levels even after CMV DNA became undetectable (Table 1).

Table 2 CMV-specific T cells (%) in the $CD3^+$ $CD8^+$ fraction

	A*02:01 dextramer	A*24:02 dextramer	Dextramer (−)
PB	0.01	0.26	0.01
CSF	1.19	0.47	0.19

Discussion

Cytomegalovirus disease of the CNS is a rare complication after allogeneic SCT in patients. Reddy et al. [4] recently summarized 11 cases of CMV disease of the CNS after SCT. According to their report, all cases developed CMV CNS disease at late onset (occurring 166 or more days after transplantation), were ganciclovir resistant, and ten of them expired despite antiviral combination therapy. Drug resistance was pointed out to be a key factor in the occurrence of CMV CNS disease [5]. In our case, the CMV disease was also suggested to be relatively FCN resistant, since CMV-ME developed during prophylactic FCN administration. In accordance with the previous report [4], there was no evidence of CMV disease in organs other than CNS. The patient did not even show CMV antigenemia or positive PCR test for CMV DNA using the plasma samples except for one (PCR data on day 42). The occurrence of CMV CNS lesion in an isolated form may reflect a relatively low penetration of FCN, as described by Reddy et al. CMV disease of the CNS is reported to develop at late onset because drug resistant virus appears after a relatively long period of drug therapy. On the other hand, CMV-ME in our case that developed in a form as related to PIR in the engraftment period is similar to post-transplant HHV-6 encephalitis, which was reported to develop in association with the production of inflammatory cytokines such as interleukin-6 [6]. Furthermore, in our case, the absence of abnormal findings of MRI of the brain may have resulted in complete recovery of this serious complication.

There have been no reports showing the presence of CMV-specific CTLs in the CFS of patients with CMV-ME. Regarding the detection of virus-specific CTLs in the CSF, JC virus-specific CTLs in patients with progressive multifocal leukoencephalopathy [7], and HIV-specific $CD8^+$ T cells in antiretroviral therapy-naïve HIV-positive subjects [8], have been reported. These studies suggest that the presence of virus-specific CTLs in the CSF has a beneficial effect in controlling these viral CNS diseases. Likewise, the presence of CMV-specific CTLs in the CSF in our case may have exerted some beneficial effects, although ganciclovir and/or cidofovir are considered to have contributed to controlling CMV-ME. In the present report, we first showed the existence of CMV-specific T cells in CSF samples of the patient with CMV-ME. In addition, we

underlined that CMV-specific T cells were of donor origin (CB derived), and that the frequency of CMV-specific T cells was higher in CSF than in PB. In macaques, activated T cells were reported to preferentially enter the intrathecal compartment and increase in frequency early after acute simian immunodeficiency virus infection [9]. Furthermore, rodent data suggest that the expression of viral antigens in the brain may upregulate endothelial cell major histocompatibility complex class I expression, contributing to $CD8^+$ T cell migration into the brain [10]. Taken together with these findings, we consider in our case that CB-derived CMV-specific T cells may develop early in transplantation and enter the intrathecal compartment.

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Conflict of interest The authors declare no competing financial interests.

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Gender differences in health-related quality of life, physical function and psychological status among patients in the early phase following allogeneic haematopoietic stem cell transplantation

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Abstract

Objective: **The aim of this study was to examine gender differences in quality of life (QOL), physical function and psychological status before and in the early phase after allogeneic haematopoietic stem cell transplantation (allo-HSCT).**

Methods: **One hundred patients (66 men, 34 women) who underwent allo-HSCT between July 2007 and June 2011 at Hyogo College of Medicine Hospital were included in this study. Patients were evaluated for health-related QOL using the Medical Outcome Study 36-item Short Form Health Survey; exercise capacity was measured with the 6-min walk test, hand grip strength and knee extensor strength. Fatigue and psychological status were measured by the Piper Fatigue Scale and Hospital Anxiety and Depression Scale, respectively.**

Results: **Women had significantly lower scores for physical function and general health on health-related QOL tests compared with men ($p < 0.01$). No difference between genders was found in decline of physical function. In women, exercise capacity was strongly associated with QOL ($p < 0.01$). In men, depression and anxiety were closely related to QOL ($p < 0.01$).**

Conclusions: **Gender-appropriate rehabilitation in allo-HSCT patients is important. Women may need more endurance exercises and training for activities of daily life. Men may need rehabilitation including a psychological approach.**

Introduction

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) has been increasingly used in treating malignant and non-malignant haematopoietic diseases [1]. Patients with malignant diseases not controlled by conventional means or in whom treatment failure is expected are candidates for allo-HSCT [2,3]. Allo-HSCT entails a conditioning regimen of frequent high-dose chemotherapy combined with total body irradiation, followed by infusion of donor-harvested bone marrow or peripheral blood stem cells [4]. Allo-HSCT patients have decreased muscle strength and exercise capacity because of nerve damage from radiotherapy or chemotherapy, fatigue, graft versus host disease (GVHD) and decreased activity levels [5]. Allo-HSCT patients have lower quality of life (QOL) after transplantation compared with that before transplantation [6]. However, gender differences in terms of physical and psychological function have not been studied in the early phase following allo-HSCT.

Gender differences in QOL have been studied in patients of colorectal cancer with intestinal ostomy [7–10], laryngectomy [11], head and neck cancers [12], craniopharyngioma [13] and primary brain tumour [14]. Women's QOL was found to be significantly lower than men's QOL. This is also true in research on various other forms of cancer [15]. Holzner *et al.* [16] studied QOL differences among genders in chronic lymphocytic leukaemia patients and found that women have lower physical, emotional and role functioning and global QOL than men. Heinonen *et al.* [17] studied gender differences in QOL among patients undergoing allogeneic bone marrow transplantation after 4–5 years and found that women's emotional functioning, fatigue and satisfaction with social support were lower compared with men.

Patients who undergo allo-HSCT experience fatigue because of chemotherapy; furthermore, long-term hospitalisation leads to mental instability in many patients, which causes them to become isolated and confined to bed all day. Women may have low tolerance for these problems and be more sensitive about certain issues than men, such as hair loss, skin rash and decreased activity during hospitalisation and after discharge. However, to our knowledge, no study has investigated gender differences with respect to QOL and physical and psychological function, as well as the relevance of physical function and QOL, in the early phase following allo-HSCT.

Therefore, we examined gender differences in health-related QOL, physical function and psychological status before and in the early phase after allo-HSCT. The second aim of this study was to investigate the relevance of changes before and after transplantation.

Methods

Study design

This is a prospective observational study investigating the relevance of changes before and after stem cell transplantation.

We investigated gender differences in terms of health-related QOL, physical function and psychological status.

Participants

Sampling methods and setting

Total 278 patients with hematologic diseases underwent allo-HSCT at Hyogo College of Medicine Hospital from July 2007 to July 2011. Patients aged 18 years or more in whom evaluation of physical function before and after HSCT was possible were selected. However, patients with active infectious diseases, severe complications and acute GVHD were excluded.

Study sample

Consequently, 100 patients were enrolled in this study (66 men and 34 women). The evaluation was performed up to 3 weeks (mean 21.7 ± 10.2 days) before and 6 weeks (mean 42.4 ± 16 days) after transplantation.

Ethical considerations

The Hyogo College of Medicine Institutional Committee on Human Research approved the study. Written informed consent was obtained from each patient.

Data collection

Patients were assessed for overall health-related QOL using the Medical Outcome Study 36-item Short Form Health Survey (SF-36). Hand grip strength, knee extensor strength and a 6-min walk test (6MWT) were measures of exercise capacity. Fatigue and psychological status were measured using the Piper Fatigue Scale (PFS) and Hospital Anxiety and Depression Scale (HADS), respectively.

The following information was gathered from patients' medical records: age, sex, height, body weight, current marital status, employment status, underlying haematological diagnosis, number of days from initial diagnosis to hospitalisation, type of transplantation, donor–recipient status, conditioning and complete remission before HSCT.

Measurements

Health-related quality of life

General health-related QOL was assessed using the SF-36. This self-administered questionnaire has been widely used and validated in the Japanese general population and in patients after HSCT [18–23]. The SF-36 assesses physical and mental health components in eight domains: physical functioning (PF), physical role functioning (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), emotional role functioning (RE) and mental health (MH). It measures the multidimensional properties of health-related QOL on a scale of 0–100 from lowest to highest.

Hand grip strength

A standard adjustable-handle dynamometer (TKK5101; TAKEI Scientific Instruments Co. Ltd., Niigata, Japan) was used to measure hand grip strength as an index of upper limb strength and was set at the second grip position for all subjects. Grip strength was measured in both hands by the same hand grip dynamometer; all examinations were performed by the same physiotherapist. Possible Valsalva effect was taken into consideration. The resulting data were used as an index of hand grip strength (kilogramme force units).

Knee extensor muscle strength

Hand held dynamometers (HHD; μ-tas MT1; ANIMA Co., Tokyo, Japan) were used to measure knee extensor muscle strength as an index of lower limb strength. The HHD was used manually (kilogramme force units). All sessions used the HHD equipped with a stabilising belt to aid the tester in applying resistance. Intraclass correlation coefficients were 0.98 using the belt and 0.04 when not used [24]. In a reliability test–retest of the belt-restrained HHD, intraclass correlation coefficients were at 0.94–0.96 [25]. Knee extension force was tested with subjects sitting with the knee flexed at approximately 60°. The dynamometer was applied proximal to the malleoli. Maximum force during 10 s of effort was recorded. The HHD was reset to kilogramme force at the start of every measurement. Measurements were carried out three times with both legs; the highest value was selected for analysis.

Exercise capacity

The 6MWT was used as a sub-maximal exercise capacity test conducted in accordance with the American Thoracic Society guidelines [26]. Patients walked along a 20-m corridor in our department for 6 min at their own pace. Patients were encouraged to cover as much distance as possible but were permitted to rest and continue walking as soon as they felt able or stop if they experienced symptoms of dyspnoea or leg pain [27]. Data were collected and analysed on the following: distance after 6 min (metres), duration (minutes) and heart rate at initiation and 6 min.

Fatigue

The PFS score captures four dimensions of subjective fatigue [28]. These four dimensions include behavioural/severity (six items), affective meaning (five items), sensory (five items) and cognitive/mood (six items). The total score is the mean of the 22 numerically scaled (0–10) items with word anchors. PFS scores were interpreted as follows: 1–3 = mild, 4–6 = moderate and 7–10 = severe. Excellent validity and reliability of the PFS have been reported previously [28]. Alpha reliabilities for the total score in this sample were 0.98 at all times.

Psychological status

The HADS was used to measure psychological status using parameters such as anxiety and depression [29]. There are two domains with seven questions in each. Responses are scored on a scale of 0–3; each sub-scale is scored from 0 to 21. Scores reflect the severity of anxiety and depression. Kugaya *et al.* [30] used the HADS to screen for psychological distress, adjustment disorder and major depressive disorder in 128 Japanese cancer patients. In their study, Cronbach's value of the Japanese version of the HADS was 0.77 for the anxiety sub-scale and 0.79 for depression.

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Statistical analysis

Results are presented as mean (SD) or median. We compared demographic and clinical characteristics between men and women using Student's *t*-test for continuous measures and Pearson's χ^2 test or Fisher's exact test for ordinal variables. Two-way repeated-measures analysis of covariance (ANCOVA) was used to detect the main effect of gender on physical function before and after HSCT. Body weight was used as a covariate in the analyses for hand grip and knee extension, and body weight and height were used in the analyses for 6MWT. Two-way repeated-measures analysis of variance (ANOVA) was used to determine differences in health-related QOL, fatigue and psychological status before and after allo-HSCT according to gender. If ANCOVA and ANOVA revealed significant differences, post hoc pairwise comparisons with Tukey adjustments were used to identify the differences. Pearson's correlation coefficients were used to evaluate potential associations between changes in health-related QOL and physical function, fatigue and psychological status before and after allo-HSCT. Statistical analysis was performed using the SPSS 19.0J statistical software (SPSS Japan Inc., Tokyo, Japan). p values ≤ 0.05 were considered statistically significant.

Results

Socio-demographic and clinical characteristics of haematopoietic stem cell transplantation patients

Socio-demographic and clinical characteristics for all patients and differences between men and women are summarised in Table 1. Mean age for men was 44.6 years (SD 13.4 years) and for women was 46.3 years (SD 13.6 years); no significant difference was found in age between genders. Right upper-limb dominance was found in 92 of the 100 patients (92%). Similarly, right lower-limb dominance was identified in 90 of the 100 patients (90%). No significant difference was observed in upper-limb and lower-limb dominance between genders. Male patients were more likely to be employed or studying ($p < 0.001$). However, no significant differences were found in their current marital status, duration of the disease, type of transplantation, donor recipient characteristics, conditioning and complete remission before HSCT.

Health-related quality of life according to gender before and after haematopoietic stem cell transplantation

Table 2 shows the mean values for male and female allo-HSCT patients on the SF-36 sub-scales. Female patients scored significantly lower on the PF and GH domains compared with men (PF = $p < 0.001$, GH = $p < 0.05$). For both genders, scores on the seven sub-scales (except GH) significantly decreased after HSCT compared with before HSCT (PF, RP, BP, VT, SF and RE: $p < 0.01$. MH: $p < 0.05$). In all eight sub-scales, differences in gender × time interaction were not statistically significant.

Physical function according to gender before and after haematopoietic stem cell transplantation

Table 3 shows the mean values for hand grip, knee extension and 6MWT before and after HSCT by gender. Hand grip and knee extension in women were significantly lower than that in men ($p < 0.01$). The 6MWT in women was lower than that in men; however, the difference was not statistically significant ($p = 0.182$). In both men and women, hand grip, knee extension and 6MWT significantly decreased after HSCT compared with before HSCT [hand grip: men = 19% and 20% (right and left, respectively), women = 20% and 21% (right and left, respectively); knee extension: men = 24% and 25% (right and left, respectively), women = 21% and 23% (right and left, respectively); 6MWT: men = 13%, women = 16%, respectively; $p < 0.001$]. Differences in gender × time interaction were not statistically significant for these three parameters.

Fatigue and psychological status according to gender before and after haematopoietic stem cell transplantation

Table 4 shows the mean values for fatigue, depression and anxiety before and after HSCT for each gender. There were no significant differences in these factors between men and women. In both genders, fatigue after HSCT was significantly higher than that before HSCT (men = 24%, women = 26%, respectively; $p < 0.001$). However, depression and anxiety after HSCT did not differ significantly compared with before HSCT. For these factors, differences in gender × time interaction were not statistically significant.

Health-related quality of life and physical function by gender

Table 5 presents the correlation coefficients between differences in QOL and physical function before and after HSCT by gender. The SF-36 sub-scales in male patients indicated that PF was significantly related to hand grip, knee extension and 6MWT ($r = 0.44$–0.56, $p < 0.01$). GH and MH were significantly related to hand grip ($p < 0.05$). The SF-36 sub-scales in female patients indicated that PF was significantly related to knee extension and 6MWT ($r = 0.34$–0.6, $p < 0.01$). VT, SF, RE and MH were significantly related to hand grip and 6MWT ($p < 0.05$).

Health-related quality of life, fatigue and psychological status by gender

Table 5 presents the correlation coefficients between differences in QOL, fatigue and psychological status before and after HSCT by gender. On the SF-36 in male patients, PF, RP, BP, GH, VT, RE and MH were significantly negative in relation to fatigue ($p < 0.01 \sim 0.05$). PF, RP, GH, VT, RE and MH were significantly negative in relation to depression and anxiety ($p < 0.01 \sim 0.05$). On the SF-36 in female patients, PF, RP, GH, VT and MH were significantly negative in relation to fatigue ($p < 0.01 \sim 0.05$). RE and MH were significantly negative in relation to anxiety ($p < 0.01 \sim 0.05$). On all sub-scales, QOL was unrelated to depression.

Discussion

In this study, female patients undergoing allo-HSCT had significantly lower scores on the PF and GH sub-scales of the SF-36 than male patients before and after HSCT.

Table 1. Socio-demographic and clinical characteristics of HSCT patients ($n = 100$)

	Men ($n = 66$)		Women ($n = 34$)		
Characteristics	**No**	**%**	**No**	**%**	***p***
Age, years					
Mean	44.6		46.3		$p = 0.569$
SD	13.4		13.6		
Median	46		48		
Height, cm					
Mean	171		156.6		$p < 0.001$
SD	6.9		5		
Body weight, kg					
Mean	67.9		49.6		$p < 0.001$
SD	13.4		9.2		
Upper-limb dominance					
Right	61	92.4	31	91.2	
Left	5	7.6	3	8.8	$p = 0.553$
Lower-limb dominance					
Right	60	90.9	30	88.2	
Left	6	9.1	4	11.8	$p = 0.46$
Current marital status					
Married/living with mate	43	65.2	26	76.5	$p = 0.246$
Single/widowed/divorced	23	34.8	8	23.5	
Current employment status					
Employed/studying	42	63.6	6	17.6	$p < 0.001$
Retired/unemployed	24	36.4	28	82.4	
Diagnosis					
Acute leukaemia	32	48.5	23	67.6	
Non-Hodgkin's lymphoma	22	33.3	7	20.6	
Myelodysplastic syndrome	10	15.2	2	5.9	
Severe aplastic anaemia	1	1.5	1	2.9	
Chronic myelogenous leukaemia	1	1.5	0	0.0	
Chronic lymphoblastic leukaemia	0	0.0	1	2.9	
Duration of disease, days					
Mean	834		588		$p = 0.217$
SD	1037		707		
Stem cell source					
Peripheral blood stem cell	38	57.6	23	67.6	$p = 0.101$
Bone marrow	13	19.7	9	26.5	
Cord blood	15	22.7	2	5.9	
Donor					
HLA-matched/related	3	4.5	2	5.9	$p = 0.255$
HLA-matched/unrelated	1	1.5	1	2.9	
HLA-mismatched/related	48	72.7	29	85.3	
HLA-mismatched/unrelated	14	21.2	2	5.9	
Conditioning					
Myeloabalative	21	31.8	10	29.4	$p = 0.805$
Reduced intensity	45	68.2	24	70.6	
Complete remission before HSCT					
Yes	14	21.2	4	11.8	$p = 0.244$
No	52	78.8	30	88.2	

Data in bold show that statistical testing at baseline was performed using an independent Student's *t*-test and Pearson's $\chi 2$ test or Fisher's exact test.
SD, standard deviation; HLA, human leukocyte antigen; HSCT, haematopoietic stem cell transplantation.

Costa-Requena *et al.* [31] showed that the SF-36 is useful to evaluate QOL in patients with various cancers. In that study, women scored lower on seven of the sub-scales (except GH) than did men. Similarly, Thomé *et al.* [32] also used the SF-12 to evaluate QOL in patients with various cancers. In their study, women had lower physical and mental scores than did men. Decline of PF means that activities such as bathing or dressing on their own become very difficult to perform for medical reasons. For women, treatment not only to improve physical fitness but also for activities of daily living may be necessary. On the other hand, both sexes showed a decline in scores on SF-36 items (except GH) in the early stages, that is, 6 weeks after HSCT. Hacker *et al.* [33] assessed QOL before and immediately after discharge in peripheral blood stem cell transplantation (PBSCT) patients. They found that before discharge (after PBSCT), nausea, vomiting, appetite loss and sleep disturbance had significantly decreased compared with before PBSCT. In the current study, nausea, vomiting, appetite loss and sleep disturbance were common problems after HSCT, which may have been associated with QOL. Further evaluation of these common problems may be necessary to define more accurately factors that contribute to the decline of QOL in these patients.

Physical function in allo-HSCT patients decreased as early as 6 weeks after transplantation. Decline in physical function can be due to loss of physical performance and fitness during bed rest, fatigue, distress, immunological/haematological changes and common somatic side effects (GVHD, infections, diarrhoea, nausea and pain) [34].

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Table 2. Health-related QOL according to gender before and after HSCT

		Before HSCT			After HSCT			Between gender		Within gender (time)		Interaction gender × time	
Variables	**Gender**	**Mean**	**95%CI lower**	**Upper**	**Mean**	**95%CI lower**	**Upper**	***F***	***p* value**	***F***	***p* value**	***F***	***p* value**
Physical functioning	Male	80.1	75.4	84.8	58.0	52.9	63.0						
	Female	66.8	60.2	73.3	44.0	36.9	51.1	14.8	<0.001	94.2	<0.001	0.0	0.885
Role—physical	Male	49.5	40.8	58.3	27.0	18.9	35.1						
	Female	47.8	35.6	60.0	36.2	24.9	47.5	0.4	0.544	19.1	<0.001	2.0	0.164
Bodily pain	Male	66.7	60.2	73.3	49.3	42.7	55.9						
	Female	72.9	63.8	82.1	51.8	42.6	61.0	1.0	0.322	27.2	<0.001	0.3	0.615
General health	Male	50.4	45.3	55.6	54.0	49.3	58.7						
	Female	44.0	36.9	51.2	45.6	39.0	52.1	4.0	0.049	1.5	0.225	0.2	0.628
Vitality	Male	58.7	53.5	63.9	52.3	46.4	58.1						
	Female	57.5	50.3	64.8	48.2	40.0	56.3	0.4	0.523	10.8	0.001	0.4	0.544
Social functioning	Male	50.2	42.9	57.5	42.0	33.9	50.2						
	Female	55.9	45.7	66.1	44.1	32.8	55.5	0.5	0.487	7.2	0.009	0.2	0.627
Role—emotional	Male	65.3	56.7	73.8	44.2	34.7	53.7						
	Female	63.7	51.8	75.6	52.7	39.5	65.9	0.3	0.601	15.2	<0.001	1.5	0.225
Mental health	Male	63.6	58.6	68.5	59.7	54.3	65.1						
	Female	61.5	54.5	68.4	56.8	49.3	64.2	0.4	0.523	4.0	0.049	0.0	0.845

Statistical analysis using two-way ANOVA. Multiple comparisons were performed using the Tukey method.
QOL, quality of life; HSCT, haemopoietic stem cell transplantation; CI, confidence interval.

Table 3. Physical function according to gender before and after HSCT

		Before HSCT			After HSCT			Between gender		Within gender (time)		Interaction gender × time	
Variables	**Gender**	**Mean**	**95% CI lower**	**Upper**	**Mean**	**95% CI lower**	**Upper**	***F***	***p* value**	***F***	***p* value**	***F***	***p* value**
Rt hand grip (kgf)	Male	33.6	32.1	35.2	27.3	25.9	28.7						
	Female	23.7	21.4	26.1	18.9	16.8	21.0	45.6	<0.001	134.7	<0.001	1.7	0.195
Lt hand grip (kgf)	Male	30.9	29.2	32.7	24.7	23.1	26.3						
	Female	22.5	20.0	25.1	17.7	15.4	20.1	25.8	<0.001	132.0	<0.001	1.6	0.205
Rt knee ext (kgf)	Male	35.0	33.1	36.9	26.5	24.6	28.3						
	Female	27.8	25.0	30.6	21.9	19.2	24.7	12.4	0.001	118.5	<0.001	2.7	0.103
Lt knee ext (kgf)	Male	34.0	31.7	36.3	25.5	23.6	27.5						
	Female	27.7	24.3	31.1	21.4	18.6	24.3	8.0	0.006	83.0	<0.001	1.3	0.261
6MWT (m)	Male	506.5	482.2	530.7	441.2	417.6	464.8						
	Female	490.3	451.8	528.8	412.6	375.1	450.0	0.9	0.345	80.4	<0.001	0.3	0.589

Statistical analysis using two-way ANCOVA. Multiple comparisons were performed using the Tukey method.
Body weight was used as a covariate in the analyses for hand grip and knee extension.
Body weight and height were used as covariates in the analyses for 6-min walking.
Rt, right; Lt, left; Knee ext, knee extension; 6MWT, 6-min walking test; HSCT, haemopoietic stem cell transplantation.

Table 4. Fatigue and psychological status according to gender before and after HSCT

		Before HSCT			After HSCT			Between gender		Within gender (time)		Interaction gender × time	
Variables	**Gender**	**Mean**	**95%CI lower**	**Upper**	**Mean**	**95%CI lower**	**Upper**	***F***	***p* value**	***F***	***p* value**	***F***	***p* value**
Fatigue	Male	63.2	53.9	72.6	78.3	67.7	88.9						
	Female	63.0	49.9	76.0	79.0	64.2	93.8	0.0	0.978	12.0	0.001	0.0	0.916
Depression	Male	5.2	4.4	6.1	5.6	4.6	6.6						
	Female	5.9	4.7	7.1	6.4	5.1	7.8	1.1	0.291	1.8	0.189	0.1	0.786
Anxiety	Male	4.6	3.9	5.4	4.8	4.0	5.7						
	Female	5.6	4.6	6.7	5.5	4.3	6.7	2.0	0.164	0.0	0.869	0.3	0.604

Statistical analysis using two-way ANCOVA. Multiple comparisons were performed using the Tukey method.
Fatigue was evaluated using the Piper Fatigue Scale.
Depression and anxiety were evaluated using the Hospital Anxiety and Depression Scale.
HSCT, haemopoietic stem cell transplantation; CI, confidence interval.

Despite height and weight being used as covariates for ANCOVA, there were significant gender differences in values of grip strength, knee extension strength and 6MWT. Men's muscle strength scores were higher than women's per kilogramme body weight [35]. The regression analysis of grip strength [36] and 6MWT [37] showed gender differences. Therefore, from the results of the current study, we cannot conclude that female allo-HSCT patients have more muscle weakness than male allo-HSCT patients. Furthermore, the interaction of gender × time was not significant in the current study; thus, no difference between genders was found in decline of physical function. Contrary

Table 5. Correlations between health-related QOL and physical function, fatigue and psychological status by gender

	Gender	Δ Rt hand grip	Δ Lt hand grip	Δ Rt knee ext	Δ Lt knee ext	Δ 6MWT	Δ Fatigue	Δ Depression	Δ Anxiety
Δ Physical functioning	Male	0.50**	0.45**	0.54**	0.56**	0.51**	−0.43**		−0.27*
	Female			0.36*	0.34*	0.60**	−0.44**		
Δ Role—physical	Male						−0.40**	−0.31*	−0.32**
	Female						−0.46**		
Δ Bodily pain	Male						−0.25*		
	Female								
Δ General health	Male		0.30*				−0.31*	−0.30*	
	Female						−0.41*		
Δ Vitality	Male						−0.55**	−0.36**	−0.32**
	Female	0.35*				0.49**	−0.54**		
Δ Social functioning	Male								
	Female	−0.34*							
Δ Role—emotional	Male						−0.29*	−0.29*	−0.26*
	Female					0.36*			−0.40*
Δ Mental health	Male	0.28*					−0.27*	−0.30*	−0.38**
	Female					0.35*	−0.40*		−0.50**

Statistical analysis using Pearson correlation coefficient.
Only significant correlation coefficients are presented.
Rt, right; Lt, left; knee ext, knee extension; 6MWT, 6-min walking test; Δ, delta, represents the difference before and after haemopoietic stem cell transplantation; QOL, quality of life.
**$p < 0.01$.
*$p < 0.05$.

to our expectations, physical function decreased to a similar degree for both genders. In the 6 weeks following HSCT, gender differences in decline of physical function may not yet have become evident.

No significant differences in fatigue were found between men and women in the current study. Fatigue increased >20% in allo-HSCT patients of both genders after transplantation. Previous studies have reported that fatigue in patients with autologous HSCT increased 14% 30 days after transplantation compared with before transplantation [38]. This increased fatigue may be due to high doses of immunosuppressive drugs in the treatment of these patients and the higher risk of GVHD. Fatigue may have persisted in the months after transplantation and may have increased similarly for men and women. Further assessment of fatigue over time is necessary in future studies.

Depression and anxiety were higher among women than men in this study, but the difference was not statistically significant. Similarly, previous studies reported that female patients have more depression and anxiety than male patients in various forms of cancer [39]. However, this difference did not reach statistical significance. In allo-HSCT patients, women may have a tendency toward worse anxiety and depression compared with men, as is the case in other forms of cancer.

There was a strong association between decreased PF and actual decline in physical function in both sexes before and after transplantation. In patients with lung cancer, increased performance status and increased QOL were significantly associated [40]. Similarly, in allo-HSCT patients, improved physical function may be associated with improved QOL. To avoid a decrease in QOL, exercise may be recommended for improving physical function as early as possible after allo-HSCT.

In addition, 6MWT was not only associated with PF in female allo-HSCT patients but also with VT, RE and MH. Decreased 6MWT indicated reduced mobility, which might decrease QOL in allo-HSCT patients. Conversely, improved mobility may lead to improved QOL. In female patients after allo-HSCT, exercise to increase endurance may be preferable than exercise to increase muscle strength.

In the current study, fatigue was strongly associated with QOL in allo-HSCT. Solberg *et al.* [41] reported that fatigue was significantly associated with QOL in patients with haematological malignancies preparing for HSCT. Our previous study also showed that fatigue was significantly associated with QOL in patients before allo-HSCT [42]. Fatigue was one of the greatest factors inhibiting QOL in patients not only before but also after allo-HSCT. Thus, physicians must always be careful to evaluate fatigue both before and after allo-HSCT.

Depression and anxiety were significantly associated with decreased QOL after allo-HSCT. Previous studies reported that depression and anxiety were significantly associated with QOL in patients with various cancers [43]. We found furthermore that depression and anxiety in male patients (depression: 5, anxiety: 5) were closely related to QOL compared with female patients (depression: 0, anxiety: 2). Decreased psychological function in men may also be related to decreased QOL in allo-HSCT patients. Thus, men may need rehabilitation that includes a psychological approach.

Strengths and limitations

The findings of this study need to be considered in light of a few limitations. The sample for this part of the study was smaller than that for the larger study (especially in the number of women), thereby reducing statistical power. The sample was obtained from a single hospital and was entirely Asian, limiting its generalisability. Despite these limitations, the longitudinal nature of this study, the gender comparisons in the factors related to QOL, as well as physical function and psychological status can be regarded as providing important considerations in planning rehabilitation for patients in the early phase following allo-HSCT.

In summary, the present study showed that in terms of QOL, women have significantly lower physical function

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and general health compared with men. However, there were no differences between genders in decline of physical function, which indicates a similar decrease. In women, 6MWT was strongly associated with QOL. In men, depression and anxiety were closely related to QOL. Finally, we suggest the importance of gender-appropriate rehabilitation rather than the current standardised rehabilitation in allo-HSCT patients. For example, women may need more endurance and activities of daily living exercises than men after allo-HSCT. Men may need rehabilitation that includes a psychological approach.

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Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Frequency of $CD4^+FOXP3^+$ regulatory T-cells at early stages after HLA-mismatched allogeneic hematopoietic SCT predicts the incidence of acute GVHD

T Fujioka, H Tamaki, K Ikegame, S Yoshihara, K Taniguchi, K Kaida, R Kato, T Inoue, J Nakata, S Ishii, T Soma, M Okada and H Ogawa

Acute GVHD (aGVHD) is a major obstacle to allogeneic hematopoietic SCT (alloHSCT). Although it is thought that aGVHD is initiated in secondary lymphoid organs at a very early stage of alloHSCT, whether $CD4^+FOXP3^+$ regulatory T-cells (Tregs) have an impact on aGVHD development during this period remains unclear. Here, we measured Tregs in peripheral blood as early as possible after HLA-mismatched alloHSCT, and assessed the incidence of aGVHD. Flow cytometric analyses revealed that at the second week after HSCT, patients with aGVHD had significantly ($P=0.018$) lower Treg:$CD4^+$T-cell ratios than those without aGVHD. As these differences were seen before the development of aGVHD, these ratios can predict the incidence of aGVHD. The cumulative incidence of aGVHD in patients with ratios of $<9\%$ was significantly higher than that in patients with ratios of $\geqslant 9\%$ ($P=0.0082$, log-rank test). Additionally, the specific ratio of Tregs:$CD4^+$T-cells was the most significant value among all other possible lymphocyte-associated ratios and absolute cell counts. These findings suggest that the ratio of Tregs:$CD4^+$T-cells at the second week post HLA-mismatched alloHSCT might be a potent predictor of aGVHD in these patients. The practical efficacy of this finding should be verified in further interventional studies.

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Keywords: regulatory T-cells; GVHD; allogeneic SCT; HLA mismatch

INTRODUCTION

Although allogeneic hematopoietic SCT (alloHSCT) has the potential to cure many hematological disorders, GVHD continues to be a major obstacle associated with morbidity and mortality. Naturally occurring regulatory T-cells (Tregs) initially found in $CD4^+CD25^{high}$T-cell fractions[1,2] suppress autoreactive[1] and alloreactive[3–5] immunoreactions. Other researchers have investigated the relationship between the frequency of $CD3^+CD4^+CD25^{high}$Tregs in peripheral blood and the incidence of GVHD after alloHSCT, but results have been inconsistent, possibly due to differences in the definition of $CD25^{high}$.[6–8] The intracellular protein derived from the *FOXP3* gene has since been detected using flow cytometry, and is recognized as both a master regulatory gene and a unique marker for these Tregs.[9,10] This procedure enables the specific measurement of Tregs, and distinguishes them from activated conventional $CD4^+CD25^+$ T-cells. Subsequent studies have applied this procedure and suggested the role of Tregs in attenuating GVHD, mostly after HLA-matched alloHSCT.[11–14]

Here we examined Treg frequencies in the peripheral blood of patients who received alloHSCT from an HLA-mismatched related donor without T-cell depletion. As donor T-cells rapidly recover under our HSCT clinical protocol,[15,16] we analyzed the frequencies of Tregs and other lymphocyte populations as early as possible following HSCT, and examined the relationship between Treg frequency and the subsequent incidence of acute GVHD (aGVHD).

PATIENTS AND METHODS

Patients and samples

Forty-seven patients who underwent alloHSCT from partially HLA-mismatched related donors without T-cell depletion were evaluated. All patients received treatment at the Hyogo College of Medicine Hospital (Nishinomiya City, Japan) between July 2007 and August 2010 in accordance with the protocols approved by the institutional review board. Of these 47 patients, 45 received HLA-haploidentical HSCT. Patient characteristics are summarized in Table 1. After the provision of written informed consent, peripheral blood samples were obtained weekly on a fixed day of the week from the first to the eighth week after transplantation. Data acquired between day 1 and 7 were accordingly defined as data of the first week, those between day 8 and 14 as data of the second week, and so on.

Transplant procedure

Thirty and seventeen patients were preconditioned with a nonmyeloablative and myeloablative regimen, respectively, as reported previously.[15,16] In brief, the nonmyeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, for 6 days), BU (3.2 mg/kg/day, for 2 days, i.v.), and either anti-T-lymphocyte globulin (Fresenius Biotech GmbH, Munich, Germany) or anti-thymocyte globulin (Genzyme, Cambridge, MA, USA) (8 mg/kg or 2–4 mg/kg of the total dose, respectively). The myeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, 4 days), cytosine arabinoside (2 g/m^2/day, 4 times over 2 days), CY (60 mg/kg/day, for 2 days) and TBI (8 Gy delivered in 4 fractions). The GVHD prophylaxis regimen for nonmyeloablative HSCT consisted of tacrolimus (0.02 mg/kg/day) and methylprednisolone (1 mg/kg/day), and that for myeloablative HSCT consisted of tacrolimus (0.03 mg/kg/day), MTX

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Table 1. Patient characteristics

	No GVHD	*GVHD*	*P-value*
Number	25	22	
Median age	37	34	0.49
Sex			*0.33*
Male	9	11	
Female	16	11	
Diagnosis			*0.91*
ALL	6	8	
AML	6	6	
Non-Hodgkin lymphoma	7	4	
Myelodysplastic syndrome	3	2	
Hodgkin lymphoma	1	1	
CLL	1	1	
CML	1	0	
Conditioning intensity			*0.98*
Nonmyeloablative	16	14	
Myeloablative	9	8	
Source of stem cells			*0.16*
PBSC	12	15	
BM	13	7	
GVHD grade			*NA*
I		11	
II		7	
III		4	
IV		0	

Abbreviation: NA = not applicable.

(10 mg/m^2 on day 1 and 7 mg/m^2 on day 3), methylprednisolone (2 mg/kg/day) and mycophenolate mofetil (15 mg/kg). After transplantation, degrees of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood were assessed by quantitative PCR for STR markers, as previously reported.[17] Assessment of aGVHD was based on clinical symptoms in accordance with commonly accepted criteria.[18,19] Unless patient condition precluded them, skin, liver and gastrointestinal tract biopsies were performed to support the diagnoses. Gastric biopsy was essentially required for the diagnosis of gut GVHD without manifest diarrhea (stage 1).

Flow cytometric analysis of Treg

Peripheral blood samples were collected using EDTA anticoagulant, and PBMCs were isolated by density-gradient centrifugation for analysis without cryopreservation. Flow cytometric analysis was performed using a Coulter cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) with CXP software (Beckman Coulter), using the following Abs: FITC-conjugated anti-CD3, phycoerythrin-Texas Red energy-coupled dye-conjugated anti-CD25, and phycoerythrin-Cy5-conjugated anti-CD4 (Beckman Coulter). For FOXP3 intracellular staining, the phycoerythrin-conjugated anti-FOXP3 Staining Set (eBioscience, San Diego, CA, USA) was used according to the manufacturer's instructions. FOXP3 staining was performed independently after staining with other Abs.

Statistical analysis

Differences in characteristics between patient groups were assessed by the Mann – Whitney *U*-test for continuous variables and the χ^2 test for categorical values. Median Treg frequencies were compared using the Mann – Whitney *U*-test. Treg frequencies were adjusted for differences between patients with and without aGVHD by multiple regression with logistic analysis. The sensitivity and specificity of Tregs in predicting aGVHD were assessed by receiver operating characteristic curve analysis. Cumulative incidences of aGVHD were plotted according to the Kaplan – Meier method and compared using the log-rank test.

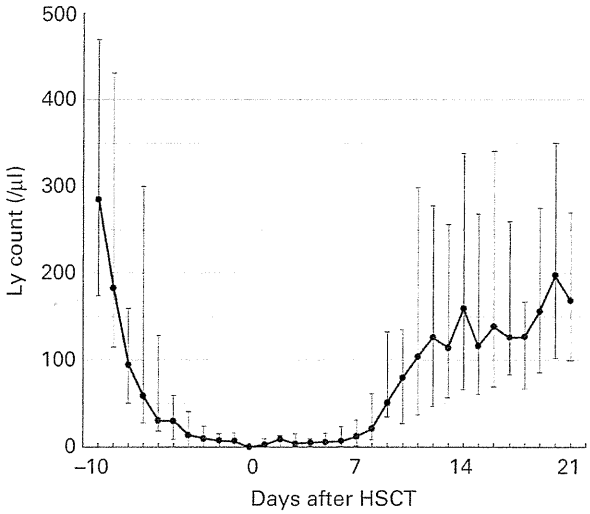

Figure 1. Recovery of lymphocyte (Ly) counts after HLA-mismatched HSCT. Median lymphocyte counts in peripheral blood after HLA-mismatched HSCT are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively.

RESULTS

Patients

Of the 47 patients, 22 presented with aGVHD *vs* 25 who did not (Table 1). The onset of aGVHD occurred at a median of 38 days after transplantation (range: 14–102). None of the characteristics examined had any significant impact on aGVHD incidence. As described previously, the degree of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood achieves the complete donor type within 2 weeks after HLA-mismatched HSCT in our hospital.[15–17] Here, assessment once per week confirmed that on average complete donor-type chimerism in T-cells was achieved on day 10 (median, range: 5–23). Lymphocytes recovered during the second week (Figure 1) were therefore considered to consist almost entirely of donor-originated lymphocytes.

Flow cytometric analysis of Tregs

Representative results of a patient 4 weeks after HSCT are shown in Figures 2a–d. FOXP3$^+$ Tregs were analyzed using a flow cytometric plot gated by CD3$^+$CD4$^+$ fractions (Figure 2a). Although CD25 staining alone showed a large overlap between CD25$^+$ and CD25$^-$ cells (Figure 2c), FOXP3 staining was able to separate FOXP3$^+$ cells as an isolated population (Figure 2d). As demonstrated previously, CD25 staining alone is frequently incapable of revealing an unequivocal boundary that discriminates Tregs from CD4$^+$CD25$^+$-activated conventional T-cells in almost all cases.[20] As FOXP3 staining has apparent objectivity and, moreover, FOXP3 is the key molecule for this type of Treg,[9,10] we defined Tregs simply as CD4$^+$FOXP3$^+$T-cells, regardless of CD25 expression.

Treg:CD4$^+$T-cell ratios at the second week were significantly lower in patients with aGVHD

As lymphocyte numbers were markedly low during the first week, as shown in Figure 1, flow cytometric analysis was unable to detect any Tregs. By the second week after HSCT, in contrast, lymphocyte numbers increased to levels that made analysis possible in almost all cases (41 of 47 patients). Representative results of patients without and with aGVHD are shown in Figures 2e and f, respectively. Of the remaining six patients with slower lymphocyte recovery, two could be assessed at the third week,

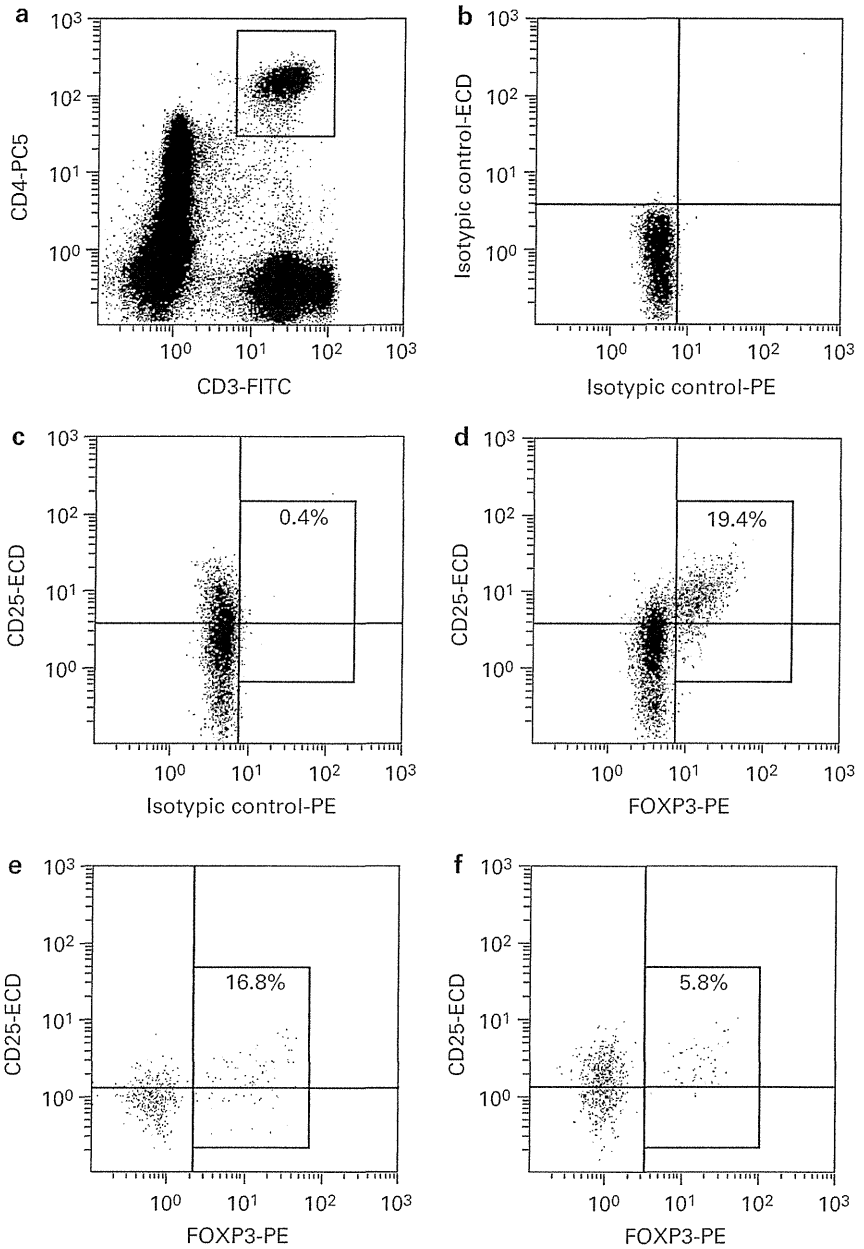

Figure 2. Flow cytometric analysis of Tregs. Representative results of four-color flow cytometric analyses performed for Tregs. Peripheral mononuclear blood cells were stained with CD3-FITC, CD4-PC5, CD25-energy-coupled dye (ECD) and FOXP3-phycoerythrin (PE). All dot plots were gated into lymphocyte populations according to forward- and side-scatter properties, and the gate of $CD3^+CD4^+$ fractions shown on plot **a** was used for the other dot plots with CD25/FOXP3 axes. The percentage of $FOXP3^+$ cells was calculated by subtracting the background percentage of the gate found in plot **c** from the gate shown on plot **d**. Plots **a**–**d** are representative results of a patient 4 weeks after HSCT. Plot **e** is a representative result of a patient without aGVHD in the second week and plot **f** is one of a patient with aGVHD in the second week.

one at the fifth week, one at the sixth week and two at the seventh week.

Figure 3 shows Treg:$CD4^+$T-cell ratios after HSCT ($n=41$), which are the most meaningful values as described in the following paragraph. On average, Tregs were collected on day 12 (median, range: 8–14) during the second week. Patients with aGVHD had significantly lower ratios in the second week after HSCT than those without aGVHD (median (range), 5.23 (0.32–44.8) *vs* 15.5 (0.00–37.1); $P=0.018$). Similar tendencies were seen during the following weeks, but the differences were not statistically significant. Multivariate analysis using logistic regression, which incorporated patient characteristics and transplantation settings, showed that Treg:$CD4^+$T-cell ratio was a unique independent and significant factor related to the incidence of aGVHD (Table 2).

Treg:$CD4^+$T-cell ratio is the most significant value among all other ratios and absolute counts

We also examined the significance of all other ratios between two major lymphocyte populations during the second week. As summarized in Table 3A, although $CD4^+$T-cell:whole T-cell ratio (median (range), 0.32 (0.09–0.78) in the aGVHD (+) group *vs* 0.16 (0.02–0.79) in the aGVHD (−) group; $P=0.026$) and

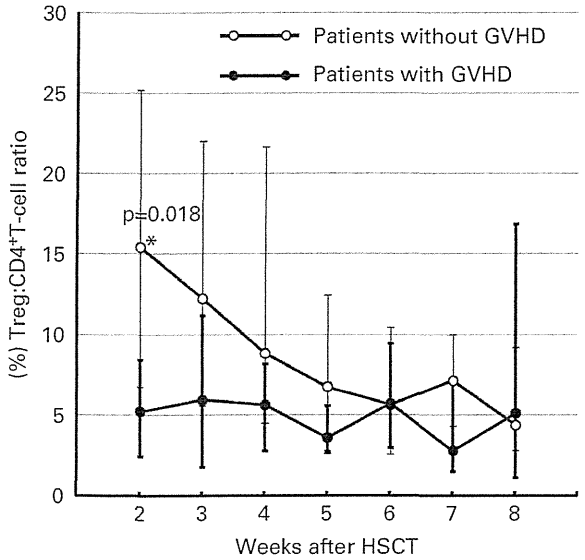

Figure 3. Frequency of Tregs in peripheral blood after HLA-mismatched HSCT. The frequencies of Tregs were assessed by flow cytometry weekly until the eighth week after HSCT. The median Treg:CD4$^+$T-cell ratios of patients with or without aGVHD are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively. Patients with aGVHD had significantly ($P=0.018$) lower median ratios at the second week after HSCT than those without aGVHD.

Table 2. Multivariate analysis

Parameter	*P-value*
Age	0.643
Sex	0.295
Diagnosis	0.774
Conditioning intensity	0.732
Source of stem cells	0.397
Treg:CD4$^+$T-cell ratio	0.032*

Abbreviation: Tregs = regulatory T-cells. *Indicates statistical significance ($P<0.05$).

CD8$^+$T-cell:CD4$^+$T-cell ratio (median (range), 1.98 (0.43–9.79) in the aGVHD (+) group *vs* 4.13 (0.38–32.3) in the aGVHD (−) group; $P=0.043$) were significant, the Treg:CD4$^+$T-cell ratio ($P=0.018$) had statistically the most significant value. Additionally, neither absolute numbers of whole lymphocytes nor the respective lymphocyte fraction (including Tregs) significantly correlated with the incidence of aGVHD (Table 3B).

Treg:CD4$^+$T-cell ratio at the second week predicts the incidence of aGVHD

As aGVHD occurred at a median of 38 days after HSCT (range, 14–102), while the significant decreases in Treg:CD4$^+$T-cell ratio were observed during the second week, this ratio can serve to predict the incidence of aGVHD. A receiver operating characteristic curve was generated by plotting the true positive rate of aGVHD against the false-positive rate for different cutoff-ratio values (Figure 4). The area under the curve was 0.73, indicating that the Treg:CD4$^+$T-cell ratio at the second week is a good predictor of aGVHD. Further analysis revealed that a cutoff-ratio value of 9% yielded the most accurate predictions of future aGVHD incidence (Figures 4, 69.6% sensitivity and 77.8% specificity). Treg:CD4$^+$T-cell ratios of $<9\%$ predicted a significantly higher incidence of aGVHD than ratios of $\geqslant 9\%$ (Figure 5, $P=0.0082$, log-rank test).

Table 3. *P*-values of each ratio (A) and absolute number (B)

A

numerator / denominator	Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
Ly		0.636	0.093	0.674	0.203	0.478
T			0.026*	0.237	0.318	0.478
CD4$^+$T				0.043*	0.774	0.018*
CD8$^+$T					0.213	0.713
B						0.139
Treg						

B

Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
0.674	0.636	0.083	0.979	0.213	0.875

*Indicates statistical significance ($P<0.05$).

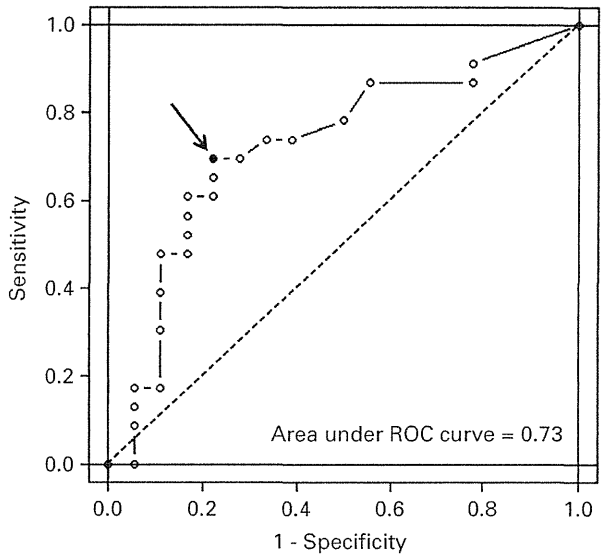

Figure 4. Receiver operating characteristic (ROC) analysis. ROC curve for the ratios of Tregs:CD4$^+$T-cells in identifying patients with aGVHD. The dashed diagonal line represents non-discrimination. Arrow, cutoff ratio at which the sensitivity and specificity resulted in a maximal Youden's index (cutoff ratio, 9%; sensitivity, 69.6%; specificity, 77.8%).

DISCUSSION

In this study, we found that ratios of Treg:CD4$^+$T-cells during the second week after HLA-mismatched HSCT without T-cell depletion accurately predicted the incidence of future aGVHD. Other investigators have used flow cytometry with intracellular staining of FOXP3 to demonstrate a relationship between aGVHD and Treg frequency in peripheral blood. Rezvani *et al.*[11] demonstrated a significant decrease in Treg frequencies at days 30 and 45 when comparing patients with and without aGVHD at the time of Treg sampling, as did Ratajczak *et al.*[14] at a mean of 3 months. Furthermore, Magenau *et al.*[13] assessed Tregs at the onset of aGVHD and demonstrated significant decreases in Treg frequencies in aGVHD patients, with comparison done using samples at GVHD onset and from patients without GVHD, such that the two groups were balanced for the time of acquisition. While their study sampled Tregs more than 4 weeks post transplantation, our study reports significant differences at less than half this time. Although further investigation is needed to determine whether early-stage Treg measurements are possible in other alloHSCT settings, our method under the condition of HLA-mismatched HSCT with rapid hematopoietic reconstitution[15–17]

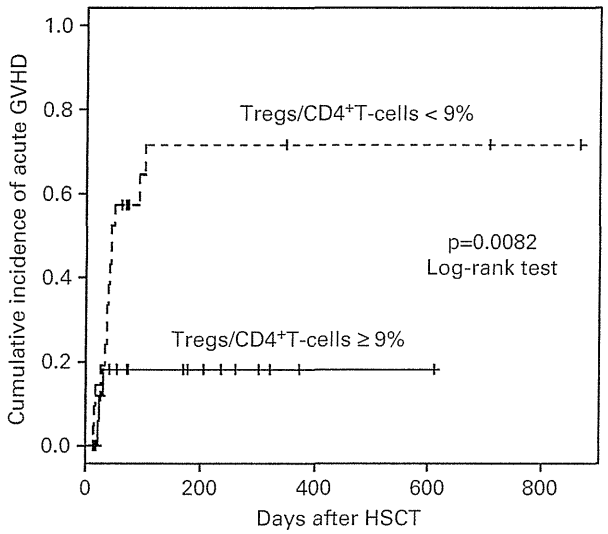

Figure 5. Cumulative incidence of aGVHD. Patients with Treg:CD4$^+$ T-cell ratios $<9\%$ had a significant higher incidence of aGVHD than those with ratios $\geqslant 9\%$ ($P=0.0082$, log-rank test). Measurements were taken in the second week after HSCT.

produced the earliest reported differences in Treg frequency, a finding with practical applications that allows for the prediction of future incidence of aGVHD.

It is a commonly accepted theory that GVHD is initiated in the priming phase, in which donor T-cells activate and proliferate in response to host APCs in secondary lymphoid organs,[21] where Tregs presumably function effectively by suppressing APC function.[22] TNFα is also well known as a central cytokine that peaks immediately after HSCT and stimulates APCs to prime T-cells during this phase.[21,23] Choi *et al.*[24] and Willems *et al.*[25] have both demonstrated that levels of TNFα receptor 1 (a surrogate marker of TNFα) at day 7 correlate with subsequent development of GVHD after myeloablative and nonmyeloablative alloHSCT, respectively. Although a number of studies using animal models have contributed to theories underlying GVHD pathogenesis,[26–30] their finding that this priming phase is limited to a very short duration immediately after HSCT in humans is particularly valuable. The most important point in our study is that conducting investigations at the earliest possible time point after HSCT enabled us to obtain our findings from a very narrow time window. Although it remains unclear whether lower Treg frequencies in peripheral blood reflect lower frequencies in secondary lymphoid organs, integrating our findings and previous studies in which the decline in Treg frequency in peripheral blood was seen during the initial phase of GVHD,[31] it is reasonable to assume that this is indeed the case, and thus that it causes the development of GVHD.

We also found that while absolute numbers of each lymphocyte population did not predict the occurrence of aGVHD, the Treg:CD4$^+$T-cell ratio was the most significant predictor among all other ratios (Table 3). As Tregs can work in cooperation with other cells, including APCs and other T-cells,[22] it is considered rational that ratio rather than absolute number is the relevant factor for predicting aGVHD. However, the reason why the specific ratio of Tregs:CD4$^+$T-cells is the most significant predictor remains uncertain. Although CD4$^+$T-cells recognize MHC class II molecules and have been shown to induce GVHD in a class II-mismatched (class I-matched) murine HSCT model,[32] the importance of CD4$^+$T-cells in GVHD pathogenesis has been demonstrated in fully MHC-mismatched (both class I and class II) murine models[33–35] and even in an HLA class I-mismatched HSCT.[36] Beilhack *et al.*[33] visualized initial proliferation of CD4$^+$T-cells followed by CD8$^+$T-cells in secondary lymphoid organs, and Ewing *et al.*[34] and Yu *et al.*[35] have demonstrated that the activity of CD4$^+$T-cells in the early phase contributes to subsequent development of aGVHD by CD8$^+$T-cells. Accordingly, CD4$^+$T-cells would likely have a leading role during the priming phase of aGVHD, and only then would activation and proliferation of CD8$^+$T-cells proceed. Our observation that both higher CD4$^+$ T-cell:whole T-cell and lower CD8$^+$T-cell:CD4$^+$T-cell ratios in the second week exhibit a significant relationship with aGVHD development does not conflict with these findings, as they both indicate a greater abundance of CD4$^+$T-cells than CD8$^+$T-cells. Furthermore, CD4$^+$T-cells have a particularly direct relationship with Tregs, with CD4$^+$T-cells being the principal targets that Tregs suppress in APC-dependent[37] and -independent[38] manners. Considering this, the high significance of the Treg:CD4$^+$T-cell ratio is reasonable.

Whereas 22 patients developed aGVHD in this study, half of those had grade 1 aGVHD. As previously described,[15] once aGVHD appears in these HLA-mismatched HSCT cases, it inevitably and rapidly progresses to more severe disease, resulting in fatal outcome. All the 11 patients with grade I aGVHD had stage 1 or 2 skin disease at onset. We were therefore obliged to treat them at the earliest time possible, usually within 24 h, with a combination of topical treatment and dose escalation of internal corticosteroid as initial treatment. Additionally, in cases where a skin biopsy was performed, we were unable to delay treatment while waiting for the results, although they would have been helpful for subsequent validation of treatment. Consequently, the disease remained at grade 1 in half of the patients, whereas progression could not be prevented in the other half. It is notable that Treg:CD4$^+$T-cell ratios are able to predict even mild cases of aGVHD, as even grade 1 aGVHD poses a high risk of causing more serious conditions, and should be avoided if possible. In contrast to previous studies,[13,14] we did not observe a significant inverse relationship between aGVHD grade and Treg:CD4$^+$T-cell ratio (data not shown). We attribute this to the early intervention and/or unequal distribution of patients for each grade.

We have demonstrated that patients who developed aGVHD had significantly lower Treg:CD4$^+$T-cell ratios at the second week after HLA-mismatched HSCT, well in advance of clinical aGVHD symptoms. The measurement of Tregs during the second week therefore provides a means to predict the development of aGVHD. Our results suggest that Tregs have a vital role in regulating aGVHD progression, and support the efficacy of early infusions of donor Tregs to prevent GVHD in HLA-haploidentical HSCT.[39] Further studies are needed to confirm whether interventions lead to improved outcomes for patients who show a high risk of aGVHD during the second week post HSCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FK506 induces endothelial dysfunction through attenuation of Akt and ERK1/2 independently of calcineurin inhibition and the caspase pathway

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ABSTRACT

Calcineurin inhibitors such as cyclosporin A (CsA) and FK506 have been used in solid organ and hematopoietic stem cell transplantations to suppress immune function. However, these immunosuppresants are associated with severe endothelial dysfunction. We investigated whether CsA and FK506 induce endothelial dysfunction using a three-dimensional culture blood vessel model, in which human umbilical vein endothelial cells form and maintain capillary-like tube and lumen structures. We found that FK506, but not CsA, induced breakdown of the tube structures and endothelial cell death. FK506 inhibited calcineurin activity, but FK506-induced tube breakdown and cell death was not suppressed by RNA interference targeting calcineurin Aα. FK506 also induced caspase activation, but caspase inhibition by zVAD(OMe)-fmk failed to suppress FK506-induced tube breakdown and cell death. FK506 induced attenuation of Akt and extracellular-regulated kinase 1/2 (ERK1/2). Furthermore, Akt inhibition by LY294002 or ERK1/2 inhibition by PD98059 induced tube breakdown and cell death. Present results suggest that FK506 induces endothelial dysfunction through attenuation of Akt and ERK1/2 independently of calcineurin inhibition and the caspase pathway.

1. Introduction

Thrombotic microangiopathy (TMA) is an infrequent but life-threatening endothelial dysfunction after solid organ and hematopoietic stem cell transplantations [1]. Although various etiologies have been postulated for posttransplant TMA, calcineurin inhibitors including cyclosporin A (CsA) and FK506, which are used for immune suppression, have been shown to be closely associated with TMA [2,3]. However, direct effects of CsA and FK506 on human endothelial cells have not been clearly determined.

CsA and FK506 inhibit calcineurin, which is a eukaryotic Ca^{2+}-dependent serine/threonine protein phosphatase with a multi-component structure consisting of a catalytic A subunit, a regulatory B subunit and calmodulin [4]. Three catalytic subunit genes of calcineurin, referred to as Aα, Aβ, and Aγ, have been identified in vertebrate species. Aα and Aβ are ubiquitously expressed, whereas Aγ is restricted to the testis and a limited region of the brain [4]. CsA and FK506 bind to cyclophilins and FK506-binding proteins (FKBPs), respectively [5,6]. The resultant complexes bind to calcineurin, leading to inhibition of dephosphorylation of nuclear factor of activated T cell (NFAT), which suppresses IL-2 expression and T cell activation. In FKBPs, complexes of FKBP-12-kD (FKBP12) and FKBP-12.6-kD (FKBP12.6) potently inhibit enzyme activity of calcineurin [7].

We have developed a three-dimensional (3D) blood vessel model, in which human umbilical vein endothelial cells (HUVEC) were stimulated with basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) to form and maintain capillary-like tube and lumen structures in type I collagen [8]. Angiogenic stimuli such as bFGF and VEGF are known to activate Akt, one of the most thoroughly investigated survival signals, and extracellular-regulated

Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; CsA, cyclosporin A; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; eNOS, endothelial nitrogen oxide synthase; ERK, extracellular-regulated kinase; FKBP, FK506-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cell; N.S., not significant; PKC, protein kinase C; shRNA, short hairpin RNA; 3D, three-dimensional; TMA, thrombotic microangiopathy; VEGF, vascular endothelial growth factor; WST-8, water tetrazolium salt 8; zVAD, zVAD(OMe)-fmk.

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kinase 1/2 (ERK1/2), a member of mitogen-activated protein kinase (MAPK) superfamily, which are mainly involved in cell proliferation, differentiation and development [9,10]. On the other hand, p38, another member of MAPK, is a key mediator of stress and inflammation responses evoked by a variety of physical, chemical, and biological stress stimuli [10]. Akt and ERK1/2 are also known to transduce survival signals in endothelial cells and prevent apoptosis through the caspase pathway by inactivating proapoptotic proteins [11,12]. Caspases are classified as cysteine proteases which, in response to apoptotic stimuli, transmit messages to effect apoptotic processes such as proteolysis (e.g., lamin) through effector caspases (e.g., caspase-3, -6, and -7) [13].

2. Materials and methods

2.1. Cell culture and reagents

HUVEC isolated from human umbilical cord were purchased from Lonza Walkersville, Inc. (MD, USA) and cultured as previously reported [8]. Briefly, HUVEC were grown in gelatin-coated 100-mm dishes (Becton Dickinson Labware, Franklin Lakes, NJ) in culture medium composed of MCDB-104 medium (Nihon Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Moregate, Brisbane, Australia), 100 ng/ml bovine brain extract, 10 ng/ml epidermal growth factor (EGF) (BD Biosciences, Bedford, MA), 100 μg/ml heparin, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 20 μg/ml neomycin. Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS [14]. Incubation was carried out at 37 °C in 95% air and 5% CO_2. Type I collagen solution (Atelocollagen Bovine Dermis, IPC-50) was purchased from Koken (Tokyo, Japan). zVAD(OMe)-fmk (zVAD), a broad-spectrum caspase inhibitor, LY294002, an inhibitor of phosphatidylinositol 3-kinase that is located upstream of Akt, and PD98059, an inhibitor of MAPK/ERK kinase that directly activates ERK, were purchased from Enzo Life Sciences inc. (Farmingdale, NY). Other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Tube formation in 3D culture

Tube formation was performed as described previously with slight modification [8]. HUVEC were trypsinized and spun down, supernatants of culture medium were aspirated, and HUVEC pellet was mixed with 0.43% type I collagen gels. For morphological observation and cell viability analysis, 42 μl of collagen gel was added to each well (1.71×10^6 cells/ml) of the 96-well culture plates. For Western blot analysis, 140 μl of collagen gel was added to each well (1.71×10^6 cells/ml) of the 48-well culture plates. The plates were then incubated at 37 °C for 1 h to solidify the collagen gel. Tube formation was induced in tube-induction medium composed of MCDB-104 medium and 199 medium at a 13:7 ratio, supplemented with 2% FBS, 30 ng/ml human recombinant bFGF (Wako Pure Chemical, Osaka, Japan), 30 ng/ml human recombinant VEGF (Humanzyme, Chicago, IL), 25 μg/ml L-ascorbic acid, 10 μg/ml penicillin, 10 μg/ml streptomycin and 20 μg/ml neomycin. Tube-induction medium was added to the 3D cultures, for morphological observation and cell viability analyses in a volume of 78 μl, and also for Western blot analysis in a volume of 260 μl. The tube-induction medium was aspirated after incubation for 24 h, and then 120 μl of fresh tube-induction medium was added to each well of the 96-well culture plates, and 400 μl of the fresh tube-induction medium to that of the 48-well culture plates. Cells were then incubated for up to 48 h to induce tube formation.

2.3. Treatments of HUVEC with additive agents

The tube-induction medium was aspirated after tube formation for 48 h. The medium was replaced every 24 h, and incubation continued for an additional 24–48 h. For morphological observation and cell viability analyses in 3D cultures, 120 μl of fresh tube-induction medium supplemented with CsA, FK506, LY294002, PD98059 or vehicle (dimethylsulfoxide, DMSO) was added. For Western blot analysis in 3D cultures, 400 μl of the fresh tube-induction medium supplemented these agents was also added. zVAD was added to the fresh tube-induction medium supplemented with FK506 or DMSO in the 3D cultures. For cell proliferation and Western blot analyses in monolayer cultures, HUVEC (2×10^4 ml) were seeded in collagen-coated 12-well culture plates and 100-mm dishes (Becton Dickinson Labware) in culture medium. The medium was changed to fresh tube-induction medium after cell adhesion for 24 h. HUVEC were then incubated further for 24–72 h in fresh tube-induction medium supplemented with CsA, FK506 or DMSO.

2.4. Morphological observation

Morphological observation was performed as described previously [8]. Briefly, HUVEC grown in the 3D cultures were fixed with 1% glutaraldehyde overnight at 4 °C after experimental treatments. The cells were stained with 0.1% toluidine blue in 30% methanol, destained,

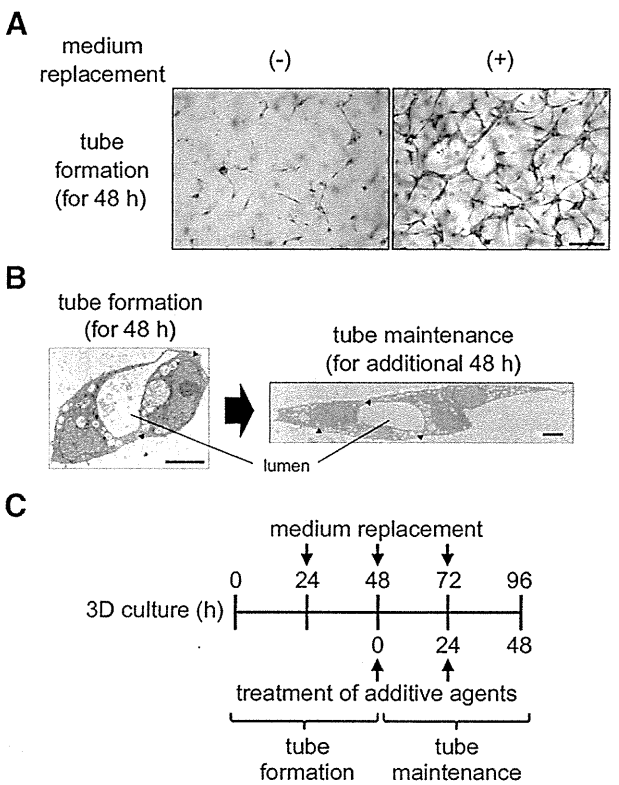

Fig. 1. Repeated replacement of tube-induction medium enables to mimic capillary-like tube structures in 3D cultures. (A) Replacement of tube-induction medium helps HUVEC to form capillary-like tube structures in 3D cultures. HUVEC at 1.71×10^6 cells/ml in 96-well culture plates were incubated in tube-induction medium in the presence of 30 ng/ml bFGF and 30 ng/ml VEGF for 24 h, then the medium was replaced to the fresh tube-induction medium, and incubation continued for an additional 24 h. The bar indicates 100 μm. (B) Replacement of tube-induction medium also helps HUVEC to form and maintain lumen structures in 3D cultures. HUVEC at 1.71×10^6 cells/ml in 96-well culture plates were incubated in tube-induction medium in the presence of 30 ng/ml bFGF and 30 ng/ml VEGF for 48–96 h with replacement of tube-induction medium every 24 h. Cells were fixed with glutaraldehyde and thin sections were prepared and examined under an electron microscope as described in Materials and methods. The arrowhead indicates a cell–cell junctional contact. The bar indicates 5 μm. (C) Schedule of replacement of tube-induction medium supplemented with or without immunosuppressants and inhibitors in 3D cultures. The experiments in A and B were performed four times and the representative data are shown.

and observed under a light microscope (Nicon, Tokyo, Japan) for bright-field images.

2.5. Electron microscopic observation

Tube-forming HUVEC in the 3D cultures were fixed with 1% glutaraldehyde overnight at 4 °C. The cells were dehydrated in a graded series of ethanol and then substituted with propylene oxide and embedded in epoxyresin (TAAB812, TAAB, England). Ultra thin sections (0.08 mm thick) were cut on a LEICA ULTRCUT UCT ultramicrotome (Leica, Eien, Austria), mounted on formvar-coated copper grids (VECO, Eerbeek, Netherlands), and stained with uranyl acetate (Merck, Frankfurt, Germany) and then with lead citrate. Sections were observed using a conventional transmission electron microscope, JEM-1220 microscope (JEOL, Tokyo, Japan).

2.6. Cell viability analysis

Cell viability was determined using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's protocol [8]. Briefly, water tetrazolium salt 8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), was added in the 3D cultures. The compound was reduced by cellular dehydrogenases to form a water-soluble orange-colored formazan dye. The intensity of color developed was quantified using a micro plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, CA). Experiments were repeated in triplicate.

2.7. Cell proliferation analysis

Cell proliferation was analyzed as described previously [15]. Briefly, HUVEC in collagen-coated 12-well culture plates were treated with CsA, FK506 or DMSO as described above, and harvested by trypsinization. Cell numbers were measured with a Coulter Counter Z1 (Coulter Japan, Tokyo, Japan).

2.8. Western blot analysis

Proteins were extracted from HUVEC as described previously with slight modification [16]. Briefly, the collagen gels, including tube-forming HUVEC with Cellytic-M, were cut into small pieces with needle tips and passed through 23-G needle 10 times. Extracted proteins in the 3D and monolayer cultures were dissolved in the SDS sampling buffer (0.05 M Tris–HCl, pH 6.8, 2% SDS, 5.88% 2-mercaptoethanol, 10% glycerol) with 1× protease inhibitor cocktail and boiled for 10 min, and Western blotting was performed as described previously [17]. All antibodies used were purchased from Cell Signaling Technology (Beverly, MA) except those against

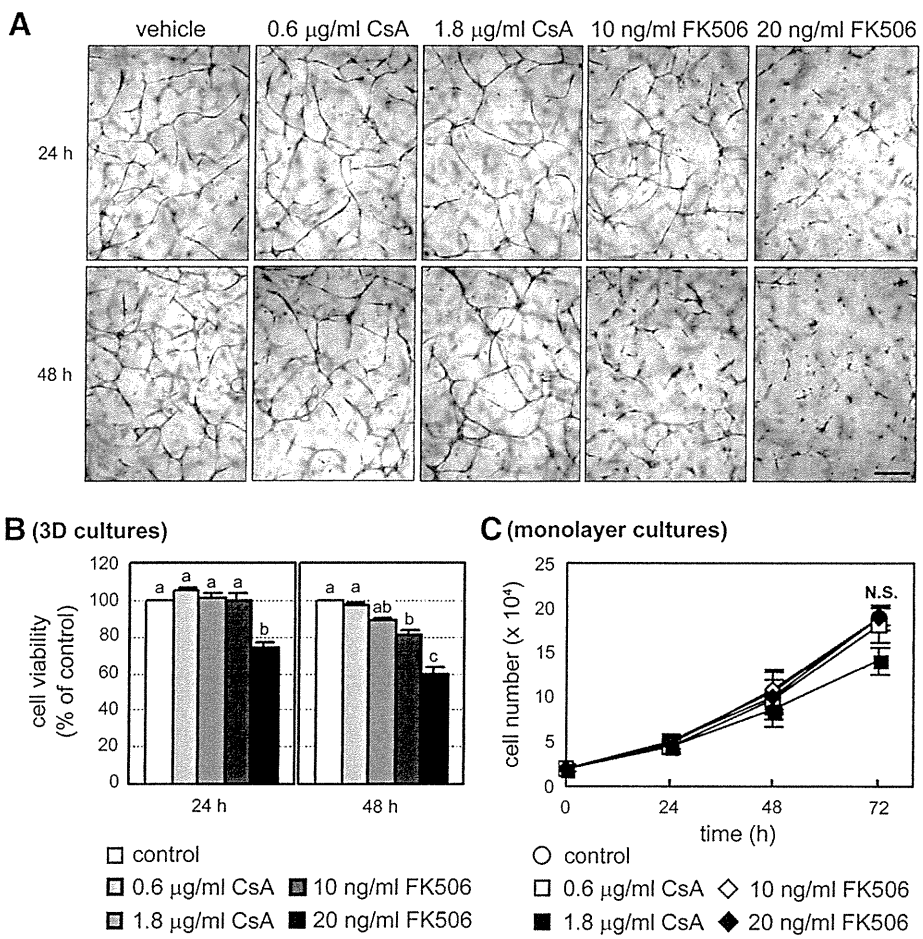

Fig. 2. FK506, but not CsA, directly induces breakdown of capillary-like tube structures and cell death in 3D cultures. (A) FK506, but not CsA, induces breakdown of capillary-like tube structures in 3D cultures. Tube-forming HUVEC were treated with CsA or FK506 at the indicated concentrations for 48 h. The bar indicates 100 μm. Representative data of four independent experiments with similar results are shown. (B) FK506, but not CsA, induces cell death in 3D cultures. Tube-forming HUVEC were treated with CsA or FK506 at the indicated concentrations for 48 h, and cell viability was assessed using WST-8 as described in Materials and methods. Each bar represents means ± SE of four independent experiments. One-way factorial ANOVA for multiple comparisons was performed for statistical differences. Values with different letters are significantly different at $P < 0.05$ (Bonferroni test). (C) CsA and FK506 have little effect on cell proliferation in monolayer cultures. HUVEC in collagen-coated 12-well culture plates were treated with CsA or FK506 at the indicated concentrations for 0–72 h, and cell numbers of HUVEC were determined with a Coulter counter. Each bar represents means ± SE of four independent experiments. One-way factorial ANOVA with Bonferroni test for multiple comparisons was performed for statistical differences. N.S., not significant.

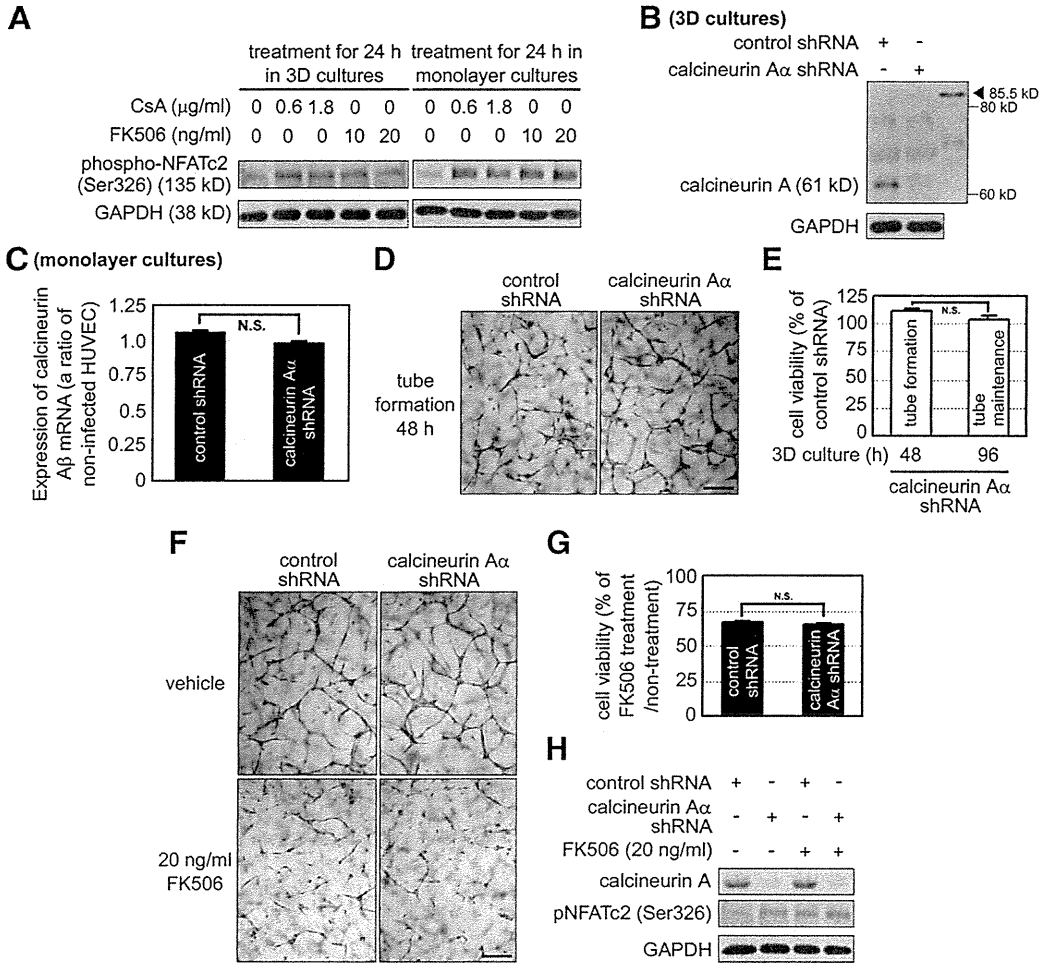

Fig. 3. Calcineurin inhibition is not involved in FK506-induced endothelial dysfunction. (A) CsA and FK506 inhibit phosphatase activity of calcineurin in 3D cultures. Cell extracts were prepared from HUVEC treated with CsA or FK506 at the indicated concentrations for 24 h in 3D and monolayer cultures. (B) Knockdown of calcineurin catalytic subunit A isoform alpha (calcineurin Aα) in HUVEC. After 48-h of treatment with 30 ng/ml bFGF and 30 ng/ml VEGF for tube formation, cell extracts were prepared from tube-forming HUVEC infected with lentivirus carrying shRNA against calcineurin Aα or Luciferase as a control. The arrowhead indicates human glutathione-S-transfecase-tagged recombinant calcineurin catalytic subunit A isoform beta (calcineurin Aβ) protein (10 ng). (C) Expression of calcineurin Aβ mRNA in HUVEC. Total RNA was isolated from HUVEC infected and not infected with lentivirus carrying the indicated shRNA as described in Materials and methods. Each bar represents means ± SE of four independent experiments (Student's unpaired *t*-test). (D) Calcineurin Aα knockdown has little effect on tube formation. HUVEC infected with lentivirus carrying the indicated shRNAs were stimulated with 30 ng/ml bFGF and 30 ng/ml VEGF for 48 h. The bar indicates 100 μm. (E) Calcineurin Aα knockdown has little effect on cell viability. HUVEC infected with lentivirus carrying the indicated shRNAs were incubated in the presence of 30 ng/ml bFGF and 30 ng/ml VEGF for 48–96 h. Each bar represents means ± SE of four independent experiments (Student's unpaired *t*-test). (F) FK506-induced tube breakdown is not suppressed by calcineurin Aα knockdown. Tube-forming HUVEC that were infected with lentivirus carrying the indicated shRNAs were treated with 20 ng/ml FK506 for 48 h. The bar indicates 100 μm. (G) Calcineurin Aα knockdown fails to suppress FK506-induced cell death. Tube-forming HUVEC that were infected with lentivirus carrying the indicated shRNAs were treated with 20 ng/ml FK506 for 48 h. Each bar represents means ± SE of four independent experiments (Student's unpaired *t*-test). (H) Enzyme activity of calcineurin is suppressed by calcineurin Aα knockdown. Cell extracts were prepared from tube-forming HUVEC that were infected with lentivirus carrying the indicated shRNAs and treated with 20 ng/ml FK506 for 24 h. The experiments in A, B, D, F and H were performed four times and the representative data are shown. N.S., not significant.

phospho-NFATc2 (Ser-326) (Santa Cruz Biotechnology, Santa Cruz, CA) and calcineurin A (BD Biosciences, San Jose, CA). Human glutathione-S-transfecase-tagged recombinant calcineurin Aβ protein was obtained from Abnova (Taipei City, Taiwan).

2.9. Construction and production of fluorescent protein- and short hairpin RNA-expressing lentiviral vectors

The mCherry-fluorescence expression vectors were kindly provided by Roger Y. Tsien (University of California, San Diego). Self-inactivating lentivirus vector expressing mCherry-fluorescent protein (mCFP) was created by replacement of sinSKcmv-enhanced green fluorescent protein with mCFP cDNA as described previously [18]. The retroviral silencing plasmids, which express shRNA targeting Luciferase, as a control shRNA, and human calcineurin Aα (NM_000944) were purchased from Origene (Rockville, MD). To construct vector plasmids expressing shRNA and mCFP, shRNA expression cassettes with U6 promoter from the retroviral silencing plasmids (Origene) were cloned into self-inactivating lentivirus vector expressing mCFP. The virus preparations were produced by transient co-transfection of 293T cells as described previously [19,20]. The titers of these vectors were determined by fluorescent protein expression using a FACS Calibur flow cytometer (Becton Dickinson Japan, Tokyo, Japan) and expressed in terms of transducing units per microliter.

2.10. Transduction of HUVEC with shRNA

HUVEC were grown in culture medium without penicillin, streptomycin, and neomycin at a density of 2×10^5 cells/ml in 100-mm dishes. The cells were then incubated with self-inactivating lentivirus expressing Luciferase-shRNA-mCFP, or calcineurin Aα-shRNA-mCFP at a multiplicity of infection of 3 for 48 h.