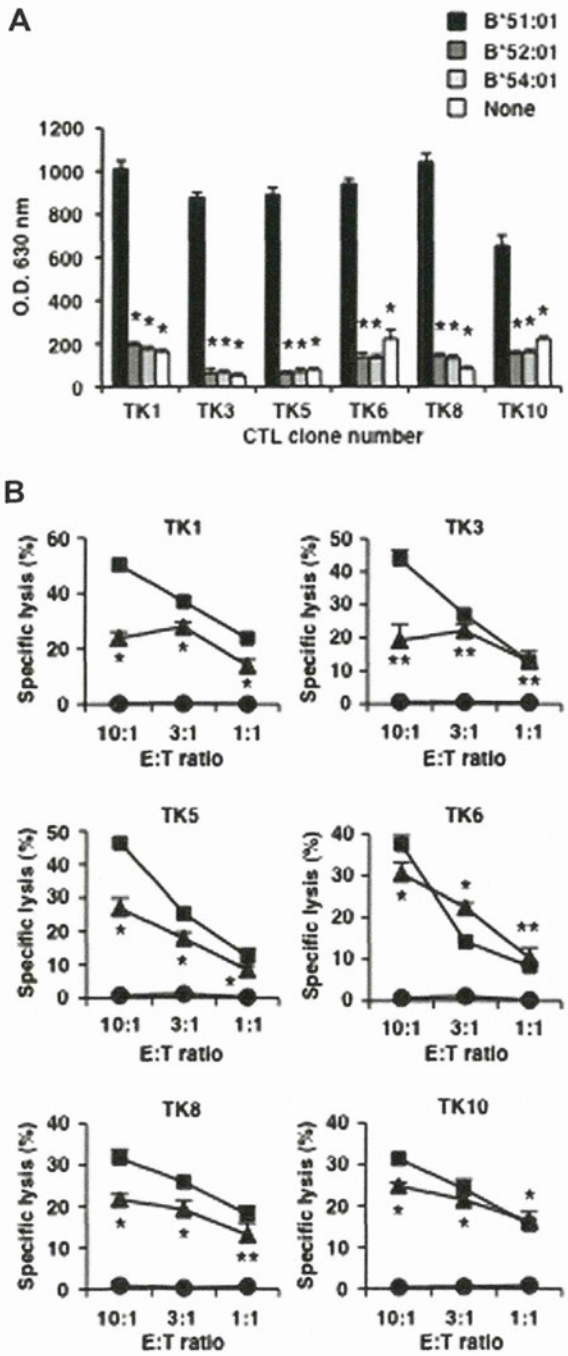

Fig. 3. Recognition of the HLA-B*51:01 molecule by CTLs. (A) COS cells were transfected with a plasmid encoding *B*51:01* cDNA, *B*52:01* cDNA, *B*54:01* cDNA, or no cDNA cocultured with CTL clones, and IFN-γ production was measured in the supernatant using ELISA. Data are the means and SD of triplicate determinations. *Significant difference ($p < 0.01$: Student's *t*-test) in the IFN-γ production stimulated by *B*52:01* cDNA, *B*54:01* cDNA or no cDNA compared with *B*51:01* cDNA. Data are representative of three experiments. (B) Recipient B-LCL (square), donor B-LCL (circle), and donor B-LCL transfected with *HLA-B*51:01* cDNA (triangle) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. Significant difference (*$p < 0.01$; **$p < 0.05$) in the lysis of *B*51:01*-transfected donor B-LCL compared with donor B-LCL (negative control). Data are representative of three experiments.

were CD3+/CD4−/CD8+ (data not shown). The nucleotide sequences of the uniquely rearranged *TCR Vβ* gene of each clone were determined by direct DNA sequencing of the amplified PCR products of TCR (Table 2). The TK2 and TK3 clones had the same nucleotide sequences in the CDR3 regions of their TCR Vβ20, suggesting that these CTLs originated from a single clone. Similarly, TK4 and TK5, as well as TK6, TK7, and TK9, had the same nucleotide sequences in the CDR3 regions of their TCR Vβ19*1 and Vβ12, respectively, suggesting that each group also originated from a single clone. Thus, the 10 isolated alloreactive CTL clones appeared to have been derived from six independent clones.

3.3. CTL clones recognized the HLA-B*51:01 molecule

To evaluate the possibility that isolated CTL clones recognize the HLA-B*51:01 molecule, COS cells were first transfected with an *HLA-B*51:01*, *–B*52:01*, or *–B*54:01* cDNA construct, COS transfectants were cocultured with six independent CTL clones, and then the production of IFN-γ in the supernatant was measured. The COS cells transfected with *HLA-B*51:01* clearly stimulated IFN-γ production by six independent CTL clones, whereas neither *B*52:01* nor *B*54:01* stimulated them (Fig. 3A). Then, donor B-LCL were transfected with an *HLA-B*51:01* cDNA construct and used as target cells in a cytotoxicity assay. The donor B-LCL transfected with *HLA-B*51:01* were lysed by six CTL clones (Fig. 3B), indicating that all clones recognized the mismatched HLA-B*51:01 molecule as an alloantigen. On the other hand, these data suggest that the CTL response toward the HLA-B*51:01 molecule accounted for the majority of the recipient's CTL alloresponse during acute GVHD.

3.4. Recognition of HLA molecules by CTL clones was peptide-dependent

Various forms of T lymphocyte recognition of the allogeneic major histocompatibility antigen, ranging from peptide-dependent to peptide-independent, have been demonstrated [20]. To confirm peptide dependency in CTL recognition, examinations were focused on the difference in the amino acid sequences of the recipient B*51:01 and the donor B*52:01. They differed in two amino acids at positions 63 and 67 (Fig. 4A), which constitute peptide binding pockets A and/or B [21,22]. In particular, B-pocket has a critical role in peptide binding to HLA-B*51:01 molecules [23], and substitution of a single amino acid constituting peptide binding pocket can affect peptide binding [24]. Two mutated *B*51:01* cDNA constructs, *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser*, in which individual amino acids were substituted with the corresponding amino acid in B*52:01 (Fig. 4A), were generated, as well as two more mutated *B*51:01* cDNA constructs, *B*51:01-Val194Ile* and *B*51:01-Ala199Val*, in which individual amino acids exist in *B*44:02* and other B alleles and localize outside the positions constituting peptide binding pockets. COS cells were then transfected with each wild or mutated cDNA construct and examined in the CTL stimulation assay. IFN-γ production of the TK3 clone was significantly decreased when stimulated by the *B*51:01-Phe67Ser* mutant in comparison with the wild-type *B*51:01* construct (Fig. 4B). IFN-γ production of all other CTL clones, TK1, TK5, TK6, TK8, and TK10, was significantly decreased when stimulated by *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser* mutants in comparison with the wild-type *B*51:01* construct (Fig. 4B). However, both *B*51:01-Val194Ile* and *B*51:01-Ala199Val* mutants stimulated all CTL clones to the same degree as the wild-type *B*51:01* construct. Thus, these data suggest that recognition of the HLA-B*51:01 molecule by CTL clones was peptide-dependent.

Furthermore, CTL clones should recognize certain peptides other than leukemia antigens, presented by HLA-B*51:01 molecules, because *B*51:01*-transfected COS cells, which are derived from monkey kidney cells, stimulated IFN-γ production of CTLs

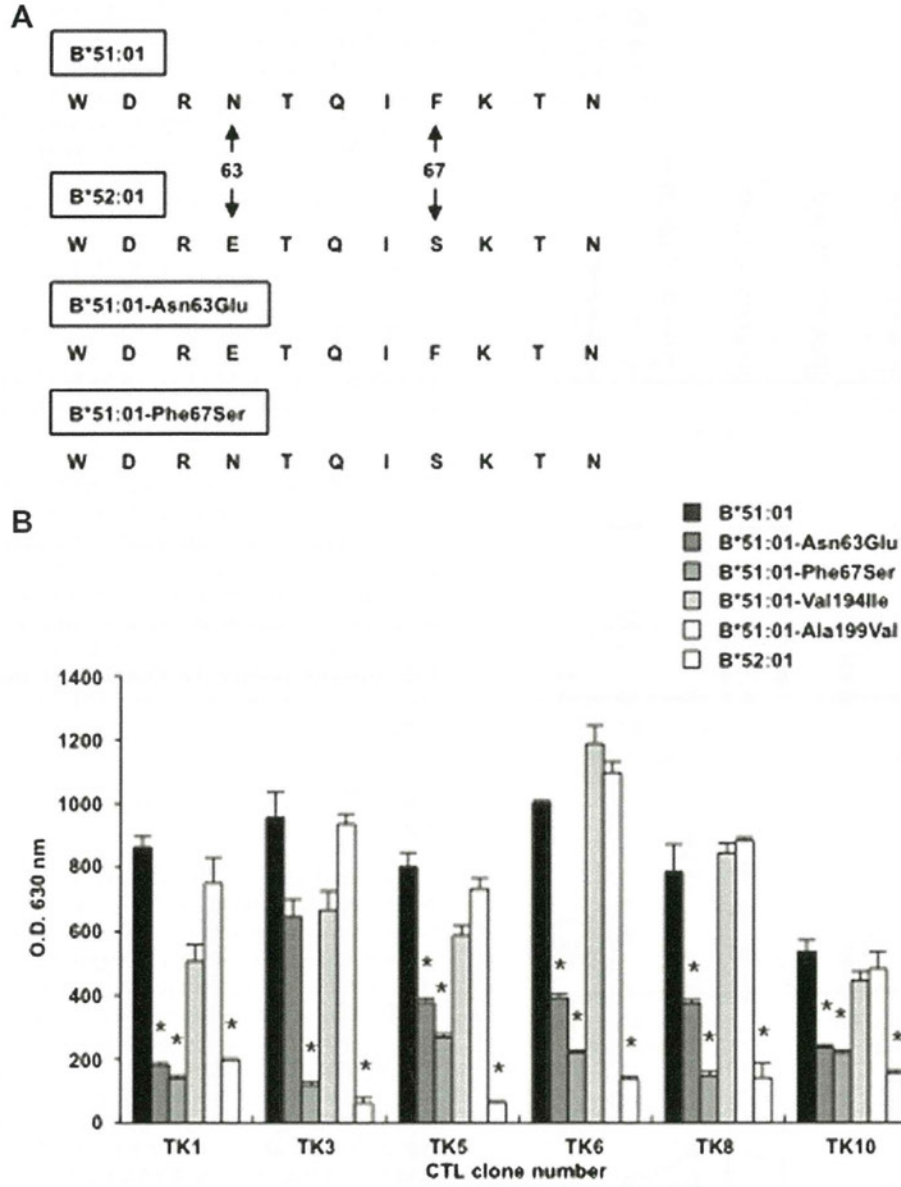

Fig. 4. Recognition of the HLA-B*51:01 molecule by CTLs is peptide-dependent. (A) The amino acid sequences at position 60 to 70 of the *B*51:01*, *B*52:01*, *B*51:01-Asn63Glu*, and *B*51:01-Phe67Ser* cDNAs are shown. Asn at position 63 was substituted with the corresponding amino acid in B*52:01, Glu, in the *B*51:01-Asn63Glu* mutant. Phe at position 67 was substituted with the corresponding amino acid in B*52:01, Ser, in the *B*51:01-Phe67Ser* mutant. (B) COS cells were transfected with a plasmid encoding *B*51:01*, *B*51:01-Asn63Glu*, *B*51:01-Phe67Ser*, *B*51:01-Val194Ile*, *B*51:01-Ala199Val* or *B*52:01* cDNA construct, cocultured with CTL clones, and IFN-γ production was measured in the supernatant using ELISA. Data are the means and SD of triplicate determinations. *Significant difference ($p < 0.05$: Student's *t*-test) in the IFN-γ production stimulated by each mutant or *B*52:01* cDNA construct compared with the wild-type *B*51:01* cDNA construct. Data are representative of three experiments.

(Fig. 3A), and *B*51:01*-transfected donor B-LCL, which are derived from B lymphocytes, were lysed by CTLs (Fig. 3B).

3.5. Leukemia blasts escaped from immunological pressure by HLA-B-specific CTLs

Whether the leukemia blasts escaped from the cytotoxicity of HLA-B*51:01-specific CTL clones was then examined. Pre-transplant and post-transplant leukemia blasts were purified by fluorescence-activated cell sorter (purity, ~62% and ~99%, respectively), and a cytotoxicity assay was performed only for the TK1 CTL clone because of the limited number of cryopreserved blasts. Weak but clear lysis of pre-transplant leukemia blasts by the TK1 CTL clone was observed, whereas post-transplant leukemia blasts were not lysed (Fig. 5A). All other CTL clones (TK3, TK5, TK6, TK8, and TK10) also did not lyse post-transplant leukemia blasts (Fig. 5B).

In addition, whether HLA-B*51:01-specific CTL pressure persisted until leukemia relapse was examined. The IFN-γ ELISPOT assay was performed to detect HLA-B*51:01-reactive CTLs in patient blood on day 232, 1 month before clinical leukemia relapse (Fig. 6). IFN-γ-producing B*51:01-reactive T lymphocytes were detected at a level nearly equal to the level of recipient B-LCL-reactive CTLs, that is, the total CTL alloresponse.

4. Discussion

The mechanism of leukemia relapse in this recipient can be explained as follows. CTLs specific for HLA-B*51:01 molecule/

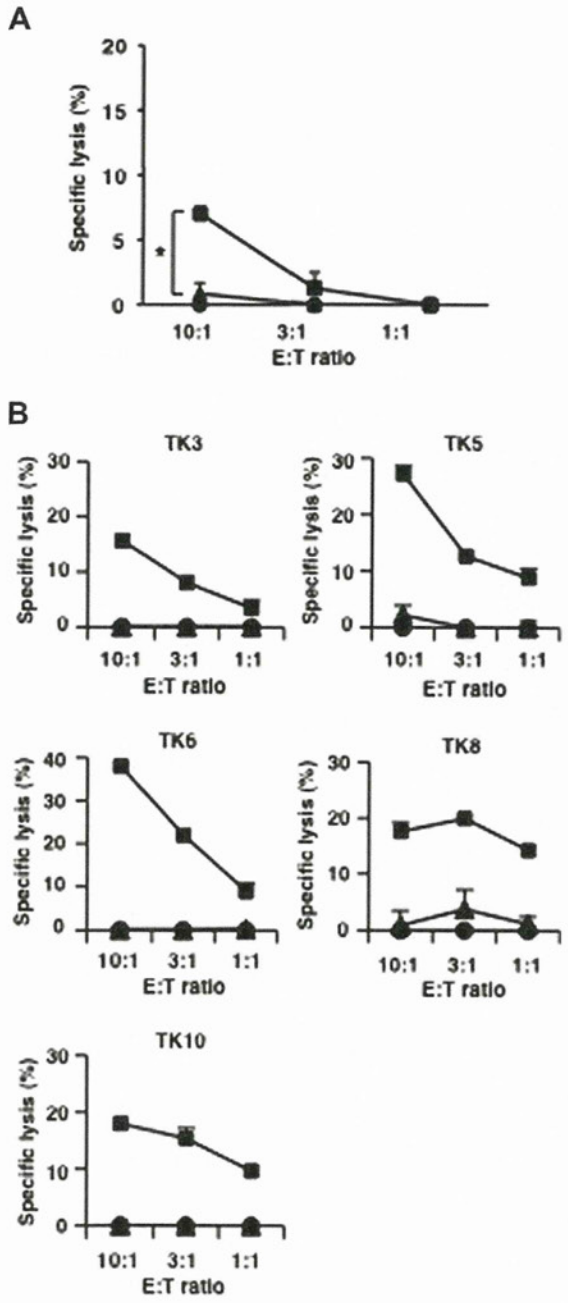

Fig. 5. Cytotoxicities of CTLs against leukemia blasts. (A) Purified pre-transplant leukemia blasts (purity, ~62%) (square), purified post-transplant leukemia blasts (purity, ~99%) (triangle) and donor B-LCL (circle) were used as targets for TK1 CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. *Significant difference ($p = 0.024$: Student's t-test) in the lysis of the pre-transplant leukemia blasts compared with the post-transplant leukemia blasts. Data are representative of three experiments. (B) Purified post-transplant leukemia blasts (purity, ~99%) (triangle), B-LCLs from the patient (square) and donor (circle) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. Data are representative of three experiments. There was no significant difference in the lysis of the post-transplant leukemia blasts compared with B51-negative donor B-LCL (negative control).

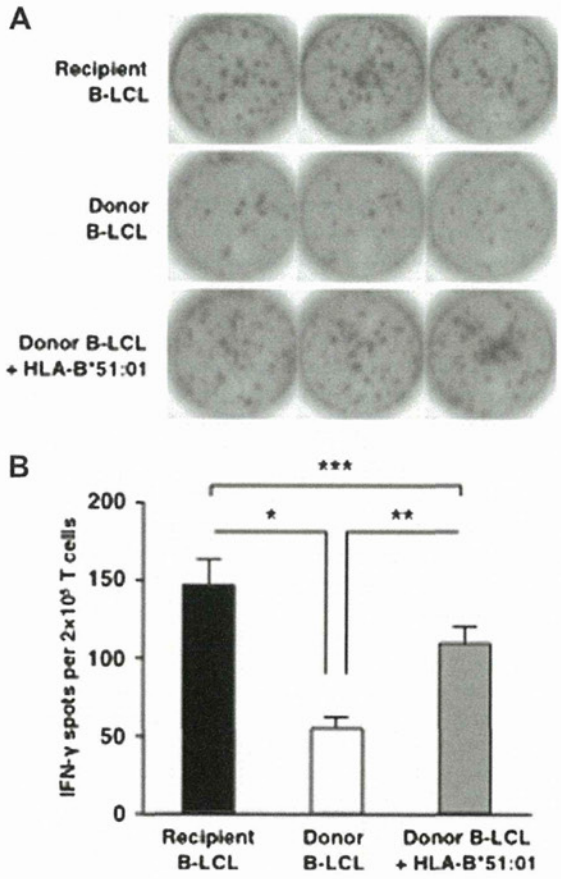

Fig. 6. Detection of HLA-B*51:01-specific CTLs in T lymphocytes obtained from the recipient on day 232 after transplantation. (A) Representative ELISPOT wells show triplicate results of T lymphocytes stimulated by recipient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. Data are representative of three experiments. (B) The frequency of CTLs in T lymphocytes recognizing the HLA-B*51:01 molecule was measured by IFN-γ ELISPOT analysis. The frequency of IFN-γ-producing cells is shown against recipient B-LCL (black), donor B-LCL (white), and *HLA-B*51:01*-transfected donor B-LCL (gray). Data are the means and SD of triplicate determinations. *$p = 0.0057$; **$p = 0.0077$; ***$p = 0.090$ (Student's t-test). Data are representative of three experiments.

non-leukemia peptide complex were generated in the recipient blood during acute GVHD, and these CTLs continued to produce immunological pressure on leukemia blasts for at least 8 months after transplantation, but B*51:01-down-regulated leukemia blasts escaped from the pressure of B*51:01-specific CTLs, and then the leukemia clinically relapsed.

CTLs recognizing mismatched HLA molecules play an important role in the immune reaction after HLA-mismatched HSCT, including graft rejection [25–27], GVHD [28], and the GVL effect [11]. In this study, the mismatched HLA-B*51:01-specific CTLs could participate both in GVHD and the GVL effect in the recipient. Ten CTL clones were isolated from the recipient's blood just after the onset of grade III acute GVHD involving skin, gut, and liver, and all clones demonstrated HLA-B*51:01-specific cytotoxicity in a non-leukemia peptide-dependent manner (Fig. 3 and 4). The patient was suffering from GVHD until his death on day 279, and in the ELISPOT assay for T lymphocytes obtained from recipient blood on day 232, HLA-B*51:01-reactive T lymphocytes accounted for the majority of alloreactive T lymphocytes (Fig. 6). Meanwhile, weak but clear lysis of pre-transplant leukemia blasts by an HLA-B*51:01-specific CTL clone was confirmed (Fig. 5A), and the primary refractory T lymphoblastic leukemia/lymphoma was in remission until day 261. These data are consistent with participation of the recipient HLA-B locus-specific CTLs both in GVHD and the GVL effect.

Selective HLA down-regulation was seen in this patient's post-transplant leukemia blasts. Mechanisms that alter HLA class I expression have been investigated and summarized as follows [29]: (1) loss of heterozygosity in chromosome 6 and/or 15, in

which class I heavy chain or β_2-microglobulin genes are located [30,31]; (2) mutations in these genes [32]; and (3) down-regulation of the antigen processing machinery, including transporter associated with antigen processing and low-molecular-weight protein genes [33]. Ten *HLA-B* cDNAs were cloned from purified post-transplant leukemia blasts (purity, ~99%) by RT-PCR; 5 (50%) clones were identical to the canonical *B*51:01* cDNA sequence, and the others (50%) were identical to the canonical *B*54:01* cDNA sequence, which was another recipient B allele (data not shown). These data suggest that down-regulation of HLA-B*51:01 expression in the post-transplant leukemia blasts resulted from mechanisms other than loss of heterozygosity of B locus and mutation of the *B*51:01* gene itself, although the entire sequence of *B*51:01* DNA including introns has not been determined. Recently, hypermethylation of the HLA-class I gene promoter regions has been identified as a mechanism for transcriptional inactivation of HLA class I genes in esophageal squamous cell carcinoma lesions [34]. We analyzed *B*51:01* promoter methylation by pyrosequencing of bisulfite-treated DNA from purified post-transplant leukemia blasts and confirmed no hypermethylation of the *B*51:01* gene (data not shown). Other possible mechanisms are down-regulation of translation and post-translational modification of the *B*51:01* gene, although, to the best of our knowledge, these mechanisms have not yet been investigated for HLA-class I genes. Further analysis is required.

The change in expression in HLA-A*11:01 between pre-transplant and post-transplant leukemia blasts was of similar magnitude, but in the opposite direction, to that observed for B*51:01. Because the expression of HLA-A11 on target cells can protect them from lysis by KIR3DL2-positive NK cells [35], the possibility that the post-transplant blasts with high expression of HLA-A11 were resistant to NK cell-mediated cytotoxicity, resulting in leukemia relapse, cannot be ruled out.

A question left unresolved is whether the present observation is unique to this recipient or can be duplicated in additional recipients who receive HLA one locus-mismatched HSCT. However, the present findings can explain, at least in part, the mechanism of how leukemia relapse occurs during persistent GVHD after HSCT. Another question is whether the present observation is unique to T lymphoblastic leukemia/lymphoma, which is a relatively rare subset of acute leukemia in adults. The relevance of this finding to other leukemias, including B lymphoblastic leukemia/lymphoma and myeloid malignancies, should be confirmed. Further efforts to identify the peptides that are presented by HLA-B*51:01 molecules and recognized by isolated CTL clones should help to elucidate the precise mechanisms of leukemia escape.

In conclusion, immune escape of leukemia blasts from CTL pressure toward a mismatched HLA molecule/non-leukemia peptide complex may lead to clinical leukemia relapse.

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Phase II study of dose-modified busulfan by real-time targeting in allogeneic hematopoietic stem cell transplantation for myeloid malignancy

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We aimed to evaluate the efficacy and safety of allogeneic hematopoietic stem cell transplantation with targeted oral busulfan (BU) and cyclophosphamide (CY) in a phase II study. Busulfan (1.0 mg/kg) was given initially in six doses. Based on the estimated concentration at steady state after the first dose of BU, subsequent (7th–16th) doses were adjusted to obtain a targeted overall concentration at steady state of 700–900 ng/mL. The primary endpoint was 1-year overall survival (OS). Fifty patients were registered and 46 (median age, 53 years; range, 18–62 years) received planned transplant, including 24 with AML, 16 with myelodysplastic syndrome, and six with CML. Fourteen patients were categorized as standard risk. Nineteen patients received transplant from human leukocyte antigen-identical siblings, 27 from unrelated donors. The BU dose required reduction in 32 patients and escalation in six patients. One-year OS was 65% (95% confidence interval, 50–77%). Cumulative incidence of hepatic sinusoidal obstruction syndrome was 11%. One-year transplant-related mortality was 18%. Both OS and transplant-related mortality were favorable in this study, including patients of older age and with high risk diseases. Individual dose adjustment based on BU pharmacokinetics was feasible and effective in the current phase II study. This trial is registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). (*Cancer Sci* 2012; 103: 1688–1694)

Busulfan is an alkylating agent widely used in high-dose chemotherapy regimens for HSCT.[1,2] The BU level in serum has been shown to be an important factor for graft rejection and regimen-related toxicity such as SOS.[3–5] Unfavorable profiles of oral BU include delayed and variable absorptive characteristics and high variability in drug metabolism.[6] Individualized dose adjustment of BU using the LSM, and its transplantation results, have been investigated widely in Caucasian patients and pediatric populations, but few prospective studies have investigated results in Asian patients.[7] Prior to the current study, we carried out a prospective PK study to analyze BU concentration using gas chromatography–mass spectrometry.[8] Nine patients were enrolled in the study, and received preparative regimen containing oral BU 1 mg/kg every 6 h for eight or 16 doses. Out of nine patients, only three met the average steady-state plasma concentration levels in the safety range of 650–1000 ng/mL[4,9] after the first and 13th dose. From the results, we developed LSM to estimate the AUC using two different formulas in order to fit even delayed clearance. Subsequently, we carried out a pilot study that used the same targeting method as the current study, and six patients with myeloid malignancy received tBU+CY conditioning with a targeting AUC of Css 700–900 ng/mL. Four patients received dose reduction after the seventh dose of BU, and overall Css of three patients met the safety range of 786–905 ng/mL (Akio Kohno, Mariko Fukumoto, Hiroto Narimatsu, Kazutaka Ozeki, Masashi Sawa, Shuichi Mizuta, Hitoshi Suzuki, Isamu Sugiura, Seitaro Terakura, Kazuko Kudo, and Yoshihisa Morishita, unpublished data, 2003).

From these results, we carried out a prospective phase II trial in Japanese patients with myeloid malignancies to evaluate the clinical results of allogeneic HSCT undergoing individualized high-dose oral BU+CY conditioning.

Materials and Methods

Eligibility criteria. Patients from 16 to 65 years old were eligible if they had a diagnosis of AML, CML, or MDS, with an Eastern Cooperative Oncology Group performance status of 0–2, and no previous history of HSCT. Standard risk was defined as AML in first complete remission, MDS in refractory anemia or refractory anemia with ringed sideroblasts, and CML in chronic phase. High risk was defined as the remaining disease type. Patients receiving T cell depletion, or those with clinically significant infection or severe abnormalities of cardiac, pulmonary, and hepatic functions were excluded. Included patient/donor pairs were either related HLA matched by serological typing of A, B, and DR locus, unrelated HLA matched, or HLA DRB1 one locus mismatched by genotypical typing of A, B, and DRB1 locus. Unrelated donors were chosen by coordination with the Japan Marrow Donor Program. Written informed consent was obtained from each patient according to the Declaration of Helsinki. The study

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protocol was approved by the Institutional Review Board of each center.

Conditioning regimen, GVHD prophylaxis, and supportive care. Patients received a conditioning regimen consisting of BU 1.0 mg/kg given orally four times a day for six doses on two consecutive days (dose 1–6). Six hours after dose 6, patients received an adjusted dose of BU four times a day for 10 doses (dose 7–16) on three consecutive days (Fig. S1). Cyclophosphamide 60 mg/kg was given i.v. on two successive days. Both BU and CY were dosed based on actual body weight if it was <120% of ideal body weight, and adjusted body weight for those exceeding 120%. Sodium valproate was given as seizure prophylaxis before and during BU treatment. Fluconazole was used as fungal prophylaxis.

Either cyclosporine or tacrolimus in combination with methotrexate was used for GVHD prophylaxis. Cyclosporine was given i.v. at a dose of 3 mg/kg per day in two divided doses starting on day −1. Tacrolimus was given i.v. at a dose of 0.025 mg/kg continuously starting on day −1. Methotrexate was given at a dose of 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6. Oral cyclosporine or tacrolimus was substituted for i.v. administration when tolerated. In the absence of GVHD, the cyclosporine and tacrolimus doses were tapered after day 50. Acute GVHD of grade 2 or more was treated with methylprednisolone 1–2 mg/kg. Chronic GVHD was treated by the protocols of each institute.

Supportive care measures were used according to institutional guidelines. Daily granulocyte colony stimulating factor was started on day 6 and continued until absolute neutrophil count exceeded 500/μL for two consecutive days.

Pharmacokinetic studies of BU. For PK studies of BU, blood samples were obtained 0, 30, 60, 120, 300, and 360 min after the first oral dose. Frozen plasma samples were sent to the laboratory at Kitasato University, and plasma BU concentrations were assayed by gas chromatography–mass spectrometry.[8] The AUC was calculated by LSM using the formulas shown in Table 1.

Average Css levels of BU were determined by the ratio of the BU AUC_{LSM} over the dosing interval to the time between doses. The BU dose after the sixth dose was adjusted when Css after the first dose was not within 700–900 ng/mL. Dose adjustment was not carried out for patients whose Css after the first dose was 700–900 ng/mL. A targeted dose was calculated to achieve an average Css after all doses of 800 ng/mL. The optimal dose of BU was calculated as follows: optimal single dose of BU (mg/kg) = 800 (ng/mL) × first dose (mg/kg)/Css of first dose (ng/mL).

The dose of the 7th to 16th BU was calculated as follows: revised dose (mg/kg) = [optimal single dose (mg/kg) × 16 (times) − first dose (mg/kg) × 6 (times)]/10 (times).

Definitions of outcomes. The study was designed as a phase II prospective trial. The primary endpoint of the study was 1-year OS after transplantation. The secondary endpoint was DFS, PK of BU, aGVHD, and cGVHD, and the frequency and severity of SOS, regimen-related toxicity up to day 28, mortality at day 100, hematological recovery, and DFS and OS of each disease category.

All patients were prospectively monitored for engraftment,[10] post-transplant toxicities, GVHD, hepatic SOS, and infection. Failure to reach an absolute neutrophil count of 0.5 × 10^9 cells/L by day 28 after transplantation was defined as graft failure, and the patient was withdrawn from the study. The aGVHD was evaluated daily until day 28 and weekly from day 29 to 100 and graded by established criteria.[11] The cGVHD was evaluated up to day 365. Treatment and the outcome of aGVHD and cGVHD were also evaluated. Sinusoidal obstruction syndrome was clinically evaluated before day 28, and diagnosed,[12–14] then graded clinically[12] according to the published criteria. Liver toxicity that occurred after day 21 and fulfilled the above criteria of SOS was defined as late-onset SOS. Clinical data after day 29 until day 100 was additionally surveyed to evaluate late-onset SOS retrospectively.

Disease monitoring was carried out by bone marrow aspiration within 1 week before or after days 30, 60, and 90 after transplantation. Relapse was defined by hematological recurrence for AML,[15,16] and by hematological or cytogenetic relapse for CML. Deaths in the absence of persistent relapse were categorized as non-relapse mortality. Additional surveillance was carried out and the onset of SOS and regimen-related toxicities from days 29 to 100 were collected retrospectively. Long-term survival data and data of relapse after day 365 were also collected retrospectively.

Statistical analysis. The primary endpoint of the study was 1-year OS after transplantation. The expected 1-year OS was estimated to be 60%, and its threshold was estimated to be 40%. With a statistical power of 90% and a one-sided, type I error of 5%, the number of eligible patients required for this study was calculated to be 46 using a binominal analysis method. The projected sample size was 50 patients, with the expectation that 10% of patients would be deemed ineligible.

Disease-free survival was calculated from the date of transplantation until the date of relapse or the date of death in complete remission. This trial has been registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). Data were analyzed with Stata 9.2 statistical software (Stata, College Station, TX, USA).

Table 1. Formulas for limited sample model (LSM) in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide

i	In cases C_6/C_2 = or <0.5
	$AUC_{LSM} = 0.5C_{0.5} + 0.75C_1 + 2.5C_2 + 2.0C_6 + 4C_6/(LnC_2 - LnC_6)$
ii	In cases $C_6/C_2 > 0.5$
	$AUC_{LSM} = 0.5C_{0.5} + 0.75C_1 + 2.5C_2 + 2.0C_6 + 2C_6/(LnC_2 - LnC_6)$

In the previous pilot study, formula (i) bore a strong approximation to actual area under the blood concentration time curve (AUC), but not in patients with an elongated absorption or a delayed elimination of busulfan. The formula of the LSM was modified in the case of $C_6/C_2 > 0.5$ and formula (ii) was used for those patients. C_x, serum busulfan level obtained at x hours after the first dose.

Results

Patient characteristics. Patients were registered from October 2003 through March 2007. Fifty patients were registered. One patient who developed severe hemorrhagic ulcer of the ileum after registration was considered to be ineligible. One patient developed metastatic breast cancer before receiving the conditioning regimen and was withdrawn. Forty-eight patients received tBU+CY conditioning. One patient developed systemic convulsion on day −6 before transplantation, and the study was discontinued. Another patient received cord blood transplantation due to unexpected emergent unavailability of the unrelated bone marrow and was included only in the PK analysis. The remaining 46 patients who completed tBU+CY conditioning and received the planned transplantation were analyzed in the subsequent outcome study. Characteristics and a transplantation summary of these 46 patients at the time of registration are shown in Tables 2 and 3, respectively.

Treatment-related toxicity and hepatic veno-occlusive disease. Forty-five of 46 patients undergoing tBU+CY conditioning (98%) experienced grade II or higher regimen-related toxicity, and 38 of 48 patients (79%) experienced grade III or more toxicity within 28 days post-transplantation (Table S1). Infection (70%), oral mucositis (52%), nausea and vomiting (30%), and

Table 2. Characteristics of patients receiving allogeneic hematopoietic stem cell transplantation (*n* = 46)

Characteristics	
Median age of patients (range), years	53 (18–62)
Sex of recipient (%)	
Male	29 (63)
Female	17 (37)
Sex, donor versus recipient (%)	
Match	25 (54)
Male to female	11 (24)
Female to male	10 (22)
Disease type (%)	
AML	24 (52)
1st CR	5
2nd CR	10
1st relapse	5
No treatment†	4
MDS	16 (35)
RA	4
RAEB	9
CMML	1
RAEB-t	2
CML	6 (13)
CP	5
AP	1
Disease risk‡ (%)	
Standard	14 (30)
High	32 (70)
Performance status§ (%)	
0	40 (86)
1	6 (13)
2	0 (0)
Donor (%)	
Related	19 (41)
Unrelated	27 (59)
HLA (%)	
HLA identical sibling	19 (41)
HLA 6/6 matched, unrelated	23 (50)
HLA mismatched, unrelated	4 (9)

†Two patients with overt leukemia from myelodysplastic syndrome (MDS) and another two patients with hypoplastic AML did not receive induction chemotherapy before transplantation. ‡Standard risk was defined as AML in 1st complete remission (CR), MDS in refractory anemia (RA) or RA with ringed sideroblasts, and CML in chronic phase (CP). §According to Eastern Cooperative Oncology Group criteria. AP, accelerated phase; CMML, chronic myelomonocytic leukemia; HLA, human leukocyte antigen; RAEB, refractory anemia with excess of blasts; RAEB-t, RAEB in transformation.

Table 3. Summary of transplantation in patients with AML (*n* = 24), myelodysplastic syndrome (*n* = 16), or CML (*n* = 6)

Stem cell source	
G-PBMC	7
Bone marrow	39
GVHD prophylaxis†	
sMTX+CyA	22
sMTX+FK	21
aGVHD, grade (%)	
None	26 (56)
I	4 (9)
II	11 (24)
III	4 (9)
IV	1 (2)
cGVHD, type (%)	
None	16 (43)
Lmt	9 (24)
Ext	12 (32)

†One patient received short-term methotrexate + tacrolimus prophylaxis and subsequently received short-term methotrexate + cyclosporine. aGVHD, acute graft versus host disease; chronic GVHD, chronic graft versus host disease; GVHD, graft versus host disease.

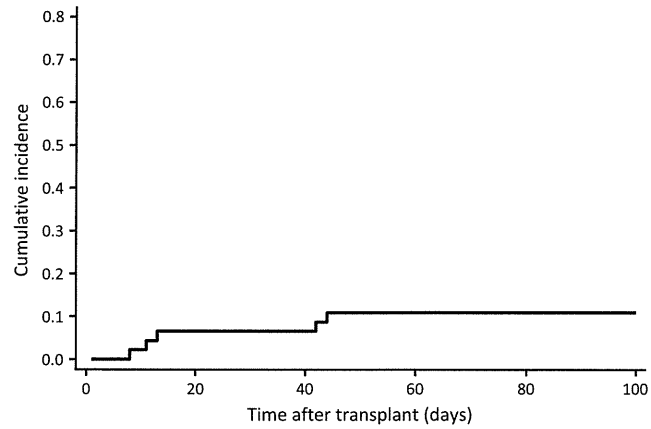

Fig. 1. Cumulative incidence of sinusoidal obstruction syndrome in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide. The cumulative incidence of overall sinusoidal obstruction syndrome was 11% (95% confidence interval, 4–22%) at day 100 after transplantation.

diarrhea (30%) were frequent grade III or more adverse reactions. Severe neurological toxicity of grade III or more was observed in five patients (11%). One patient developed subarachnoid hemorrhage and died on day 1 after transplantation. Another patient developed tacrolimus encephalopathy on day 23 after transplantation. This patient died of acute bleeding from gastric ulcer on day 57. Another patient developed neurological toxicity during the course of septic shock and died on day 15. One patient who received dose reduction had delayed engraftment, but subsequently engrafted on day 31.

Among 46 patients undergoing planned transplantation, four patients experienced grade III or IV liver toxicity before day 28 (Table S1). Grade III or more long-term liver toxicity between days 29 and 100 was observed in nine patients (Table S2). Three patients were reported to have SOS before day 20, and two were reported to have late-onset SOS from days 21 to 100. Cumulative incidence of overall SOS was 11% (95% CI, 4–22%) at day 100 after transplantation (Fig. 1). Two patients had mild SOS on day 8 and 11 after transplantation, and both improved. One of these patients died of an unrelated cause (acute renal failure and infection). The third patient was reported to have moderate SOS on day 13. This patient died of an unrelated cause (septic shock) on day 15 after transplantation. Two patients developed severe SOS on days 42 and 44. These patients died of hepatic failure on day 64 and 81, respectively.

Graft versus host disease. The cumulative incidence of grade II–IV and III/IV aGVHD at day 100 were 35% and 11%, respectively. The cumulative incidence of grades II–IV aGVHD in the recipients who underwent transplant from an HLA-identical related donor or unrelated donor was 26% and 41%, respectively, and those of grades III/IV aGVHD was 11% and 11%, respectively. The cumulative incidence of cGVHD at 1 year after transplantation was 52%. Of the 21 patients who developed cGVHD, 12 had extensive disease and nine had limited disease.

Survival outcome. Twenty-six patients were alive with a median follow-up of 43 months (range, 11.9–65 months) after

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transplant. Overall survival was 65% (95% CI, 50–77%) at 1 year after transplantation, 66% (95% CI, 47–79%) for high risk and 64% (95% CI, 34–83%) for standard risk patients (Fig. 2a). Overall survival of AML was 71% (95% CI, 48–85%; $n = 24$) 1 year after transplantation, 50% (95% CI, 25–71%; $n = 16$) for MDS, and 83% (95% CI, 27–97%; $n = 6$) for CML patients. Two patients died before day 28 as described above. From days 28 to 100, seven patients died due to treatment-related mortality (four patients), infection (two patients), and relapse (one patient). Of the four patients who died of TRM, two died from hepatic toxicity, one from gastrointestinal bleeding, and one from thrombotic microangiopathy.

Disease-free survival was 57% (95% CI, 41–69%) 1 year after transplantation, 56% (95% CI, 38–71%) for high risk and 57% (95% CI, 28–78%) for standard risk patients (Fig. 2b). Disease-free survival of AML was 58% (95% CI, 36–75%) at 1 year, 44% (95% CI, 20–66%) for MDS, and 83% (95% CI, 27–97%) for CML patients.

Relapse and TRM. Thirteen patients (28%) experienced disease recurrence. Cumulative incidence of relapse was 24% at 1 year after transplantation. Cumulative incidence of relapse was 22% among patients with high risk disease, and 14% among patients with standard risk disease (Fig. 3a). Cumulative incidence of TRM was 18% at 1 year after transplantation (Fig. 3b).

Pharmacokinetic studies and dose modification. Among the 47 patients who completed the 16 BU doses, Css of the first dose was 1090 ± 318 ng/mL (range, 593–1673). The mean AUC_{inf} estimated after the first dose of BU was 6760 μg·h/L (range, 3656–13058 μg·h/L). The mean values of oral clearance, distribution volume, and elimination half-life were 0.159 L/h/kg (0.079–0.263 L/h/kg), 0.55 L/kg (0.178–0.989 L/kg), and 2.54 h (0.98–5.49 h), respectively. Six patients received dose escalation of BU, and 32 received dose reduction (Fig. 4a). Median decreasing dose of BU was 4.5 mg/kg (28% of 16 mg/kg). Mean actual dose of BU was 12.7 ± 3.7 mg/kg (range, 7.6–21.3 mg/kg).

One patient was excluded from the analysis due to systemic convulsions on day −6, as described above. The Css of the first dose was 683.1 ng/mL in this patient. Although dose escalation was carried out to receive 18.7 mg/kg, the conditioning regimen was not completed.

Busulfan targeting and transplant outcome. Overall survival was not different between patients who received dose reduction, no modification, or escalation of BU (68%, 67%, and 50% at 1 year, respectively). Significantly more grade III–IV toxicities from days 29 to 100 were observed in patients who received dose escalation (Fisher's exact test, $P = 0.023$) (Table S2). No difference in TRM was observed among these three groups.

All three patients who developed early-onset SOS within 20 days after transplant had received dose reduction of BU. Two developed grade II liver toxicity, and another developed grade IV liver toxicity before day 28 (Fig. 4b). Two patients who had late-onset SOS and died had received dose escalation (Fig. 4c).

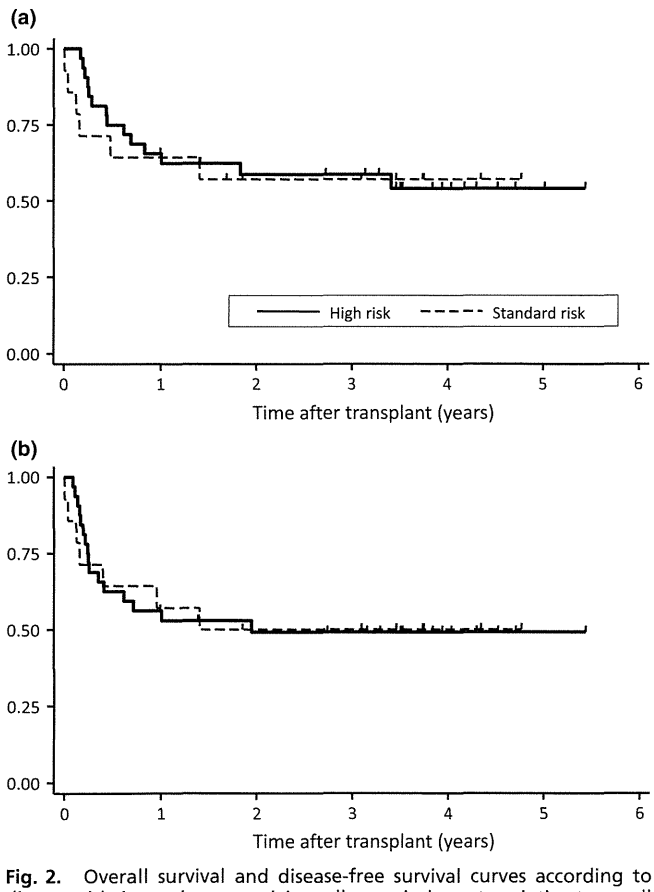

Fig. 2. Overall survival and disease-free survival curves according to disease risk in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide. Overall survival (a) and disease-free survival (b), each stratified according to disease risk. Data were analyzed with the Kaplan–Meier method.

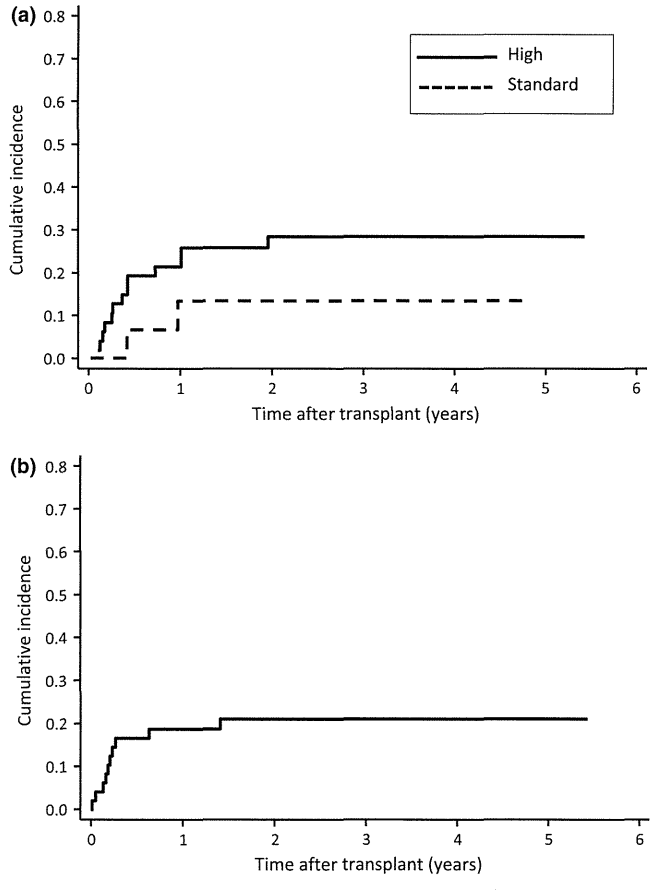

Fig. 3. Cumulative incidence of relapse and transplant-related mortality in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide. Cumulative incidence of relapse with (a) high risk disease (22% at 1 year), standard risk disease (14%), and (b) cumulative incidence of treatment-related mortality (18%).

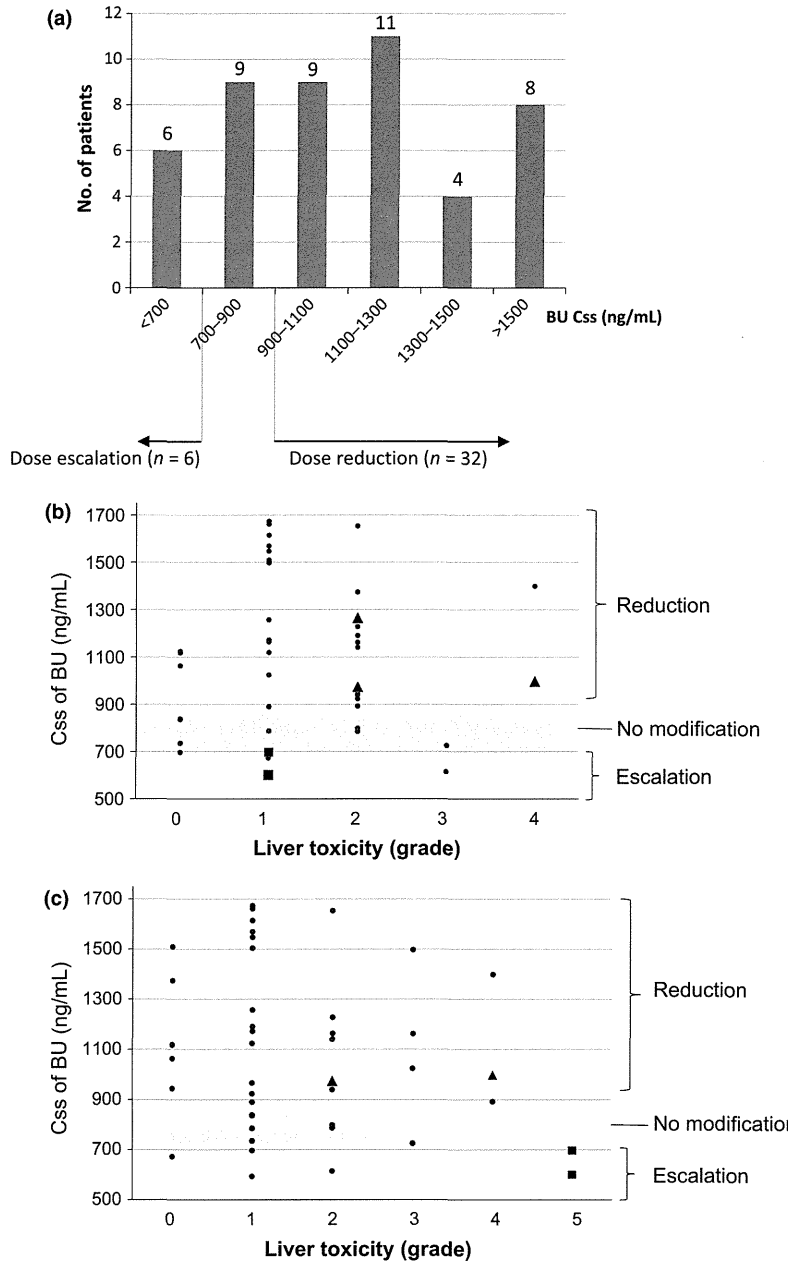

Fig. 4. Pharmacokinetic studies of busulfin (BU) in patients receiving allogeneic hematopoietic stem cell transplantation. (a) Number of the patients reaching concentration at steady state (Css) with the first BU dose. Six patients received dose escalation, nine patients received no modification, and 32 patients received dose reduction. (b) Liver toxicity in the first 28 days, sinusoidal obstruction syndrome (SOS), and BU dose modification. Triangles (▲) indicate patients diagnosed with SOS before day 20. Two of these patients developed grade II liver toxicity, and another developed grade IV liver toxicity. Two of these patients with early onset SOS died of an unrelated cause. Squares (■) indicate two patients who developed grade I liver toxicity in the first 28 days and were later diagnosed with severe late-onset SOS. (c) Liver toxicity from days 29 to 100, SOS and BU dose modification. Triangles (▲) and squares (■) indicate the same patients as (b). Two patients who had late-onset SOS and died had received dose escalation.

Discussion

We carried out a phase II study of individualizing the oral BU and CY conditioning regimen for adult allogeneic HSCT for myeloid malignancies. In the current study, 1-year OS (65%; 95% CI, 50–77%) clearly exceeded the threshold level of 40%.

Oral administration of BU had been associated with erratic gastrointestinal absorption and resulted in unpredictable systemic drug exposure.[3–5,17] Pharmacokinetic studies of BU and subsequent dose adjustment strategies for the BU and CY conditioning regimen have been reported, mainly among pediatric patients.[18–22] Although no essential difference in PK analysis has been reported between data from Japan and North America,[23] survival data and information on the benefit of the tBU+CY regimen for Asian adult populations are limited.[7] In this phase II study to target the BU Css range of 700–900 ng/mL, 32 patients received BU dose reduction and the median dose of total BU was reduced. Nevertheless, no increase in relapse was observed and the incidence of TRM was comparable to the BU+CY regimen using the i.v. form.[23] Notably, the incidence of SOS (11% at day 100) was relatively lower than in the previous report of an adult population receiving the CY+total body irradiation regimen[24] or oral non-targeted BU+CY.[6] Severe SOS was not observed within 20 days after transplantation, and this targeting strategy may contribute to reduce the severity of early-onset SOS. Our positive results could be a consequence of adjusting the BU dose, considering that 38 of 47 patients (81%) actually had not achieved optimal Css after the first dose. That is, the fixed dose of BU was not optimal in 81% of these Japanese patients.

In our previous study, SOS was not observed among patients whose Css range was within the target dose or when the BU dose

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was reduced (Akio Kohno, Mariko Fukumoto, Hiroto Narimatsu, Kazutaka Ozeki, Masashi Sawa, Shuichi Mizuta, Hitoshi Suzuki, Isamu Sugiura, Seitaro Terakura, Kazuko Kudo, and Yoshihisa Morishita, unpublished data, 2003). In the current study, three patients in the BU reduction group developed early-onset SOS, although the estimated cumulative Css remained within the targeted range. Liver toxicity in these patients might also be related to increased exposure to toxic metabolites of CY.[24] A dose-escalation study using test dose PK also showed that patients who showed a high level of AUC in the first dose developed severe toxicity, including hepatic SOS.[25]

Two of the six patients who received dose escalation experienced late-onset severe SOS. We may need to be cautious of possible late-onset severe SOS after dose escalation of BU. However, the causal relationship between dose escalation of BU based on low initial Css and SOS needs to be further evaluated, because individual oral BU PK are influenced by many factors. Glutathione S-transferase-mediated conjugation with GSH is the main mechanism to detoxify BU. Accumulation of the active metabolite of CY through depletion of the cellular GSH pool may contribute hepatic toxicity.[26] Hepatic GST activity and GST gene polymorphisms have been shown to be associated with BU clearance as well as transplant outcome. Polymorphism of GSTM1 is reported as a risk factor of SOS.[27] The heterozygous variant of GSTA1 (GSTA1*A/*B), which is observed in 26% of the Japanese population, resulted in slower elimination of BU than the wild-type.[28] Analysis using the Japan Marrow Donor Program showed a higher risk of TRM among recipients with the GSTM1-positive genotype, which was different from the Caucasian population.[29] We are currently investigating gene polymorphisms reported to be related with the risk factors of transplantation, such as GST genes and the UDP glucosyltransferase gene family[30] in a prospective trial.

Dose targeting possibly improves the OS by alleviating the variable absorptive characteristics among individuals. However, our results also suggest that dose modification might increase the chance of toxicity after day 28, especially in the case of dose escalation, although we should be careful of this interpretation. Dose reduction could generally lead to rejection of the graft. However, in this study, only one patient had 3 days' delay of engraftment in spite of the large number of patients in the study who received a dose reduction of BU. Busulfan in i.v. form has enabled us to accomplish narrow-ranged dose adjustment.[31] Careful validation of the clinical efficacy of PK-based targeting using i.v. BU is warranted.

In conclusion, individual dose adjustment based on BU PK was feasible and effective in the current phase II study.

Acknowledgments

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Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

aGVHD	acute graft versus host disease
AUC	area under the blood concentration time curve
BU	busulfan
cGVHD	chronic graft versus host disease
CI	cumulative incidence
Css	concentration at steady state
CY	cyclophosphamide
DFS	disease-free survival
GSH	glutathione
GVHD	graft versus host disease
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
LSM	limited sample model
MDS	myelodysplastic syndrome
OS	overall survival
PK	pharmacokinetic
SOS	sinusoidal obstruction syndrome
tBU+CY	targeting BU+CY
TRM	transplant-related mortality

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Study scheme.

Table S1. Regimen-related toxicity before day 28 in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.

Table S2. Regimen-related toxicity (day 29–100) in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.

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ORIGINAL ARTICLE

Efficacy and safety of human adipose tissue-derived mesenchymal stem cells for supporting hematopoiesis

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Abstract We have demonstrated that adipose tissue-derived mesenchymal stem cells (ADSCs) from mice are capable of reconstituting the hematopoietic microenvironment, and facilitate hematopoiesis more effectively than bone marrow-derived mesenchymal stem cells (BMSCs) in mouse. The ready accessibility of fat tissue rich in MSCs and the higher hematopoiesis-supporting capacities of ADSCs suggest that ADSCs might represent a new therapeutic modality for the regeneration of impaired hematopoiesis. As a further step towards their use in clinical practice, we established human BMSCs and ADSCs from healthy volunteers of similar age, and compared their proliferation capacities, hematopoiesis-supporting properties, and safety. In vitro cell proliferation studies revealed that ADSCs have a higher population doubling number than BMSCs. In vitro co-culture assays showed that ADSCs not only support human $CD34^+$ peripheral blood stem cells (PBSCs), but also yield significantly more non-adherent hematic cells than BMSCs. In vitro progenitor assays revealed that ADSCs promote a higher frequency of early progenitors than do BMSCs. Interestingly, BM cellularity in irradiated mice that had received ADSCs tended to be higher than that of mice treated with BMSCs. When MSCs were injected into the BM cavity of tibiae, we observed no evidence of MSC-induced toxicity either during or after treatment. In addition, no microscopic abnormalities were observed in the bone marrow and major organs.

Keywords Human adipose tissue-derived mesenchymal stem cells · Hematopoiesis-supporting properties · Safety · Cell therapy

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Introduction

Hematopoiesis is a dynamic process that involves self renewal of hematopoietic stem cells in the bone marrow, generation of lineage-committed cells, and mobilization of mature cells into the bloodstream. Mesenchymal stem cells (MSCs) present in bone marrow (BM) are thought to give rise to cells that constitute the hematopoietic microenvironment. MSCs produce a number of cytokines and extracellular matrix proteins and express cell adhesion molecules, all of which are involved in the regulation of hematopoiesis [1].

The hematopoietic microenvironment can be damaged by various pathophysiological mechanisms such as chemotherapy, irradiation, aging and malignant disease [2, 3]. Not only intensive chemotherapy but also chemotherapeutic drugs alone disrupt the hematopoietic microenvironment [4, 5]. In the elderly, bone marrow structures tend to be replaced with adipocytes that can be negative regulators of the hematopoietic microenvironment [6].

Long-lasting damage to the hematopoietic microenvironment impairs hematopoiesis, causing infection, bleeding, anemia and subsequent mortality.

The emerging field of regenerative medicine seeks to repair or restore lost or damaged tissue function due to the effects of injury, disease, and aging. Compelling studies showed that BM-derived mesenchymal stem cells (BMSCs), when directly injected into the BM, could reconstitute the hematopoietic microenvironment [7, 8]. These facts clearly suggest that BMSCs can be a new modality for regeneration of the hematopoietic microenvironment. However, there are several drawbacks in the use of BMSCs for clinical application. Even though it is desirable to establish MSCs from the patient to whom they will be administered due to the possibility that an immune response and subsequent adverse effects could be provoked by administration of allogeneic MSCs [9], BMSCs are only available in a limited number. To make matters worse, the number, differentiation potential, and maximal life span of BMSCs decline with increasing age [10, 11].

The discoveries that a large number of nonadipocyte stem cells exist in fat tissue (adipose tissue-derived MSCs; ADSCs) and that these cells can be rapidly expanded ex vivo suggested that ADSCs might be useful for clinical applications [12]. We recently reported that ADSCs are a better alternative to BMSCs for reconstitution of the hematopoietic microenvironment in a mouse model [13]. Since then, we have maintained an ongoing commitment to explore the potential of ADSCs for clinical applications. In this study, we provide evidence that human ADSCs support hematopoiesis better than human BMSCs.

Materials and methods

Animal studies

The animal experiments were approved by the institutional ethics committee for Laboratory Animal Research, Nagoya University School of Medicine, and were performed according to the guidelines of the institute.

Reagents and cells

RPMI 1640, heat-inactivated fetal bovine and horse serum, and α-minimal essential medium were purchased from Gibco-BRL (Carlsbad, CA, USA). Human BMSCs and ADSCs were established from healthy volunteers of similar age (20–30 years). Briefly, bone marrow cells and fat tissues were obtained with informed consent from four and five individuals, respectively, and were then processed as described elsewhere [14]. Before experimental use, we confirmed that the MSCs possessed the ability to differentiate into adipocytes and osteoblasts. Cultures between passages 4–8 were used. $CD34^+$ hematopoietic stem cells were mobilized by G-CSF into the periphery, collected and frozen at −130 °C until use.

In vitro cell proliferation studies

Human BMSCs and ADSCs were plated (5×10^3 cells/well, three independent determinations per MSC) onto 24-well plates. After 72-h incubation, the cells were trypsinized and viable cells were counted using trypan blue exclusion.

Co-culture of $CD34^+$ progenitor cells with BMSCs or ADSCs

1×10^5 human $CD34^+$ PBSCs suspended in long-term culture medium were applied (1×10^5 cells in 2 ml) to feeder layers comprising human BMSCs or human ADSCs, as described previously [13]. The co-cultures were incubated for 4 weeks with replenishment of the culture medium twice per week. Non-adherent viable cells were counted at the indicated time points and were analyzed by FACS at the end of incubation as described elsewhere [13]. Co-culture experiments were repeated three times.

In vitro progenitor assays

Effects of human MSCs on progenitor cells were analyzed using a colony-forming cell assay. The following human cells (5×10^2 each) were plated in 0.5 mL of methylcellulose media (Stemcell Technologies, Vancouver, Canada): BMSCs, ADSCs, $CD34^+$ PBSCs, PBSCs plus BMSCs and PBSCs plus ADSCs. Colonies of >50 cells were scored after 8-days of incubation. Experiments were repeated three times.

Intra-bone marrow transplantation

Human BMSCs, human ADSCs (1×10^5 cells each in 10 μl of RPMI 1640), or 10 μl of RPMI 1640 were injected into the right tibiae of irradiated (3.0 Gy) 6- to 8-week-old NOD/SCID mice (4–5 mice per subgroup, Chubu Kagaku Shizai, Nagoya, Japan) using a Hamilton syringe. All mice were killed 5 weeks after injection, and the tibiae and major organs were excised for histological evaluation. For quantitative analysis of BM cellularity, 4 fields were randomly selected and the number of nucleated cells was scored under a microscope.

Statistical analysis

Statistical significance of group differences was evaluated using Student's t test and Excel software (Microsoft, Redmond, WA, USA).

Results

Human ADSCs can be expanded faster than human BMSCs

An in vitro proliferation assay showed that the number of BMSCs increased 1.16- to 2.32-fold above the input cell number (5000 cells/well) after 72-h incubation. In contrast, the fold increase in the number of ADSCs (3.5–4.0) was significantly higher (Fig. 1a).

Human ADSCs support human $CD34^+$ PBSCs to a greater degree than human BMSCs

To analyze the ability of human ADSCs to induce granulocyte differentiation of human $CD34^+$ PBSCs, co-culture assays were performed. Human ADSCs yielded significantly more non-adherent cells from human $CD34^+$ PBSCs than human BMSCs (Fig. 1b, upper panel). As noted previously [13], round-shaped hematic cells grew in clusters, suspended in the culture supernatant or loosely attached to supportive stroma (Fig. 1b, lower right panel). FACS analysis of non-adherent cells showed that these cells differentiated into $CD33^+$ granulocytes derived from human $CD34^+$ PBSCs (Fig. 1b, lower right panel). These results suggest that human ADSCs significantly enhance proliferation of myeloid cells from human $CD34^+$ PBSCs compared with human BMSCs.

In vitro progenitor assays revealed that human ADSCs promoted a higher frequency of early progenitors than human BMSCs (Fig. 1c), whereas neither human BMSCs nor human ADSCs alone generated colonies (not shown). The major lineages of the colonies were colony-forming unit (CFU) granulocytes and CFU granulocyte-macrophages. Few erythrocyte colonies were observed. However, there was no significant difference in the percentage of CFU granulocytes or CFU granulocyte-macrophages within $CD34^+$ PBSCs in the presence or absence of BMSCs or ADSCs (data not shown). Representative results from three independent experiments are shown.

Safety of human ADSCs in vivo

To ensure the safety of human ADSCs in vivo, we injected human BMSCs or ADSCs (1×10^5 cells each) into the right tibiae of irradiated 6–8 weeks old NOD/SCID mice (one mouse per each individual MSC). We observed no evidence of MSC-induced toxicity (e.g., body weight loss or death) either during or after treatment. Histological evaluation 5 weeks after injection showed neither gross morphological nor microscopic change of heart, lung, liver, kidney or spleen (Fig. 2a). Similarly, no microscopic abnormalities such as fatty change or fibrosis were observed in the bone marrow of mice that received MSCs (Fig. 2b). Interestingly, BM cellularity in mice that had received human ADSCs was significantly higher than that of mice that had received human BMSCs (Fig. 2b).

Discussion

We have recently demonstrated that ADSCs from mice can facilitate hematopoiesis more effectively than BMSCs [13]. These data suggested that ADSCs possess clinical potential to facilitate hematopoiesis. However, human cells and mouse cells do not always behave similarly. To determine the hematopoiesis-supporting properties of human ADSCs, we established BMSCs and ADSCs from a number of individuals of similar age, because the number, differentiation potential, and maximal life span of BMSCs decline with increasing age [11] while ADSCs are abundant even in the elderly [15]. Although we were not able to establish BMSCs and ADSCs from the same individual, in vitro coculture and progenitor assays clearly showed that human ADSCs generated significantly more granulocytes and progenitor cells from human hematopoietic stem cells (HSCs) than human BMSCs (Fig. 1b, c). Intra-bone marrow transplantation experiments revealed that BM cellularity in mice that had received human ADSCs was significantly higher than that of mice that had received human BMSCs (Fig. 2b). These data clearly suggest that human ADSCs are superior to human BMSCs in terms of hematopoiesis-supporting properties. Bone marrow failure is a heterogeneous disease that is caused by various pathophysiological mechanisms including immune destruction of hematopoiesis, quantitative and qualitative defects in hematopoietic stem cells in addition to perturbation of the hematopoietic microenvironment [2, 16]. ADSCs are useful, not only for supporting hematopoiesis, but also for modulation of immunoreactions [17, 18]. In addition, ADSCs freshly isolated from fat tissue (adipose-derived stem and regenerative cells) using the Celution® system (Cytori Therapeutics, Inc. http://www.cytori.com/Home.aspx) may contain hematopoietic stem and progenitor cells [19]. Again, our data and these facts suggest that fat tissue is a good source of cells that can be used for therapy to reconstitute impaired hematopoiesis.

Analysis of the proliferation capacities showed that ADSCs possessed higher population doubling numbers than BMSCs, which is consistent with a previous report [20]. A prospective randomized study using MSCs as first-line therapy for graft failure after hematopoietic stem cell transplantation (HSCT) showed that two out of six patients with poor hematopoietic recovery after HSCT responded to the infusion of BMSCs (1×10^6/kg) and their blood cell counts increased [21]. The authors speculated that

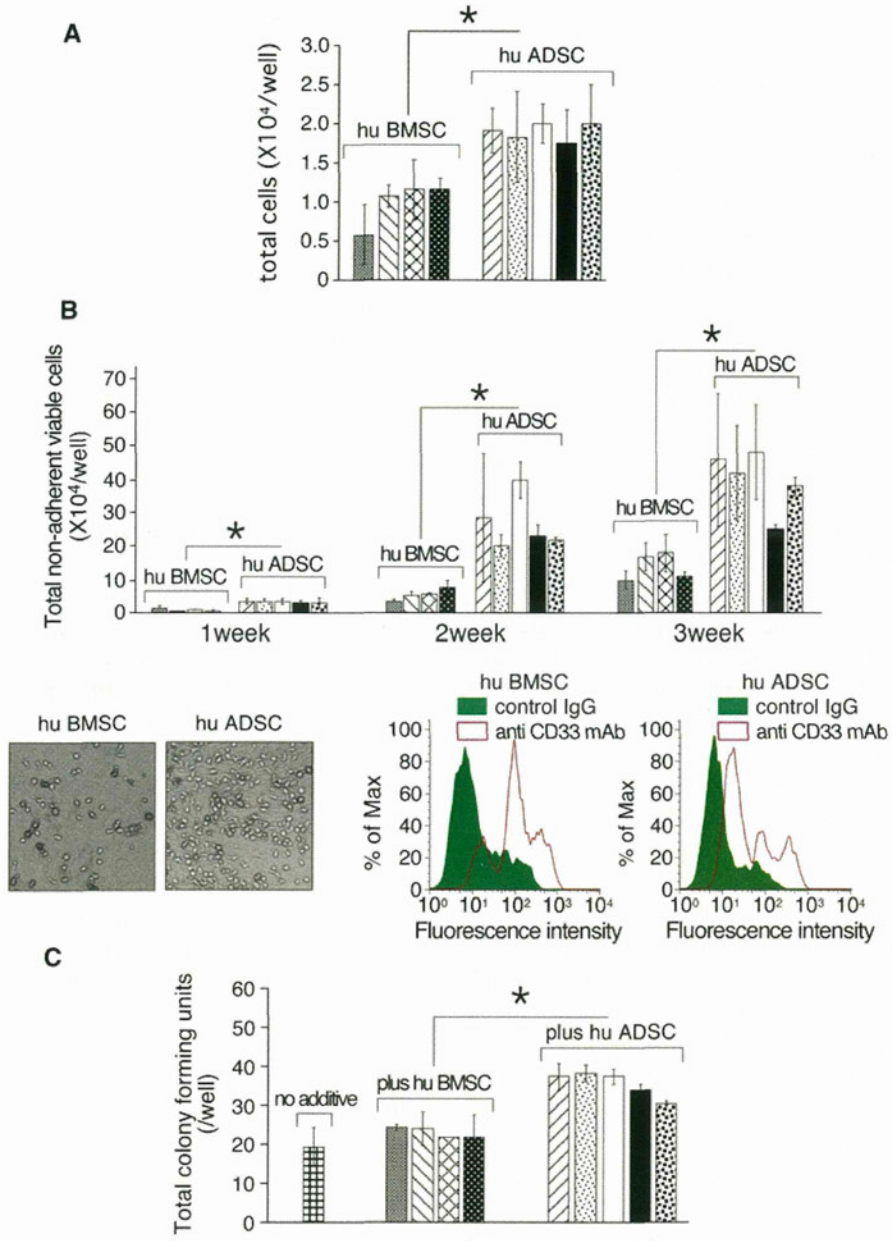

Fig. 1 In vitro comparison of human BMSCs and ADSCs. **a** In vitro proliferation of human BMSCs and ADSCs. Human BMSCs and human ADSCs were plated (5×10^3 cells/well, three independent determinations per MSC) onto 24-well plates. After 72-h incubation the cells were trypsinized and viable cells were counted using trypan blue exclusion. *Each bar* represents cells from one individual. The results are presented as the mean ± SD. The *asterisk* denotes statistical significance ($*P < 0.05$). **b** Co-culture of $CD34^+$ PBMCs with human MSCs. BMSCs and ADSCs were established from four and five individuals, respectively. Cell layers of BMSCs and ADSCs were established on 0.5 % gelatin pre-coated 24-well plates (80 % confluent). $CD34^+$ PBSCs were applied onto the stromal layers (4 wells/each MSC). The cocultures were incubated for 3 weeks with replenishment of the culture medium twice a week. Non-adherent viable cells were counted at the indicated time points (*upper panel*), photographed (*lower left panel*, representative photographs), and analyzed by FACS at the end of the incubation (*lower right panel*, representative results). Each point represents the mean (±SD) of four replicates. The *asterisk* denotes statistical significance ($*P < 0.05$). **c** In vitro progenitor assays using human MSCs. Human $CD34^+$ peripheral blood stem cells (PBSCs: 500 cells, no additive), PBSCs plus BMSCs (500 cells each) or PBSCs plus ADSCs (500 cells each) were plated in 0.5 mL of methylcellulose media containing human recombinant IL-3, SCF, and Epo (3 wells/each MSC). The plates were incubated for 8 days following which progenitors were scored. The results represent the mean (±SD) of three replicates. The *asterisk* denotes statistical significance ($*P < 0.05$)

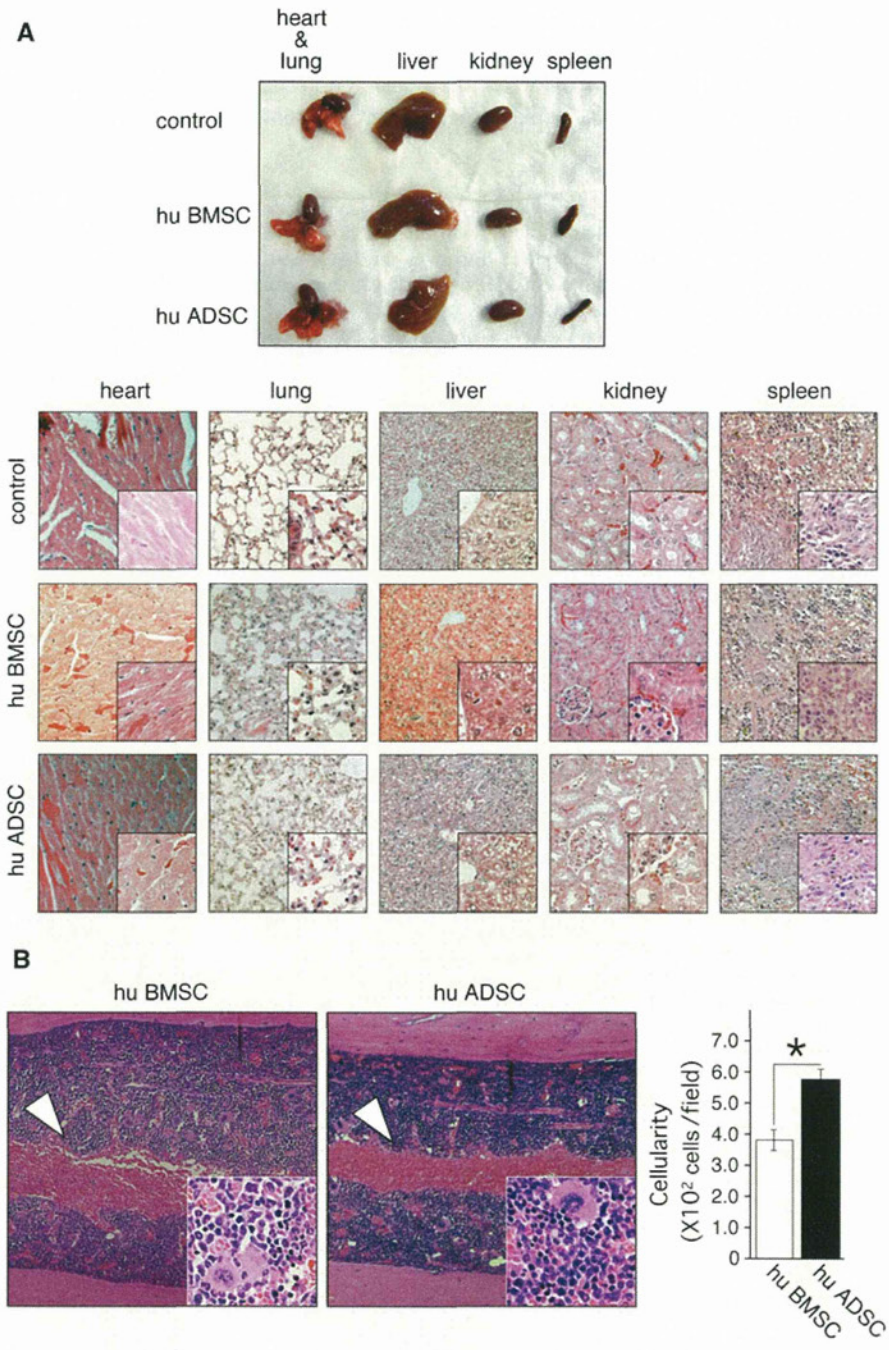

Fig. 2 Intra-bone Marrow Transplantation of human MSCs. Human (*hu*) BMSCs (1×10^5 cells), hu ADSCs (1×10^5 cells) or RPMI 1640 (control) were injected into the right tibiae of irradiated (3.0 Gy) 6- to 8-week-old NOD/SCID mice (one mouse per each MSC). All mice were humanely killed 5 weeks after injection, and the tibiae and major organs were excised for histological evaluation. **a** Representative gross morphology (*upper panel*) and microscopic histology (*lower panel* ×20; *inset* ×40) of heart, lung, liver, kidney, and spleen from control, BMSC- and ADSC-treated mice. **b** Representative microscopic histology of the BM stained with H&E stain (×10). *Inset* BM cellularity in mice that had received human BMSCs or ADSCs (×60). The cavities into which the 31-gauge needle had been inserted were filled with red blood cells (*arrowheads*). For quantitative analysis of BM cellularity, 4 fields were randomly selected and the number of nucleated cells was scored under a microscope. The mean nucleated cells per field ± SD is shown for each group. Statistical significance: $^*P < 0.05$

administration of 1×10^6 cells/kg body weight might be insufficient to restore a functional hematopoietic microenvironment. According to our recent findings, 1×10^5 ADSCs per mouse (about 25 g in weight) were required to facilitate hematopoiesis [13], which would be equivalent to 4×10^6 ADSCs per kilogram for adult humans. These data suggest that large quantities of infused cells are required for treatment. Thus, the rapid expansion capacity of ADSCs will also be advantageous for their clinical exploitation.

We observed no evidence of human ADSC-induced toxicity either during or after treatment. Histological evaluation 5 weeks after injection showed neither gross morphological nor microscopic changes in major organs of mice that received human ADSCs, which are sometimes a clue to the presence of latent adverse effects [22] (Fig. 2a). It has been reported that MSCs that persist in vivo may be tumorigenic [23]. However, we found that human ADSCs alone formed no colonies in semi-solid cultures (not

shown) and no tumors in vivo (Fig. 2). These data indicate the clinical safety of human ADSCs.

In summary, these data provide an important step in the regeneration of a perturbed hematopoietic microenvironment by ADSCs in a clinical setting.

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A significant association of viral loads with corneal endothelial cell damage in cytomegalovirus anterior uveitis

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ABSTRACT

Aim The aim of the study was to investigate the correlation between the clinical manifestation and the cytomegalovirus (CMV) viral load in the aqueous humour of patients with CMV anterior uveitis.

Methods Seven patients with CMV-associated iridocyclitis and four patients with CMV-associated corneal endotheliitis were enrolled. Presence of CMV, but not other human herpes viruses, was confirmed by multiplex polymerase chain reaction (PCR). Viral load was measured using real-time PCR. Clinical manifestations were examined using a slit-lamp microscope and ophthalmoscope, applanation tonometer and specular microscope.

Results All 11 patients had unilateral recurrent anterior uveitis with high intraocular pressure and mutton fat keratic precipitates with pigmentation. Stromal oedema of the cornea was found in CMV-associated endotheliitis, but not in CMV-associated iridocyclitis patients. A significant corneal endothelium cell loss was recorded in all 11 patients with CMV-associated endotheliitis and iridocyclitis patients. High viral loads of CMV were detected in the aqueous humour of all 11 patients. A significant association was found between the corneal endothelial cell loss intensity and CMV viral load in the aqueous humour.

Conclusion There is a significant correlation between the CMV viral load and corneal endothelial cell loss in both CMV-associated iridocyclitis and corneal endotheliitis.

INTRODUCTION

Cytomegalovirus (CMV) is a member of the human herpes virus family and is found in latent infections in the majority of the adult population. In immunocompromised hosts, the virus causes necrotising retinitis,[1] but has been thought not to cause any diseases in immunocompetent hosts. However, a previous study showed local production of anti-CMV antibodies in the aqueous humour of an immunocompetent patient with iridocyclitis with elevated intraocular pressure (IOP).[2] In addition, recent studies using qualitative PCR have demonstrated that genomic CMV DNA is present in the aqueous humour of immunocompetent patients with unilateral iridocyclitis[3–6] as follows. Markomichelakis *et al*[3] reported two cases of iridocyclitis with sectoral iris atrophy in which CMV was detected by PCR, and de Schryver *et al*[4] also reported five similar cases. In the recent report by Chee *et al*,[5] they studied if there was a relationship between the CMV viral load in the aqueous and clinical manifestation of the diseases such as either acute or chronic iridocyclitis, eg Posner–Schlossman syndrome and Fuchs heterochromic iridocyclitis. CMV genomic DNA was also detected in the aqueous humour of immunocompetent patients with another inflammatory condition of the eye, ie corneal endotheliitis, in three previous reports.[7–9] Corneal endotheliitis is an inflammatory condition at the corneal endothelium in which keratic precipitates (KPs) develop together with severe stromal oedema in the cornea, whereas iridocyclitis has cells and flare in the anterior chamber with or without KPs but no stromal oedema in the cornea.

The real-time PCR made it possible to measure the viral load quantitatively. Thus, the use of this assay makes it possible to determine the clinical significance of the viral infection in the pathogenesis of human diseases. Our previous report showed a high CMV genomic DNA load in the aqueous humour in an immunocompetent patient with unilateral iridocyclitis with high IOP.[6] However, the correlation between the viral load in the aqueous humour and the clinical manifestation of the disease (iridocyclitis versus corneal endotheliitis) was not investigated. Therefore, we examined if there was any correlation between the CMV viral load in the aqueous humour and the clinical manifestation of anterior inflammatory diseases associated with CMV. We showed a significant correlation between the CMV viral load in the aqueous humour and the endothelial cell damage of the cornea in patients with iridocyclitis and corneal endotheliitis associated with CMV.

MATERIALS AND METHODS

Subjects

Between 2006 and 2008, 11 patients with CMV-associated inflammation in the anterior segment of the eye, ie seven patients with CMV-associated iridocyclitis and four patients with CMV-associated corneal endotheliitis, were enrolled. These patients were from Tokyo Medical and Dental University Hospital (Tokyo, Japan), Miyata Eye Hospital (Miyakonojo, Miyazaki, Japan) and Kyoto Prefectural University Hospital (Kyoto, Japan). Diagnosis was made based on clinical manifestations and the qualitative detection of the CMV genomic DNA in the aqueous humour by the multiplex PCR. The viral load in the aqueous humour was further measured quantitatively by the real-time PCR.

An aliquot of 0.1 ml of the aqueous humour was aspirated with a 30G needle after disinfection and

processed for PCR. Anti-viral therapy was not given before the PCR assay, but topical corticosteroids were given by local ophthalmologists to treat intense anterior uveitis. The interval between the disease onset and the aqueous humour sampling varied among the patients.

Polymerase chain reaction

The aqueous humour samples were centrifuged at 1000 *g* for 5 min and used for multiplex PCR and real-time PCR.[10 11] Multiplex PCR was designed to qualitatively measure the genomic DNA of eight human herpes viruses: herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), CMV, and human herpes virus type 6 (HHV-6), type 7 (HHV-7) and type 8 (HHV-8). DNA was extracted from the aqueous humour samples using a DNA minikit (Qiagen, Valencia, California, USA). Multiplex PCR was performed using LightCycler (Roche, Basle, Switzerland). The primers of the glycoprotein gene sequences for CMV were TACCCCTATCGCGTG TGTTC (forward) and ATAG-GAGGCGCCACGTATTC (reverse). The probes used included 3′-fluorescein isothiocyanate: TCGTCGTAGCTACGCTTACAT and LcRed705-5′: ACACCACTTATCTGCTGGGCAGC. Specific primers for the virus were used in conjunction with Accuprim Taq (Invitrogen, Carlsbad, California, USA). PCR amplification conditions used in the current study have been reported previously.[12]

Real-time PCR was only performed for the HHV, with multiplex PCR used to detect the genomic DNA. Amplitaq Gold, with a Real-Time PCR 7300 system (ABI, Foster City, California, USA), was used to perform the procedure. The forward and reverse primers of immediate early (IE)-1 were CATGAAGGT-CTTTGCCCAGTAC and GGCCAAAGTGTAGGCTACAATAG, respectively. FAM-TGGCCCGTAGGTCATCCACACTAGG-TAMRA was used as the probe. The PCR amplification conditions used in the current study were previously reported by Sugita *et al*.[11] When more than 50 copies per tube (5×10^3/ml) were observed, the value of the sample's viral copy number was considered to be significant.

Clinical evaluation

Clinical manifestations of the eye were determined by a slit-lamp microscopic and ophthalmoscopic examination. Each patient underwent best corrected visual acuity (BCVA) measurement using a Japanese standard decimal visual acuity chart (Landolt ring chart) after treatment. Anterior chamber flare was measured by a laser flare photometer (FC-1000; Kowa Electronics, Nagoya, Japan). A photograph of the central cornea using a specular microscope (NONCON ROBO FA-3509; Konan Medical, Nishinomiya, Japan) was used for evaluation of the corneal endothelial cells. In cases of corneal endotheliitis, intense corneal oedema disturbed the measurements of the corneal endothelium, and we measured corneal endothelial cell counts after the inflammation was reduced by the treatment.

Evaluation of corneal endothelial cell loss

The relationship between the CMV viral load in the aqueous humour and the intensity of the corneal endothelial cell loss was assessed. The corneal endothelial cell loss was determined according to the following formula:

$$\text{Corneal endothelial cell loss}(\%) = 100 - (\text{endothelial cell counts in affected eye})/(\text{endothelial cell counts in the fellow eye})\times 100$$

Statistical analysis

Statistical analysis was performed using the Mann–Whitney U test. Statistical significance was set at $p<0.05$. Linear regression analysis was performed using the Spearman's correlation coefficient by rank test.

RESULTS

Clinical manifestations

Nine men and two women ranging in age from 23 to 71 years (mean age 60.6 years) were enrolled in the study. No abnormalities were found in the systemic investigations and laboratory tests. Serology examinations for human immunodeficiency virus were all negative. None of the patients had any history of eye surgery prior to the onset of uveitis. Clinical findings of the CMV-associated iridocyclitis patients (n=7) and corneal endotheliitis patients (n=4) are shown in table 1. A unilateral mild anterior uveitis with high IOP was noted in all 11 patients. There were no significant differences between the iridocyclitis and corneal endotheliitis groups in the cells and flare values in the anterior chamber, nor were there any differences noted for the elevated levels of IOP, KPs, gonioscopic findings and iris atrophy. Stromal oedema of the cornea was seen in all corneal endotheliitis but not in iridocyclitis patients. While the stromal oedema was diffuse in three out of the four patients, it was localised at upper cornea in one of the corneal endotheliitis patients. Representative cases for iridocyclitis and corneal endotheliitis are shown in figures 1 and 2, respectively. As for the IOP elevation, all 11 eyes required anti-glaucoma medications, with two eyes (cases 1 and 2) requiring trabeculectomy. With regard to the iris atrophy, no sectorial iris atrophy was seen in all 11 eyes, although four eyes (two each in the iridocyclitis and the corneal endotheliitis groups, respectively) presented diffuse iris atrophy.

Systemic valganciclovir therapy (1800 mg/day for longer than 3 weeks) in conjunction with topical corticosteroids and

Table 1 Clinical findings in patients with CMV anterior uveitis

Case	Age (years)	Sex	Eye	Diagnosis	Corneal oedema	KPs	Cells in AC	Flare in AC	IOP (mmHg)	Pigmentation in the AC angle	Iris atrophy
1	66	M	R	Iridocyclitis	-	Mutton-fat	1+	17	38	Depigmentation	None
2	62	M	R	Iridocyclitis	-	Mutton-fat	1+	26	40	PAS and pigment	Diffuse
3	56	M	L	Iridocyclitis	-	Mutton-fat	1+	13	44	Depigmentation	Diffuse
4	53	F	R	Iridocyclitis	-	Mutton-fat	1+	13	36	Depigmentation	None
5	71	M	L	Iridocyclitis	-	Mutton-fat	2+	28	25	PAS	None
6	63	M	R	Iridocyclitis	-	Fine	1+	Nt	50	Depigmentation	None
7	23	M	R	Iridocyclitis	-	Fine	1+	Nt	25	Depigmentation	None
8	71	M	R	Endotheliitis	+ (diffuse)	Mutton-fat	2+	151	37	PAS	None
9	67	M	R	Endotheliitis	+ (diffuse)	Fine	1+	14	25	Depigmentation	Diffuse
10	64	F	L	Endotheliitis	+ (superior)	Fine	1+	21	28	Depigmentation	None
11	71	M	R	Endotheliitis	+ (diffuse)	Mutton-fat	1+	12	43	PAS	Diffuse

Information from 11 patients with CMV anterior uveitis were reviewed. Data collected included intraocular pressure and clinical manifestation of the anterior segments in the affected eye.
AC, anterior chamber; F, female; KP, keratic precipitate; M, male; Nt, not tested; PAS, peripheral anterior synechia.