age) who were expected to survive beyond 100 days after HSCT were eligible. The eligibility criteria also included serum creatinine less than twice the upper normal limit, as well as serum total bilirubin less than 1.5 times, and aspartate aminotransferase, alanine aminotransferase and gamma-glutamyltranspeptidase less than three times the upper normal limit. Left ventricular ejection fraction $\geqslant 50\%$ or arterial blood oxygen saturation $\geqslant 94\%$, and in adult patients a carbon monoxide lung diffusing capacity $\geqslant 60\%$, were required. Patients with arrhythmia, hypertension or diabetes mellitus that was difficult to control despite medication, severe cardiopulmonary or renal disease, chronic active hepatitis, liver cirrhosis, acute hepatitis, ascites more than 1 l, central nervous system disorders, active infection; positive hepatitis B surface antigen, hepatitis B core antibody, hepatitis C virus antibody or human immunodeficiency virus antigen/antibody; or prior HSCTs were all excluded. Patients were also required to have either BM available from an HLA-matched related or unrelated donor or G-CSF-mobilized PBSCs available from an HLA-matched related donor without T-cell depletion. The study was conducted in conformity with ICH-GCP and the Declaration of Helsinki. The protocol and informed consent forms were approved by each institution's Research Ethics Committee. All patients gave written informed consent prior to their participation in the study.

Conditioning regimen

The i.v. BU (KRN246; Kirin Pharma Co. Ltd., Tokyo, Japan) was given at 0.8 mg/kg through a central venous catheter for 2 h every 6 h at a total of 16 doses for 4 days on days −7 to −4. CY 60 mg/kg was administered through a central venous catheter for 3 h at a total of two doses for 2 days on days −3 and −2. After a rest on day −1, BM or G-CSF-mobilized PBSC without T-cell depletion was infused on day 0. A fixed-dose regimen for BU was calculated based on either the ideal body weight or actual body weight, whichever was less, for adults (18–55 years of age) and the actual body weight for children (over 5 and less than 18 years of age).

Supportive care

For seizure prophylaxis, phenytoin was administered at 5–10 mg/kg/day (upper limit of 300 mg/kg/day) in 2–3 divided doses starting from 2 days before initiation (day −9) to 48 h after completion of BU administration (day −2). G-CSF was administered on day 1 or 5 until engraftment. For patients undergoing allogeneic HSCT, GVHD prophylaxis consisted of CYA (3 mg/kg/day by continuous i.v. infusion from day −1 in related and 3–5 mg/kg/day in unrelated transplantation) and short-term methotrexate, that is, 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6 in related pairs or 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3, 6 and 11 in unrelated pairs. Mesna was administered at a dose equivalent to 120% of CY on days −3 and −2. Other supportive treatments including antiemetic administration, antibiotic treatment, transfusion support, GVHD treatment and VOD treatment were given according to the standards of each hospital.

Evaluation of clinical data

The efficacy variables were myeloablation, engraftment, relapse, overall survival (OS) and disease-free survival (DFS). The safety variables were non-relapse mortality and adverse events included convulsive seizure, VOD, acute GVHD and other organ toxicities. Engraftment was defined as an absolute neutrophil count of 0.5×10^9/l for three consecutive days. Engraftment failure was defined as the failure to reach an absolute neutrophil count of 0.5×10^9/l by day 28 after transplantation. OS was measured as the time from the day of transplantation until death from any cause, and DFS as the time from the day of transplantation until disease relapse or death from any cause. Relapse, OS and DFS were calculated using the Kaplan–Meier method.[23] non-relapse mortality was defined as any death without progression of the underlying disease. Patients were monitored daily for adverse events, hematology and transplant-related complications. After discharge, patients were followed weekly for adverse events and transplant-related complications, and monitored weekly for hematologic and biochemical data through 100 days after transplantation. The appearance of VOD by day 30 was evaluated based on any two of the major criteria as established by McDonald *et al.*[24] and Jones *et al.*[25] GVHD was graded according to the consensus criteria.[26,27] Kirin Pharma Co. Ltd. provided financial support for the medical costs associated with the conditioning regimen, including i.v. BU for enrolled patients, monitored source data and entered these data in a database. Statistical analysis was performed using SAS software (version 8.02; SAS Institute, Cary, NC, USA).

PK sampling and analysis

The objective of this study was to describe the PK characteristics of i.v. BU, with parameters including BU concentrations for the first and ninth administrations and the accumulation of i.v. BU. Plasma samples were collected from all patients at designated times, in conjunction with the first and ninth doses as follows: immediately before drug infusion and at 15, 30 and 45 min after the start of infusion, at 5 min before the end of infusion and at 15, 30, 60, 120, 180 and 240 min after completion of infusion. In addition, one sample was taken immediately before the 13th infusion and 5 min before its completion. The plasma was assayed using a gas chromatographic-mass spectrometric detection method.[10]

Plasma concentrations for first and ninth dose in individual subjects were analyzed by the non-compartmental method using WinNonlin (version 3.3; Pharsight Corp., Mountain View, CA, USA). The maximum plasma concentration (C_{max}) and the time to reach maximum plasma drug concentration (t_{max}) were observed values. The terminal half-life ($t_{1/2}$) was calculated as $\ln 2/k_{el}$, where k_{el} was the elimination rate constant, determined by log-linear regression of the terminal phase data points. The area under the plasma concentration–time curve from time 0 to infinity (AUC_{inf}) for the first dose was calculated as $AUC_{0-t} + C_t/k_{el}$, where AUC_{0-t} was the AUC from time 0 to the last detectable time, calculated using linear trapezoidal rule, and C_t was the plasma concentration at

the last detectable time. AUC at steady state (AUC_{ss}) for the ninth dose was calculated by the linear trapezoidal rule. Clearance (CL) was calculated as dose/AUC. Volume of distribution (V_z) was calculated as CL/k_{el}. CL and V_z were normalized to actual individual body weight (CL/ABW and V_z/ABW) on the day of dosing. Summary statistics were obtained for C_{max}, t_{max}, $t_{1/2}$, AUC, CL/ABW and V_z/ABW at the first and ninth dose. The AUC at dose 1 (AUC_{inf}) and dose 9 (AUC_{ss}) and the trough concentration ($C_{p,trough}$) and peak concentration ($C_{p,peak}$) at doses 9 and 13 were calculated and compared by preparing each plot.

Results

Patient characteristics

Thirty Japanese patients were registered in this prospective trial between July 2002 and October 2003. The disease characteristics and status at transplantation are given in Table 1. The median age of the patients was 30 years (range, 7–53 years). The median body mass index (BMI) was 22.65 (14.4–29.1), and the mean BMI was 22.32 ± 3.47. There were no patients with moderate or severe obesity (BMI <30). The diseases were AML in 13 patients (43%), ALL or CML in chronic phase in five patients each (17%), non-Hodgkin lymphoma (NHL) in four patients (13%) and MDS in three patients (10%). In total, 11 of the 12 patients with AML were in CR. Four of the five patients with ALL were in CR. Three patients with MDS included refractory anemia, refractory anemia with excess blasts and refractory anemia with excess blasts in transformation. Four patients with NHL included diffuse large B-cell lymphoma in CR ($n=2$), primary refractory peripheral T-cell lymphoma ($n=1$) or suspected extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue in CR ($n=1$). One patient with AML who was in remission at registration was subsequently withdrawn from protocol treatment due to onset of cardiac myopathy on day −3, and CY was changed to fludarabine. Owing to an additional protocol violation, this patient was excluded from the objective group in the analysis.

Table 1 Patient characteristics

Variables	n *(%)*	
	Allogeneic HSCT (n=28)	*Autologous HSCT (n=2)*
Patient age (years) (range, median)	7–53, 30	48–50, 49
5–17	3 (11)	0
18–49	20 (71)	1 (50)
50–55	5 (18)	1 (50)
Gender		
Men	18 (64)	2 (100)
Women	10 (36)	0
Disease		
AML	12 (43)	1 (50)
ALL	5 (18)	0
CML	5 (18)	0
Myelodysplastic syndrome	3 (11)	0
Non-Hodgkin lymphoma	3 (11)	1 (50)
Disease status		
CR, CP, RA	23 (82)	2 (100)
NR, RAEB, RAEB-t	5 (18)	0
Prior chemotherapy	26 (93)	2 (100)
Prior radiotherapy	2 (7)	0
Source of stem cells		
BM	18 (64)	0
Peripheral blood cells	10 (36)	2 (100)
Related or unrelated donor		
Related	19 (68)	NA
Unrelated	9 (32)	NA
Cell dose infused		
Nucleated (× 10^8/kg, median, range)	2.6 (0.7–4.4)	NA
CD34 positive (× 10^6/kg, median, range)	2.7 (2.1–6.3)	2.9 (2.7–3.1)

Abbreviations: CP = chronic phase; HSCT = hematopoietic SCT; NA = not applicable; NR = non-remission; RA = refractory anemia; RAEB = refractory anemia with excess of blasts; RAEB-t = refractory anemia with excess of blasts in transformation.

Engraftment

Twenty-eight patients (97%) achieved engraftment at a median of 14 days (range, 9–20 days) and 11 days after allogeneic and autologous HSCT, respectively (Table 2). One patient who received unrelated BMT for CML had graft failure. No secondary engraftment failure was observed.

Toxicity and complications

All adverse events were those that are commonly observed in HSCT and no characteristic events related to i.v. BU were observed. None of the patients had to interrupt i.v. BU treatment because of adverse events. The number of observed adverse events was 714 in 27 patients who received allogeneic HSCT and 19 in two patients who received autologous HSCT. The most frequent adverse events in the 27 allogeneic HSCT patients were vomiting and nausea in 20 patients each (74%), anorexia in 19 patients (70%), stomatitis and diarrhea in 18 patients each (67%) and headache in 17 patients (63%; Table 2). Both of the autologous HSCT patients showed stomatitis, vomiting, catheter-related infection, anorexia and dysgeusia. No seizures were observed, and with regard to other neuropsychological profiles, seven patients experienced mild dysgeusia, one moderate systemic burning sensation, one severe tremor, one severe mood change and one severe insomnia in an allogeneic setting. With regard to cardiovascular profiles, one patient experienced mild cardiac failure and the other developed moderate cardiomyopathy due to CY in the allogeneic setting, as described above. This patient had completed i.v. BU administration for 4 days and CY once. When the patient complained of chest discomfort, the heart rate was 101 beats/min, and her electrocardiography showed ST depressions in leads II, III, aVF and V_1–V_6 1 h after the completion of the first dose of CY, which made suspected diagnosis of CY-induced cardiomyopathy. The signs and symptoms subsided shortly, and the second dose of CY on day −2

Table 2 Regimen-related toxicity, engraftment, GVHD and death

Outcome	*Allogeneic HSCT (n = 28) (%)*	*Autologous HSCT (n = 2) (%)*
Toxicity		
Vomiting	21 (75)	2 (100)
Nausea	21 (75)	1 (50)
Anorexia	19 (68)	2 (100)
Stomatitis	18 (64)	2 (100)
Diarrhea	18 (64)	0 (0)
Headache	18 (64)	0 (0)
Seizure	0 (0)	0 (0)
VOD	1 (4)	0 (0)
	Allogeneic HSCT (n = 27) (%)	*Autologous HSCT (n = 2) (%)*
Engraftment	26 (96)	2 (100)
Median (days)	14	11
Range (days)	9–20	11
Graft failure	1 (4)	0 (0)
Acute GVHD	13 (48)	—
Grade I	4 (15)	—
Grade II	5 (19)	—
Grade III	2 (7)	—
Grade IV	2 (7)	—
Chronic GVHD	16 (59)	—
Death	8 (30)	0 (0)
Relapse	4 (15)	0 (0)
Non-relapse	4 (15)	0 (0)

Abbreviations: HSCT = hematopoietic SCT; VOD = venoocclusive disease.

was substituted by fludarabine with no subsequent complications.

One patient who received allogeneic HSCT was diagnosed with mild VOD on day 1 based on two diagnostic criteria,[24,25] which resolved on day 3. In another patient, elevated total bilirubin and body weight gain were found on days 60–69, and this was not confirmed to be VOD based on these criteria. Opportunistic infection occurred in 16 of 27 patients (59%), with a median onset of day 113 (range, 7–399). Pulmonary complications occurred in 7 of 27 patients (26%), with a median onset of day 149 (range, 65–335).

GVHD

Acute GVHD occurred in 13 of the 27 patients (48%) who received allogeneic HSCT; four (15%) had grade I, five (19%) grade II and two each (7%) grades III or IV (Table 2). Acute GVHD was documented in 7 of the 19 patients (37%) who received related transplantation (six had grades II–IV), and in six of the eight patients (75%) who received unrelated transplantation (three patients had grades II–IV). Acute GVHD occurred with a median onset of day 45 (range, 7–98). Chronic GVHD occurred in 16 of 27 patients (59%) with a median onset of day 133 (range, 39–239).

Causes of death

Four patients (15%) died of non-relapse causes (Table 2). One patient who received allogeneic HSCT died of multi-

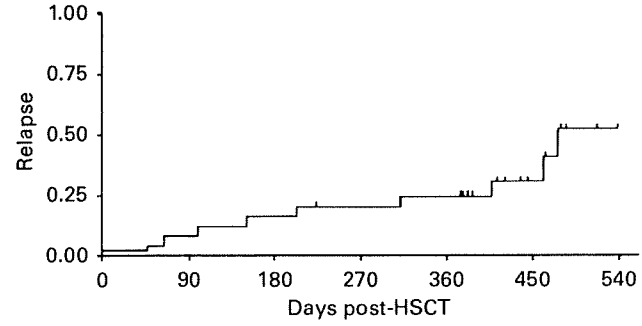

Figure 1 Disease relapse after i.v. BU and CY prior to allogeneic hematopoietic SCT in patients with leukemia and lymphoma.

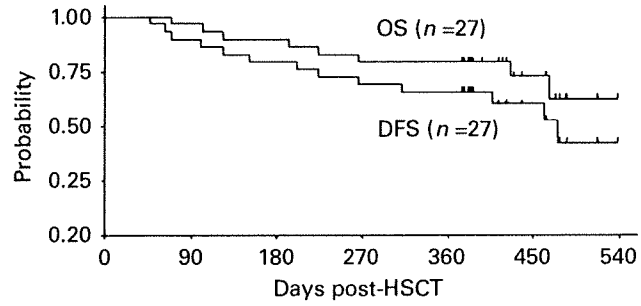

Figure 2 Overall survival and disease-free survival after i.v. BU and CY prior to allogeneic hematopoietic SCT in patients with leukemia, myelodysplastic syndrome and lymphoma.

organ failure due to aggravated GVHD on day 69. Three patients who received allogeneic HSCT died of chronic GVHD on day 223, hepatic failure due to unknown reasons on day 266 (with extensive chronic GVHD and methicillin-resistant *staphylococcus aureus* (MRSA) pneumonia) and pneumonia due to adenovirus and cytomegalovirus on day 124. Four patients (15%) died of relapse.

Relapse and survival

Relapse occurred in 9 of the 23 evaluable allogeneic HSCT patients with leukemia and lymphoma (39%). None of the 23 evaluable patients had central nervous system relapse. The relapse rates at days 100 and 365 were 18% (95% confidence interval (CI), 0–38%) and 26% (95% CI, 8–45%), respectively (Figure 1). The median day of relapse was day 202 (range, 46–476).

OS at days 100 and 365 in allogeneic HSCT was 96% (95% CI, 88–100%) and 78% (95% CI, 62–94%), respectively, with the median follow-up of 413 days (range, 69–537 days) (Figure 2). The median day of death in eight allogeneic HSCT patients was day 208 (range, 69–467). DFS at days 100 and 365 in allogeneic HSCT was 81% (95% CI, 63–99%) and 63% (95% CI, 45–81%), respectively (Figure 2). The two autologous HSCT patients were alive disease-free at day 365.

PK analysis

Intensive PK sampling was assessed at doses 1 and 9 of i.v. BU, and peak and trough levels were obtained at dose 13. Although these analyses were completed in all 30 patients,

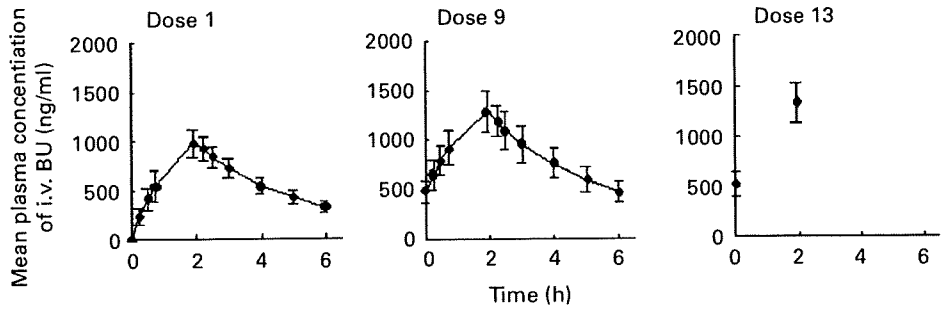

Figure 3 Pharmacokinetic results of i.v. BU at doses 1, 9 and 13 ($n=30$).

data from one patient were excluded from the objective analysis group as noted above. All PK parameters for dose 1 were obtained from 29 patients. For dose 9, all PK parameters except for C_{max} and t_{max} were obtained from 28 patients because the last sample for one patient was collected after initiation of the next dose (Figure 3). The documented plasma concentration of i.v. BU increased over the 2-h period of infusion, with C_{max} observed in the last 5 min, and this was followed by a rapid decrease. The profile of trough and peak levels was essentially the same between doses 9 and 13.

The resulting parameters are listed in Table 3. The mean AUC for doses 1 and 9 was 1171 μmol min/l (coefficient of variation (CV) = 19%) and 1242 μmol min/l (CV = 17%), and the mean C_{max} was 994 ng/ml (CV = 12%) and 1311 ng/ml (CV = 15%), respectively. The mean CL/ABW was 2.66 ml/min/kg (CV = 17%) and 2.46 ml/min/kg (CV = 15%), respectively. V_z/ABW was 0.60 l/kg (CV = 9%) and 0.60 l/kg (CV = 11%), respectively. The AUC of the initial dose was below 1500 μmol min/l in 27 patients (90%), and this was within the range of 900–1350 μmol min/l in 21 of the 29 patients (72%).

The AUC for doses 1 and 9 are compared in Figure 4, which supports both intra- and interpatient predictability and consistency. In the patient who developed VOD, the AUC for doses 1 and 9 was 1102 and 1181 μmol min/l, respectively, whereas for the remaining patients without VOD, it was 1173 μmol min/l (CV = 19%) and 1244 μmol min/l (CV = 17%).

Pediatric patients

A 7-year-old girl with AML in first remission received allo-BMT from a matched unrelated donor. Her body weight and BMI were 17.8 kg and 14.4, respectively. Her AUC was 963.9 μmol min/l. Her regimen-related toxicities were grade 3 vomiting and grade 2 acute hemorrhagic gastritis and hypoalbuminemia. She is alive without graft failure or relapse.

A 13-year-old boy with CML in first chronic phase received allo-BMT from a matched unrelated donor. His body weight and BMI were 46.7 kg and 18.8, respectively. His AUC was 932.6 μmol min/l. His regimen-related toxicities were grade 4 anorexia and grade 2 fatigue and vomiting. He did not achieve engraftment by day 28, and he soon received a second allo-BMT from a mismatched

Table 3 Pharmacokinetics of i.v. BU ($n=30$[a])

	C_{max} (ng/ml)	$t_{1/2}$ (h)	AUC (μmol min/l)	CL/ABW (ml/min/kg)	V_z/ABW (l/kg)
Dose 1					
Mean	999	2.64	1171	2.67	0.596
Median	997	2.66	1144	2.65	0.596
s.d.	124	0.41	216	0.44	0.054
Maximum	1320	3.52	1698	3.72	0.716
Minimum	796	1.97	811	1.94	0.483
Dose 9					
Mean	1317	2.86	1247	2.46	0.601
Median	1315	2.82	1198	2.36	0.605
s.d.	192	0.37	205	0.36	0.068
Maximum	1720	3.59	1686	3.05	0.786
Minimum	964	2.27	889	1.80	0.466

Abbreviations: ABW = actual body weight; AUC = area under the plasma concentration–time curve; CL = clearance; C_{max} = maximum plasma concentration; s.d. = standard deviation; $t_{1/2}$ = terminal half-life; t_{max} = time to observed maximum plasma concentration from dosing; V_z = volume of distribution.

[a]For dose 9, all PK parameters except for C_{max} and t_{max} were obtained from 29 patients because the last sample for one patient was collected after initiation of the next dose.

For dose 1, AUC_{inf} is shown; for dose 9, AUC_{ss} for the 6-h dosing interval is presented.

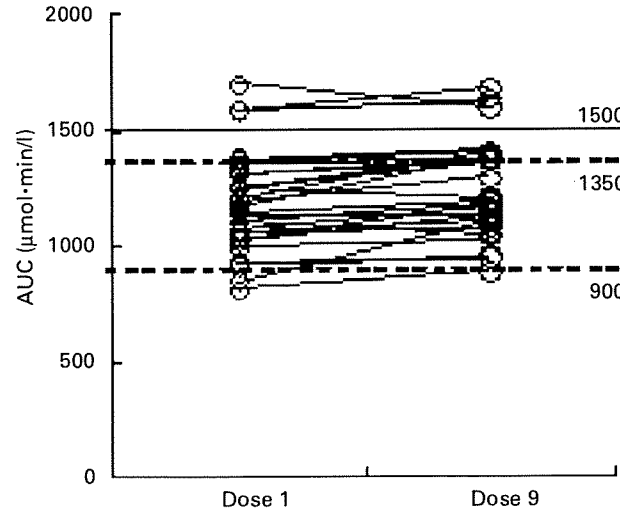

Figure 4 Individual patient area under the plasma concentration–time curve (AUC) values of i.v. BU at doses 1 and 9 ($n=29$).

related donor. He is alive without graft failure or relapse after the second transplant.

A 17-year-old woman with AML in first relapse received allo-BMT from a matched unrelated donor. Her body weight and BMI were 43.2 kg and 17.3, respectively. Her AUC was 902.7 μmol min/l. Her regimen-related toxicities were grade 4 thrombocytopenia, grade 3 febrile neutropenia and grade 2 nausea, vomiting and stomatitis. She died of disease progression on day 193.

Discussion

It has been reported that a high steady-state concentration of BU causes toxicities including VOD,[5–10] whereas a low steady-state concentration leads to graft rejection[10–15] or relapse/progression of the disease.[11] Targeted dose adjustment of BU to maintain the overall systemic exposure within a proper range may reduce these risks.[4–7,14,15] Although it has been reported that there are ethnic differences in PK for a wide range of drugs,[28] this has not been seriously examined with i.v. BU. Therefore, we conducted this drug bioavailability study in a Japanese population. The data obtained were compared with those published mostly overseas. In this study, all observed treatment-related toxicities were as expected, with a low incidence of severe complications. One patient was clinically diagnosed with VOD. This patient showed body weight gain, liver enlargement and right upper abdominal pain, but had no jaundice. As his body weight returned to the baseline within 2 days, this could have been due to overhydration. One patient who developed graft failure had CML and underwent unrelated BMT following interferon therapy, all of which are well-known risks of graft failure.[10,29] The incidence of relapse and the survival rate in this study were similar to those in previous studies.[11,19]

In studies with an oral preparation of BU, it was unclear whether plasma levels of BU correlate with severe regimen-related toxicities.[4,6–8,11] In the pivotal study for US approval of i.v. BU, plasma levels of BU exceeded 1500 μmol min/l in two of the five patients who developed VOD,[19] whereas in our study there was no case of VOD in three patients who had a level over 1500 μmol min/l. This may suggest an ethnic difference in the PK of BU. On the other hand, a population pharmacokinetic analysis of i.v. BU is rare.[30] Our earlier small-scale study revealed high inter- and intrapatient consistency for i.v. BU pharmacokinetics.[22] However, the value of therapeutic drug monitoring remains crucial. Our study demonstrated no essential difference in PK analysis from earlier published Western data,[19] and this supports the notion that racial factors may not seriously influence the bioactivity of i.v. BU.

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CASE REPORT

Cord blood transplantation using minimum conditioning regimens for patients with hematologic malignancies complicated by severe infections

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Abstract Patients with severe infections are thought to be ineligible for cord blood stem cell transplantation (CBT) because the conventional 5–6 day-conditioning regimens potentially makes them susceptible to fatal infections by the time neutrophil engraftment occurs. Two patients were treated with minimum conditioning regimens consisting of 30 mg/m^2 fludarabin (Flu) and 2 g/m^2 cyclophosphamide (CY) on day-1 and total body irradiation (TBI) of 2 or 4 Gy on day −1 or 0 followed by single unit CBT. The reasons for adopting such weak regimen were febrile neutropenia due to the rejection of the first cord blood (CB) graft given to a patient with follicular lymphoma resistant to chemotherapy and pulmonary aspergillosis in another patient with AML who relapsed after CBT. The AML patient received 40 mg/m^2 of melphalan on day-2 to reduce the leukemia burden. Both patients achieved 100% donor chimerism by day 19 and day 20 after CBT without an apparent exacerbation of the infections and remained in remission at 23 and 18 months after the CBT. These findings suggest that the 1–2 day regimens excluding antihuman thymocyte globulin may be sufficiently potent to ensure engraftment of CB in immunocompromised patients and safely administered even when patients are complicated by active infections.

Keywords Cord blood transplantation · Active infection · Minimum intensity conditioning regimen

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1 Introduction

Cord blood (CB) is becoming a major source of allogeneic hematopoietic stem cell transplantation [1, 2]. The success of reduced intensity CB transplantation has accelerated the use of CB for treatment of aged patients with hematologic malignancies [3]. However, patients complicated by severe documented infections are still considered ineligible for cord blood transplantation (CBT) even if reduced intensity regimens are adopted because the preconditioning causes severe neutropenia which usually lasts until day 20 after transplantation [2, 3] and exacerbates infections leading to treatment related-death. As a result, some patients with hematologic malignancies who failed to achieve remission after chemotherapy or those who failed to engraft after allogeneic stem cell transplantation cannot benefit from CBT.

One possible measure to solve this problem is to shorten the time for preconditioning in addition to reducing the intensity. Since most conventional preconditioning regimens take more than 4 days, they need to be started at least 5 days prior to the day of transplantation [4]. Shortening the time for preconditioning to 1 or 2 days may help patients to survive a neutopenic period from the start of preconditioning to neutrophil engraftment. Goggins et al. used a 1-day conditioning regimen consisting of fludarabin (Flu), alemtuzumab and cyclophosphamide (CY) to treat five leukemia patients with allogeneic peripheral blood stem cell transplantation (PBSCT) and observed stable engraftment in three patients. A similar 1-day regimen consisting of Flu, CY and antihuman thymocyte globulin (ATG) was used to treat a myelodysplastic syndrome (MDS) patient with a second allogeneic PBSCT (K. Mochizuki et al., in preparation). The patient suffered from a high fever suggestive of bacteremia due to

persistent neutropenia following the rejection of the first PBSC graft. The second PBSC of another HLA-identical sibling from the original donor successfully engrafted and the patient has been in remission for more than 4 years. However, all of these cases used PBSC grafts containing a high number of hematopoietic stem cells as well as T cells which are thought to be helpful to accelerate the engraftment of donor stem cells and rapid neutrophil recovery. It is still unclear whether CB can engraft after such a very weak regimen and eventually rescue neutropenic patients complicated by severe infections.

This report describes two patients with a devastating condition who were successfully treated with a minimum intensity regimen of 1–2 days followed by single unit CBT.

2 Patients

2.1 Patient 1

In January 2005, a 56-year-old man was diagnosed to have a clinical stage IV follicular lymphoma. He achieved only PR after standard chemotherapy consisting of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone and was refractory to other chemotherapy regimens for salvage. He underwent CBT following a reduced conditioning regimen consisting of cladribine, CY and 4 Gy of total body irradiation (TBI). His neutrophil count remained at 0 on day 21 after the CBT. A chimerism analysis of the bone marrow cells preformed on the same day revealed 100% cells to be recipient-type, thus indicating graft rejection. There was no sign of autologous hematologic recovery and a high fever persisted. There was no sign of an autologous hematologic recovery and a high fever persisted despite the administration of meropenem 1.0 g twice daily and micafungin 300 mg daily. The patient's CRP rose to 25.9 mg/dl on day 25. On day 27 after CBT, he received 30 mg/m^2 Flu and 2 g/m^2 CY followed by 2 Gy of TBI in the morning of the next day. HLA 2 locus-mismatched CB containing 2.6 $\times$ 10^7/kg cells and 9.8 $\times$ 10^6 CD34$^+$ cells/kg was infused 13 h after the completion of CY infusion. Clinical data including HLA alleles of the patient, the first CB donor, and the second CB donor are shown in Table 1. Tacrolimus was given from day-1 for prophylaxis of GVHD. The high fever started abating on day 16 after the second CBT and his neutrophil count surpassed 0.5 $\times$ 10^9/l on day 19. A chimerism analysis performed on day 26 revealed the 100% of the peripheral blood leukocytes were donor-type. Although grade I GVHD occurred, it resolved without treatment. CT scanning on day 33 after the second CBT showed a marked reduction of cervical lymph node swelling in comparison to that at 29 days before the first CBT (Fig. 1). He remains well in partial remission 30 months after the second CBT.

Table 1 Clinical data and HLA alleles of the patients and cord blood donors

	Sex	Blood type	HLA-A	HLA-B	HLA-DR
Patient 1	M	O+	0206/3303	3901/4403	1302/1501
First CB for patient 1	M	O+	0201/3303	3501/4403	1302/1501
Second CB for patient 1	M	A+	1101/3303	3901/4403	0803/1501
Patient 2	F	A+	2402/–	3501/4001	0901/1302
First CB for patient 2	M	AB+	0201/2402	3501/4006	0901/1302
Second CB for patient 2	M	B+	2402/–	4001/4006	0901/1501

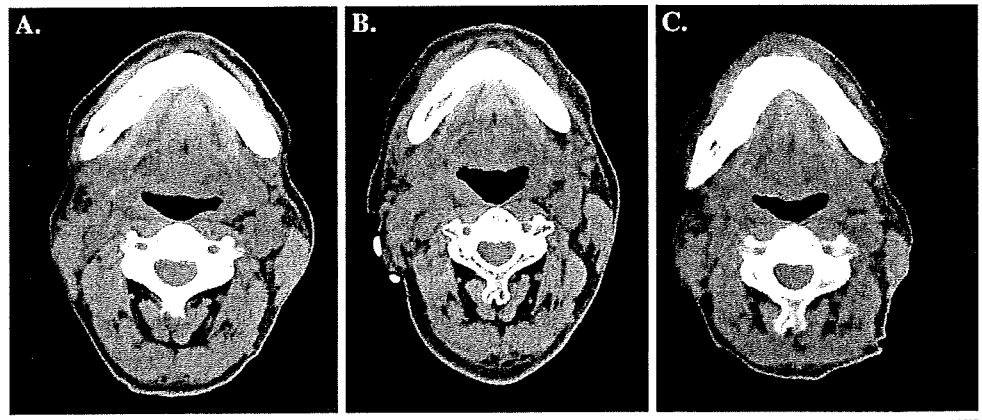

Fig. 1 Changes in the cervical lymphoma lesions after CBT in patient 1. CT scan on 23 months after the second CBT showed a marked reduction in size of the cervical lymph nodes in comparison to those before the first and the second CBT

A. 29 days before the first CBT B. 25 days after the first CBT C. 23 months after the second CBT

2.2 Patient 2

In April 2005; a 66-year-old female was diagnosed to have AML evolving from MDS. Chemotherapy consisting of idarubicin (IDA) and cytosine arabinoside (Ara-C) failed to induce remission and severe pancytopenia persisted. She underwent CBT following a conditioning regimen with fludarabine, melphalan, rabbit ATG and 4 Gy of TBI. The CB was 2-loci mismatched and contained 2.9×10^7/kg cells. Engraftment was confirmed on day 18 and she achieved complete remission. However, the AML relapsed in 18 months after the CBT. Remission induction with IDA and Ara-C only induced marrow hypoplasia with 33% residual leukemia cells. On day 18 of the chemotherapy, invasive aspergillosis developed in the left lung. Liposomal amphotericin B, 2.5 mg/kg daily, was administered from the same day without any appreciable effects. The neutrophil count remained at 0 on day 22 of the chemotherapy. She received melphalan 40 mg/m^2 to reduce leukemic cell burden, followed by 30 mg/m^2 Flu and 2 g/m^2 CY on the next day. In the morning of the following day, she received 4 Gy of TBI and underwent a second CBT 12 h after the completion of CY infusion. The CB was 2-loci mismatched, and contained 2.9×10^7/kg cells and 1.9×10^6 CD34$^+$ cells/kg. HLA alleles of the patient, the first CB donor, and the second CB donor are shown in Table 1. Tacrolimus was given from day-1 for prophylaxis of GVHD. Liposomal amphotericin B was switched to voliconazole, 4.0 mg/kg daily, on day 48 after the second CBT due to a rise in the creatinine level. Although her pulmonary aspergillosis was transiently exacerbated on day 6 after the second CBT, the high fever abated on day 17 and engraftment of donor cells was confirmed on the same day. The aspergillosis lesion was encapsulated with time after the second CBT (Fig. 2).

She underwent a left upper lobectomy on day 113 and presently remains in CR at 24 months after the second CBT.

3 Discussion

Treatment of the patients with hematologic malignancies complicated by severe neutropenic infections with no hope of prompt hematologic recovery is challenging. Although immunoablative conditioning followed by allogeneic stem cell transplantation is the only measure to rescue patients with such devastating conditions, this treatment may also tend to sometimes hasten the patients' death by aggravating the preexisting infections. Even if reduced intensity regimens are adopted, severe neutropenia which lasts from the day of preconditioning until 2–3 weeks after SCT greatly increases the risk of infectious death [3, 5, 6]. In order to solve this dilemma, Goggins et al. pioneered a very weak conditioning regimen, known as the 1-day regimen [7]. They treated five infirmed patients with 30 mg/m^2 Flu, 2 g/m^2 CY, 20 mg/kg alemtuzumab, TBI 2 Gy on day-1 and infused PBSC from family donors who were HLA 1–3 loci mismatched. Engraftment occurred in three patients, two of whom achieved long-term remission. According to their protocol, an MDS patient who suffered febrile neutropenia due to rejection of the first PBSCT was treated with Flu (30 mg/m^2), CY (2 g/m^2), horse ATG (15 mg/kg) and TBI (2 Gy) followed by PBSCT from a second HLA-identical sibling donor. The neutrophil count promptly recovered and the patient achieved complete donor chimerism. This experience indicated that the alemtuzumab in the 1 day regimen can be replaced with low dose ATG and that the minimum conditioning regimen coupled with PBSCT from a second donor can overcome the rejection after SCT.

Cord blood transplantation is associated with a higher incidence of engraftment failure [8–12] and a slower neutrophil recovery [2, 9, 13] than BMT or PBSCT due to the low number of hematopoietic stem cells and mature T cells in the CB graft. The disadvantages of CBT has precluded the use of CB for treatment of patients with very low intensity regimens for allogeneic stem cell transplantation such as 2 Gy TBI alone [14] or ATG + total lymphoid

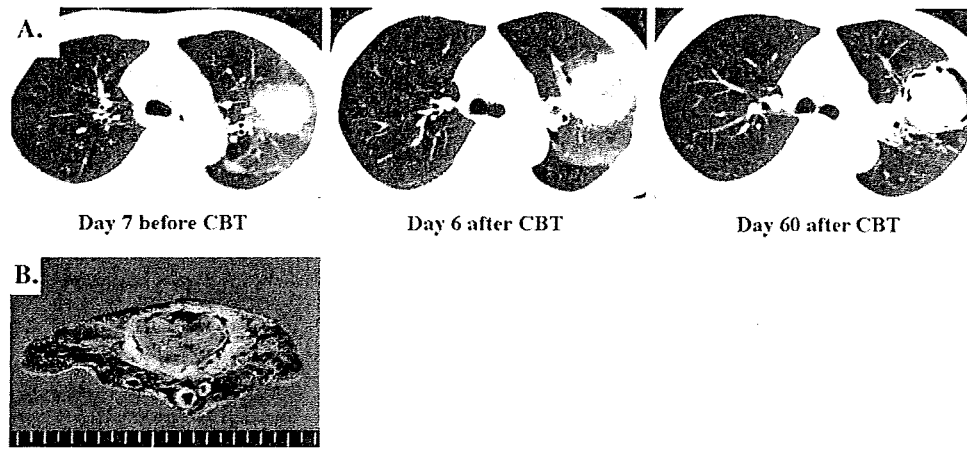

Fig. 2 Pulmonary aspergillosis lesion of patient 2. **a** Changes in the CT findings before and after CBT. **b** *Left upper* lung resected on day113 after CBT

irradiation regimens [15]. However, there were no options other than CBT for the two current patients because they did not have matched family donors and could not afford to wait until an HLA-matched unrelated donor was available. ATG was not included in the conditioning regimen for those patients because they could have succumbed to their infections which became exacerbated by the administration of ATG. Despite their devastating conditions and the elimination of ATG from the conditioning regimen, both patients achieved engraftment of CB without any apparent exacerbation of their infections or the development of severe GVHD. Therefore, in vivo purging of T cells using anti-T cell antibodies may not be a prerequisite for engraftment of CB after the 1–2-day regimen. However, it should be noted that both patients had been previously treated with conditioning regimens for allo-SCT. Prior conditioning regimens used for the first CBT may therefore be necessary for patients to take CB following such a minimum conditioning regimen. Other reduced-intensity regimens have been successfully used as preconditioning for a second transplantation using CB to treat graft rejection after allo-SCT [16–20]. However, all such regimens were administered for over 5 days and were not as weak as the regimens we used for the above described two patients.

Sustained engraftment of CB after the weak regimen in the current patients may therefore have important implications in the management of patients with hematologic malignancies refractory to chemotherapy. Patients who fail chemotherapy often suffer from severe infections due to persistent neutropenia and are therefore excluded as candidates for hematopoietic stem cell transplantation, particularly CBT, which is associated with delayed neutrophil recovery. Following very weak preconditioning, the patients not only circumvented life threatening infections but also achieved hematologic remission possibly with the help of the graft-versus-leukemia/lymphoma effects of CBT. CB can be utilized for patients with severe complications because of its easy accessibility and prompt availability [21]. Therefore, CBT following the minimum intensity conditioning may provide a chance to achieve complete chimerism in patients suffering from severe infections associated with profound neutropenia due to graft rejection or chemotherapy for leukemic relapse after the first allo-SCT.

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Anti-Moesin Antibodies in the Serum of Patients with Aplastic Anemia Stimulate Peripheral Blood Mononuclear Cells to Secrete TNF-α and IFN-γ[1]

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Moesin is an intracellular protein that links the cell membrane and cytoskeleton, while also mediating the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane. To determine the roles of anti-moesin Abs derived from the serum of patients with aplastic anemia (AA) in the pathophysiology of bone marrow failure, we studied the expression of moesin on various blood cells and the effects of anti-moesin Abs on the moesin-expressing cells. The proteins recognized by anti-moesin mAbs were detectable on the surface of T cells, NK cells, and monocytes from healthy individuals as well as on THP-1 cells. The peptide mass fingerprinting of the THP-1 cell surface protein and the knock-down experiments using short hairpin RNA proved that the protein is moesin itself. Both the anti-moesin mAbs and the anti-moesin polyclonal Abs purified from the AA patients' sera stimulated THP-1 cells and the PBMCs of healthy individuals and AA patients to secrete 60–80% as much TNF-α as did LPS 100 ng/ml. Although the polyclonal Abs induced IFN-γ secretion from the PBMCs of healthy individuals only when the PBMCs were prestimulated by anti-CD3 mAbs, the anti-moesin Abs were capable of inducing IFN-γ secretion from the PBMCs of AA patients by themselves. Anti-moesin Abs may therefore indirectly contribute to the suppression of hematopoiesis in AA patients by inducing myelosuppressive cytokines from immunocompetent cells. *The Journal of Immunology,* **2009, 182: 703–710.**

Acquired aplastic anemia (AA)[4] is a syndrome characterized by pancytopenia and bone marrow (BM) hypoplasia (1). The T cell-mediated suppression of hematopoiesis is considered to be the most important mechanism responsible for the development of this syndrome because approximately 70% of AA patients respond to immunosuppressive therapy, such as antithymocyte globulin and cyclosporine (2, 3). In addition to a large body of evidence for T cell involvement in the pathogenesis of AA (4–7), recent studies have revealed the presence of Abs specific to self-Ags in the serum of AA patients (8–11). Although some of these Abs are directed toward Ags that are abundant in hematopoietic cells (e.g., kinectin (Ref. 8) and DRS-1 (Ref. 9)), their roles in the pathophysiology of AA are unclear.

Of the various autoAbs detected in the autoimmune diseases, some are known to exhibit stimulatory effects on the target cells rather than inhibitory effects, such as anti-thyroglobulin Abs in Basedow's disease (12) and anti-desmoglein Abs in pemphigus vulgaris (12, 13). The autoAbs specific to platelet-derived growth factor receptors in patients with scleroderma and those with extensive chronic graft-vs-host diseases trigger an intracellular loop, involving Ha-Ras-ERK 1 and 2 (ERK 1/2)-reactive oxygen species (Ha-Ras-ERK 1/2-ROS), and augment collagen gene expression as well as myofibroblast phenotype conversion of normal human primary fibroblasts (14, 15). The anti-proteinase 3 Abs detected in Wegener's granuloma stimulate monocytes through the binding of cell surface proteinase 3 to secrete IL-8 (16). The autoAbs detected in AA patients may also be involved in the pathophysiology of BM failure by way of other mechanisms than the direct toxicity against the hematopoietic cells, though there has been no evidence for such functional autoAbs in AA patients.

We previously demonstrated that Abs specific to moesin, a membrane-cytoskeleton linker protein in the cytoplasm, were detectable in approximately 40% of AA patients (11). Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane (17). On the other hand, some reports have identified molecules that were recognized by anti-moesin mAbs on the surface of blood cells such as T cells and macrophages (18, 19). Because these immune cells are an important source of myelosuppressive cytokines such as TNF-α and IFN-γ, it is conceivable that anti-moesin Abs in AA patients may bind such moesin-like molecules on these immune cells and affect the cytokine secretion from these cells.

To test these hypotheses, we studied the expression of moesin on blood cells and the effects of anti-moesin Abs on the moesin-expressing

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[4] Abbreviations used in this paper: AA, aplastic anemia; BM, bone marrow; pAb, polyclonal Ab; PB, peripheral blood.

cells. The present study revealed a novel function of autoAbs, which may contribute to the pathophysiology of BM failure.

Materials and Methods

Study subjects (patients)

Sera were obtained from 19 patients with AA and 4 healthy individuals. BM plasma was obtained from five patients with AA and three healthy individuals. All AA patients had severe AA and were positive for anti-moesin Abs. The samples were cryopreserved at −80°C until use. Peripheral blood (PB) was obtained from 7 patients with AA and 10 healthy individuals and BM was aspirated from 3 healthy individuals. The PBMCs were isolated using lymphoprep (Nycomed). All patients and healthy volunteers provided an informed consent before sampling according to the Declaration of Helsinki. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science.

Cell lines

Molt-4, THP-1, U937, K562, Daudi, and Jurkat cell lines were purchased from the Health Science Research Resources Bank. A megakaryoblastic leukemia cell line UT-7, a myeloid leukemia cell lines OUN-1, and a myelodysplastic syndrome cell line TF-1 were provided by Dr. N. Komatsu of Jichi Medical School, Dr. M.Yasukawa of Ehime University, and Dr. S. Ogawa of the University of Tokyo, respectively.

Purification of anti-moesin Abs in the sera of patients with AA

The anti-moesin polyclonal Abs (pAbs) were purified from the patients' serum with affinity chromatography using a protein G column (mAb Trap kit, no. 17-1128-0; GE Healthcare) and recombinant moesin protein (11) fixed on an agarose-gel column (1 ml, HiTrap NHS-activated HP, no. 17-0716-01; GE Healthcare) according to the manufacturer's instruction. In brief, 20 ml of serum from AA patients was applied to the Protein G column. After washing with the binding buffer, the whole IgG was eluted with the elution buffer and neutralized with the neutralizing buffer. The purified IgG was then applied to the recombinant moesin-fixed affinity chromatography column. After washing with the binding buffer (75 mM sodium phosphate (pH 8.0)), anti-moesin pAbs were eluted with the elution buffer (100 mM glycine-HCl and 500 mM NaCl (pH 2.7)). The purified anti-moesin pAbs were dialyzed in PBS at 4°C overnight using a spectra/Por Float-A-Lyzer column (Spectrum Laboratories; no. 235118). The purity of the isolated anti-moesin pAbs was confirmed by PAGE followed by Coomassie Brilliant Blue staining.

Flow cytometry

Mouse anti-moesin mAb (clone 38/87; Neomarkers) which was labeled with FITC (Immuno-Biological Laboratories) was used in combination with mAbs specific to CD3-PE (BD Pharmingen; no. 555333), CD19-PE (BD Pharmingen; No. 555413), CD4-PE (BD Pharmingen; No. 347327), CD-8-PE (BD Pharmingen; no. 555367), CD14-PE (BD Pharmingen; No. 555398), CD11b-PE (BD Pharmingen; No. 555388), CD34-PE (BD Pharmingen; no. 348057), and CD3-Cy-Chrome (BD Pharmingen; no. 555334). Isotype-matched control mAbs (BD Pharmingen) were used as negative controls. For the detection of moesin-like molecules on leukocytes and leukemia cell lines, 1 μl of anti-moesin mAbs and 2 μl of PE-labeled mAbs were added to 50 μl of cell suspension containing 1×10^6 cells and the suspension was incubated on ice for 30 min. For the detection of moesin-like molecules by pAbs, 1×10^6 THP-1 cells were washed twice with PBS containing 1% BSA (Sigma-Aldrich; no. A8022) and resuspended in 200 μl of a PBS containing 2% FCS, 2% goat serum, and 2% BSA. The cell suspension was incubated for 30 min at 4°C. Then, 5 μg/ml anti-moesin pAbs or isotype control human IgG pAbs isolated from healthy individuals were added to the cell suspension and incubated for 1 h at 4°C. The cells were washed twice with PBS containing 1% BSA followed by incubation with a secondary Ab (goat anti-human IgG FITC-labeled Ab; Sigma-Aldrich; no. F5512) diluted 1/100 in PBS containing 2% goat serum and were incubated at 4°C for 30 min. Finally, the cells were washed twice with PBS containing 1% BSA and subjected to flow cytometry.

Stimulation of THP-1 cells with PMA/LPS

THP-1 cells (10^6) were suspended in 2 ml of RPMI 1640 containing 10% FCS and 20 ng/ml PMA (Wako Chemicals; no. 545-00261) and incubated for 24 h at 37°C in a CO_2 incubator. A total of 10 ng/ml LPS (Sigma-Aldrich; no. L2880) was added to the cell suspension and further incubated for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry using anti-moesin mAbs (clone 38/87; Neomarkers).

Isolation of monocytes and T cells

Monocytes were isolated by plastic adherence as previously described (20). In brief, 5×10^6 PBMCs/well were distributed into 12-well plates (Corning) and allowed to adhere in a 5% CO_2 incubator at 37° for 2 h in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin - 0.1 mg/ml streptomycin (Invitrogen; No.15140-148) and 10 μg/ml polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538). Nonadherent cells were removed and the remaining adherent cells on the plates were used as monocytes. T cells were purified by negative selection using the Human T Cell Enrichment Columns (R&D Systems; no. HTCC-500) following the manufacturer instructions. The purity of enriched T cells and monocytes was approximately 90% as determined by flow cytometry using anti-CD3-PE and anti-CD-14-PE mAbs, respectively.

Stimulation of THP-1 cells, PBMCs, monocytes, and T cells with anti-moesin Abs

THP-1 cells, PBMCs, monocytes, or T cells were suspended in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS. Polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538) was added at 10 μg/ml to eliminate any contaminating endotoxin. The endotoxin concentration in the pAbs and the reagents used for culture was <10 pg/ml as demonstrated by chromogenic *Limulus* amebocyte lysate assay (Seikagaku). The cells (5×10^5) were incubated for 48 h in the presence of 5 μg/ml of anti-moesin mouse mAbs (clone 38/87, IgG1; Neomarkers) or 5–10 μg/ml of pAbs isolated from the serum of AA patients as described above. Mouse mAbs (Coulter Clone; IgG1, no. 6602872) and control human IgG pAbs isolated from healthy individuals were used as negative controls. This Ab concentration was selected based on an estimated concentration of anti-moesin pAbs in the serum of an AA patient. For prestimulation of PBMCs to induce IFN-γ secretion, the cells were incubated for 1 h in the presence of 100 ng/ml of anti-CD3 mAbs (Clone OKT3) (eBioscience; functional grade no. 16-0037). For co-stimulation of isolated T cells to induce IFN-γ secretion, the cells were cultured for 48 h on a 48-well tissue culture plate that was coated with 100 ng/ml of anti-CD3 mAbs (clone OKT3) (eBioscience; functional grade no. 16-0037) overnight at 4°C and washed. Then, 100 ng/ml LPS or 10 μg/ml PHA (Sigma-Aldrich; no. L4144) was included instead of anti-moesin Abs as the positive controls for the induction of TNF-α or IFN-γ secretion, respectively, and control human IgG pAbs isolated from healthy individuals were added as a negative control.

Western blotting

Western blotting was performed using THP-1 cell lysates. The specific bands were visualized by anti-moesin mAbs, pAbs from AA patients, or control human IgG pAbs from healthy individuals as described in a previous report (11). The expression levels of α-tubulin were determined as an internal control using Western blotting with anti-α-tubulin mAbs (Sigma-Aldrich; no. T 5168).

Isolation of proteins on the surface of THP-1 cells

The THP-1 cells were treated with sulfo-NHS-SS-biotin, and the cell surface proteins were isolated with avidin-fixed columns according to the manufacturer's instructions (Pierce). Thereafter, 1×10^7 cells were washed twice with 8 ml of ice-cold PBS. The cells were suspended in 10 ml PBS containing 2.5 mg sulfo-NHS-SS-Biotin and incubated for 30 min at 4°C. Then, 500 μl of quenching solution was added to the cell suspension and the cells were washed with 10 ml TBS twice. The cell pellet was lysed in 500 μl of lysis buffer containing 60 μl of preotease inhibitor cocktail (Sigma-Aldrich; no. P-8340) and then disrupted by sonication. The biotin-labeled membrane proteins were isolated by an immobilized NeutrAvidin Gel column. The isolated membrane proteins were subjected to Western blotting and peptide mass fingerprinting.

Peptide mass fingerprinting

Mass spectrometric identification of 80- and 75-kDa proteins on the surface of the THP-1 cells was performed as previously described (21). In brief, the proteins fractionated by SDS-PAGE were visualized by Coomassie Brilliant Blue staining and the 80- and 75-kDa bands were excised from gels, followed by in-gel digestions with trypsin (Promega) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C. Molecular mass analyses of the tryptic

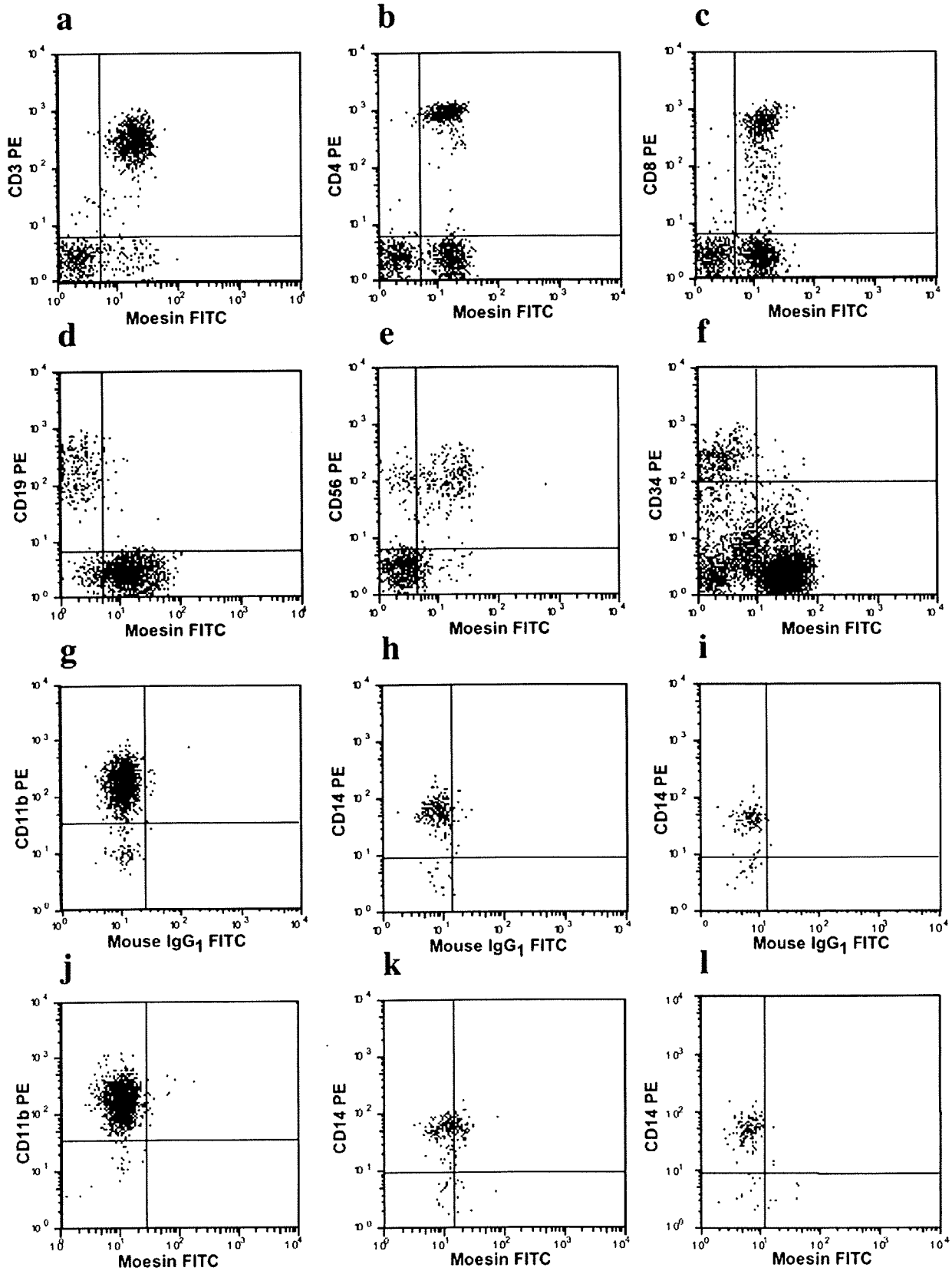

FIGURE 1. Expression of moesin-like molecules on the surface of various blood cells. PB lymphocytes, granulocytes, and monocytes, as well as BM mononuclear cells, of a healthy individual and a patient with AA were analyzed by flow cytometry. The gate was set up for lymphocytes (*a–d*), $CD3^-$ lymphocytes (*e*), granulocytes (*g* and *j*), and monocytes (*h* and *k*) derived from a healthy individual and monocytes (*i* and *l*) derived from an AA patient. BM mononuclear cells (*f*) of a healthy individual were included in the analysis. One representative result of three experiments is shown.

peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an ultraflex TOF/TOF (Bruker Daltonics). The proteins were identified by comparisons between the molecular weights determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the theoretical peptide masses of proteins registered in NCBInr.

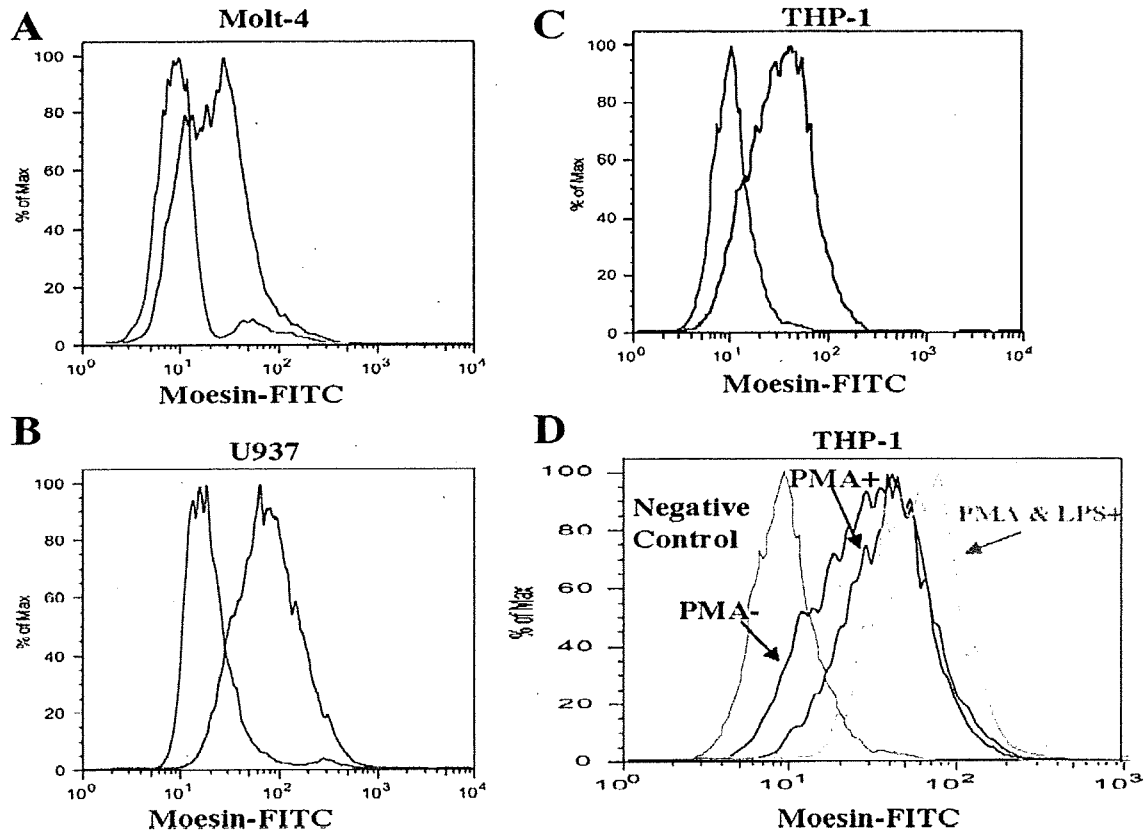

FIGURE 2. Expression of moesin-like molecules on the surface of T cell and monocytic leukemia cell lines. *A–C*, Three leukemia cell lines were examined for the cell surface expression of moesin-like molecules. Left lines, mouse IgG used as negative control; right lines, FITC-labeled anti-moesin mAbs. *D*, THP-1 cells were cultured in the presence or absence of 20 ng/ml PMA for 24 h and then the PMA-stimulated cells were further cultured in the presence of 10 ng/ml LPS for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry. One representative result is shown.

Transfection of moesin short hairpin (shRNA)

Moesin shRNA plasmid (pENTR/moesin-shRNA-264) (22) was kindly provided by Dr. G. M. Kelly of the University of Western Ontario (Ontario, Canada). THP-1 cells were transfected by electroporation using a Gene Pulser II Electroporation System (Bio-Rad). In brief, 3–5 μg of moesin shRNA plasmid or control shRNA (pENTR/U6-GW/lacZ^{shRNA}) was mixed with 800 μl of Opti-Mem I medium (Invitrogen) containing 1 × 10^6 THP-1 cells and incubated on ice for 10 min. The cells were electroporated in a 4-mm cuvette (Bio-Rad) at the setting of 300 V of voltage pulse and 960 μF of capacitance. Immediately after electroporation, the transfected THP-1 cells were left on ice for 10 min and then 3 ml of RPMI 1640 containing 10% FCS was added to the cell suspension followed by overnight incubation at 37°C. The cells were rinsed and cultured in 3 ml of fresh RPMI 1640 containing 10% FCS for 72 h at 37°C in a CO_2 incubator and were analyzed for the expression of moesin-like molecules by flow cytometry using FITC-labeled anti-moesin mAb (clone 38/87; Neomarkers).

ELISA

The TNF-α and IFN-γ concentration in the culture supernatant, as well as in PB serum and BM plasma was measured using ELISA kits (Mabtech;

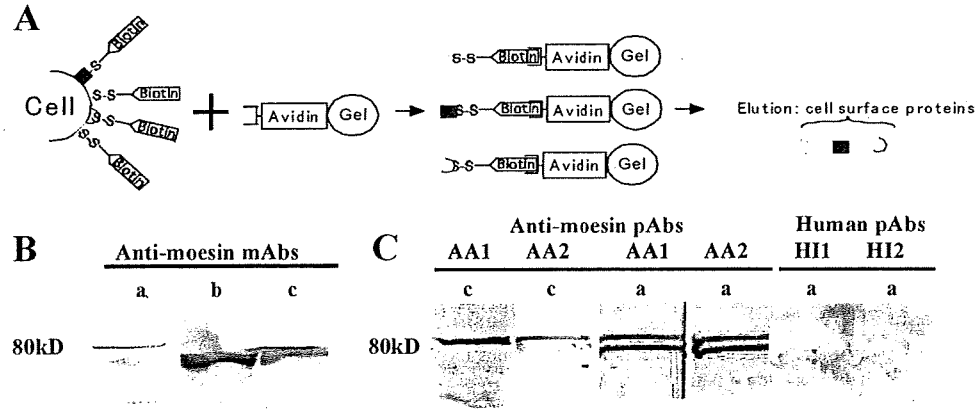

FIGURE 3. Isolation and identification of proteins on THP-1 cells recognized by anti-moesin Abs. *A*, THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns. *B*, Three different protein lysates (*a*, whole cells; *b*, cytoplasmic proteins; and *c*, surface proteins) were subjected to Western blotting with anti-moesin mAbs. *C*, THP-1 cell lysates (*a*) and surface proteins (*c*) isolated from THP-1 cells were subjected to Western blotting using anti-moesin pAbs purified from two AA patients' sera (AA1 and AA2) or non-specific control human IgG pAbs purified from two healthy individuals' sera (HI1 and HI2).

AB, No. 3510-1H-20, and Mabtech; AB, No. 3420-1H-6) according to the manufacturer's instructions. The OD absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments). For determination of cytokine levels in the PB serum and BM plasma, the following additional procedures were performed. Samples were centrifuged at 10,000 rpm for 10 min. ELISA plates were covered with 200 μl/well of TNF-α assay diluent (eBioscience; No. 00-4202-AD) or IFN-γ assay diluent (Mabtech; No. 3652-D) for 1 h at room temperature before adding samples to block nonspecific reactions. TNF-α assay diluent (eBioscience; No. 00-4202-AD) and IFN-γ assay diluent (Mabtech; No. 3652-D) were used to dilute biotinylated mAb TNF-α-II solution and biotinylated mAb 7-B6-1, respectively.

Statistics

The results are given as the mean ± SD. Comparisons were made using the paired *t* test.

Results

Expression of moesin-like molecules on the surface of various blood cells

To confirm the expression of moesin-like molecules on the PB and BM cells, various leukocyte subsets were examined using flow cytometry with anti-moesin mAbs. Fig. 1 shows the representative results of flow cytometry on one healthy individual. Moesin-like molecules were detectable on T cells, NK cells, and monocytes on their surface but not on B cells, neutrophils, and BM $CD34^+$ cells as shown in Fig. 1. All three healthy individuals and the three AA patients showed similar results except that moesin-like molecules were not detectable on monocytes derived from the three AA patients. The mean fluorescence intensity values of the monocytes from healthy individuals and AA patients were 11.5 ± 2.2 and 6.6 ± 2.1, respectively, and the difference was significant (mean fluorescence intensity ± SD, $p < 0.05$, unpaired *t* test). In addition to the leukocyte subsets from the healthy individuals, moesin-like molecules were detectable on a T cell leukemia cell line Molt-4 as well as on monocytic leukemia cell lines U937 and THP-1 (Fig. 2), while they were undetectable on myeloid leukemia cell lines such as K562, UT-7, OUN-1, and TF-1. They were either undetectable on the Burkitt lymphoma cell line, Daudi, or T cell lymphoma cell line, Jurkat (data not shown). The treatment of THP-1 with 20 ng/ml PMA for 24 h and/or 10 ng/ml LPS for 20 h augmented the expression of moesin-like molecules (Fig. 2*D*), thus, indicating an up-regulation of the moesin-like molecules associated with the differentiation of THP-1 cells into macrophages.

Identification of moesin on the surface of THP-1 cells

To identify the proteins on THP-1 cells recognized by anti-moesin Abs, the THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns (Fig. 3*A*). Western blotting of the isolated proteins with anti-moesin mAbs showed two clear bands of which the sizes were 75 and 80 kDa (Fig. 3*B*). Mass fingerprinting of the eluted protein revealed the 80 kDa protein to be moesin. The 75 kD band proved to be nucleolin and eukaryotic translation elongation factor 2. To confirm that anti-moesin pAbs in the serum of AA patients can bind to this cell surface moesin, anti-moesin pAbs were purified from the AA patients' sera (AA1 and AA2) with recombinant moesin proteins using affinity chromatography and then were used for Western blotting. As shown in Fig. 3*C*, the serum-derived anti-moesin pAbs bound to moesin derived from the surface proteins of THP-1.

Effect of moesin-specific shRNA on the expression of moesin on THP-1 cells

To further confirm the expression of moesin on the surface of THP-1 cells, the cells were transfected with moesin shRNA using electroporation. Flow cytometry showed a decrease in the moesin

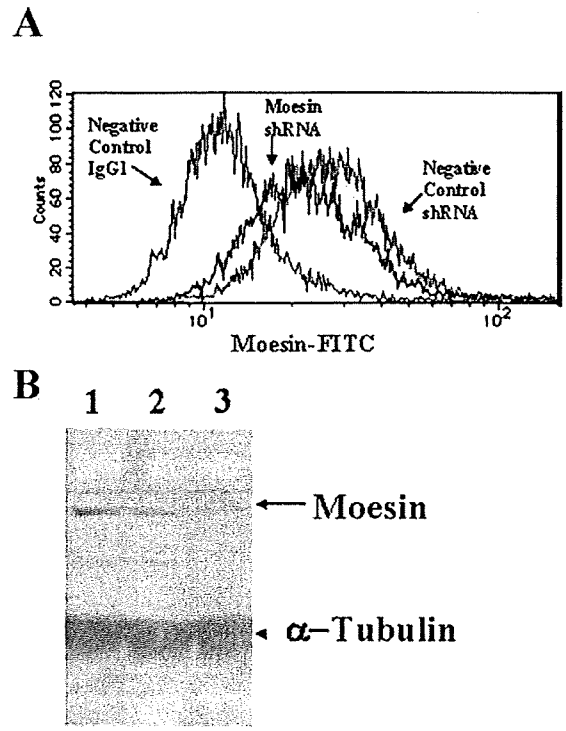

FIGURE 4. Effect of moesin shRNA transfection on the expression of moesin by THP-1 cells. *A*, THP-1 cells transfected with 5 μg of moesin shRNA or control shRNA were examined for the expression of moesin with flow cytometry. The blue line, non-transfected THP-1 cells stained with control mouse IgG1 mAbs; the green line, moesin shRNA transfected cells stained with anti-moesin IgG1 mAbs; the red line, negative control shRNA transfected cells stained with anti-moesin IgG1 mAbs. *B*, Negative control shRNA or moesin-specific shRNA transfected THP-1 cell lysates were examined by Western blotting. 1, 5 μg control shRNA; 2, 3 μg moesin shRNA; 3, 5 μg moesin shRNA.

expression level on the surface of the THP-1 cells transfected with moesin shRNA in comparison to the THP-1 cells transfected with negative control shRNA (Fig. 4*A*). When the THP-1 cells transfected with different dosages of moesin-specific shRNA were examined by Western blotting, the moesin expression by the THP-1 cells was decreased in a dose-dependent manner. The control shRNA specific to LacZ had no effect on moesin expression.

Effect of anti-moesin Abs on THP-1 cells

To determine whether anti-moesin Abs have some effects on THP-1 cells, the THP-1 cells were cultured in the presence of anti-moesin Abs or control IgG for 48 h and the TNF-α concentration of the culture supernatant was measured using ELISA. Both the anti-moesin mAbs and pAbs induced a significantly greater amount of TNF-α from the THP-1 cells than did the control IgG (Fig. 5*A*). The amount of TNF-α induced by anti-moesin pAbs (5 μg/ml) was almost comparable to that induced by LPS (100 ng/ml) (Fig. 5*A*). The anti-moesin pAbs' binding to moesin on the THP-1 cells was ascertained by flow cytometry (Fig. 5*B*).

Effect of anti-moesin pAbs on PBMCs, monocytes, and T cells from healthy individuals and AA patients

The expression of moesin on the T cells and monocytes as well as the TNF-α secretion from the THP-1 cells induced by anti-moesin pAbs suggested that anti-moesin pAbs in the AA patients' sera might also stimulate these immune cells from healthy individuals and AA patients to secrete cytokines. When the PBMCs from healthy individuals were incubated for 48 h in the presence of 5

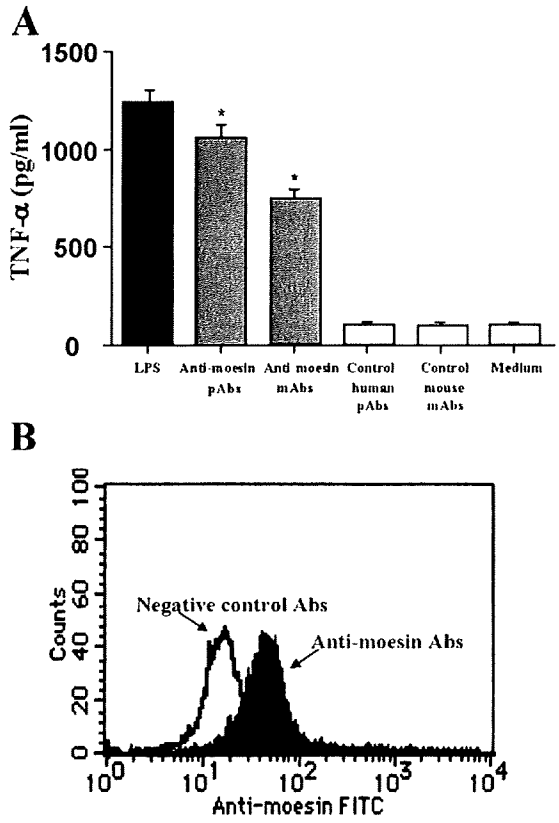

FIGURE 5. TNF-α release from THP-1 cells stimulated by anti-moesin Abs. *A*, THP-1 cells were cultured for 48 h with 5 μg/ml of anti-moesin Abs or control Abs. Anti-moesin pAbs, anti-moesin polyclonal IgG isolated from the serum of AA patients; control human pAbs, control human IgG pAbs isolated from healthy individuals; anti-moesin mAbs, anti-moesin mouse IgG1 mAbs (clone 38/87); control mouse mAbs, control mouse IgG1 mAbs. Then, 100 ng/ml LPS was used as a positive control. The data represent the mean TNF-α concentration ± SD of three experiments. *, $p < 0.01$ vs control Abs. *B*, The detection of moesin on THP-1 cells by anti-moesin pAbs purified from the serum of an AA patient.

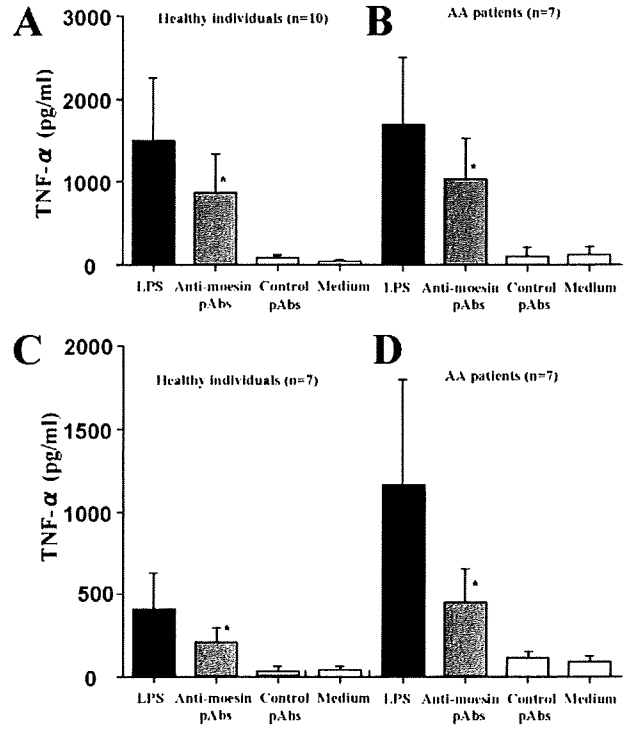

FIGURE 6. TNF-α release from PBMCs or monocytes stimulated by anti-moesin pAbs. The PBMCs or isolated monocytes were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 100 ng/ml of LPS was used as a positive control. PBMCs isolated from 10 healthy individuals (*A*) and 7 AA patients (*B*), and monocytes separated from the PBMCs of 7 healthy individuals (*C*) and 7 AA patients (*D*), were used as targets. The data represent the mean TNF-α concentration ± SD. *, $p < 0.005$ vs control Abs.

μg/ml of anti-moesin pAbs, the amount of TNF-α in the culture medium was approximately 10 times more than those of control cultures and was more than half of that of the culture stimulated by 100 ng/ml of LPS (Fig. 6*A*). The same concentration of anti-moesin pAbs induced a similar amount of TNF-α from the PBMCs from AA patients (Fig. 6*B*). On the other hand, when monocytes isolated from the PBMC of healthy individuals or AA patients were used as a target, anti-moesin pAbs induced less than half the amount of TNF-α of that induced from whole PBMCs (Fig. 6, *C* and *D*).

The unexpectedly high inducibility of TNF-α secretion from the PBMCs by the anti-moesin pAbs prompted studies on the inducibility of IFN-γ secretion from the PBMCs by the Abs. Fig. 7, *A* and *B*, shows the effect of anti-moesin Abs on the IFN-γ secretion from PBMCs. Although anti-moesin pAbs alone could not induce IFN-γ secretion from the PBMCs derived from healthy individuals, the Abs stimulated PBMCs that were prestimulated with anti-CD3 mAbs to secrete nearly as much IFN-γ as that PHA did. In contrast, the PBMCs from the AA patients could secrete IFN-γ in response to anti-moesin pAbs without the prestimulation by anti-CD3 mAbs, and the amount of IFN-γ was approximately 40% as much as that of the culture stimulated by 10 μg/ml of PHA. In contrast, T cells isolated from the PBMC of healthy individuals or AA patients could not secrete a significantly larger amount of IFN-γ in response to anti-moesin pAbs compared with that in response to control IgG pAbs (Fig. 7, *C* and *D*), and the amount of IFN-γ secreted by T cells was one-tenth as much as that by PBMCs.

When the sera of the 16 AA patients comprising 7 anti-moesin Ab-positive and 9 anti-moesin Ab-negative patients were examined using ELISA, no significant differences in TNF-α and IFN-γ concentrations were observed between the 2 groups (TNF-α: 88.0 ± 106.3 pg/ml in anti-moesin Abs-positive patients, 90.1 ± 161.3 in anti-moesin Abs-negative patients; IFN-γ: 44.6 ± 33.8 pg/ml in anti-moesin Abs-positive patients, 47.5 ± 44.9 pg/ml in anti-moesin Abs-negative patients). None of the sera derived from four healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml). On the other hand, when the BM plasma from five patients with AA was examined using ELISA, three anti-moesin Abs-positive patients showed higher levels of TNF-α (129, 338, and 349 pg/ml) compared with those of TNF-α (13 and 128 pg/ml) in two anti-moesin Abs-negative patients. IFN-γ concentrations of three anti-moesin Abs-positive patients were 29, 123, and 133 pg/ml, while those of two anti-moesin Abs-negative patients were 13 and 80 pg/ml. None of the BM plasma derived from three healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml).

Discussion

The present study revealed that the proteins recognized by the anti-moesin Abs are detectable on the surface of various leukocytes subsets including T cells, NK cells, and monocytes as well as on T lymphocytic and monocytic leukemia cell lines. Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites

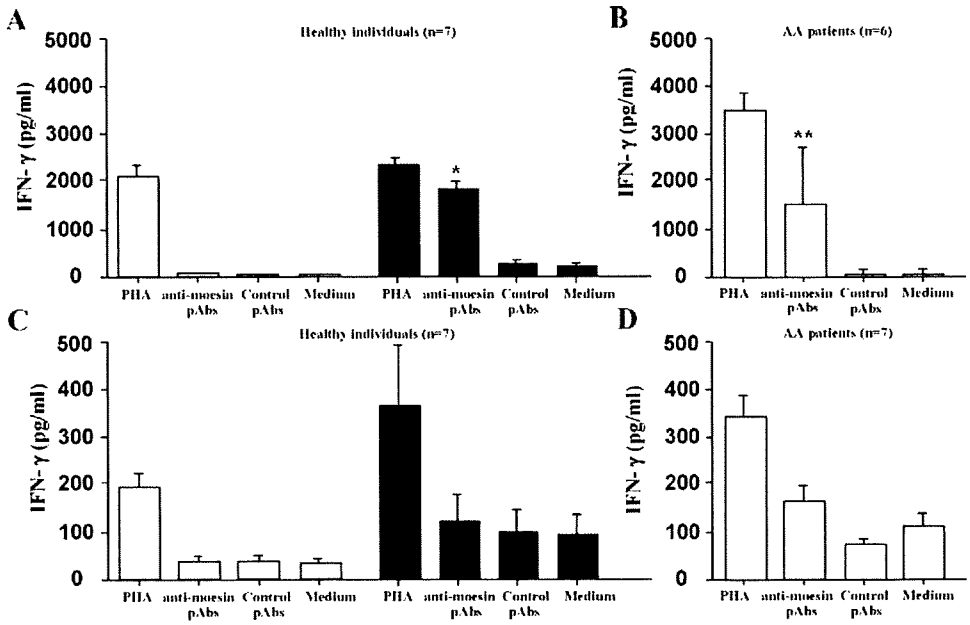

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as a positive control. Unprimed PBMCs (□) or CD3-primed PBMCs (■) were used for the culture. PBMCs were isolated from seven healthy individuals (*A*) and six AA patients (*B*). Uncostimulated T cells (□) or CD3-costimulated T cells (■) were used for the culture. T cells were isolated from the PBMCs of seven healthy individuals (*C*) and seven AA patients (*D*). The data represent the mean IFN-γ concentration ± SD. *, $p < 0.0001$ vs control Abs; **, $p = 0.04$ vs control Abs.

as well as ruffling of the cell membrane (17). This membrane-linking protein is expressed by various blood cells including megakaryocytes and granulocytes (23), but its expression was thought to be localized inside the cell membrane and not on the cell surface. Some studies revealed that anti-moesin Abs could bind to the surface of T cells (18) and macrophages (19) in keeping with our observation. However, none of the previous studies characterized the cell surface protein recognized by the anti-moesin Abs. Using biotin-labeled membrane proteins coupled with an avidin gel column and peptide massfinger printing, the present study identified the cell surface 80 kDa protein to be moesin. The decrease in the cell surface moesin induced by moesin shRNA has substantiated the presence of moesin on the cell surface of THP-1 cells.

Little is known about the function of anti-moesin Abs in vitro and in vivo. In contrast to our results, Amar et al. (24, 25) found that anti-moesin mAbs (clone 38) suppressed LPS-induced TNF-α secretion from monocytes through binding of moesin-like molecules on the cell surface. They used a different anti-moesin mAbs (clone 38) from the mAbs (clone 38/87) used in the present study. When we examined the effect of clone 38 mAbs on TNF-α secretion from THP-1 cells induced by LPS using the same condition as the one described by Amar et al. (24), a dose-dependent inhibition of TNF-α secretion was observed (data not shown). In contract to clone 38/87 mAb and pAbs from AA patients' sera, the clone 38 mAbs alone did not induce TNF-α secretion from THP-1 cells. Because the clone 38 preparation contains 1.5 mM sodium azide as a preservative, it is most likely that the dose-dependent inhibition of TNF-α secretion by clone 38 mAbs was due to toxic effect of sodium azide. Alternatively, clone 38 mAb which recognizes the C-terminal portion (554–564 amino acid residues) of moesin may exert a different effect on THP-1 cells from the effect of mAb clone 38/87 which recognizes the middle portion (317–398 amino acid residues) of moesin and from the effect of pAbs purified from AA patients' sera.

The present study revealed that both mAbs and pAbs specific to moesin stimulated the THP-1 cells to secrete TNF-α at an Ab concentration compatible to that in the serum of the AA patients. Moreover, anti-moesin pAbs were as potent as LPS in inducing TNF-α secretion from the PBMCs derived from healthy individuals and the AA patients. Our preliminary analyses on the activation of signaling pathways leading to TNF-α secretion from THP-1 cells showed the phosphorylation of ERK1/2 kinase induced by anti-moesin Abs (49th American Society of Hematology annual meeting abstract #1690, 2007 and submitted). In two patients from whom anti-moesin pAbs were purified, the Abs induced TNF-α release from autologous PBMCs. High concentrations of TNF-α were indeed present in the BM sera of two patients with high anti-moesin Ab titer. Although no difference in the serum TNF-α level was observed between anti-moesin Ab-positive and -negative patients, these findings suggest that anti-moesin Abs may induce a subtle amount of TNF-α from the monocytes or macrophages in the BM, thereby contributing to the pathogenesis of AA.

In contrast to TNF-α, IFN-γ was not induced by the anti-moesin pAbs alone from the PBMCs from healthy individuals, though anti-moesin pAbs augmented IFN-γ secretion from the PBMCs prestimulated with anti-CD3 mAbs. On the other hand, anti-moesin pAbs stimulated the PBMCs from the AA patients to secrete as much IFN-γ as did PHA. It has been shown that T cells from AA patients are in an activated state and are prone to produce IFN-γ in response to suboptimal stimuli (26). The amount of secreted TNF-α from isolated monocytes as well as the amount of secreted IFN-γ from isolated T cells was greatly reduced compared with those from unfractionated PBMCs. The inability to secrete a sufficient amount TNF-α and IFN-γ of isolated monocytes and T cells suggests that the interaction between monocytes and T cells may be required to efficiently respond to extrinsic stimuli as described by previous reports (27, 28). When the anti-moesin Abs titers in the serum were longitudinally measured in three patients, the Abs titer decreased in two patients in association with the response to immunosuppressive therapy, while the Abs titer increased in one patient who became dependent on transfusions due to the relapse of AA in comparison to the titer detected in remission (data not shown). The high titer TNF-α levels in BM plasma of patients showing high anti-moesin Abs titers and the decrease in the Ab titers in parallel with disease amelioration support the hypothesis that anti-moesin Abs are involved in the pathogenesis of AA by way of myelosuppressive cytokine induction from immunocompetent cells. One may wonder why high titer anti-moesin Abs in some AA patients do not induce hypercytokinemia. However, inability of T cell-stimulating Abs such as anti-CD3 Abs to induce IFN-γ secretion in vivo has been shown by previous reports (29, 30). There may be some regulatory mechanisms that mitigate T cell activation by stimulating Abs in vivo.

A previous study demonstrated the presence of anti-moesin Abs in 14–34% of patients with rheumatoid arthritis (11, 31), and a

case-control study on AA conducted by the International Agranulocytosis and Aplastic Anemia Study Group revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA (32). The anti-moesin pAbs derived from patients with rheumatoid arthritis also enhanced TNF-α secretion from THP-1 cells (data not shown). It is, therefore, possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin. Anti-TNF-α therapy has been successfully used for patients with rheumatoid arthritis (33–35) as well as for some patients with myelodysplastic syndrome (36, 37). Recent reports have shown the efficacy of anti-CD20 Abs in restoring hematopoietic functions of AA (38, 39). Therefore, autoAbs capable of inducing cytokine secretion like anti-moesin Abs may be a new target of therapy for AA.

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Disclosures

The authors have no financial conflict of interest.

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NKG2D gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies

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ABSTRACT

Background

NKG2D, an activating and co-stimulatory receptor expressed on natural killer cells and T cells, plays pivotal roles in immunity to microbial infections as well as in cancer immunosurveillance. This study examined the impact of donor and recipient polymorphisms in the *NKG2D* gene on the clinical outcomes of patients undergoing allogeneic T-cell-replete myeloablative bone marrow transplantation using an HLA-matched unrelated donor.

Design and Methods

The *NKG2D* polymorphism was retrospectively analyzed in a total 145 recipients with hematologic malignancies and their unrelated donors. The patients underwent transplantation following myeloablative conditioning; the recipients and donors were matched through the Japan Marrow Donor Program.

Results

In patients with standard-risk disease, the donor *NKG2D-HNK1* haplotype, a haplotype expected to induce greater natural killer cell activity, was associated with significantly improved overall survival (adjusted hazard ratio, 0.44; 95% confidence interval, 0.23 to 0.85; p=0.01) as well as transplant related mortality (adjusted hazard ratio, 0.42; 95% confidence interval, 0.21 to 0.86; p=0.02), but had no impact on disease relapse or the development of grade II-IV acute graft-versus-host disease or chronic graft-versus-host disease. The *NKG2D* polymorphism did not significantly influence the transplant outcomes in patients with high-risk disease.

Conclusions

These data suggest an association between the donor *HNK1* haplotype and better clinical outcome among recipients, with standard-risk disease, of bone marrow transplants from HLA-matched unrelated donors.

Key words: *NKG2D*, *HNK1*, *LNK1*, unrelated donor; bone marrow transplantation, single nucleotide polymorphism.

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