good response was a change from code 3 to code 2, and no response was no change in code or progression. An overall response to treatment was defined as a marked or good response in at least one organ, without progression in any other organs. We planned to include 18 patients with target and lower response rates of 40% and 10% and alpha and beta errors of 5% and 10%, respectively.

The trough blood concentrations of cyclosporine or tacrolimus and the peak plasma concentration of pravastatin were measured every 2 weeks to evaluate interaction between pravastatin and these immunosuppressants. Immunologic changes were evaluated at weeks 2, 4, 8, and 12 by quantification of the CD4/CD8 ratio, the T-helper (Th)1/Th2 ratio, and the expression of human leukocyte antigen-DR on T cells, B cells, and monocytes. Immunologic data were compared between responders and nonresponders using a repeated measures analysis of variance after logarithmic transformation.

Eighteen patients with a median age of 44 years (range 20–68 years) were included in the study. There were 14 men and 4 women. The underlying disease was acute myeloblastic leukemia in seven, chronic myeloid leukemia in four, non-Hodgkin's lymphoma in three, acute lymphoblastic leukemia in two, myelodysplastic syndrome in one, and aplastic anemia in one. Thirteen and five patients received grafts from a related or an unrelated donor, respectively. Ten of them demonstrated chronic GVHD of progressive onset. All patients but one demonstrated extensive chronic GVHD before starting pravastatin, and nine patients were receiving prednisolone. The grade of chronic GVHD at study entry according to Akpek's prognostic model is shown in Table 1. Seven patients, 10 patients, and 1 patient were grouped into the low-, intermediate-, and high-risk groups, respectively.

Pravastatin was well tolerated, and no patients developed grade 3 or 4 adverse events attributable to pravastatin. Treatment was discontinued in three patients between 14 and 41 days after starting pravastatin because of unrelated causes, including painful oral chronic GVHD, infection, and interstitial pneumonitis. According to each organ, a response was observed in the skin score in two patients, mouth score in five patients, eye score in two patients, liver score in three patients, and platelet count score in one patient (Table 1). An overall response was seen in five patients (28%). Pravastatin did not act through the interaction with cyclosporine or tacrolimus, because an increase in these blood levels was not observed after the administration of pravastatin (data not shown). The serum pravastatin concentration on day 42 was not different between responders and nonresponders (median 157.5 ng/mL vs. 253.1 ng/mL, $P=0.53$). The serum total cholesterol level significantly decreased from 6.37 mmol/L (standard deviation [SD] 1.79) before treatment to 5.67 mmol/L (SD 1.40, $P=0.0095$) and 4.77 mmol/L (SD 1.99, $P=0.0001$) on days 14 and 84 after starting pravastatin, respectively. The initial cholesterol response (ratio between cholesterol level on day 14 and before treatment) was significantly better in GVHD responders (0.78 vs. 0.95, $P=0.029$).

The Th1/Th2 ratio before the administration of pravastatin was greater than 1.0 in all but one patient. The Th1/Th2 ratio at study entry tended to be lower in responders than in nonresponders and became even lower after pravastatin treatment in responders, but not in nonresponders, although these differences were not statistically significant (Fig. 1, $P=0.22$). The CD4/CD8 ratio and the expression of human leukocyte antigen-DR on T cells, B cells, and monocytes did not change after treatment (data not shown).

This study demonstrated that pravastatin at 40 mg/day can be safely administered in patients with refractory chronic GVHD, including those taking cyclosporine. The overall response of 28% was similar to that with other alternative salvage treatments including tacrolimus, mycophenolate mofetil, thalidomide, and so on (*3*). However, considering the safety profile of pravastatin, it may be worthwhile for patients with chronic GVHD, especially in those with a coexisting infection that precludes severely immunosuppressive treatments. We chose pravastatin among many statins because it is hydrophilic and was considered to be less likely to cause rhabdomyolysis than other lipophilic statins (*10*, *11*). However, atorvastatin, lovastatin, and simvastatin have stronger in vitro immunosuppressive effects than pravastatin, and thus they may also have greater in vivo effects against chronic GVHD (*7*, *8*).

There is some controversy whether human chronic GVHD is a Th1 or Th2 disease. The immunophenotypic analyses in this study clearly showed that Th1 cells were dominant in patients with chronic GVHD. The efficacy of statin against rheumatoid arthritis, a Th1 disease, has been demonstrated clinically (*12*). In a mouse model of chronic and relapsing

TABLE 1. Severity of chronic graft-versus-host disease in each organ and the response to pravastatin

	Severity code before treatment				Response to treatment				
Each organ	1	2	3	NE	Marked	Good	NC	PD	NE
Performance status	16	2	0	0	0	0	18	0	0
Skin and fascia	6	6	4	2	1	1	13	2	1
Mouth	5	11	2	0	3	2	12	1	0
Eye	7	8	3	0	2	0	13	2	1
Liver enzyme	4	5	9	0	2	1	13	2	0
Thrombocytopenia	14	1	3	0	0	1	15	2	0

Overall response	Responder	5 (28%)
	Nonresponder	13

NE, not evaluable; NC, no change; PD, progressive disease.

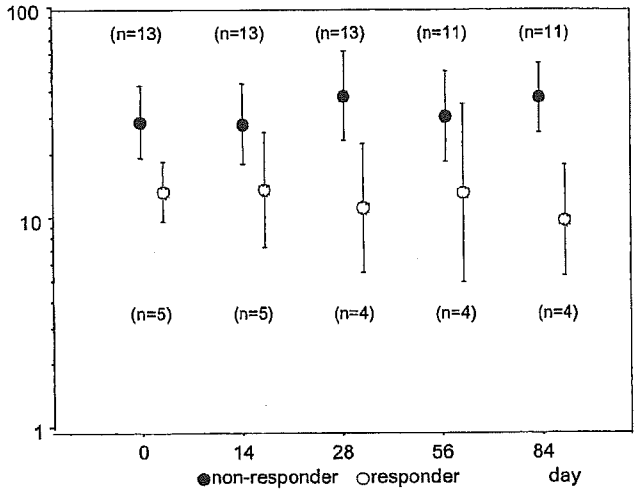

FIGURE 1. Serial changes in the T-helper (Th) 1/Th2 ratio in responders and nonresponders. Data are shown as geometric mean and standard error.

experimental autoimmune encephalomyelitis, oral atorvastatin promoted a Th2 bias and reversed paralysis through the inhibition of STAT4 phosphorylation and the induction of STAT6 phosphorylation (*13*). Although we did not find a statistically significant association between the Th1/Th2 ratio and the response to pravastatin, pravastatin might have ameliorated chronic GVHD by inducing a Th2 shift.

In conclusion, our experience suggests that pravastatin may be safe and effective for the treatment of refractory chronic GVHD. However, a double-blind, randomized, controlled trial is needed to evaluate its true efficacy against refractory chronic GVHD.

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Post-transplant events

Value of surveillance blood culture for early diagnosis of occult bacteremia in patients on corticosteroid therapy following allogeneic hematopoietic stem cell transplantation

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Summary:

Bloodstream infection (BSI) is a significant complication following allogeneic hematopoietic stem cell transplantation (allo-SCT). Corticosteroids mask inflammatory responses, delaying the initiation of antibiotics. We reviewed medical records of 69 allo-SCT patients who had been on >0.5 mg/kg prednisolone to investigate the efficacy of weekly surveillance blood cultures. A total of 36 patients (52%) had positive cultures, 25 definitive BSI and 11 probable BSI. Pathogens in definitive BSI were *Staphylococcus epidermidis* ($n=7$), *S. aureus* ($n=4$), *Entrococcus faecalis* ($n=3$), *Pseudomonas aeruginosa* ($n=5$), *Acenitobacter lwoffii* ($n=4$), and others ($n=10$). The median interval from the initiation of corticosteroids to the first positive cultures was 24 days (range, 1–70). At the first positive cultures, 15 patients with definitive BSI were afebrile. Four of them remained afebrile throughout the period of positive surveillance cultures. Patients with afebrile BSI tended to be older ($P=0.063$), and had indwelling central venous catheters less frequently than febrile patients ($P<0.0001$). Bloodstream pathogens were directly responsible for death in two patients with afebrile BSI. This study demonstrates that cortisosteroid frequently masks inflammatory reactions in allo-SCT recipients given conrticosteroids, and that surveillance blood culture is only diagnostic clue for 'occult' BSI.

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Bloodstream infection (BSI) remains a significant complication following allogeneic hematopoietic stem cell transplantation (allo-SCT). The incidence of post transplant BSI has varied from 12.5 to 41%.[1,8] The frequency of bacterial infection has decreased with the widespread use of antimicrobiotic prophylaxis and empiric administration of broad-spectrum antimicrobiotics. However, better control of Gram negative infections in neutropenic patients has fostered the rise of infections caused by both Gram positive organisms and Gram negative organisms, which are resistant to beta-lactams or fluoroquinolones. These organisms now account for most bacterial infections.[1,9,12]

Although blood culture is the standard method for obtaining a definitive diagnosis of bacteremia, the usefulness of surveillance blood culture has not been adequately evaluated in patients who are on antibiotic prophylaxis.[13,15] Serody *et al*[15] found that blood culture failed to isolate causative organisms in patients developing neutropenic fever while on prophylactic antibiotics. Furthermore, in allo-SCT recipients, inflammatory reactions are frequently absent during neutropenia, and are easily masked by immunosuppressive agents, particularly corticosteroids. Since corticosteroids are commonly used for the prevention and treatment of graft-versus-host-disease (GVHD), asymptomatic bacteremia may be common in the setting of allo-SCT. Few studies reported the benefit of surveillance blood culture.[16] Although a guideline to prevent opportunistic infection among neutropenic patients after allo-SCT has been published,[17] a suitable procedure for the management of infections in asymptomatic patients receiving immunosuppressive therapy remains to be established.

We investigated the frequency and clinical features of subclinical bacteremia in allo-SCT recipients, and evaluated usefulness of surveillance blood culture to diagnose it.

Patients and methods

Patients' characteristics

We reviewed the medical records of 69 patients, who were being treated with >0.5 mg/kg prednisolone following allo-SCT and obtained surveillance blood cultures between August 1998 and August 2002 (Table 1). Patients with acute myeloblastic leukemia (AML) in first or second

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Table 1 Patients' characteristics

Variables	*Number*
Age	
Median (range)	42 (6–67)
Sex	
Male/female	50/19
Primary diseases	
Acute myeloblastic leukemia	20
Acute lymphoblastic leukemia	10
Chronic myelocytic leukemia	14
Malignant lymphoma	15
Myelodysplastic syndrome	6
Others	4
Risk of underlying diseases[a]	
High/standard	45/24
Preparative regimens	
Conventional/reduced-intensity[b]	39/30
GVHD prophylaxis	
cyclosporine alone/others	27/42
HLA	
Matched/mismatched	49/20
Donor	
Related/unrelated	44/25
Cycles of chemotherapy prior to transplant	
5>	33
5⩽	36
History of bacteremia	
yes/no	18/51
Central venous catheters	
Yes/no	57/12
Duration of neutropenia prior to corticosteroid use (days)	
Median (range)	0 (0–55)[c]
Initial grading of acute GVHD	
0–I/II–IV	18/42
Initial grading of chronic GVHD	
Limited/extensive/none	2/6/1
Reasons for steroid use	
Acute GVHD	50
Chronic GVHD	8
Cyclosporine intolerance	5
Engraftment syndrome	3
Others	3
Response to corticosteroid for GVHD treatment	
Yes/no	38/20

[a]We defined acute myeloblastic leukemia in first or second remission, acute lymphoblastic leukemia in first remission, chronic myelocytic leukemia in chronic phase, and malignant lymphoma in first or second complete or partial remission as standard risk, and the others as high risk.
[b]Conventional preparative regimens consisted of cyclophosphamide (CY)/total body irradiation (TBI) ($n=30$), and busulfan (BU)/CY ($n=9$); reduced intensity regimens consists of cladribine-based ($n=6$), fludarabine-based ($n=21$), and fludarabine and low-dose TBI-based regimens ($n=3$).
[c]In all, 53 patients had not become neutropenic.

remission, acute lymphoblastic leukemia (ALL) in first remission, chronic myelocytic leukemia (CML) in chronic phase, and malignant lymphoma (ML) in second complete or partial remission were classified as standard risk, and others as high risk.

Blood cultures

Surveillance blood cultures were obtained weekly in patients who were given >0.5 mg/kg prednisolone. When patients had central venous catheters, cultures were drawn through these lines. Blood samples were drawn after the skin or catheter hub was swabbed with 10% povidone-iodine. When cultures were positive, they were repeated. We obtained 10 to 20 ml of blood in each culture bottle throughout the study. Culture bottles were incubated until flagged as positive or negative for 7 days in an instrumented blood culture system (BACTEC 9240, Becton Dickinson, Cockeysville, MD, USA) using septic-check biphasic blood media.

Definition

There are no widely used criteria for the diagnosis of BSI in patients receiving corticosteroids. We used the Centers for Disease Control definition of nosocomial infection,[18] referring to the data from Weinstein *et al*[19] on the probability that isolated microorganisms are actual pathogens. Under immunosuppression, symptoms such as fever, with or without chills, and purulence around an intravascular device are often masked and not reliable for use as criteria. Therefore, only the number of positive blood cultures and the species of the detected organisms are considered to be reliable.

We defined definitive infection as follows:

(a) Common skin contaminants such as diphtheroids, *Bacillus* spp., *Propionibacterium* spp., coagulase-negative staphylococci (CNS), and micrococci were cultured in at least two consecutive blood cultures drawn on separate occasions.
(b) For rare contaminants such as *Haemophilus influenzae, Streptococcus pneumoniae, Neisseria meningitidis,* and group B *Streptococcus*, one positive blood culture was sufficient for a diagnosis of BSI. One positive culture was sufficient for organisms that Weinstein *et al*[19] reported to be actual pathogens in more than 60% of positive cultures that were obtained from patients without a related infection at any other site.

Control patients were those who had negative blood cultures throughout the immunosuppressive period. Other cases were defined as probable infections. In most cases, the common skin contaminants were cultured only once.

A new infectious episode in afebrile patients was defined as the first positive blood culture, or any positive culture that was drawn 96 h or later after a previous positive culture. Blood cultures positive for different organisms were regarded as separate episodes of BSI when blood samples were drawn on different days. Blood cultures in which multiple organisms grew on the same day were regarded as a single polymicrobial BSI.

Mortality was considered to be directly attributable to a bloodstream pathogen if the patient died within 7 days after the last positive blood culture without any other probable cause of death.[1]

Clinical management strategy

Patients were managed in reverse isolation in a laminar airflow-equipped room. All patients received oral prophylactic agents: that is, trimethoprim/sulfamethoxazole from day −21 to day −7, and then twice a week after engraftment, and ciprofloxacin 600 mg per day orally for bacterial prophylaxis from the initiation of the conditioning regimen to engraftment. Fluconazole 200 mg was administrated orally as fungal prophylaxis from the initiation of the conditioning regimen to the discontinuation of immunosuppressive agents. Acyclovir 1000 mg per day was given orally from day −7 until day 35, and then given at 400 mg per day until the discontinuation of immunosuppressive agents.[20]

Neutropenic fever was managed as previously reported.[21] Briefly, we empirically started beta-lactam antibiotics, and amphotericin B was added at a dose of 0.5 mg/kg/day when the fever persisted for more than 5 days. Neutropenia was defined as neutrophil counts less than 500 per cubic millimeter.

Study design, data collection and statistical analysis

The aims of this study were to (1) determine the frequency of bacterial and fungal infections in allo-SCT recipients who were receiving corticosteroid therapy, (2) identify risk factors for the development of BSI under corticosteroid therapy and (3) evaluate the usefulness of surveillance blood culture in asymptomatic patients on immunosuppressive therapy. Microbiological results were obtained from physicians' medical records, nursing flow sheets, and computerized reports. We collected information including sex, age, complete blood cell counts, body temperature, GVHD grade, antimicrobiotic use, dose of corticosteroids, additional use of other immunosuppressive agents, and day of last follow-up.

Risk factors for BSI were determined by comparing patients with definite evidence of BSI to controls. Risk factors included age, sex, primary disease, preparative regimen, GVHD prophylaxis, HLA-matching, type of donor cycles of chemotherapy prior to transplant, history of bacteremia, insertion of central lines, presence of neutropenia, grade of acute GVHD, presence of chronic GVHD, and initial dose of corticosteroids. These variables were evaluated at the initiation of corticosteroid. Patients with probable BSI were excluded from the analysis of risk factors, since it was unclear whether these patients actually had BSI.

A univariate analysis using the χ^2 test and Mann–Whitney test was performed to evaluate risk factors for BSI. We added a multiple regression analysis to assess the individual contributions of potential predictive factors. For the multivariate analysis, stepwise selection was used to identify the prognostic factors for BSI. The level of significance was set at $P<0.05$.

Results

Positive blood cultures

We obtained 968 blood samples from 69 patients, with a median of 11 blood cultures (range, 2 to 35) per patient. Of the total 110 positive blood cultures from 36 patients, 86 cultures from 25 patients were definitive BSI. The number of infectious episodes was 40, and 12 patients experienced multiple episodes. One patient developed polymicrobial BSI. Identified microorganisms were 12 bacterial strains from 24 patients and two fungal strains from one patient (Table 2). The microorganisms cultured from blood and catheter tips were identical in three episodes: *Staphylococcus epidermidis* ($n=2$) and *S. aureus* ($n=1$). Gram positive and Gram negative bacteremia developed in 14 and 15 patients, respectively.

In all, 15 positive cultures drawn from 11 patients were regarded as probable BSI. Repeated blood cultures after initial positive cultures were negative in 10 patients. A second blood culture was not obtained in the remaining patient because the patient died early of gastrointestinal bleeding due to acute GVHD. Surveillance blood cultures remained negative throughout the study period in the remaining 33 patients. These patients served as a negative control.

Patients' characteristics at the initiation of corticosteroids

Clinical features at the initiation of corticosteroids are shown in Table 3. Patients with definitive BSI had more profound neutropenia than controls ($P=0.0014$). No significant differences in other variables were observed between the two groups.

Characteristics of the first BSI

When the first positive cultures were drawn, 15 of the 25 patients with definitive BSI were afebrile (Table 4). Four of

Table 2 Organisms cultured from blood and catheter tips

Isolated organisms	*Number of patients (number of episodes)*	
	Blood	*Catheter*
Gram positive		
Staphylococcus epidermidis	7 (7)	4 (4)
Staphylococcus aureus	4 (5)	1 (1)
Entrococcus faecalis	3 (3)	
Entrococcus faecium	1 (1)	
Staphylococcus hominis	1 (1)	
Splenius capitis Splenius ureo		1 (1)
Bacillus cereus		1 (1)
Gram negative		
Pseudomonas aeruginosa	5 (7)	
Acenitobacter lwoffii	4 (4)	
Bacillus. sp.	2 (4)	
Escherichia coli	2 (2)	
Stenotrophomonas maltophilia	2 (2)	1 (1)
Proteus. mirabilis	1 (1)	
Clostridium innocum	1 (1)	
Fungi		
Candida parapsilosis	1 (1)	
Candida krusei	1 (1)	

Table 3 Clinical characteristics of patients with definitive and probable BSI, and controls[a]

Variables	*Definitive BSI (n = 25)*	*Controls (n = 33)*	*Probable BSI (n = 11)*
Age			
Median (range)	44 (6–61)	42 (16–67)	36 (19–70)
Sex			
Male/female	16/9	26/7	8/3
Primary diseases			
Acute myeloblastic leukemia	7	10	3
Acute lymphoblastic leukemia	4	5	1
Chronic myelocytic leukemia	4	7	3
Malignant lymphoma	7	5	3
Myelodysplastic syndrome	1	5	0
Others	2	1	1
Risk of underlying diseases[b]			
High/standard	19/6	19/14	7/4
Preparative regimens			
Conventional/reduced-intensity[c]	11/14	22/11	6/5
GVHD prophylaxis			
Cyclosporine alone/others	12/13	10/23	5/6
HLA			
Matched/mismatched	18/7	24/9	7/4
Donor			
Related/unrelated	17/8	20/13	7/4
Cycles of chemotherapy prior to transplant			
5 > /5 ⩽	11/14	18/15	4/7
History of bacteremia			
Present/absent	7/18	11/22	0/11
Central venous catheters			
Present/absent	23/2	25/8	9/2
Duration of neutropenia (days)			
Median (range)	0 (0–55)	0 (0–5)	0 (0–9)
Doses of corticosteroids			
0.5 mg/kg	2	5	1
1.0 mg/kg	14	18	6
2.0 mg/kg	7	8	2
Pulsed (1 g/body)	2	2	2
Acute GVHD			
0–I/II–IV	8/12	5/25	5/5
Chronic GVHD			
Present/absent	4/1	3/0	1/0

BSI = blood stream infection; GVHD = graft-versus-host disease.
[a]Patients' characteristics were compared between patients with definite BSI and controls.
[b]We defined acute myeloblastic leukemia in first or second remission, acute lymphoblastic leukemia in first remission, chronic myelocytic leukemia in chronic phase, and malignant lymphoma in first or second complete or partial remission as standard risk, and the others as high risk.
[c]Conventional preparative regimens consisted of cyclophosphamide (CY)/total body irradiation (TBI) ($n = 30$), and busulfan (BU)/CY ($n = 9$); reduced intensity regimens consists of cladribine-based ($n = 6$), fludarabine-based ($n = 21$), and fludarabine and low-dose TBI-based regimens ($n = 3$).

the 15 patients remained afebrile throughout the period of positive surveillance cultures. Patients with afebrile BSI tended to be older ($P = 0.063$), and were inserted central venous catehters less frequently than febrile patients ($P < 0.0001$).

Gram negative rods were cultured in eight of the 15 patients. Five patients remained asymptomatic in their

Table 4 Clinical characteristics of patients with documented bloodstream infections at the first positive blood cultures

Variables	*Afebrile patients (n = 15)*	*Febrile patients (n = 10)*
Sex		
Male/female	8/7	8/2
Age		
Median (range)	50 (6–61)	35 (19–56)
Risk of underlying diseases		
High/standard	4/11	2/8
Interval between initiation of corticosteroid and the first positive blood cultures		
Median (range)	23 (6–70)	26.5 (1–74)
Presence of neutropenia[a]		
Yes/no	1/14	4/6
Central venous catheters[a]		
Yes/no	2/13	10/0
Doses of corticosteroid[a]		
0.5 mg/kg	4	4
1.0 mg/kg	7	3
2.0 mg/kg	3	3
Pulsed (1 g/body)	1	0
Acute GVHD[a,b]		
0–I/II–IV	6/4	2/5
Chronic GVHD[a]		
Limited/extensive	1/3	1/1
Use of prophylactic antimicrobiotics[a]		
Yes/no	11/4	8/2
Causative organisms		
Gram-positive cocci	7[c]	4[d]
Gram-negative rods	8[e]	5[f]
Fungi	0	1[g]
Sensitivity to prophylacitic antibiotics		
Sensitive/resistant (unknown)	4/7 (0)	2/4 (2)
Reccurence of BSI	6	6
Mortality due to bloodstream infection	2	3

[a]These variables were evaluated at the first positive blood cultures.
[b]Two patients who developed documented BSI before engraftment were not included in the analysis of GVHD.
[c]These cocci included *S. epidermidis* ($n = 3$), *S. aureus* ($n = 3$), and *E. faecalis* ($n = 1$).
[d]These included *S. epidermidis* ($n = 2$), *S. aureus* ($n = 1$), and *S. hominis* ($n = 1$).
[e]These included *P. aeruginosa* ($n = 1$), *A. lwoffi* ($n = 3$), *E. coli* ($n = 1$), *S. maltiphilia* ($n = 1$), *Bacteroides* spp. ($n = 1$), and *P. mirabilis* ($n = 1$).
[f]These included *P. aeruginosa* ($n = 3$), *A. lwoffi* ($n = 1$), and *S. maltophilia* ($n = 1$).
[g]*C. parapsilosis*.

clinical courses. Two presented mild diarrhea. With the initiation of antibiotics and/or removal central lines, diarrhea subsided and the following blood culture became negative. The other patient developed pneumonia, which was successfully treated with antibiotics.

Characteristics and management of recurrent BSI

After the initial BSI episode, 15 recurrent BSI episodes were observed in 12 patients. In all, 10 patients had two BSI episodes each, one had three, and one had four. The median interval between a previous episode and recurrent BSI was 21 days (2–31 days). When BSI recurred, patients were febrile in six episodes and afebrile in nine. The dose of corticosteroids was 0.5–0.9 mg/kg in 11 episodes, 1.0–1.9 mg/kg in three, and $\geqslant$2.0 mg/kg in one. The causative organism was the same as the previous pathogen in five episodes, and included *S. aureus* ($n=1$), *Bacteroides sp.* ($n=2$), and *Pseudomonas aeruginosa* ($n=2$). In the 15 recurrent BSI episodes, all of the patients received some antimicrobiotics, and the cultured organisms were either sensitive ($n=8$) or resistant ($n=4$). Susceptibility was not examined in the remaining three episodes.

Mortality

Of the 25 patients with definitive BSI, 15 died during the study period. BSI was directly associated with the death of five patients, to give a mortality rate of 20%. The causative organisms included *S. epidermidis, P. aeruginosa, Entrococcus faecalis, Clostridium innocum*, and *Candida krusei* until discharge.

Risk factors of BSI during corticosteroid administration

A univariate analysis showed presence of neutropenia at the initiation of corticosteroid (odds ratio; 1.26, 95% confidence interval (CI); 1.03–1.53, $P=0.032$) and dosage of corticosteroid (per gram) (odds ratio; 4.39, 95% CI; 1.28–15.1, $P=0.019$) as risk factors. A multiple regression analysis revealed that dosage of corticosteroid (per gram) was significantly associated with BSI (odds ratio; 5.16, 95% CI; 1.50–17.7, $P=0.0091$).

Discussion

The present study showed that BSI remains a common and important problem. It occurred in 25 of 69 (36%) patients. Previous studies identified some predisposing factors for post transplant BSI: mucositis induced by the high-dose preparative regimens,[5] use of central intravenous catheters,[22] presence of GVHD with or without treatment using corticosteroid,[11] and prolonged neutropenia.[23] While all the patients received corticosteroid in this study, most patients were not neutropenic, and mucositis was rarely observed. These findings suggest that use of corticosteroid is a risk factor of BSI even after neutrophil recovery. Since corticosteroid was given for the treatment of GVHD, it is difficult to differentiate the effect of GVHD from that of concomitant use of corticosteroid as a cause of BSI. At present, we can safely mention that patients who receive corticosteroid for the treatment of GVHD are at high-risk of BSI. This study urges us to revise our prophylactic and treatment strategy of bacterial infection in allo-SCT recipients at high risk of bacterial infection.

It is evident that early diagnosis and prompt initiation of aggressive antibacterial treatment is essential to improve prognosis of patients with BSI. However, they are frequently difficult in patients who are given corticosteroid, which masks subjective and objective symptoms associated with BSI. Surveillance blood cultures might be useful for establishing early diagnosis of BSI in these patients. Yet, the usefulness of surveillance blood culture remains in the setting of allo-SCT, in which most patients receive prophylactic antibiotics. While guidelines exist regarding the number and timing of sampling for blood cultures before antibiotic therapy is initiated,[24] they are not established in patients on prophylactic antibiotics. Blood culture rarely isolates causative organisms after initiation of antibiotic therapy.[15,25] Antibiotics decrease the yield of blood cultures, and routine collection of blood samples may not be helpful in allo-SCT recipients. However, our study revealed a high incidence of 'occult' BSI in these patients. Except for patients' ages and uses of central venous catheters, there were no significant differences in clinical characteristics between an 'occult' BSI and a 'febrile' one. It should be noted that the some patients with definite BSI remained afebrile at the time of blood sampling, and that surveillance blood culture was only diagnostic clue for this complication. Considering that blood stream pathogens were directly responsible for deaths in two patients with 'occult' BSI, surveillance blood culture seemed to be beneficial in making an early diagnosis and initiating aggressive antibacterial treatment.

Concerning the causative organisms, 17, 21, and two of the 40 isolates were Gram positive bacteria, Gram negative bacteria, and fungi, respectively. In Gram positive bacteria, CNS is the most common isolate (seven patients), followed by *S. aureus* (four patients), and *E. faecalis* (three patients). One of the seven patients with definite BSI caused by CNS died of bacteremia. These findings indicate that CNS is the most common microorganism isolated from blood cultures in allo-SCT recipients, and that BSI caused by the organism is sometimes fatal. While determining whether there is true bacteremia or merely contamination is a clinical dilemma, it is critical to make an early diagnosis and prompt initiation of appropriate treatments for BSI caused by CNS. It should be noted that removal of inserted catheters is frequently sufficient, because CNS infections are usually related to the long-term central venous indwelling catheters for venous access.[26] Surveillance blood cultures might have contributed to make an early diagnosis of CNS bacteremia and prompt initiation for adequate antibacterial treatment in these patients.

Concerning Gram negative bacteria, five and four patients developed BSI caused by *P. aeruginosa* and *Acenitobacter lwoffii*, respectively. Fewer episodes of BSI were caused by these organisms than by CNS, although gastrointestinal involvement of acute GVHD, a risk factor of Gram negative bacteremia following HSCT, was observed in 25 of the 69 patients. Prophylactic use of oral

fluoroquinolone might reduce the incidence of Gram-negative bacteremia as reported previously.[27] Alternatively, concomitant antimicrobiotics might have influenced the sensitivity of blood cultures.[14] Compared with *S. aureus* and streptococci, Gram negative bacilli are less frequently isolated from antimicrobiotic blood cultures. Although the incidence of Gram negative bacteremia was not high in this study, Gram negative bacteremia was fatal in two patients, suggesting that current approaches using fluoroquinolone-based prophylaxis may be insufficient to prevent life-threatening Gram negative bacteremia.

This study has several limitations, including the fact that this was a retrospective study of patients with heterogeneous backgrounds. In most cases of BSI, the first positive blood cultures were drawn through central venous catheters, and subsequent cultures were obtained by venipuncture. However, we believe that the results are still informative, since little information has been reported to date. BSI is a significant problem in allo-SCT recipients, especially when they are receiving corticosteroid treatment, which masks early signs of inflammatory responses. The data observed in this study support the notion that surveillance blood culture is useful for an early diagnosis of BSI and effective early intervention. Future prospective clinical trials are warranted to confirm this point.

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Research

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Clinical response in Japanese metastatic melanoma patients treated with peptide cocktail-pulsed dendritic cells

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Abstract

Background: Metastatic, chemotherapy-resistant melanoma is an intractable cancer with a very poor prognosis. As to immunotherapy targeting metastatic melanoma, HLA-A2$^+$ patients were mainly enrolled in the study in Western countries. However, HLA-A24$^+$ melanoma patients-oriented immunotherapy has not been fully investigated. In the present study, we investigated the effect of dendritic cell (DC)-based immunotherapy on metastatic melanoma patients with HLA-A2 or A24 genotype.

Methods: Nine cases of metastatic melanoma were enrolled into a phase I study of monocyte-derived dendritic cell (DC)-based immunotherapy. HLA-genotype analysis revealed 4 cases of HLA-A*0201, 1 of A*0206 and 4 of A*2402. Enriched monocytes were obtained using OptiPrep™ from leukapheresis products, and then incubated with GM-CSF and IL-4 in a closed serum-free system. After pulsing with a cocktail of 5 melanoma-associated synthetic peptides (gp100, tyrosinase, MAGE-2, MAGE-3 and MART-1 or MAGE-1) restricted to HLA-A2 or A24 and KLH, cells were cryopreserved until used. Finally, thawed DCs were washed and injected subcutaneously (s.c.) into the inguinal region in a dose-escalation manner.

Results: The mean percentage of DCs rated as lin$^-$HLA-DR$^+$ in melanoma patients was 46.4 ± 15.6 %. Most of DCs expressed high level of co-stimulatory molecules and type1 phenotype (CD11c$^+$HLA-DR$^+$), while a moderate number of mature DCs with CD83 and CCR7 positive were contained in DC products. DC injections were well tolerated except for transient liver dysfunction (elevation of transaminases, Grade I-II). All 6 evaluable cases except for early PD showed positive immunological responses to more than 2 melanoma peptides in an ELISPOT assay. Two representative responders demonstrated strong HLA-class I protein expression in the tumor and

very high scores of ELISPOT that might correlate to the regression of metastatic tumors. Clinical response through DC injections was as follows : 1 CR, 1 PR, 1 SD and 6 PD. All 59 DC injections in the phase I study were tolerable in terms of safety, however, the maximal tolerable dose of DCs was not determined.

Conclusions: These results suggested that peptide cocktail-treated DC-based immunotherapy had the potential for utilizing as one of therapeutic tools against metastatic melanoma in Japan.

Background

Despite many attempts in the last few years to target cancer-specific antigens, a breakthrough in terms of clinical response has yet to be achieved mainly because of a scarcity of effective genuine cancer antigens, immunological evasion, or an immunosuppressive state.

Melanoma-associated antigens are categorized as class I human leukocyte antigen (HLA)-restricted cancer/testis antigens [1] which are considered to be tolerable to the immune system because they are also expressed in normal tissues. However, malignant melanoma is the most well known cancer in which multiple tumor-specific antigens have been defined and utilized in vaccination strategies as peptide vaccines or peptide-pulsed DC vaccines [2-9].

From a clinical point of view, several vaccination strategies for stage IV melanoma using a combination of several (more than 3) peptides with a restriction to HLA-A2 have been reported to date [10,11]. However, little immunotherapeutic study regarding HLA-A24-restricted multiple peptides has been conducted because HLA-A24 is not a common allele in Caucasians. Several studies have demonstrated the identification of many HLA-A24-restricted CTL epitopes from various cancer-related antigens including p53, CEA, telomerase, tyrosinase, MAGE proteins etc. [12-18]. When it comes to melanoma, our group demonstrated the feasibility of using a combination of 5 melanoma-associated peptides with restriction of HLA-A24 (peptide cocktail) as a specific cancer vaccine in an immunotherapeutical trial (Akiyama et al, Anticancer Res., 2004). Based on basic research results, a phase I clinical trial of HLA-A2 or A24-restricted melanoma peptide cocktail-pulsed dendritic cell-based immunotherapy has been performed. Here we describe the safety and efficacy of DC-based immunotherapy against metastatic melanoma.

Materials and methods

Patient characteristics and eligibility criteria

Nine patients with metastatic melanoma were enrolled in a phase I clinical trial of a peptide cocktail-pulsed DC-based vaccine approved by the Institutional Review Board (No. 12–93 and 12–94) of the National Cancer Center, Tokyo. All patients gave written informed consent. All patients had received prior surgery, chemotherapy and radiation (Table 1). Three subjects had metastatic lesions in the brain and been given radiation to control them. Inclusion criteria were: i) biopsy-proven stage IV metastatic melanoma, ii) age ≥ 18 years, iii) performance status ≤ 2, iv) HLA-A2 or A24 phenotype and v) measurable target lesions. Exclusion criteria were : i) prior therapy < 4 weeks before trial entry, ii) untreated CNS lesion, iii) pregnancy, iv) autoimmune disease, and v) concurrent corticosteroid/immunosuppressive therapy. All the patients, who gave written informed consent, received subcutaneously (s.c) 3 DC vaccines at the inguinal region weekly and toxicity was checked. DCs were injected in dose-escalation design at a dose level per cohort of 1.0, 2,0 and 5.0 × 10^7/body/shot (Table 1). The injected DC number was calculated from the percentage of Lin$^-$HLA-DR$^+$ gated populations in a FACS analysis.

Table 1: Phase I study of DC-based therapy against melanoma

Patient No.	Age	Sex	Previous therapy	Measurable lesions	DC injection (times)	Side effect	DTH		Response
							peptide	KLH	
1	41	F	ST, CT, RT, IFNβ	lung, LN	1 × 10^7(10)	Hepatic (II)	-	++	PR
2	75	M	ST, CT, IFNβ	LN	1 × 10^7(10)	-	+	+	SD
3	49	F	ST, CT, IFNβ, RT	lung, liver	1 × 10^7(3)	-	-	-	(PD)*
4	49	M	ST, CT	lung, liver	2 × 10^7(6)	-	-	-	PD
5	50	M	ST, CT, IFNβ	lung, liver, LN	2 × 10^7(6)	Hepatic (I)	-	-	PD
6	69	M	ST, CT, IFNβ	LN	2 × 10^7(10)	-	+	+	CR
7	61	M	ST, CT, RT	liver, LN	5 × 10^7(8)	Hepatic (I)	+	++	PD
8	64	F	ST, CT, RT	lung	5 × 10^7(3)	Fever (I)	-	-	(PD)
9	66	F	ST, CT,	lung, LN	5 × 10^7(3)	-	-	-	(PD)

* The (PD) patients represent those who received fewer than 4 DC injections because of an early progression of the disease.

Preparation of DCs and peptides

Leukapheresis products from 7 L of processed blood were washed and centrifuged using density-adjusted OptiPrep™ (Axis-Shield PoC, Oslo, Norway), then the monocyte layer at the top was retrieved. Cells were transferred to an X-fold culture bag (Nexell, Irvine, CA) and cultured in the presence of GM-CSF at 50 ng/ml (CellGenix, Freiburg, Germany) and IL-4 at 50 ng/ml (CellGenix) in X-VIVO15 serum-free medium (Biowhittaker, Walkersville, MD). After 7 days, harvested cells were pulsed with a cocktail of 5 melanoma-specific synthetic peptides (25 μg/ml each) restricted to HLA A2 or A24 and KLH (25 μg/ml, Intracell, Frederick, MD). DC-enriched cells were washed and cryopreserved in Cryocyte bags (Baxter Healthcare Co., Deerfield, IL) until used. The purity of $CD14^+$ cells was evaluated with a flow cytometer (FACSCalibur, Becton-Dickinson Co., CA) before and after OptiPrep™ separation. The percentage of DCs was rated as the $lin^-HLA\text{-}DR^+$ population (lineage antibodies including CD3, CD14, CD16, CD19, CD20, CD56 ; Becton-Dickinson Co.). The additional DC-related markers were determined on gated $lin^-HLA\text{-}DR^+$ cells. The following peptides restricted to HLA-A2 or A24 were synthesized according to GMP standards by Multiple Peptide Systems, CA. HLA-A2: MART-1_{27-35} (AAGIGILTV), $gp100_{209-217}$ (IMDQVPFSV), $tyrosinase_{368-376}$ (YMDGTMSQV), MAGE-$2_{157-166}$ (YLQLVFGIEV), MAGE-$3_{271-279}$ (FLWGPRALV) ; HLA-A24: $gp100_{152-160}$ (VWKTWGQYW), $tyrosinase_{206-214}$ (AFLPWHRLF), MAGE-$1_{135-143}$ (NYKHCFPEI), MAGE-$2_{156-164}$ (EYLQLVFGI), MAGE-$3_{195-203}$ (IMPKAGLLI).

Characterization of tumor specimens before DC vaccines

Skin metastatic lesions were obtained from patients who gave written informed consent. The expression of melanoma tumor antigens was investigated using RT-PCR as described previously [19]. HLA protein expression was also evaluated using an immunohistochemical (IHC) analysis with anti-HLA-A2 or A24 monoclonal antibody (One Lambda Inc., Canoga Park, CA). A phenotypical analysis of lymphocytes infiltrating the tumor site was also performed using IHC.

Clinical and immunological monitoring

Adverse effects were evaluated according to the NCI Common toxicity criteria after 3 DC injections. Standard conventional definitions of major (complete or partial) objective responses were used. Stable disease (SD) was defined as less than a 25% change in size with no new lesions lasting at least 4 weeks. Clinical response was rated as maximal through the DC vaccinations. The patients received up to 10 injections on the condition that at least one measurable lesion showed more than stable disease (SD) response and/or an ELISPOT assay performed after 4 injections indicated a positive response for more than 1 melanoma-associated peptides. PBMC samples were harvested before and 29, 78, 134 and 190 days after the 1 st DC injection, and frozen prior to use for immunological monitoring tests. All patients were followed up for 2 years after the enrollment into the study.

ELISPOT assay

The ELISPOT assay was performed using in vitro re-stimulations. Briefly, PBMCs were incubated in a 24-well culture plate at 4×10^6 per ml and divided into non-adherent and adherent cells. Adherent cells were treated with a peptide cocktail and β2-microglobulin for 2 hrs, and co-cultured with non-adherent cells in the presence of IL-2 at 15 U/ml and IL-7 at 10 ng/ml. On day 7, non-adherent cells were re-stimulated with peptide-pulsed adherent cells. On day14, responder cells (1×10^4/well) were incubated with peptide-pulsed target cells (1×10^5/well; .221A201 cells for HLA-A2 peptide or TISI cells for HLA-A24 peptide) in a 96-well culture plate coated with anti-IFN-γ antibody (MABTECH AB, Nacka, Sweden) overnight. Finally positive spots stained with anti-IFN-γ antibody were measured using the KS ELISPOT system (Carl Zeiss AG, Overkochen, Germany). HLA-A2-restricted Influenza M1 peptide (GILGFVFTL) or HLA-A24-restricted EBNA3A peptide (RYSIFFDY) was used as a negative control.

Tetramer staining

PBMCs were re-stimulated twice in vitro and utilized for tetramers staining. $CD8^+$-enriched T cells were obtained by the depletion of $CD4^+$ T cells using Dynabeads M-450 CD4 (Dynal, Oslo, Norway) and used for tetramers staining. The staining was performed according to the method reported by Kuzushima et al [20]. The PE-labeled tetramers used in the present study were as follows: HLA-A*0201 MART1 (Beckman Coulter Inc., San Diego, CA), HLA-A*0201 gp100, HLA-A*2402 tyrosinase, HLA-A*2402 MAGE-1, HLA-A*2402 HIV (RYLRDQQLL) and HLA-A*0201 Influenza M1 tetramers (MBL, Nagoya, Japan).

Intracellular cytokine staining

PBMCs were stimulated with 25 ng/ml of PMA (Sigma) and 1 μg/ml of ionomycin (Sigma) for 5 hrs in a 96-well culture plate. Breferdin A (10 μg/ml) was also added to cultures in the last hour. After the stimulation, cells were stained with FITC-anti-CD4 MoAb, and subsequently intracellular staining was performed with fix/permealization buffer and PE-labeled anti-IFN-γ or anti-IL-4 MoAb (Pharmingen, San Diego, CA). Finally, the ratio of Th1 ($IFN\text{-}\gamma^+$) and Th2 ($IL\text{-}4^+$) was calculated in PBMC samples obtained before and after DC vaccination.

DTH reactions

The HLA-A2 or A24 peptide cocktail solution diluted to a dose of 5 μg/ml (each peptide) and KLH (50 μg/ml) were injected intradermally on the patient's forearm and the

Table 2: Immunological monitoring in melanoma patients

Patient No.	HLA	Tumor antigen, HLA expression	ELISPOT	Tetramer	Th1/Th2 balance
1	A*2402	3/5(Tyr,M1,M2), A24(+)	3/5(Tyr,M1,M2)	Tyrosinase (0.34%)	5.19 (1.45)[a]
2	A*0201	A2(+)	2/5(MART1,gp100)	MART1 (0.64%)	3.68 (1.49)
3	A*2402	A24(-)	N. D[b]	N. D.	-
4	A*0206	A2(-)	2/5(MART1, M2)	-	3.05 (2.57)
5	A*0201	A2(-)	2/5(MART1, M2)	MART1 (1.48%)	2.83 (3.68)
6	A*2402	2/5(M2,M3), A24(+)	2/5(M2, M3)	-	3.76 (2.00)
7	A*0201	4/5(MART1,Tyr,gp100,M2), A2(+)	2/5(gp100, M2)	-	2.64 (1.79)
8	A*2402	A24(-)	N. D.	N. D.	-
9	A*0201	A2(+)	1/5(gp100)[c]	N. D.	N. D.

[a]The value in the parenthesis shows Th1/Th2 ratio prior to DC vaccination. [b]N. D. ; not done.
[c]The value shows the one obtained prior to DC vaccines.

redness and induration at the injection site was measured. PPD was used as a positive control.

Statistical analysis

Statistical differences were analyzed using Student's paired two-tailed *t*-test. Values of $p < 0.05$ were considered significant.

Results

DC characterization

The mean percentage of DCs rated as lin$^-$HLA-DR$^+$ in melanoma patients was 46.4 ± 15.6 %, not different from that in healthy volunteers (data not shown). The frequencies of the DC-related markers were determined on gated lin$^-$HLA-DR$^+$cells : HLA-class I 97.5 ± 0.9 %, CD80 87.6 ± 6.9 %, CD86 85.5 ± 7.4 %, CD1a 55.2 ± 24.2 %, CD83 29.9 ± 13.3 %, CCR7 32.4 ± 13.7 %, DC SIGN 78.2 ± 19.3 %, CD11c$^+$HLA-DR$^+$ 90.6 ± 6.0 %, CD123$^+$HLR-DR$^+$ 0.99 ± 1.3 %. Most of DCs expressed high level of co-stimulatory molecules and type1 phenotype (CD11c$^+$HLA-DR$^+$), while a moderate number of mature DCs with CD83 and CCR7 positive were contained in DC products. On the other hand, the T cell-stimulating activity of DCs investigated in the MLR assay using allogeneic T cells was as strong as that of DCs obtained from healthy volunteers (data not shown).

Characterization of tumor specimen

An analysis of melanoma antigen expression by RT-PCR was performed in 3 cases. The expression of more than 2 antigens in the tumor was verified in all cases. HLA protein expression was positive in 5 out of 9 cases (Table 2). Patient 1 who showed a remarkable clinical response (PR), was representative of HLA protein-positive cases (Fig. 1). In contrast, in patient 7, HLA-A2 protein expression in the tumor was lost in the course of treatment.

ELISPOT assay

CTL precursors of more than 2 melanoma peptides were recognized after DC vaccines in 6 of 9 cases. Two HLA-A2$^+$ cases (patients 5 and 9) showed HLA-A2 peptide-specific CTL responses before the vaccination. Patients 1 and 6, which showed remarkable clinical responses, exhibited many CTL precursors against a HLA-24 restricted peptide-cocktail (Table 3, Figure 2). Notably, in patient 1, a remarkable increase in the CTL response to the HLA-A24 peptide cocktail was seen in accordance with the regression of metastatic tumor of the lung (Fig. 3). On the other hand, patient 7 also demonstrated a high CTL precursor frequency, but showed no significant clinical response.

Tetramer staining

After CD4$^+$ T cell depletion, the frequency of CD8$^+$ cells was more than 85%. The proportion of PE-labeled tyrosinase-HLA-A24 tetramer-positive cells among gated CD8$^+$ cells was 0.34% in patient 1 (Table 2). HIV-A24 tetramer (negative control)-positive cells were not detected. The percentage of PE-labeled MART1-HLA-A2 tetramer-positive cells was 0.64% and 1.48% in patients 2 and 5, respectively. On the other hand, that of Influenza M1-HLA-A2 tetramer (negative control)-positive cells was 0.04%.

Th1 and Th2 balance after DC vaccination

In 5 of 6 evaluable cases, the balance of Th1 and Th2 shifted more to Th1 after 4 DC injections compared with prior to vaccination. (Table 2). The amplitude of the shift seemed to be larger in clinical responders (patients 1, 2, 6) than non-responders (patients 4, 5, 7) (% of ratio increase; 264 ± 86 vs. 114 ± 35).

DTH

Three of 6 evaluable cases showed positive DTH to a peptide-cocktail after DC injections (Table 1). On the other hand, 4 of 6 cases developed a DTH response to KLH pro-

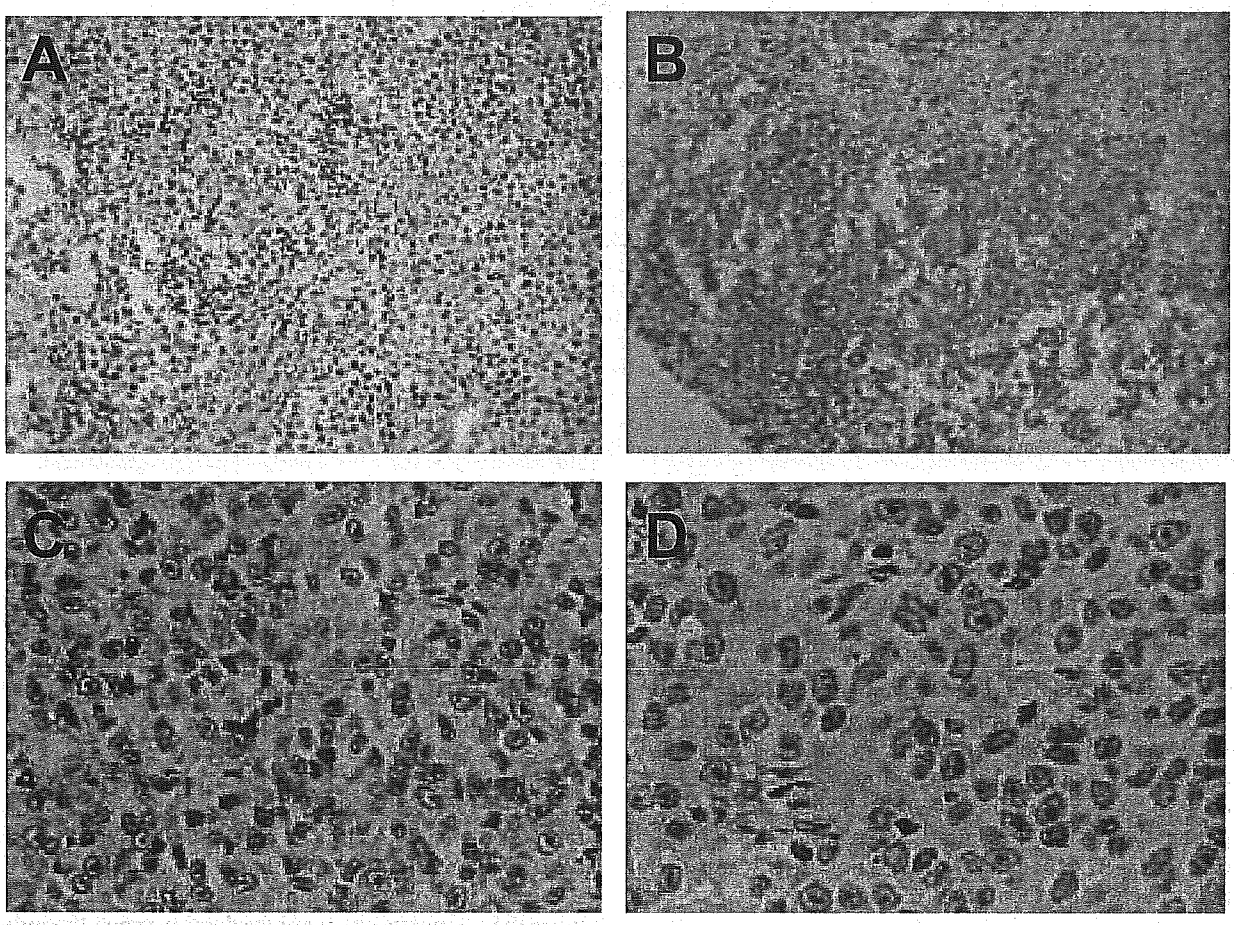

Figure 1
Immunohistochemical analysis of metastatic tumor tissue from responder patient 1 and non-responder patient 7. A; H-E stain and B; anti-HLA-A24 MoAb from patient 1. C; anti-HLA-A2 MoAb before DC vaccination and D; anti-HLA-A2 MoAb after 4 DC injections from patient 7. Magnification × 200.

tein. There were stronger reactions to KLH in patients 1 and 7.

Adverse effects of DC vaccine

Safety was assessed after 3 DC injections in all 9 cases. Three of 9 patients developed mild hepatic dysfunction (grade I-II), however it was only transient and disappeared in spite of the continuance of DC injections. Rheumatoid factor and anti-nuclear antibody were negative before the injection, but increased to 1:160 and 1:40, respectively after the injections finished in patient 1. No clinical symptoms of autoimmune disease were found in patient 1 (Table 1).

Clinical response

Clinical response was rated as maximal through the DC vaccinations. In 6 evaluable cases except for 3 cases of early PD cases due to a rapid progression of the disease, 1CR (patient 6), 1PR (patient 1), 1SD and 3 PD were obtained (Table 1). Large metastatic lesions in the lung and hilar nodes in patient 1 dramatically decreased in size after 4 DC injections, and almost disappeared after treatment finished (Fig. 3). Moderate sized cervical metastatic lesions in patient 6 finally started to decrease after 8 DC injections and disappeared surprisingly rapidly after the finish of DC therapy. In contrast, patient 7 who exhibited good immunological responses in the ELISPOT assay and

Table 3: Peptide cocktail-specific CTL precursor frequency during DC vaccination

Patient No.	DC injection (times)	Spot No./CD8+ T cell (%)[a] before	day29	day78	day134	day190
1	1×10^7(10)	1.19/0.45	6.96/0.06	8.82/0.63	8.81/0.08	5.4/0.08
2	1×10^7(10)	0.07/0.05	0.07/0.2	0.02/0	0.02/0	0.29/0.03
3	1×10^7(3)	N.D.[b]	N.A.[c]	N.A.	N.A.	N.A.
4	2×10^7(6)	0.39/0.53	1.29/0.03	1.12/0	N.A.	N.A.
5	2×10^7(6)	1.74/0.05	0.51/0.2	1.25/0.04	N.A	N.A.
6	2×10^7(10)	0.21/0.27	0.31/0.28	1.18/0.24	7.80/0.19	9.82/0.30
7	5×10^7(8)	0.62/0.20	6.52/0.1	7.33/0.11	N.A.	N.A.
8	5×10^7(3)	N.D.	N.A.	N.A.	N.A.	N.A.
9	5×10^7(3)	3.09/1.24	N.D.	N.A.	N.A.	N.A.

The percentages represent IFN-γ-positive spot No. divided by total $CD8^+$ cell No. from 1×10^4 PBMCs. [a]Each value represents the percentage with peptide cocktail/without peptide cocktail. [b]N.D. ; not done, [c]N.A. ; sample not available.

DTH, showed no shrinkage of the tumor, resulting in cessation after 6 DC injections.

Characterization of infiltrated lymphocytes in the tumor

IHC analysis of infiltrated lymphocytes in the tumor after DC vaccines was performed only in patient 1 and 7. The obvious infiltration of a larger number of $CD4^+$ or $CD8^+$ T cells and a small number of $CD20^+$ B cells were shown in patient 1 (Fig. 4). In contrast, no significant cell infiltration was seen in patient 7 who did not develop any therapeutical effect on the tumor (data not shown).

Discussion

Clinical trials of specific immunotherapy against metastatic melanoma using peptide-pulsed Mo-derived DCs have been performed in mainly Western countries, and some fruitful results were obtained [7,10,11]. In those cases, most of the patients belonged to the HLA-A*0201 type. In the present study, we investigated the effect of peptide-pulsed DCs on 4 cases of HLA-A^*2402^+ metastatic melanoma patients besides 4 cases of HLA-A^*0201^+ patients in a clinical phase I trial. This is the first report to demonstrate that peptide-pulsed DCs were effective in some $HLA\text{-}A24^+$ melanoma patents in Japan. It is well known that HLA-A*2402 is a common genotype and around 60% positive in Asians. There was one case of HLA-A*0206 patient among 5 $HLA\text{-}A2^+$ patients (Table 2). Sidney et al. [21] demonstrated that over 70% of the peptides that bound A0201 with high affinity were found to bind at least two other supertype molecules like A*0202, A*0203 or A*0206. Taking it into considerations, the HLA-A*0206 patient was finally enrolled into the study. With regard to other $HLA\text{-}A24^+$ solid cancers, stomach, colon and bladder cancers have been treated with peptide (MAGE-3)-pulsed DC vaccines, and showed a limited response [22-24]. Considering that melanoma is highly immunogenic and probably a good model for tumor-specific immunotherapy despite being an unusual tumor in Asian countries, it deserves a phase I study using peptide-pulsed DCs.

In our study, peptide cocktails combining 5 peptides for each HLA type (HLA-A2 or A24) were prepared and used for DC pulsing. Our clinical study revealed positive ELISPOT responses against more than 2 peptides in all 6 evaluable cases. In previous reports, clinical DC therapy using more than 3 melanoma peptides demonstrated the induction of a specific CTL response against multiple melanoma peptides [10,11]. However,, there is still some controversy over the efficacy of multiple epitope-based vaccinations and Smith *et al.* [25] demonstrated that, although polyepitope vaccines are an effective way of priming polyvalent CTLs, continual stimulation with polyepitope vaccines might restrict CTL induction as a result of immunodominance. The results of our study are thought to answer that question, but testing of the peptide cocktail vaccine in more patients will be needed.

To refine the quality and protocol of the tumor-specific immunotherapy for clinical trials, the prediction of clinical response in an individual is important [26] and should be discussed. In our study, the correlation between immunological parameters and clinical response was investigated in a limited number of cases.

First of all, as to HLA expression in the tumor, patients 1, 2, 6 and 7 were positive, and patients 4 and 5 were negative. HLA-negative cases showed a progression of the tumor. Even in positive cases, patient 7 turned negative in the course of DC therapy, showing tumor progression.

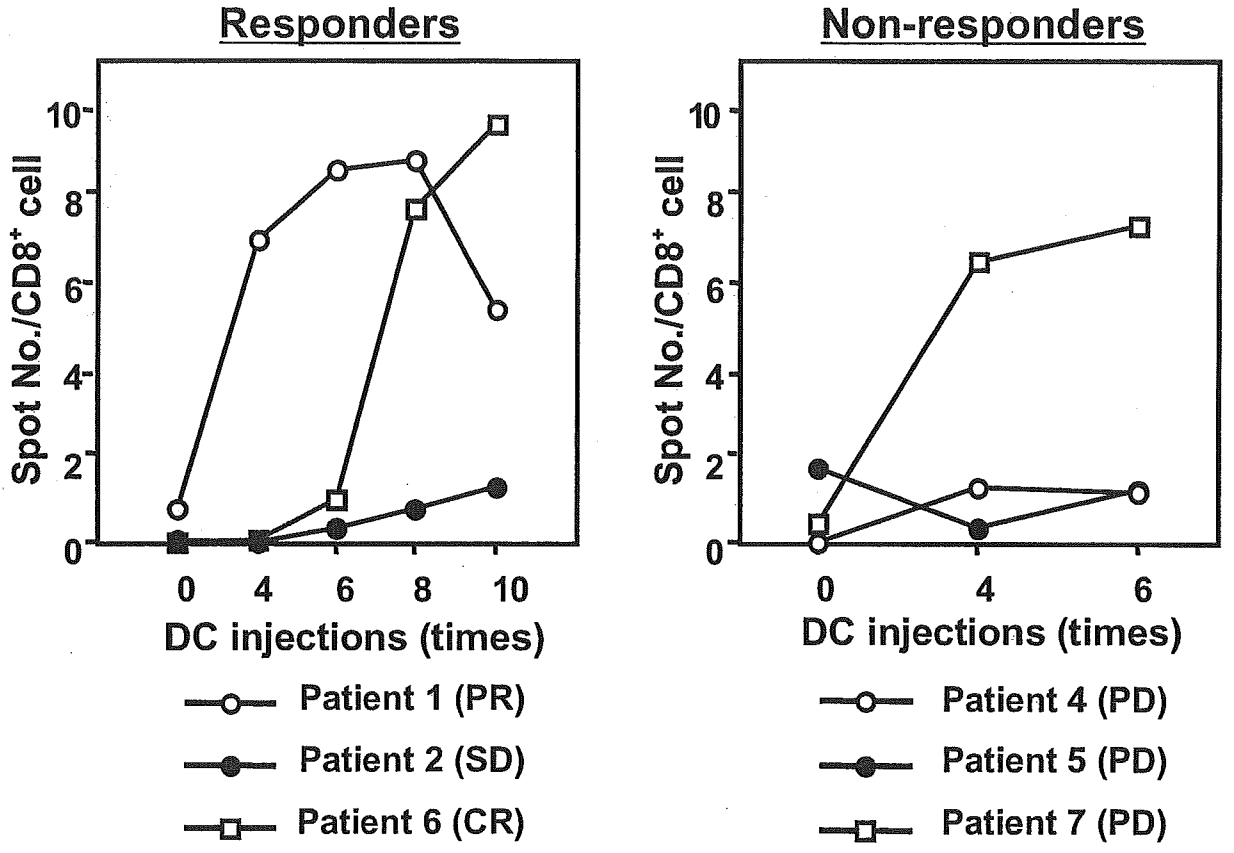

Figure 2
CTL responses in the course of DC injections in 6 evaluable cases. Patients 1, 2 and 6 were responders and patients 4, 5 and 7 were non-responders. Responders (cases 1,6) showed remarkable CTL expansion in PBLs compared with before DC vaccination. In contrast, non-responders (patients 4,5) showed no significant CTL responses except in patient 7.

Loss of HLA expression in melanoma is reported to be a complex phenomenon associated with melanoma antigen loss [27], β2-microglobulin gene mutation [28] or loss of heterozygosity (LOH) in chromosome 6 and may lead to tumor progression and metastasis. As to patient 7, considering that the melanoma antigen expression was maintained, the functional expression of β2-microglobulin should be investigated. All the other HLA-positive cases showed CR, PR and SD, respectively. There was a tendency for HLA expression to be associated with tumor response, and some researchers reported a positive correlation of HLA-expression to tumor response in immunotherapy against melanoma. However, despite the positive correlation of HLA-expression in the tumor with antitumor response, Nestle et al. demonstrated that HLA-expression in the tumor did not correlate to survival in melanoma patients [29].

Second, the amplitude of the CTL response in the ELISPOT assay seems to be another key factor predicting antitumor response. Patients 1, 6 and 7 showed large responses to peptide cocktail in ELISPOT, and patients 2, 4 and 5 showed small responses. The former exhibited a remarkable regression of tumor except patient 7. On the other hand, the latter showed a poor response. There was a likely tendency that the amplitude of the CTL response was associated with tumor regression. Also, it was difficult to predict when immunological responses like CTL induc-

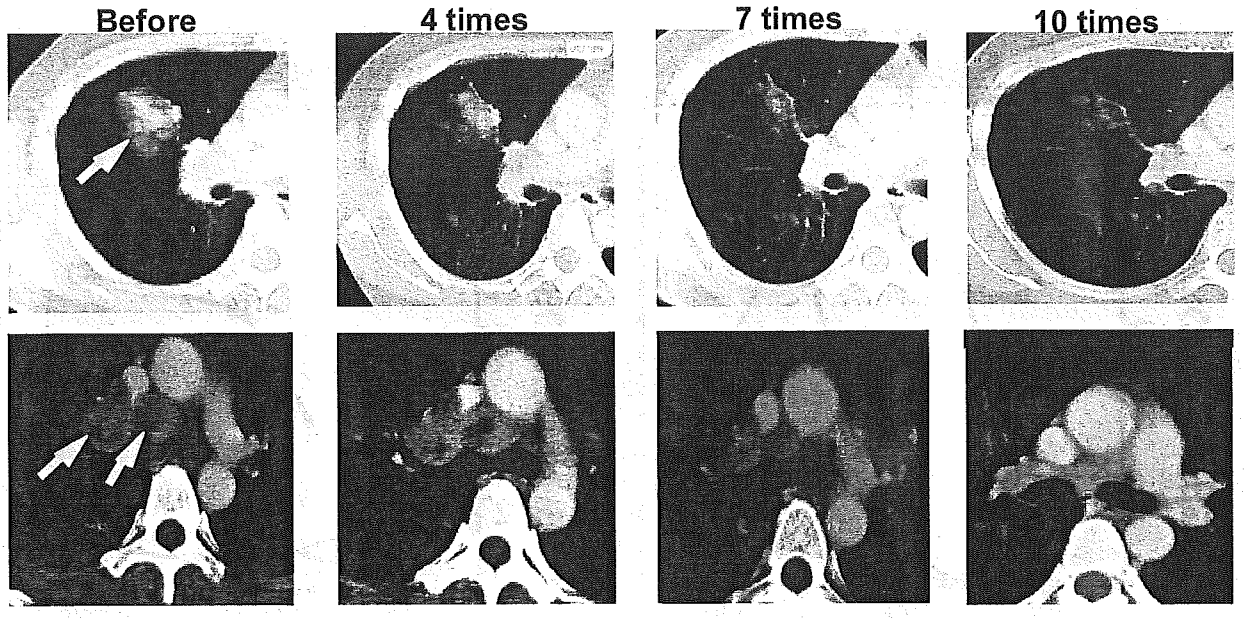

Figure 3
Impact of DC vaccines on metastatic lesions of the lung in responder patient 1. Upper and lower panels show a lung and hilar lymph node metastatic lesion (arrow), respectively. The CT scan was made before therapy and after 4, 7 and 10 DC vaccinations.

tion start to be activated in vivo during DC vaccination, and this question needs to be answered. In the present study, because of a limited number of patients given DC vaccines, the tendency that HLA-class I protein expression in the tumor and the amplitude of ELISPOT responses are seemingly associated with tumor regression is not convincing.

Finally, in order to improve tumor response in the present study, there are still some issues regarding clinical DC preparation. First of all, the purity of $CD14^+$ cells after Opti-prep separation is still low and may not be reproducible. Therefore, other clinical grade-monocyte separation methods using an elutriator or negative selection with CD2 and CD19 MoAbs [30] should be tried. Second, considering that the amplitude of the CTL response was associated with tumor regression, and that even a remarkable increase of CTL frequency inevitably diminished in spite of the repetition of DC vaccinations, it seems to be crucial to maintain increased CTL frequency in blood leading to TIL in the tumor and expand more than enough to develop a substantial number of memory $CD8^+$ CTL in lymph nodes. Such a novel method will be needed to develop an effective cancer vaccine.

Conclusions

In the present study, we investigated the effect of dendritic cell (DC)-based immunotherapy on metastatic melanoma patients with HLA-A2 or A24 genotype. Nine cases of metastatic melanoma were enrolled into a phase I study using HLA-A2 or A24-restricted peptide cocktail-pulsed DCs. All 6 evaluable cases showed positive immunological responses to more than 2 melanoma peptides in an ELISPOT assay. Clinical response through DC injections was as follows : 1CR, 1 PR, 1SD and 6 PD. All 59 DC injections in the phase I study were safely administered to patients. These results suggested that peptide cocktail-treated DC-based immunotherapy had the potential for utilizing as one of therapeutic tools against HLA-A2 or $A24^+$ metastatic melanoma.

Abbreviations

DC, dendritic cell ; HLA, human leukocyte antigen ; GM-CSF. granulocyte macrophage-colony-stimulating factor ; IL, interleukin ; KLH, Keyhole limpet hemocyanin ; CTL, cytotoxic T cell ; DTH, delayed-type hypersensitivity ; CR, complete remission : PR, partial remission ; SD, stable disease ; PD, progressive disease ; RT-PCR, reverse transcription-polymerase chain reaction ; IFN, interferon ; PBMC, peripheral blood mononuclear cell.

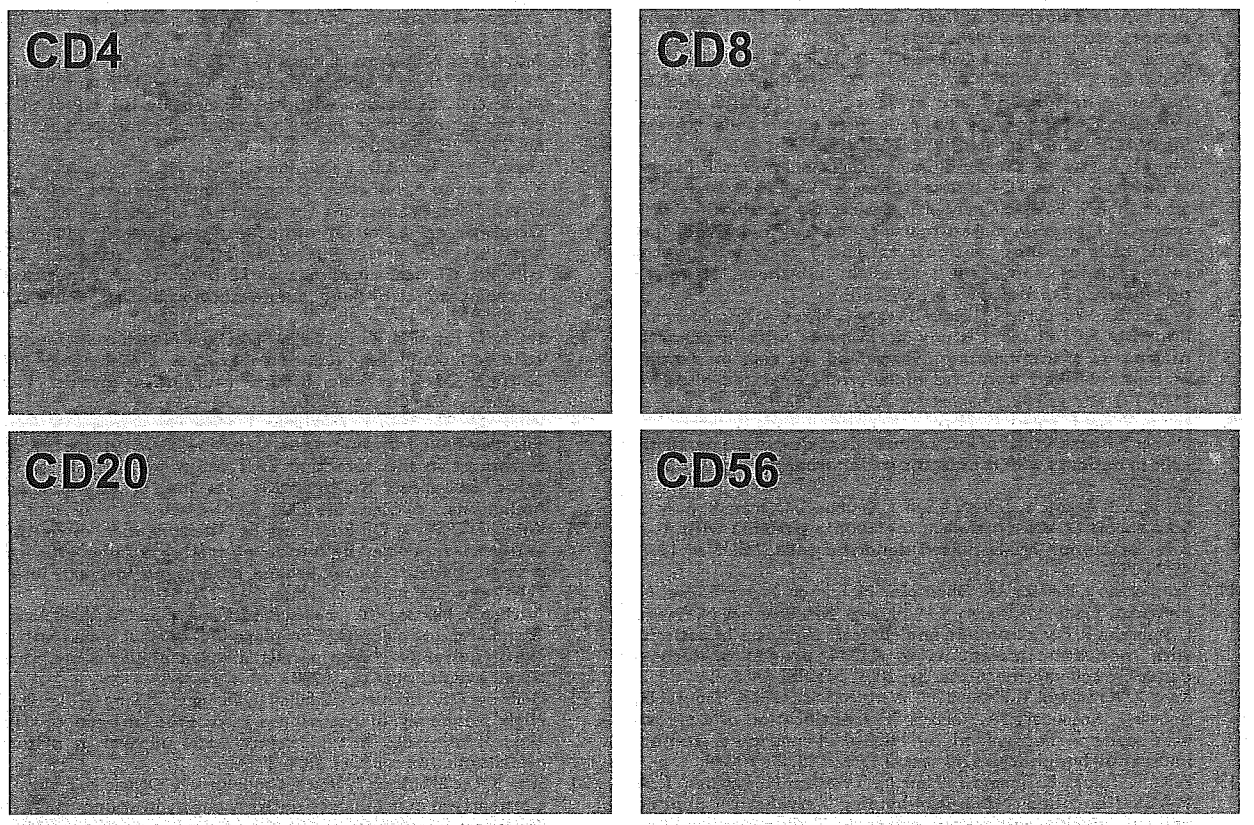

Figure 4
Phenotype analysis of lymphocytes infiltrating the tumor site in responder patient 1. Obvious infiltration of a larger number of $CD4^+$ or $CD8^+$ T cells and a small number of $CD20^+$ B cells is shown. Indirect staining using anti-CD4, CD8, CD20 or CD56 MoAb as primary Ab and goat anti-mouse Ab as secondary Ab was performed. Magnification × 200.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
YA participated in the design of the study and drafting the manuscript and were responsible for completing the study. RT, NI, MS, YH carried out apheresis and cell processing and were responsible for DC production. AY and NY were responsible for the clinical side of the study. IK, IN, KT and KM participated in the design of the study and performed biological assays. YT and KY reviewed the manuscript. All authors read and approved the final manuscript.

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A multidisciplinary treatment strategy that includes high-dose chemotherapy for metastatic retinoblastoma without CNS involvement

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Summary:

The prognosis of patients with metastatic retinoblastoma is poor with conventional chemotherapy and radiation. Since retinoblastoma is highly chemosensitive, dose-escalation of chemotherapeutic agents with stem cell support should be promising. We report our experience with high-dose chemotherapy (HDC) and autologous stem cell transplantation (SCT) in patients with metastatic retinoblastoma. Five patients with metastatic retinoblastoma underwent HDC with autologous SCT following conventional chemotherapy and local radiation therapy. Stem cells (bone marrow in four and peripheral blood stem cells in one) were collected after marrow involvement was cleared. Melphalan was a key drug in all patients, and was administered in combination with other agents such as cisplatin, cyclophosphamide, carboplatin or thiotepa. Three patients are currently alive disease-free at 113, 107 and 38 months, respectively, from the time of SCT. They had no central nervous system (CNS) involvement. The two patients who died of disease had CNS involvement. No long-term sequelae of HDC have been noted. Our treatment strategy using HDC appears to be effective for treating metastatic retinoblastoma without CNS involvement.

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Keywords: retinoblastoma; metastasis; high-dose chemotherapy; autologous stem cell transplantation; melphalan

Retinoblastoma, the most common ocular malignancy in childhood, develops in infants, and the incidence is one in 160 000–20 000 births in Japan.[1] Many therapeutic modalities have been employed, and retinoblastoma has become one of the curable pediatric solid tumors. Nevertheless, the prognosis of extraocular retinoblastoma with metastasis to bone/bone marrow (BM) or the central nervous system (CNS) remains very poor.[2] Such high-risk populations include involvement of the cut end of the optic nerve, extrascleral spread into the orbit, lymphatic or hematogenous dissemination, CNS involvement and trilateral retinoblastoma. The overall occurrence of extraocular retinoblastoma was 4.8% of all patients at an institution.[3] Since retinoblastoma is highly chemosensitive, a treatment strategy that includes the dose-escalation of chemotherapeutic agents and stem cell support should be promising. We treated five patients with metastatic retinoblastoma using high-dose chemotherapy (HDC) followed by autologous stem cell transplantation (SCT), and three patients are currently alive and disease-free. Although our experience is very limited, our experience suggests the feasibility of a prospective study.

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Patients and methods

Five patients received HDC for extraocular retinoblastoma between March 1986 and November 2000 at the National Cancer Center Hospital of Japan (NCCH), and the data reported reflect the last patient contact as of January 2004. All patients originally were treated with radiation therapy and/or enucleation for intraocular disease at NCCH. The clinical characteristics of the patients are described in Table 1. After completion of the initial series of local ophthalmic therapies in NCCH, four of the five patients developed metastatic recurrence, as reported elsewhere.[4–6] Only one patient had BM metastasis at the initial diagnosis. Staging studies included computed tomography and magnetic resonance imaging of orbits and brain, histopathologic evaluation of BM aspiration and cytologic examination of cerebrospinal fluid (CSF). All patients were classified as having stage III/IV disease by the grading system of Grabowski and Abramson.[7] After the diagnosis of metastatic diseases was established, all patients were treated with conventional chemotherapy with or without radiotherapy and surgical enucleation (Table 2). Systemic chemotherapy included courses of vincristine, cyclophosphamide and doxorubicin with or without cisplatin alternating with cisplatin and cyclophosphamide, or

Table 1 Patients characteristics

UPN	*Sex*	*Age at diagnosis*	*Involvement*	*Metastases at diagnosis*	*Treatment*	*Metastases after therapy*
1	F	3 months	Bilateral	None	Right: 50.7 Gy radiation Left: enucleation	Brain (optic chiasm), spinal cord (L1)
2	M	10 months	Bilateral	None	Right: 49.4 Gy radiation Left: enucleation	Brain (ethmoid and sphenoid sinus), bilateral cervical LNs
3	F	41 months	Left	None	Left: 46 Gy radiation + HIT	Right temporal bone, marrow (70%)
4	F	16 months	Right	Marrow	Right: enucleation + 6 Gy radiation + chemotherapy	
5	F	18 months	Right	None	Right: 46 Gy radiation + enucleation + HIT + PC + CTT + IVI	Right orbit, marrow (50%)

UPN = unique patient number; HIT = heat-inducing thermotherapy; PC = photocoagulation; CTT = chemothermotherapy; IVI = intravitreal injection.

Table 2 Therapy and outcome

UPN	*Cx. after Mets*	*Rx. after Mets*	*SCT from relapse (mos)*	*Conditioning (mg/m²)*	*Stem cell source*	*Result*	*Meta. after SCT (mos)*	*Sequelae*
1	VCR/CY/ADR × 2 CY/CDDP × 1	Spine 40 Gy, cranium 25 Gy + boost 15 Gy	5	CDDP 90, CY 120 mg/kg, L-PAM180	BM	DOD	Spinal cord at Th12-L1 level (24 mos)	NE
2	VCR/CY/ADR × 3 CDDP/ETO × 2	Cranium 40 Gy + boost 20 Gy, spine 21 Gy, cervical LNs 40 Gy	5	CDDP 90, CY 120 mg/kg, L-PAM180	BM	DOD	Rt. cervical LN (4 mos)	NE
3	VCR/CY/ADR × 4 CDDP/ETO × 2	Focal site 40 Gy	7	L-PAM 180, VP-16 800, CBDCA 1600	BM	NED (113+)	None	None
4	VCR/CY/ADR × 3 CDDP/ETO × 3	—	6	L-PAM 180, VP-16 800, CBDCA 1600	BM	NED (107+)	None	None
5	VCR/CY/ADR/ CDDP × 3 CBP/ ETO × 4	—	7	L-PAM 160, CY 120 mg/kg, TEPA 500	PBSC	NED (38+)	None	None

SCT = stem cell transplantation; BM = bone marrow; CNS = central nervous system; LN = lymph node; NED = no evidence of disease; DOD = dead of disease; NE = not evaluable; VCR/CY/ADR = vincristine 1.5 mg/m²/day × 1, cyclophosphamide 600 or 800 mg/m²/day × 2, doxorubicin 40 mg/m²/day × 1; CDDP/CY = cisplatin 90 mg/m²/day × 1, cyclophosphamide 1200 mg/m²/day × 1; CDDP/ETO = cisplatin 20 mg/m²/day × 5, etoposide 100 mg/m²/day × 5; VCR/CY/ADR/CDDP = vincristine 1.5 mg/m²/day × 1, cyclophosphamide 1200 mg/m²/day × 1, doxorubicin 40 mg/m²/day × 1, cisplatin 18 mg/m²/day × 5; CBP/ETO = carboplatin 120 mg/m²/day × 5, etoposide 100 mg/m²/day × 5; L-PAM = melphalan; VP-16 = etoposide; CBDCA = carboplatin; TEPA = thiotepa.

cisplatin and etoposide, or carboplatin and etoposide. After complete response of tumor involvement in the BM, autologous BM cells were collected from four patients, autologous blood stem cells from one patient, respectively. The nonpurged stem cells were cryopreserved. All patients also received one to five intrathecal injections of methotrexate at a variable dose of 5–12.5 mg/dose, concomitant with systemic chemotherapy. Radiation therapy was given in four patients to sites that had harbored bulky disease at early stage after the diagnosis of metastasis. All patients were prepared for HDC with SCT after achieving complete remission, which was evaluated by imaging studies, BM aspiration and/or CSF examination. We harvested BM cells or peripheral blood stem cells, if a BM aspirate had no tumor cells on morphologic analysis before harvesting. We did not apply minimum residual disease (MRD) studies on BM cells or peripheral blood stem cells. Conditioning regimens for all patients contained melphalan 180 mg/m² as a key drug. Concomitant agents were cisplatin 90 mg/m² and cyclophosphamide 120 mg/kg (case 1, 2), etoposide 800 mg/m² and carboplatin 1600 mg/m² (case 3, 4), or thiotepa 500 mg/m² and cyclophosphamide 120 mg/kg (case 5). The collected BM cells ($1.0–1.7 \times 10^8$ total nucleated cells/kg) or peripheral blood stem cells (4.7×10^6 CD34+ cells/kg), which were unmanipulated, were infused approximately 24 h after completion of the conditioning chemotherapy. Granulocyte-colony stimulating factor was administrated intravenously once daily from day +5 or +7, and was continued until engraftment of neutrophils was established (case 3–5).

Results

Engraftment

Engraftment of neutrophils, defined as the first of two consecutive days of an absolute neutrophil count of at least 0.5×10^9/l, occurred 18, 26, 10, 14 and 11 days, respectively, after stem cell rescue. Platelet engraftment, defined as the first of 2 consecutive days of an absolute platelet count of at least 50×10^9/l sustained without transfusion, occurred 67, 32, 11, 51 and 16 days, respectively, after stem cell rescue.