A*02 or HLA-A*24 serotype who had undergone allogeneic HSCT between 2002 February and 2004 May at National Cancer Center Hospital (Tokyo, Japan). Patients who received non-T-cell-depleted HSCT from a serologically HLA full-matched donor were eligible for this study. Both conventional (CST) and reduced-intensity HSCT (RIST) were included. Those who received a regimen containing antithymocyte globulin (ATG) were excluded since the immunorecovery of T cells is delayed with ATG.

Patients who could be followed up until day 160 after transplantation were eligible for analysis. The characteristics of the 66 eligible patients are shown in Table 1. Of the 34 HLA-A*02-positive patients, 15 were also positive for HLA-A*24 (A*02/A*24 patients). The other 32 patients were only positive for HLA-A*24. Genotyping for HLA-A*02 was performed in 19 patients: nine had HLA-A*0201, nine had HLA-A*0206 and one had both the HLA-A*0201 and HLA-A*0206 genotypes. Since a great majority of the A*24 serotype is A*2402 genotype among Japanese, genotyping for HLA-A*24 was omitted.

In all, 13 patients received bone marrow transplantation (BMT) from an unrelated donor (U-BM), three received BMT from a related donor (R-BM) and the remaining 50 received peripheral blood HSCT from a related donor (R-PB). With regard to the conditioning regimen, 21 patients received a CST regimen that included 120 mg/kg cyclophosphamide (CY) plus 16 mg/kg busulfan (BU) or 120 mg/kg CY plus 12 Gy of total body irradiation (TBI), whereas 45 received an RIST regimen with 0.66 mg/kg cladribine plus 8 mg/kg BU or 180 mg/m^2 fludarabine plus 8 mg/kg BU with or without 4 Gy of TBI.

Diagnostic tests for CMV infection and CMV disease

CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. The presence of CMV-specific IgG antibodies in donors or patients before HSCT was taken as a marker for CMV carrier status. CMV antigenemia was monitored weekly after engraftment to day 90, and at longer intervals thereafter by using the immunocytochemical detection of pp65 antigen in leuko-

Table 1 Patient characteristics

	All patients (n=66)		*Patients with HLA-A*02 (n=34)*		*Patients with HLA-A*24 only (n=32)*	
Sex						
Male	37	56.1%	22	64.7%	15	46.9%
Female	29	43.9%	12	35.3%	17	53.1%
Mean age (range)	46.8	(21–67)	47.8	(21–67)	45.7	(22–64)
Diagnostic indication for HSCT						
AML	16		7		9	
ALL	5		4		1	
CML	3		2		1	
MDS	18		10		8	
NHL	17		9		8	
MF	1		1		0	
ATL	2		0		2	
Solid tumor	4		1		3	
Conditioning regimen						
CST	21	31.8%	11	32.4%	10	31.3%
BU/CY	16		8		8	
CY/TBI	5		3		2	
RIST	45	68.2%	23	67.6%	22	68.8%
Flu/BU	27		11		16	
Flu/BU/TBI	4		4		0	
CdA/BU	12		7		5	
CdA/BU/TBI	2		1		1	
GVHD prophylaxis						
CSP	25		9		16	
CSP+MTX	37		23		14	
Tacrolimus	4		2		2	
Stem cell source						
Related PB	50	75.8%	24	70.6%	26	81.3%
Related BM	3	4.5%	2	5.9%	1	3.1%
Unrelated BM	13	19.7%	8	23.5%	5	15.6%
CMV serology before HSCT						
High risk (recipient+)	59	89.4%	33	97.1%	26	81.3%
Intermediate risk	5	7.6%	0	0%	5	15.6%
Low risk (recipient−, donor−)	2	3.0%	1	2.9%	1	3.1%

cytes. Test results were considered to be positive when more than one leukocyte was positively stained. CMV disease was diagnosed clinically, with confirmation by biopsy of the involved organ.

Peptides and CMV antigen

The following >80% pure peptides, which were assessed as CMV antigen epitope, were obtained using high-performance liquid chromatography (Qiagen, Tokyo): HLA-A*0201 binding peptide NLVPMVATV (AA 495–503, referred to as A*02NLV) or HLA-A*2402 binding peptide QYDPVAALF (AA 341–349, 'A*24QYD') from the CMV pp65 phosphoprotein. HLA-A*0201 binding peptide YLEPGPVTA from glycoprotein 100 (gp100 AA 280–288)[18] and HLA-A*2402 binding peptide AFLPWHRLF from tyrosinase,[19] which is an antigen for melanoma, were used as negative controls.

Tetramer staining

CD8-FITC-, CD4-PC5-, CD19-PC-5-, CD13-PC5- and PE-conjugated tetrameric HLA-A*0201 (A*02NLV-tetramer) or HLA-A*2402 CMV peptide complexes (A*24QYD-tetramer), both purchased form Beckman Coulter Inc. (Fullerton, CA, USA), were added to 200 μl heparinized blood or cultured cell suspension, and incubated for 30 min at room temperature in the dark. When whole-blood samples were used, the red blood cells were lysed after antibody staining. After being washed twice with BSA-containing PBS, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson). Analysis was performed using Cellquest software. The CD4−, CD19−, CD13− and CD8+ tetramer-positive fraction of the lymphocyte gate was defined as CMV-CTL.

Intracellular cytokine assay

Intracellular cytokine staining was performed asdescribed recently,[20] with the following modifications. Briefly, peripheral whole blood (1 ml) was stimulated for 6 h at 37°C with 10 μg/ml A*02NLV or A*24QYD, in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson; 1 μg/ml each). Breferdin A (Sigma; 10 μg/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μg/ml staphylococcal enterotoxin B or PBS. Samples were lysed, permeabilized and stained with CD69-FITC, IFN-γ-PE, CD3-APC and CD8-PerCP, and analyzed using a FACS Calibur. IFN-γ-positive cells in the CD3+ and CD8+ fraction of the lymphocyte gate were defined as peptide-specific IFN-γ-secreting T cells. CD69 was used as a marker for activated T cells.

ELISPOT assay

Peripheral blood mononuclear cells (PBMCs) were frozen in FBS containing 10% dimethyl sulfoxide (DMSO) at −140°C until use. Frozen PBMCs were thawed and washed in RPMI 1640 (Sigma) supplemented with 10% FBS, gentamicin and streptomycin (hereafter referred to as culture medium), and incubated overnight at 37°C prior to use. PBMCs (0.5×10^5 to 2.0×10^5/well) were stimulated for 24 h at 37°C with A*02NLV or A*24QYD at a concentration of 10 μg/ml. As a negative control, gp100 or tyrosinase was used.

PVDF-bottomed 96-well plates (Millipore) were coated overnight with antibody to human IFN-γ (1-D1K, Mabtech). After the plates were washed and blocked, the cells were transferred to the plate and incubated for 24 h at 37°C After removing the cells, a biotinylated mAb (7-B6-1 Biotin) was applied at 1 μg/ml and the wells were further incubated for 2 h at room temperature. After washing, streptavidin-alkaline phosphatase diluted 1:1000 was added and the wells were incubated for 1 h at room temperature. After washing, the substrate was added and the wells were incubated until spots emerged. The spots were counted using a phase-contrast microscope.

Expansion of CMV-specific CTL

PBMCs were separated from heparinized blood by Ficoll–Hypaque (IBL, Japan) density-gradient centrifugation, and diluted at 1×10^6 cells/ml in culture medium. PBMC (2×10^6 cells) were seeded in a 24-well plate, and A*02NLV or A*24QYD was added to a final concentration of 5 μM on day 0. The peptide was diluted to 10 mg/ml in DMSO prior to use, and the same amount of DMSO as was contained with peptide was used as a negative control. The culture was fed on days 4 and 7 by replacing half of the medium with fresh culture medium containing 20 U/ml IL-2, and 10 μM CMV peptide. Cells were cultured for 14 days.

Statistical analysis

The significance of differences in the frequency of CMV reactivation, high CMV antigenemia and CMV disease was evaluated by the χ^2 test or Fisher's exact test according to the sample size. Values of $P<0.05$ were considered significant.

Results

CMV infection in relation to CMV serology and HLA-A locus

The CMV reactivation status after HSCT is shown in Table 2. Among all 66 patients, 44 (67%) showed reactivation as evidenced by the appearance of pp65+ leukocytes in the blood, and 23 (35%) had an elevated level of antigenemia >10/50 000. When the patient and donor were both CMV seropositive, the CMV reactivation rate was 74%, while only one of seven CMV-seronegative recipients had CMV reactivation with low titers of antigenemia. All of the six patients who developed CMV disease had been CMV seropositive before transplantation and had undergone RIST (five R-PB, one U-BM). The CMV reactivation rate was higher among unrelated HSCT (85%) than among related pairs (62%). All of the seven patients who received U-BM with an RIST regimen developed CMV antigenemia, and six had antigenemia

Table 2 CMV reactivation status after HSCT

	Total	*CMV reactivation*		*Antigenemia >10/50000*		*CMV disease*	
All patients	66	44 (66.7%)	$P=0.394$	23 (34.8%)	$P=0.898$	6 (9.1%)	$P=1.000$
Donor and patient CMV seropositive	50	37 (74.0%)		18 (36.0%)		5 (10.0%)	
Related donor	53	33 (62.3%)	$P=0.192$	15 (28.3%)	$P=0.048$*	5 (9.4%)	$P=1.000$
Unrelated donor	13	11 (84.6%)		8 (61.5%)		1 (7.7%)	
Patients with HLA-A*02	34	24 (70.6%)	$P=0.486$	13 (38.2%)	$P=0.552$	4 (11.8%)	$P=0.673$
Patients with only HLA-A*24	32	20 (62.5%)		10 (31.3%)		2 (6.3%)	

*Statistically significant.

>10/50000, including one who developed CMV disease. The rate of CMV reactivation, antigenemia >10/50000 and CMV disease tended to be higher in HLA-A*02 (71, 38 and 12%, respectively) than in HLA-A*24 patients (63, 31 and 6%, respectively), but these differences were not significant.

Enumeration of CMV-specific CTL by tetramer assay

Peripheral blood samples were taken at least 4 weeks after transplantation with confirmed engraftment. Additional requirements for sampling included complete remission in leukemia patients and PR or stable disease without bone marrow involvement in those with lymphoma or solid tumor. The absolute number of CMV-CTL, and the percentage of CMV-CTL per lymphocyte and per CD8+ lymphocyte were assessed by tetramer assay. A*02NLV- or A*24QYD-tetramer was chosen according to the patient's HLA subtype. For those with A*02/A*24, both A*02NLV- and A*24QYD-tetramer stainings were performed independently (Figure 1a and b). A total of 185 samples from 33 HLA-A*02 patients and 147 samples from 44 HLA-A*24 patients, including 78 samples from 14 A*02/A*24 patients, were obtained. One to 10 samples (median, 5) were obtained from each patient. Samples were not obtained from one A*02/A*24 patient and two HLA-A*24 single-positive patients who had been entered into this study since they did not meet the sampling criteria mentioned above.

For the 185 samples assessed for A*02NLV-tetramer and 147 samples assessed for A*24QYD-tetramer, the median number of days from transplantation at the time of sampling and the mean lymphocyte count in the samples was, respectively, 233 (28–622) and 217 (28–633), and 1.94×10^9/l and 1.93×10^9/l.

The mean numbers of CMV-CTL for A*02NLV- and A*24QYD-tetramer among all of the samples were, respectively, 23×10^6/l and 0.4×10^6/l, and the mean percentage of CMV-CTL was 1.59 and 0.03% per lymphocyte (3.5 and 0.06% per CD8+ T-lymphocyte, respectively). With regard to A*02NLV-tetramer, 176 of the samples (95%) had detectable CMV-CTL and 83 (45%) showed more than 1% positive cells per lymphocyte (Figure 1c), whereas only 80 (54%) had detectable CMV-CTL, and none showed more than 1% for A*24QYD-tetramer (Figure 1d). All of the nine samples that were negative for A*02NLV-tetramer were also negative for A*24QYD-tetramer. Samples that were negative for A*02NLV-tetramer were taken from patients who received transplantation from seronegative donor and did not have CMV reactivation. Otherwise, a detectable level of CMV-CTL was present in all of the HLA-A*02 patients immediately after engraftment. However, 36% of the patients did not achieve detectable levels of CMV-CTL with A*24QYD-tetramer, and only 11% of the HLA-A*24 patients obtained a sufficient level (0.05% or more) of CMV-CTL throughout the course. Similarly, in the 78 samples from A*02/A*24 patients, the mean percentage of CMV-CTL for A*02NLV- and A*24QYD-tetramer was 1.47 and 0.01% per lymphocyte. (Figure 1e and f). While 73 of the samples (94%) were positive for A*02NLV-tetramer and only 16 (21%) were below 0.05%, only 37 (47%) were positive for A*24QYD-tetramer ($P<0.0001$) and 75 (96%) were below 0.05% ($P<0.0001$). While all of the five samples that were negative for A*02NLV-tetramer were also negative for A*24QYD-tetramer, 36 of the 41 A*24QYD-tetramer-negative samples were positive for A*02NLV-tetramer.

The staining of A*02NLV-tetramer was dull in samples from HLA-A*0206 genotype compared to that in HLA-A*0201, and samples with both HLA-A*0201 and HLA-A*0206 showed mixed tetramer bright and dull populations in the tetramer-positive fraction (Figure 2). The frequency of A*02NLV-tetramer-positive CMV-CTL in the samples from HLA-A*0206 patients was as high as that in samples from HLA-A*0201 patients. The mean percentage of A*02NLV-tetramer-positive cells per lymphocyte and the positive rate of A*02NLV-tetramer in 75 samples from 10 HLA-A*0201 patients and 57 samples from 10 HLA-A*0206 patients were, respectively, 1.5 and 2.7, and 99% (74 samples) and 93% (53 samples).

Intracellular cytokine assay

Intracellular cytokine was assessed in 11 samples from 11 A*02/A*24 patients. The mean percentages of intracellular IFN-γ-positive cells per CD8+ lymphocyte when stimulated with A02*NLV, A*24QYD and PBS were, respectively, 2.5% (0.05–7.3%), 0.11% (0.01–0.31%) and 0.16% (0.02–0.55). The percentages of IFN-γ-positive cells in samples from eight HLA-A*0206 patients were as high as those in three samples from HLA-A*0201 patients. All of the 11 samples showed a higher percentage of IFN-γ-positive cells when stimulated with A02*NLV than with

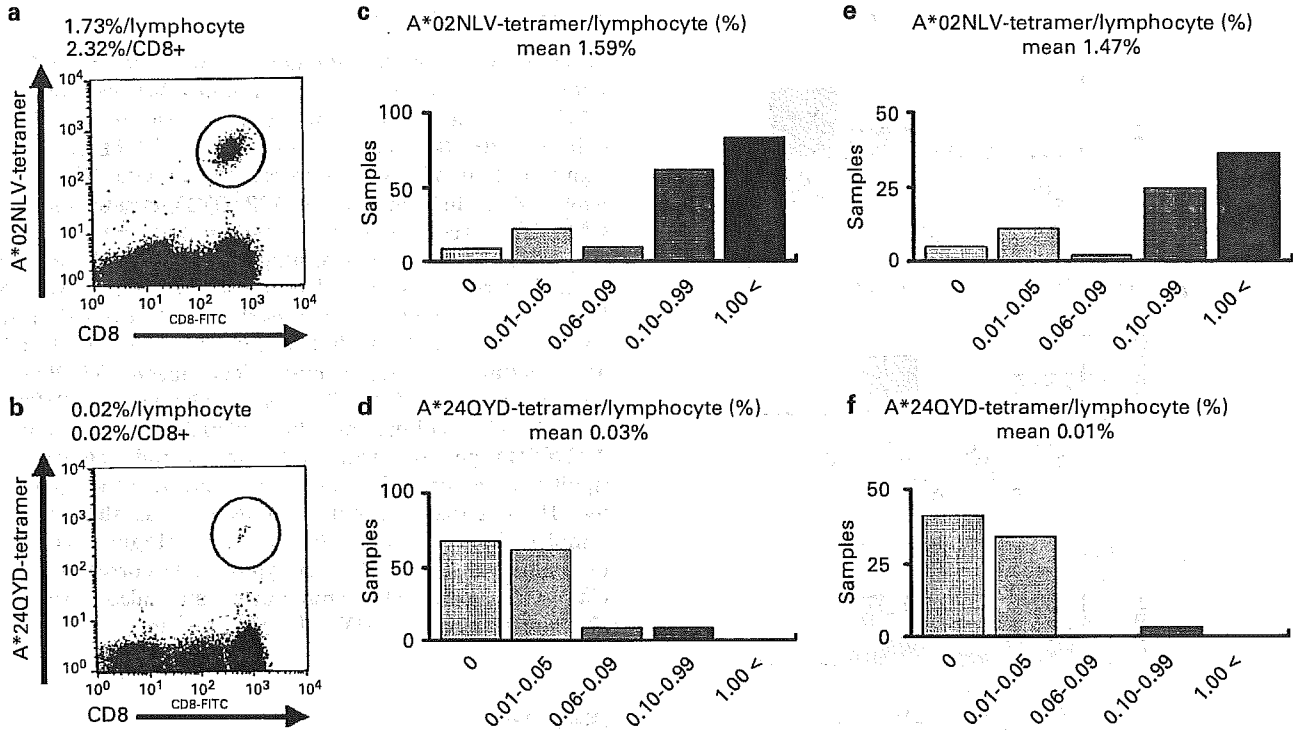

Figure 1 Staining of A*02NLV-tetramer and A*24QYD-tetramer. The percentage of CMV-CTL was higher for A*02NLV-tetramer (1.7%/lymphocyte) (**a**) than with A*24QYD-tetramer (0.02%/lymphocyte) (**b**) in a patient with both the HLA-A*0201 and HLA-A*24 phenotypes. The percentage of CMV-CTL was measured by tetramer assay in 185 samples taken from 33 patients with HLA-A*02 (**c**), 147 samples from 44 patients with HLA-A*24 (**d**) and 78 samples taken from 14 patients with both the HLA-A*02 and HLA-A*24 phenotypes (**e** and **f**). The mean percentage of tetramer-positive cells per lymphocyte was 1.6% for A*02NLV (**c**), 0.02% for A*24QYD (d), 1.5% for A*02NLV (**e**) and 0.01% for A*24QYD (**f**).

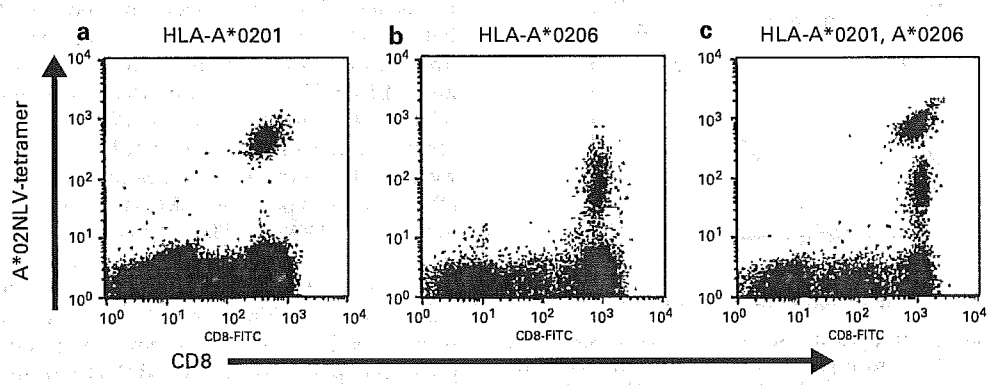

Figure 2 A*02NLV-tetramer staining in patients with different HLA-A genotypes. Tetramer staining is brighter in HLA-A*0201 (**a**) than in HLA-A*0206 (**b**). Patients with both the HLA-A*0201 and HLA-A*0206 genotypes show both tetramer bright- and dull-positive CMV-CTL (**c**).

A*24QYD. When stimulated with A*24QYD, the percentage of IFN-γ-positive cells did not differ from that in the negative control (Figure 3a–c).

A simultaneous tetramer assay also showed a higher CMV-CTL rate in A*02NLV-tetramer (average, 4.3%/CD8) than in A*24QYD-tetramer (0.02%/CD8). The number of IFN-γ-positive cells stimulated with A02*NLV correlated with the number of A*02NLV-tetramer-positive CMV-CTL (Figure 3d).

Intracellular cytokine and tetramer assay were performed in one A*02/A*24 patient with relapsed AML after RIST (not listed in Table 1 since he did not achieve clinical remission after RIST). This patient showed a higher percentage of A*24QYD-tetramer-positive CMV-CTL (1.2%/CD8) than A*02NLV-tetramer-positive CMV-CTL (0%/CD8), and also showed a higher percentage of IFN-γ-positive cells when stimulated with A*24QYD (0.5%/CD8) than with A02*NLV (0.03%/CD8).

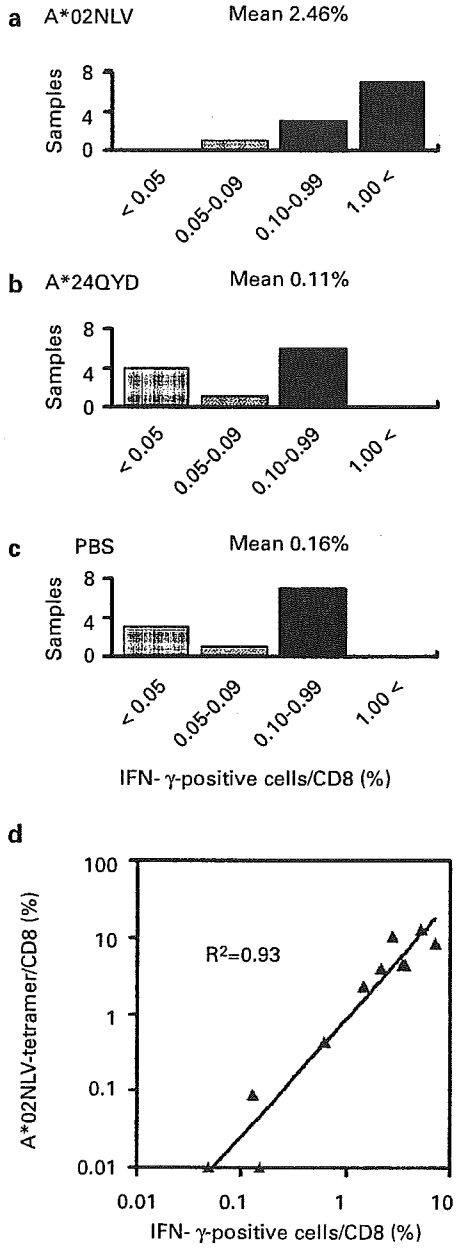

Figure 3 Proportion of IFN-γ-producing cells as assessed by intracellular cytokine staining. The percentage of IFN-γ-positive cells per CD8 lymphocyte was assessed in 11 samples obtained from 11 patients with both the HLA-A*02 and HLA-A*24 phenotypes who were not on steroid treatment. Cells stimulated with A*NLV showed more than 1%/CD8 IFN-γ-positive cells in seven samples (**a**), while cells stimulated with A*24QYD (**b**) were not significantly different from the negative control (**c**). The percentage of A*02NLV-tetramer-positive cells per CD8 correlated with the percentage of IFN-γ-producing cells per CD8 when stimulated with A*02NLV peptide (**d**).

ELISPOT assay

ELISPOT assay was performed in 12 PBMC samples obtained from seven A*02/A*24 patients. The average number of spots was $50/10^5$ PBMC when stimulated with A02*NLV, while this was $4/10^5$ PBMC for A*24QYD, $1/10^5$ PBMC for gp100 and $2/10^5$ PBMC for tyrosinase.

Expansion of CMV-specific CTL

Seven PBMC samples obtained from seven A*02/A*24 patients were cultured with or without CMV peptide, and CMV-CTL was analyzed by tetramer assay on day 14 of culture. A*02NLV-tetramer-positive CMV-CTL increased significantly in all seven samples cultured with A*02NLV, while only a slight increase in A*24QYD-tetramer-positive CMV-CTL was observed in two samples cultured with A*24QYD. The mean percentage of A*02NLV-tetramer-positive CMV-CTL per lymphocyte was 2% (0.09–8) before culture, and this increased to 19% (0.4–63) after culture with A*02NLV, but decreased to 0.4% (0.01–0.9) after culture without peptide. The mean A*24QYD-tetramer-positive CMV-CTL per lymphocyte was 0.004% (0–0.01) before culture and 0.04% (0–0.2) after culture with A*24QYD peptide, with only one sample showing a significant increase. The expansion rate in samples from five HLA-A*0206 patients was as high as that in two samples from HLA-A*0201 patients (Figure 4). The percentage of CMV-CTL per lymphocyte correlated with CMV-CTL per CD8 lymphocyte, and also with the absolute number of CMV-CTL after culture.

Discussion

Our study showed that the frequency of A*24QYD-tetramer-positive CMV-CTL was extremely low compared to that of A*02NLV. We also found that A*24QYD is not a potential epitope for monitoring CMV-specific CTL, since more than 1% A*02NLV-tetramer-positive CMV-CTL was detected in 45% of the samples, while the frequency of A*24QYD-tetramer-positive CMV-CTL was below 0.05 in 88% of the samples: 46% had an undetectable level. Both an intracellular cytokine assay and ELISPOT assay showed that the stimulation potential of A*24QYD for the secretion of IFN-γ was weak, and the results of the intracellular cytokine assay for A*24QYD were similar to those in the negative control. Moreover, the efficiency at expanding CMV-CTL was also significantly lower in A*24QYD. These results support the notion that the low frequency of A*24QYD-tetramer-positive CMV-CTL is not a false-negative result due to the quality of the analytical procedures, and the presence of one tetramer-positive patient served as a functioning internal control to prove the quality of the A*24QYD-tetramer.

In our study, the frequency and severity of CMV reactivation did not differ between HLA-A*02 and HLA-A*24 patients. A recent study with renal transplant recipients also showed that neither HLA-A*02 nor HLA-A*24 was related to the risk of CMV infection.[21] These results suggest that epitopes other than A*24QYD may play a major role in HLA-A*24 patients. Although we cannot rule out the possibility that a CMV-specific response is not restricted to HLA-A*24, we could at least show that the frequencies of other HLA alleles including HLA-B*07, which is known to have a strong CMV epitope, were not significantly different between HLA-A*02 and HLA-A*24 individuals. Furthermore, the frequency of CMV reactivation was not higher among HLA-A*24

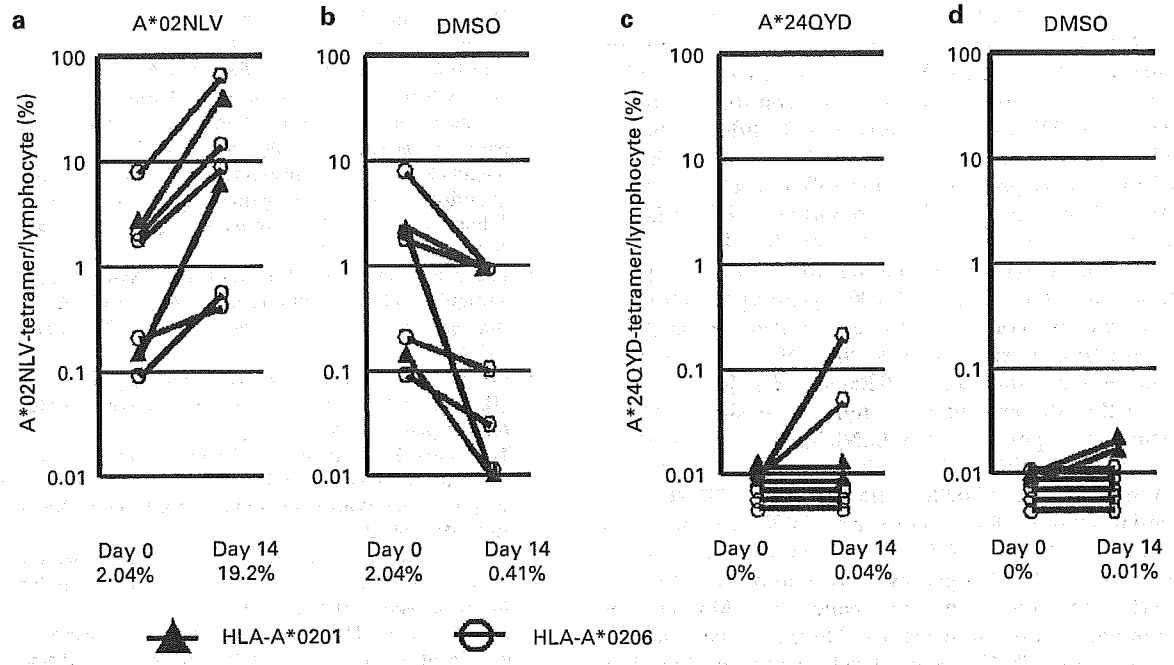

Figure 4 CMV-CTL culture in patients with both the HLA-A*02 and HLA-A*24 phenotypes. CMV-CTL was cultured with A*02NLV (**a**), A*24 QYD (**c**) or DMSO as a negative control (**b**, **d**) in seven samples from seven patients with the HLA-A*02 and HLA-A*24 phenotypes. A*02NLV-tetramer-positive cells were significantly increased in both the HLA-A*0201 and HLA-A*0206 genotypes when cells were cultured with A*02NLV (**a**), and they decreased when cells were cultured without peptide in all seven samples (**b**). Only two samples showed an increase in A*24QYD-tetramer-positive cells when cultured with A*24QYD (**c**).

homologous patients. These facts suggest the presence of a potent CMV epitope that is restricted to HLA-A*24. The contributions of other HLA-A*24 epitopes, such as VYALPLKML (VYA),[13,16,22] AYAQKIFKIL (AYA)[23] and FTSQYRIQGKL (FTS),[10] have been reported, but tetramer-based analysis has only been performed in A*24QYD.[5] Furthermore, it has been reported that IFN-γ secretion after stimulation with VYA was significantly lower than that with A*24QYD,[16] which does not have the capacity to sensitize T cells even at a higher concentration of 10 μM.[13] It has also been reported that no CTL response was detected by IFN-γ ELISPOT assay using AYA peptide.[23] To confirm this, we performed an ELISPOT assay and intracellular IFN-γ assay with A*24QYD, VYA, AYA and FTS in HLA-A*24 samples. As a result, none of the peptides except for A*24QYD showed a positive result, which demonstrates that A*24*QYD is among the strongest epitopes currently known.

One A*02/A*24 patient (not included in the analysis of this study because of relapsed AML), who had a high titer of A*24QYD-tetramer-positive CMV-CTL and no A*02NLV-tetramer-positive CMV-CTL, showed the HLA-A*0207 and HLA-A*2402 genotypes. The reason that A*24QYD was the dominant epitope in this patient may be that A*02NLV cannot be presented in the A*0207 genotype. To support this, Lacey *et al*[24] suggested that a higher frequency of tetramer-positive cells of less-favored epitopes was observed in patients lacking an immunodominant allele. Since there seems to be significant interpatient variation in the corresponding epitope, negative data with a single epitope may simply imply that the target is out of focus. Several groups have measured the T-cell response to CMV by flow cytometry using overlapping CMV peptide mixtures.[25,28] It has also been reported that with the use of such peptides of various lengths, both CD4+ and CD8+ cells could be stimulated. Since CD4+ and CD8+ CTL to multiple epitopes are both important for CMV immunity, we recognize that it would be more ideal to pulse PBMC with a CMV peptide mixture, instead of single peptides to monitor CMV immunity after HSCT.

Intracellular cytokine staining showed that not only CD3+ and CD8+ lymphocytes but also CD8-negative cells produced IFN-γ when stimulated by CMV peptide. It is assumed that cells other than CMV-CTL were influenced by the cytokines secreted from stimulated cells to non-specifically produce IFN-γ. Although ELISPOT is widely used for detecting antigen-specific cells,[29,32] it is impossible to distinguish whether the spots are specific from CTL or nonspecific from other functioning mononuclear cells, including CD4+ lymphocytes, NK or NK-T cells, since no other information to specify the cytokine-secreting cells can be obtained. In our study, the sensitivity of the ELISPOT assay did not differ from that of the intracellular cytokine assay, with a lower frequency of positive cells compared to the tetramer assay, which was compatible with the previous report.[33] Based on these results, the intracellular cytokine assay seems to be suitable for evaluating the function of CTL.

Our study proved that A*02NLV may be used for monitoring and in the expansion of CMV-CTL not only in HLA-A*0201 but also in the HLA-A*0206 genotype, which suggests that A*02NLV is crosspresented by HLA-

A*0201 and A*0206. This is very important, since the HLA-A*0206 genotype accounts for a considerable population of the HLA-A*02 serotype in certain ethnic groups.[10,11] The crosspresentation of epitopes by related alleles of the HLA-A*02 supertype with different binding affinities has been reported,[34] and the possibility of A*02NLV being presented by five different allelic variants of HLA-A*02 has also been reported.[10] The relatively dull staining of A*02NLV-tetramer for HLA-A*0206 may be due to the lower binding affinity of the epitope peptide in the HLA-A*0206 genotype. However, this should be confirmed by experiments using HLA-A*0206-tetramer, since we also used HLA-A*0201-tetramer in the assay for HLA-A*0206 samples. In either case, the fact that the percentage of tetramer-positive cells and the stimulation potential of A*02NLV for producing IFN-γ and expanding CTL did not differ significantly between HLA-A*0201 and A*0206 confirms that A*02NLV is a potential epitope for monitoring CMV-CTL not only in patients with the HLA-A*0201 genotype but also in those with the A*0206 genotype. It is important to know the HLA restriction and dominance of CMV epitopes for immunotherapeutic strategies. There have been successful studies on CMV-specific T-cell expansion that have involved sorting IFN-γ-producing cells on stimulation with CMV epitope peptides,[20] or the collection of CMV-CTL using tetramer.[35,36] These procedures have the advantage that CTL could be expanded from a small amount of PBMC obtained from a single blood draw. Tetramer-based cell expansion has thus far been performed only on HLA-A*0201 patients, but our results suggests that it may also be possible to apply this to HLA-A*0206 patients. Furthermore, these procedures could be applied to more than 90% of the population if a dominant HLA-A*24 epitope were identified. More effort should be focused on the investigation of CMV epitope for HLA-A*24 serotype.

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Allografting

Comparison between reduced intensity and conventional myeloablative allogeneic stem-cell transplantation in patients with hematologic malignancies aged between 50 and 59 years

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Summary:

To evaluate the efficacy of reduced-intensity stem-cell transplantation (RIST), we retrospectively compared outcomes of 207 consecutive Japanese patients aged between 50 and 59 years with hematologic malignancies who received RIST ($n=70$) and conventional stem-cell transplantation (CST) ($n=137$). CST recipients received total body irradiation (TBI)-based or busulfan/cyclophosphamide-based regimens. RIST regimens were purine analog-based ($n=67$), 2 Gy TBI-based ($n=2$), and others ($n=1$). Most CST recipients (129/137) received calcineurin inhibitors and methotrexate as graft-versus-host (GVHD) prophylaxis, while 32 RIST recipients received cyclosporin. In all, 23 CST and five RIST recipients died without disease progression within 100 days of transplant. Grade II to IV acute GVHD occurred in 56 CST and 38 RIST recipients. There was no significant difference in overall survival (OS) and progression-free survival between CST and RIST. On multivariate analysis on OS, five variables were significant: preparative regimens (CST *vs* RIST) (hazard ratio = 1.92, 95% confidence interval, 1.25–2.97; $P=0.003$), performance status (2–4 *vs* 0–1) (2.50, 1.51–4.16; $P<0.001$), risk of underlying diseases (1.85, 1.21–2.83; $P=0.004$), acute GVHD (2.57, 1.72–3.84; $P<0.001$), and CML (0.38, 0.21–0.69; $P=0.002$). We should be careful in interpreting results of this small-sized retrospective study; however, reduced regimen-related toxicity might contribute to better survival in RIST. The low relapse rates following RIST suggest a strong antitumor activity through allogeneic immunity.

Bone Marrow Transplantation (2005) **36,** 667–674. doi:10.1038/sj.bmt.1705122; published online 22 August 2005

Keywords: allogeneic hematopoietic stem-cell transplantation; regimen-related toxicity; graft-versus-host disease; nonrelapse mortality; graft-versus-leukemia effect

Allogeneic hematopoietic stem-cell transplantation (autologous stem-cell transplantation (allo-SCT)) is a therapeutic option for advanced hematologic malignancies. A small but significant proportion of these patients can be cured with allo-SCT.[1] Conditioning regimens have been developed to maximize dose intensity, escalating the dose-limiting toxicity in nonhematopoietic tissues.[2] Conventional stem-cell transplantation (CST) using a myeloablative preparative regimen is associated with severe regimen-related toxicities (RRT), resulting in high nonrelapse mortality (NRM) especially for old patients.[3] NRM tends to be higher in patients with refractory or advanced diseases, who have been treated heavily, compared with those who have achieved remission.[3] Considering that high-dose chemotherapy followed by allo-SCT is ineffective for these patients,[4] and that intensification of preparative regimens usually leads to severe RRT and high NRM,[5] it remains unknown whether myeloablative preparative regimens are beneficial to improve survival of patients with advanced chemorefractory leukemia.

A new strategy for transplantation using a reduced-intensity stem-cell transplantation (RIST) or nonmyeloablative preparative regimen has been developed to reduce RRT while preserving an adequate antileukemia effect.[4,6] This strategy decreases the risk of NRM and allows

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Received 28 February 2005; accepted 23 June 2005; published online 22 August 2005

transplantation in elderly patients or those with organ dysfunction. RIST appears to be promising for a variety of hematologic diseases, if disease activity is controlled prior to transplant.[7] Most physicians believe that RIST is insufficient in controlling advanced hematologic malignancies, and that intensification of preparative regimens is required to improve their prognosis. Small pilot studies showed that RIST had been unsuccessful for advanced hematologic malignancies,[5,8] yet, efficacy of RIST has not been fully evaluated. Few comparative studies have been reported between RIST and CST for hematologic malignancies.[9]

Patients older than 50 years are regarded as candidates for RIST, yet, patients younger than 60 years frequently undergo CST. Either RIST or CST is offered to patients aged between 50 and 59 years according to doctors' preferences or based on patients' conditions. To evaluate the efficacy of RIST for hematologic malignancies in the elderly patients, we retrospectively compared the outcomes of 207 consecutive patients aged between 50 and 59 years with hematologic malignancies who had received either RIST ($n=70$) or CST ($n=137$).

Patients and methods

Data collection

We conducted a nation-wide retrospective survey of 207 adult Japanese patients aged between 50 and 59 years who received allo-HSCT from an HLA-identical sibling for the treatment of acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), and myelodysplastic syndrome (MDS) from February 1998 to November 2002 in 55 participating hospitals. Patients with a history of previous transplantation were excluded from this study.

All the CST and RIST recipients who were eligible in this study were included in each hospital. In Japan, approximately 2000 transplants are performed annually. The types of transplantations are autologous (40%), myeloablative allogeneic (45%), and reduced intensity or nonmyeloablative allogeneic transplantation (15%).[10] RIST recipients are generally treated as clinical studies in Japan. Most patients were incurable with conventional treatments and were considered inappropriate for conventional allo-SCT because they were age >50 years old and/or due to organ dysfunction (generally attributable to previous intensive chemo- and/or radiotherapy).

Data from participating centers were derived from questionnaires distributed to each center. Minimum data required for the inclusion of a patient in this study were age, performance status (PS) according to the Eastern Cooperative Oncology Group (ECOG) criteria before conditioning, medical complications at transplant, diagnosis of underlying diseases, treatment prior to allo-HSCT, disease status at transplant, preparative regimens, GVHD prophylaxis, date of transplant, date of follow-up, disease status at follow-up, development of acute and/or chronic GVHD, date of acute and/or chronic GVHD, date of disease progression/death, and causes of death. We have not collected information on the types of chronic GVHD (limited *vs* extensive).

Definition

Reduced-intensity regimens were defined as reported previously.[11,12] The upper limits of busulfan, melphalan, and TBI were 8, 140 mg/m^2, and 2 Gy for consideration as reduced-intensity preparative regimens. Neutrophil recovery was defined as an absolute neutrophil count of more than 0.5×10^9/l for two consecutive days. Patients were divided into two groups based on their disease status at transplant. Low-risk patients were defined as those with acute leukemia in first remission, CML in chronic phase, and myelodysplastic syndrome refractory anemia. The others were classified into the high-risk group. NRM was defined as death without progression of the underlying disease. Overall survival (OS) was defined as the duration of survival between transplant and either death or last follow-up. Progression-free survival (PFS) was defined as the duration of survival after transplant without disease progression, relapse, and death.

End points and statistical analysis

The primary end points were 2-year OS and PFS. The secondary end points included NRM within 100 days and 1-year of transplant, incidence of acute GVHD, and relapse rates. These end points were compared between CST and RIST recipients. For the analysis of OS and PFS, patients were stratified according to the risk of the underlying disease.

OS and PFS were determined using the Kaplan–Meier method. The last follow-up was on 1st August 2003. Median follow-up of surviving patients was 26.6 months (range, 9.5–63.6). Surviving patients were censored on the last day of follow-up. Acute GVHD was analyzed in patients who achieved initial engraftment. Cumulative incidence of acute GVHD, relapse rates, and NRM was calculated using Gray's method, considering each other event as a competing risk.[13]

Clinical characteristics were compared between CST- and RIST recipients using Fisher's exact test or the Mann–Whitney test. A multivariate Cox proportional hazards model was used to identify independent and significant prognostic factors on OS. The variables entered in each analysis were patient age, sex, primary disease, their risks, PS, and type of preparative regimens (CST *vs* RIST). Acute and/or chronic GVHD was included as a time-dependent covariate. A significance level of 5% was set as the limit for inclusion in the model. Prognostic factors, significant at $P<0.05$ in the stepwise proportional model analysis, were considered to be of importance in influencing survival.

Results

Patient characteristics and transplantation procedures

Types of transplants were CST ($n=137$) and RIST ($n=70$). Patient characteristics and transplantation procedures are shown in Table 1. Between the two groups, there were significant differences in age, sex, types of stem cells, presence of infectious complications at transplant, and PS.

Table 1 Characteristics of patients

Variables	*CST (n = 137)*	*RIST (n = 70)*	*P-value*
Pretransplant factors			
Age			
Median (range)	52 (50–59)	57 (50–59)	<0.01*
Sex			
Male/female	93/44	35/35	0.012*
Underlying diseases			
AML	56 (41%)	33 (47%)	0.42
ALL	27 (20%)	8 (11%)	
CML	34 (25%)	16 (23%)	
MDS	20 (15%)	13 (19%)	
Risk of underlying diseases[a]			
Total: low/high	63/74	25/45	0.18
AML: low/high	19/37	7/26	
ALL: low/high	14/13	5/3	
CML: CP/BC/AP	19/3/4	12/3/2	
MDS: RA/RAEB/RAEB in T/CMMoL	0/0/0/1	1/1/1/1	
Stem cells[b]			
Peripheral blood/bone marrow	57/80	68/2	<0.01*
Complications			
Cardiac impairment	5	3	0.72
Liver dysfunction	10	6	0.78
Respiratory dysfunction	6	6	0.22
Infection	9	11	0.028*
Performance status (PS)			
0–1/2–4	123/12	54/13	0.033*
Sex mismatch			
Donor → Recipient; F → M	35	12	0.17
Transplantation procedures			
Conditioning regimen	12 Gy TBI-based	74 (54%)	
BU/CY-based	51 (37%)		
TBI/BU/CY	12 (9%)		
Cladribine-based		6 (9%)	
Fludarabine-based		61 (87%)	
2 GY TBI-based		3 (4%)	
GVHD prophylaxis			
CSP	3 (2%)	32 (46%)	
CSP + sMTX	124 (91%)	23 (33%)	
FK506 + sMTX	5 (4%)	8 (11%)	
Others	5 (4%)	7 (10%)	

*Statistically significant.
[a]We divided the risk of transplantation into two groups. The low-risk group was as follows: acute myeloid or lymphoid leukemia in first remission, chronic myelogeneous leukemia in chronia phase, and myelodysplastic syndrome refractory anemia.
[b]Four patients were infused both peripheral and bone marrow.
CST = conventional stem cell transplantation; RIST = reduced-intensity stem cell transplantation; TBI = total body irradiation; CY = cyclophosphamide; BU = busulfan; 2-CdA = cladribine; Flu = fludarabine; Mel = melpharan; CSP = cyclosporine; sMTX = short-term methotrexate; AML = acute myeloid leukemia; ALL = acute lymphoid leukemia; CML = chronic myelocytic leukemia; MDS = myelodysplastic syndrome; RA = refractory anemia; RAEB = refractory anemia with excess blasts; RAEB in T = refractory anemia with excess blasts in transformation; CMMoL = chronic myelomonocytic leukemia.

RIST recipients had poorer characteristics than CST recipients.

All the CST recipients received either TBI-based or busulfan/cyclophosphamide-based regimens. RIST regimens were purine analog based ($n = 67$), and 2 Gy TBI based ($n = 3$).

Most CST recipients (129/137) received a combination of calcineurin inhibitors (cyclosporin or tacrolimus) and short-term methotrexate as GVHD prophylaxis, while 32 of the 70 RIST received cyclosporin alone as GVHD prophylaxis (Table 1).

Engraftment

Six CST recipients (9%) died of NRM before engraftment. Neutrophils did not decrease below 0.5×10^9/l in 6 RIST recipients (9%). The other 131 CST recipients (96%) and 64 RIST recipients (91%) achieved primary neutrophil engraftment. The median intervals between transplant and neutrophil engraftment were 15 days (range, 5–27) and 12 days (range, 9–30) in CST and RIST, respectively.

Secondary graft failure developed in three patients (CST 2 and RIST 1) 3–9 months after transplant. All the three patients died of infectious complication during neutropenia.

NRM

In all, 23 CST (17%) and five RIST recipients (7%) died of NRM within 100 days of the transplant. Cumulative incidences of 100 days NRM following CST and RIST were 16% (95% confidence interval (CI), 10–22%) and 7% (95% CI, 1–14%), respectively ($P = 0.040$). As of August 2003, 46 CST (34%) and 16 RIST recipients (23%) died of NRM. The median onset of NRM following CST and RIST was day 95.5 (range, 2–967) and day 254 (range, 49–724), respectively. Cumulative incidences of 1-year NRM following CST and RIST were 31% (95% CI, 23–39%) and 15% (95% CI, 6–23%), respectively ($P = 0.0062$, Figure 1). Primary causes of NRM following CST and RIST are

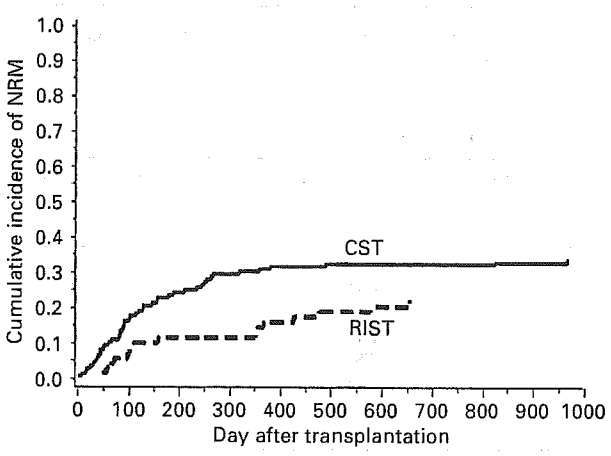

Figure 1 Cumulative incidences of NRM following CST and RIST. Cumulative incidences of NRM following CST and RIST were 31% (95% CI, 23–39%) and 15% (95% CI, 6–23%), respectively.

shown in Table 2. NRM attributable to RRT occurred in 12 and one patient following CST and RIST, respectively.

Graft-versus-host disease

A total of 130 CST and 68 RIST recipients were evaluable. There was no difference in the cumulative incidences of grade II–IV acute GVHD between CST and RIST (Figure 2).

In CST, grade II–IV and grade III–IV acute GVHD occurred in 56 (43%) and 24 patients (18%), respectively. The median onset of grade II–IV acute GVHD was day 23 (range, 3–146 days). GVHD was fatal in 13 of the 56 patients. Of the 104 patients who survived longer than 100 days, 60 patients (58%) developed chronic GVHD.

In RIST, grade II–IV and grade III–IV acute GVHD developed in 38 (56%) and 16 (24%), respectively. The median onset of grade II–IV acute GVHD was day 44 (range, 7–109). GVHD was fatal in 11 of the 38 patients. Of the 57 patients who survived longer than 100 days, 37 (65%) developed chronic GVHD.

Survival

As of August 1, 2003, median follow-ups of surviving patients following CST and RIST were 31.6 months (range, 9.5–63.6) and 20.3 months (range, 9.5–38.4), respectively. Disease-specific outcomes are shown in Table 3.

In all, and low-risk patients, significant differences were not observed in OS between CST and RIST ($P=0.25$, $P=0.69$) (Figures 3 and 4). Among the high-risk patients, there was a significant difference between the two groups ($P=0.044$). The 2-year OS following CST and RIST was 27 and 37%, respectively (Figure 5). There was no significant difference in PFS between CST and RIST among all and low-risk patients ($P=0.39$, $P=0.77$). Among high-risk patients, there was a trend toward better PFS after RIST ($P=0.063$). The 2-year PFS following CST and RIST was 30 and 56%, respectively.

Underlying diseases relapsed in 38 CST and 23 RIST recipients. There was no significant difference in the cumulative incidence of 1-year relapse rates between the two groups; CST 24% (95% CI, 17–32%) and RIST 29% (95% CI, 19–40%) ($P=0.21$, Figure 6).

Risk factors

A univariate analysis revealed that CML ($P<0.0001$), risk of underlying diseases ($P=0.0002$), PS ($P<0.0001$), and

Table 2 Causes of deaths

	CST	*RIST*
Relapse	28	16
Graft-versus-host disease	13	11
Infection	4	0
Bacteria	5	0
Virus	4	1
Fungi		
Idiopathic pulmonary syndrome	5	0
Thrombotic microangiopathy	5	1
Hepatic venoocclusive disease	2	0
Secondary malignancy	2	1
Cardiac failure	1	1
Cerebral infarction	1	0
Others	4	1

CST = conventional stem cell transplantation; RIST = reduced-intensity stem cell transplantation.

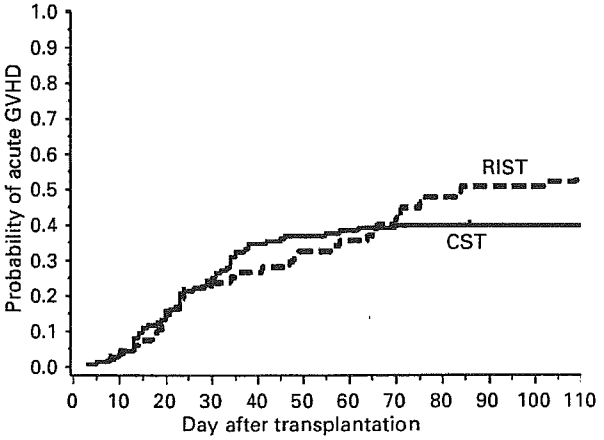

Figure 2 Cumulative incidences of grade II–IV acute GVHD. There was no difference in the cumulative incidences of grades II–IV acute GVHD between CST and RIST.

Table 3 Disease-specific outcomes

Underlying disease	*Type of transplant*	*Number of patients*	*Nmber of patients who died of TRM*	*Number of patients who developed disease progression*	*2-year overall survival*[a]
AML	CST	56	19	20	38.7 (25.8–51.6)
	RIST	33	8	12	69.3 (53.4–85.2)
ALL	CST	27	11	10	33.3 (15.5–51.1)
	RIST	8	2	3	50.0 (15.3–84.7)
MDS	CST	34	8	5	45.0 (23.2–66.8)
	RIST	16	5	3	53.8 (26.8–80.8)
CML	CST	20	8	3	73.4 (58.5–88.3)
	RIST	13	1	5	93.3 (80.8–100)

[a]Each column denotes a rate of 2-year overall survival and its 95% confidence interval.
AML = acute myeloid leukemia; ALL = acute lymphoid leukemia; MDS = myelodysplastic syndrome; CML = chronic myelocytic leukemia; TRM = transplant-related mortality; CST = conventional stem-cell translantation; and RIST = reduced intensity stem cell transplantation.

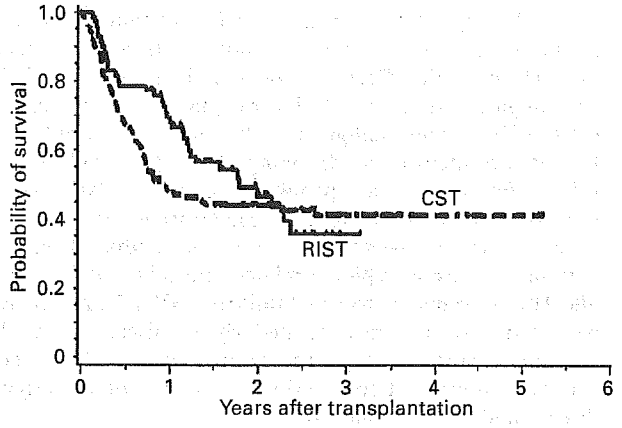

Figure 3 Overall survival (OS) following CST and RIST in all patients. There was no significant difference in OS between CST and RIST ($P=0.25$).

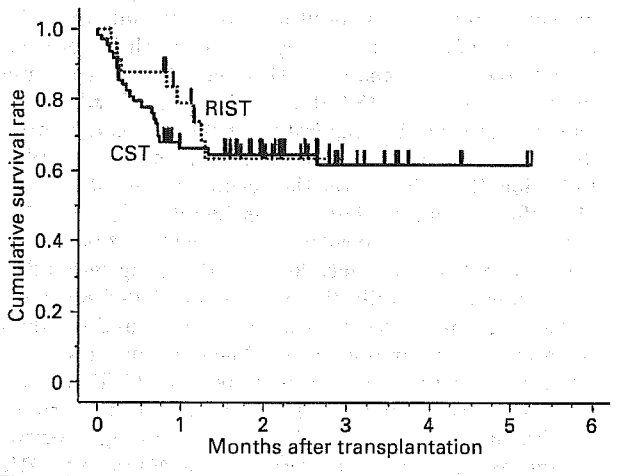

Figure 4 OS following CST and RIST in patients with low-risk diseases. There was no significant difference in OS between CST and RIST ($P=0.69$).

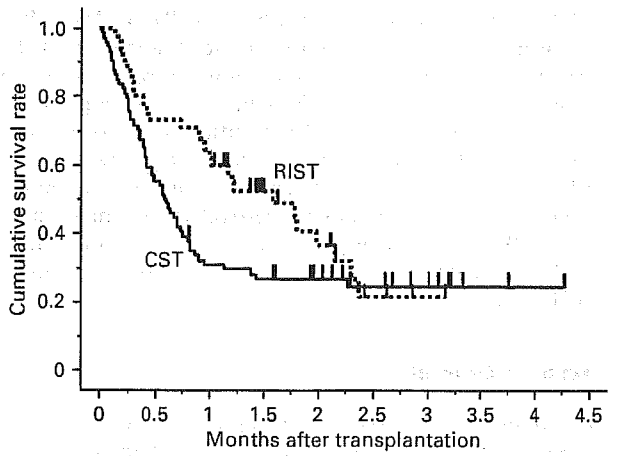

Figure 5 OS following CST and RIST in patients with high-risk diseases. There was a significant difference in OS between CST and RIST ($P=0.044$). The 2-year OS following CST and RIST were 27 and 37%, respectively.

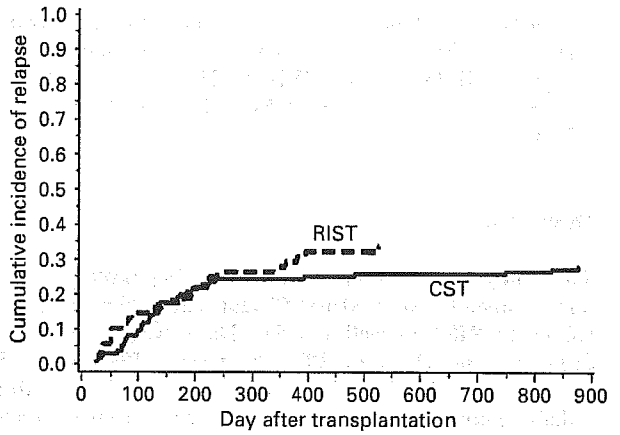

Figure 6 Cumulative incidences of relapse following RIST and CST. There was no significant difference in cumulative incidences of relapse between RIST and CST.

Table 4 Risk factors for overall survival following allogeneic hematopoietic stem-cell tranplantation

	Hazard ratio	*95% confidence interval*	*P-value*
Factors			
Univariate analysis			
Pretransplant factors			
Sex: Female	0.85	0.58–1.25	0.40
Age: 56–59 *vs* 50–51 years	1.11	0.72–1.70	0.63
Donor: female to male recipient	1.22	0.80–1.88	0.35
Disease	1.00		0.0002
CML	0.29	0.15–0.58	
ALL	1.30	0.73–2.31	
AML	0.91	0.55–1.51	
Risk of underlying diseases; high	2.30	1.58–3.37	<0.0001*
PS: 2–4	3.49	2.16–5.64	<0.0001*
Preparative regimen; CST	1.26	0.85–1.88	0.25
Posttransplant factor			
Grade II–IV acute GVHD; presence	2.58	1.76–3.79	<0.0001*
Variables			
Multivariate analysis			
Preparative regimen; CST *vs* RIST	1.92	1.25–2.97	0.003*
PS; 2–4 *vs* 0–1	2.50	1.51–4.16	<0.001
Disease; CML	0.38	0.21–0.69	0.002
Risk of underlying diseases; high	1.85	1.21–2.83	0.004
Grade II–IV acute GVHD; presence	2.57	1.72–3.84	<0.001*

*Statistically significant.
AML = acute myeloid leukemia; CML = chronic myelogeneous leukemia; MDS = myelodysplastic syndrome; ALL = acute lymhoid leukemia; PS = performance status; CST = conventional stem-cell transplantation; GVHD = graft-versus host disease.

development of GVHD ($P<0.001$) were significant risk factors for OS (Table 4). On multivariate analysis, five variables were significant: preparative regimens (CST *vs* RIST) (hazard ratio (HR) = 1.92, 95% CI, 1.25–2.97; $P=0.003$), PS (2–4 *vs* 0–1) (HR = 2.50, 95% CI,

1.51–4.16; $P<0.001$), risk of underlying diseases (HR = 1.85, 95% CI, 1.21–2.83; $P=0.004$), development of grade II–IV acute GVHD (HR = 2.57, 95% CI, 1.72–3.84; $P<0.001$), and CML (HR = 0.38, 95% CI, 0.21–0.69; $P=0.002$).

Discussion

This study suggests that patients with hematologic malignancies aged between 50 and 59 years can achieve remission following RIST as well as CST. There was no significant difference in OS and PFS between RIST and CST (Figure 3). Follow-up of this study was too short to draw a definite conclusion; however, short-term survivals tended to be better in RIST recipients than in CST recipients in the high-risk group (Figure 5). These situations were in contrast to the low-risk group, in which OS and PFS were similar between the two groups (Figure 4). Myeloablative preparative regimens might have been intolerable for high-risk elderly patients. Patients with more progressive diseases might have received CST rather than RIST.

Most physicians believe that it is difficult to control advanced hematologic malignancies with RIST.[5,7] Yet, feasibility of myeloablative preparative regimens has not been fully investigated in patients aged between 50 and 59 years. It is questionable whether intensification of preparative regimens is beneficial for controlling advanced or chemoresistant hematologic malignancies in these patients, because patients with high-risk hematologic malignancies frequently have organ damage due to repeated cytotoxic chemotherapies prior to transplantation.[14] These patients are at high risk of NRM.[15,16] As shown in this study, a myeloablative preparative regimen is not necessarily beneficial in allo-HSCT for elderly patients with high-risk hematologic diseases. In contrast, patients aged between 50 and 59 years in good physical condition are able to tolerate a high-dose preparative regimen. Variables such as CML, low-risk underlying disease, and good PS were independent good prognostic factors for OS. We should tailor preparative regimens considering the patient's condition and risk of the underlying disease.

There are two types of complications associated with allo-HSCT. One is RRT, which often occurs within 30 days of transplantation.[3] The other is GVHD, which is frequently complicated with infections.[14,17] In the present study, there was a significant difference in NRM attributable to RRT between CST and RIST (16 *vs* 7%, $P=0.04$). Reduced-intensity regimens cause less organ damage, contributing to less NRM. These findings were comparable to previous reports.[4,16,18]

GVHD is the most significant concern after allo-HSCT. This study confirmed the previous studies on GVHD following RIST.[19,20] There was no significant difference in the incidence of GVHD between CST and RIST (43 *vs* 56%), and onset of GVHD was delayed in RIST compared with CST. Mortality of GVHD was similar between CST and RIST (23 *vs* 29%). Development of grade II to IV acute GVHD was an independent poor prognostic factor for OS (HR = 2.57, 95% CI, 1.72–3.84; $P<0.001$). These findings demonstrate that GVHD is a significant complication following RIST as well as CST, and that its optimal management awaits further investigation. Balancing GVHD and GVL effects is a delicate issue in allo-HSCT. The augmentation of GVHD prophylaxis may hamper GVL effects, and malignant cells cannot be eradicated by reduced-intensity conditioning alone. Augmentation of GVL effects such as prophylactic donor lymphocyte infusion, vaccination, and administration of cytotoxic T-cells[21] may be beneficial to control residual leukemia without increasing regimen-related mortality. At present, allo-HSCT recipients received uniform GVHD prophylaxis irrespective of the risk of underlying diseases and the patient's condition. In the future, management of GVHD should be optimized considering the risk of the underlying disease and patient conditions.

Relapse is another concern in RIST. This study did not show significant differences in relapse rates between CST and RIST (Figure 6). The unexpectedly low relapse rates following RIST suggest that it has a strong antitumor activity through allogeneic immunity. Augmentation of allogeneic immunity without increasing the intensity of the arative regimen is promising for controlling advanced hematological malignancies. However, late relapse might increase following RIST due to the lack of reduction of leukemic cells by the preparative regimen. It is too early to draw definite conclusions about the incidence of late relapse following RIST based on the results of this study, since allo-HSCT recipients have a considerable risk of relapse within 3 years of transplant[22] and median follow-up of surviving patients was only 26.7 months. Long-term follow-up is required to clarify the prognosis of RIST recipients.

This is a small-sized retrospective study, and we should be careful in interpreting results. The most important was a difference in patient backgrounds between CST and RIST recipients. To minimize unrecognized biases, patients enrolled in this study were limited to those aged between 50 and 59 years who had leukemia or MDS. Yet, RIST recipients were significantly older, and their disease status and PS were significantly worse than CST recipients. These variables influence survival following RIST[7,23] as well as CST.[24–26] Furthermore, there was a wide difference in GVHD prophylaxis between CST and RIST. Most RIST recipients received cyclosporin alone. Short-term methotrexate, and cyclosporin or tacrolimus were given to CST recipients. The median follow-up of surviving patients enrolled in this study was 26.6 months, and thus too short, requiring further observation. Considering these facts, it is difficult to make an accurate comparison between reduced-intensity and myeloablative preparative regimens in this study. We are now planning a prospective randomized study to compare RIST with CST for hematologic malignancies.

Acknowledgements

This study was supported by a Grant-in-aid of the Ministry of Labor and Welfare in Japan. We thank T Fukuda for scientific discussions and for critically reviewing the manuscript. We thank all the staff and resident members of the transplant centers in Japan. A complete list of participating institutions appears in the Appendix.

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Appendix

This study was conducted at the following institutions under the auspices of the following investigators in Japan: Tanimoto E Tetsuya (Kyusyu University Graduate School of Medical Sciences, Fukuoka), Iida H (Meitetsu Hospital, Aichi), Matsue K (Kameda General Hospital, Chiba), Kato K (Hamanomachi Hospital, Fukuoka), Shinagawa K (Okayama University Medical School, Okayama), Abe Y (Kyusyu University Graduate School of Medical Sciences, Fukuoka), Nakajyo T (Kanazawa University Graduate School of Medicine, Kanazawa), Uike N (National Kyushu Cancer Center, Fukuoka), Okamoto S (Keio University School of Medicine, Tokyo), Hirabayashi N (Nagoya Daini Red Cross Hospital, Aichi), Komatsu T (Tsukuba Memorial Hospital, Ibaraki), Tamaki S (Yamada Red Cross Hospital, Mie), Izumi Y (Kokura Memorial Hospital, Fukuoka), Karasuno T (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka), Yamane T (Osaka City University, Osaka), Ashida T (Kinki University Hospital, Osaka), Wakita A (Nagoya City University Graduate School of Medical Science, Aichi), Furukawa T (Niigata Uniersity Medical Hospital, Niigata), Teshima H (Osaka City General Hospital, Osaka), Yamashita T (National Defense Medical College Hospital, Saitama), Miyazaki Y (Kansai Medical University Hospital, Osaka), Kobayashi Y & Taniwaki M (Kyoto Prefectural University of Medicine, Kyoto), Kobayashi H (Nagano Red Cross Hospital, Nagano), Ito T (Nihon University School of Medicine, Tokyo), Ishida Y (Iwate Medical University Hospital, Iwate),

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Cell Cycle Arrest and Apoptosis Induced by SART-1 Gene Transduction

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Abstract. *The biological function of the SART-1 gene product is demonstrated and its potential as a target for cancer gene therapy is discussed. Materials and Methods: The SART-1 gene was transduced by a recombinant adenovirus vector and its expression was promoted by a CMV promoter. Results: The transduction efficiency by recombinant adenoviruses in A549 and MCF-7 cells was determined using a vector expressing luciferase, which showed high expression in the cells. Cell count analysis using Trypan-Blue dye exclusion showed that SART-1 gene transduction inhibited cell growth. Flow cytometry analysis suggested that SART-1 gene transduction induced cell cycle arrest followed by apoptosis. Western blot analysis confirmed that the apoptosis pathway was activated by SART-1 gene transduction. Conclusion: These results show that SART-1 gene transduction induces cell cycle arrest leading to apoptosis and suggest the possibility of gene therapy against cancer. In addition, SART-1 is known to be a tumor antigen in a range of cancers recognized by T cells, thus a potential strategy would be the combination of suicide gene therapy with immuno-gene therapy.*

Since the MAGE-1 gene product was identified as a tumor rejection antigen by Boon *et al.*, numerous tumor rejection antigens recognized by cytotoxic T lymphocytes (CTLs) have been identified (1-3). Computer software to predict antigen peptide sequences from cDNA databases has recently become available and researchers seeking tumor antigens have reported that some identified sequences are active *in vivo*. SART-1 is ubiquitously expressed in various cancers, including breast, esophagus, lung and uterine cancers (4-10). SART-1 encodes both the SART-1_{259} antigen, expressed in the cytosol of epithelial cancers and the SART-1_{800} antigen, expressed in the nuclei of most proliferating cells (11-15). Peptides derived from the gene are known to be tumor-derived antigens recognized by HLA A2601- and A2402-restricted CTLs. There have been several reports on SART-1-derived peptides capable of inducing CTLs (16, 17). The HLA-A26 allele is found in 22% of the Japanese population, 17% of Caucasians and 16% of Africans, while the HLA-A24 allele is found in 60% of Japanese, 20% of Caucasians and 12% of Africans (18). Therefore, SART-1 may be an ideal target molecule in specific immunotherapy for cancer patients. However, in contrast to its immunological properties, the function of SART-1 has not been elucidated.

Gene transfer into mammalian cells using viral vectors provides a powerful tool for gene therapy. Vectors include retroviruses derived from the Mouse Moloney Leukemia virus, human immunodeficiency virus and herpes virus (19, 20). With regard to safety, efficiency and specificity, adenoviruses are superior to other vectors. Adenovirus vectors are able to transduce genes into non-replicating / poorly-replicating cells, while other viral vectors, including retrovirus vectors, require cell proliferation for sufficient gene transduction. This suggests that adenovirus vectors have many advantages when used as vehicles for gene transfer (21).

The RGD-fiber-modified recombinant adenovirus, in which the Arg-Gly-Asp (RGD)-containing peptide is incorporated into the HI-loop of the fiber knob domain, exhibits high transduction and expression efficiency when

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Key Words: Tumor rejection antigen, SART-1, cell cycle, apoptosis.

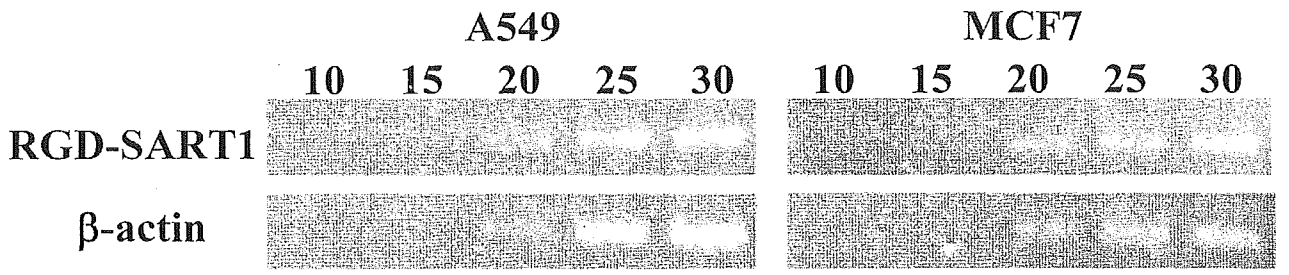

Figure 1. *Expression of SART-1 mRNA after Ad-RGD-SART-1 treatment. Cells were treated with Ad-RGD-SART-1 at 30 M.O.I for 24 h. RNA from the cells was then isolated and SART-1 mRNA was quantified by RT-PCR. The data indicate the mean and standard deviation of three independent results.*

compared with conventional recombinant adenoviruses, increasing transduction efficiency into cancer cells by two to three orders of magnitude (22).

In our experiments, the SART-1 gene was transduced into two different cancer cell lines using the RGD-fiber-modified recombinant adenovirus (Ad-RGD-SART-1) and the biological functions of the gene product, particularly on the cell cycle, cell viability and cell cycle/apoptosis-associated proteins, were analyzed. The possibility of SART-1 gene therapy is also discussed.

Materials and Methods

Cells. 293 cells (human kidney epithelial cells transformed with adenovirus 5 DNA), MCF7 cells (human breast cancer cells) and A549 cells (human non-small cell lung cancer cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The 293 and MCF7 cells were cultured in DMEM/F12 supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA, USA), penicillin (100 U/ml) (Invitrogen Corp.) and streptomycin (100 mg/ml) (Invitrogen Corp.), in a humidified 5% CO_2 atmosphere at 37°C. The A549 cells were cultured in RPMI1640 supplemented with 10% v/v heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml), in a humidified 5% CO_2 atmosphere at 37°C.

All experiments using recombinant adenovirus were performed in a biological safety cabinet (Level 2, Sanyo, Tokyo, Japan), in accordance with institutional regulations.

Preparation of Ad-RGD-SART-1 and Ad-RGD-Luc. Ad-RGD-SART-1 and Ad-RGD-Luc are E1-deleted recombinant adenoviruses that encode SART-1 and luciferase, respectively, under the control of a hybrid promoter consisting of a cytomegalovirus (CMV). They also contain fibers with the RGD motif in the HI loop. Purification and concentration of the recombinant adenoviruses was performed using conventional CsCl gradient methods and an ultracentrifuge (Beckman, Fullerton, CA, USA), as described previously (23). Purified adenovirus was dialyzed against phosphate-buffered saline (PBS) without calcium and magnesium (PBS(-)) containing 10% glycerol, at 4°C for 12 h. Dialyzed viral solutions were either used immediately or stored at –130°C until used.

The number of viral particles within each solution was determined according to the following formula:

1OD260 = 7x10e11 viral particles.

The multiplicity of infection (M.O.I) was determined by plaque assay using 293 cells.

Evaluation of SART-1 gene expression by RT-PCR. Transfer and expression of SART-1 by Ad-RGD-SART-1 was tested at 30 M.O.I. Twenty-four h after transduction, the A549 and MCF7 cells were lysed and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). The expression levels of SART-1 mRNA were evaluated by reverse transcription polymerase chain reaction (RT-PCR) using specific primers (5'-TCACTTGGTGATGG TGTTCG-3' and 5'-AAGCAGCTGGAGAAGGGACG-3'). The expression of β-actin mRNA was also evaluated as an internal control using specific primers (5'-CTAGAAGCATTTGCGG TGGA-3' and 5'-ATGGATGATGATATCGCCGC -3'). Amplification was performed with 30 cycles of 1 min at 95°C, 1.5 min at 57°C and 2 min at 72°C, with samples being taken every 5 cycles. Amplified DNA was electrophoresed in 1% agarose gels and PCR bands were detected and quantified.

Effects of SART-1 gene transduction on cell growth. To determine the effects of SART-1 transduction on cell growth, the viable cells were counted at 24, 48 and 72 h after treatment with 30 M.O.I of Ad-RGD-SART-1 and Ad-RGD-Luc. The percentage of cell growth was calculated using the following formula:
Number of viable cells treated with virus on day X / Number of viable cells without virus on day X x100(%).

Analysis of cell cycle by flow cytometry. The cells were treated with 30 M.O.I of Ad-RGD-SART-1 or Ad-RGD-Luc and were cultured for 24 or 48 h. Cells in 6-well plates were collected by Trypsin-EDTA (Invitrogen Corp.) treatment and were re-suspended in culture medium. The re-suspended cells were washed with PBS(-), and were fixed with 70% ethanol at 4°C for 48 h. The fixed cells were incubated in lysis buffer containing 0.1% TritonX-100 and 0.1% RNaseA at 4°C for 24 h. To evaluate DNA content, propidium iodide (P.I.) solution was added to the samples (final concentration; 25 μg/ml). P.I. fluorescence of nuclei was measured with a FACScan (Becton Dickinson Co., Franklin Lakes, NJ, USA) and data were obtained from 10^4 cells per sample.

Analysis of cell cycle and apoptosis by Western blot analysis. The cells were treated with Ad-RGD-SART-1 or Ad-RGD-Luc at 30 M.O.I for 1 h, followed by additional cultures. At 0, 24, 48 and 72 h after transduction, the cells were washed twice with PBS(-), scraped off the plate and lysed in cell lysis buffer (1% SDS, 1 mM sodium ortho-vanadate, 10 mM Tris-HCl, pH 7.4). Twenty μg of lysed material was

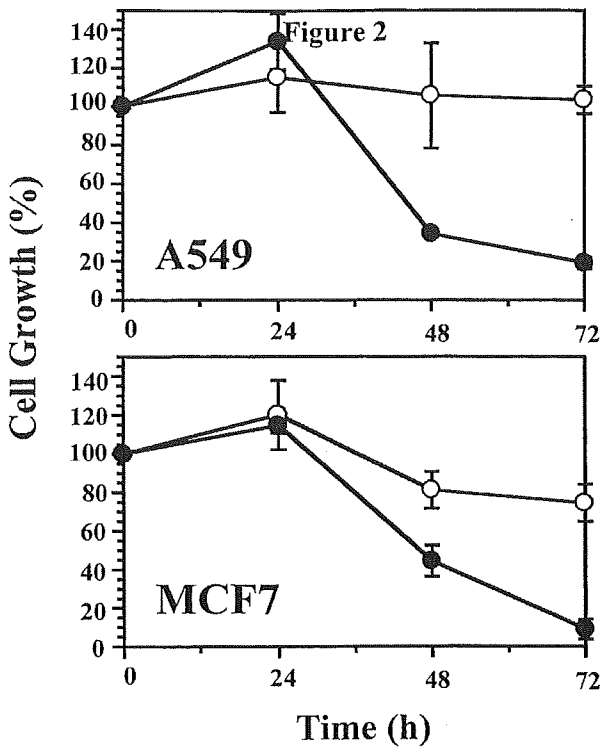

Figure 2. *Effects of SART-1 gene transduction on cell growth. To determine the effects of SART1 gene transduction on cell growth, the viable cells after treatment with 30 M.O.I of Ad-RGD-SART1 (●) and Ad-RGD-Luc (○) were counted. Twenty-four hours after infection, cells were cultured for a further 72 h. Each point represents the mean and standard deviation of triplicate cultures.*

electrophoretically separated on 7.5%, 10% or 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). Primary antibodies supplied with Cell Cycle Sampler Kits I and II, and Apoptosis Sampler Kits I and II (Transduction Laboratories, Lexington, KY, USA) were used. The membranes were treated with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions in order to analyze protein expression.

Results

Transduction efficiency of adenovirus. In preliminary experiments, we tested the transduction efficiency of Ad-RGD in A549 and MCF7 cells using Ad-RGD-Luc (Data not shown). The results suggested that transduction at 30 M.O.I induced sufficient gene expression in both cells. Figure 1 shows the expression of SART-1 mRNA in the cells. The expression of SART-1 mRNA in the MCF7 and A549 cells was detected at 20, 25 and 30 cycles, but was not detected in the Ad-RGD-Luc-treated cells. SART-1 expression in Ad-RGD-SART-1 was also detected by Northern hybridization (Data not shown).

Cell growth inhibition by SART-1 gene transduction. Figure 2 shows the growth inhibition induced by SART-1 gene transduction. SART-1 gene transduction at 30 M.O.I induced strongly inhibited cell growth in a time-dependent manner. The growth inhibition rates of the A549 and MCF7 cells at 72 h after infection were 81.0% and 91.2%, respectively.

Cell cycle inhibition after SART-1 gene transduction. At 24 and 48 h after infection, the cells were collected and cell cycle status and apoptosis were analyzed by flow cytometry. Figure 3 shows that G1 and G2 arrest followed by cell death was observed after Ad-RGD-SART-1 treatment.

Western blot analysis of molecules associated with cell cycle and apoptosis. The expression of 24 cell cycle-related proteins and 22 apoptosis-related proteins was evaluated by Western blot analysis. Figure 4 shows the results for proteins influenced by treatment with Ad-RGD-SART-1. Figure 5 summarizes the results for cell cycle (A549, Figure 5A; and MCF7, Figure 5B) and apoptosis (A549, Figure 5C; and MCF7, Figure 5D) cascades. The expressions of Cyclin A, Cyclin B, CDK2, Rb and Rb2 increased, while the expressions of CDC25B, Kip1/p27, Mad2, p53 and RBBP decreased in A549 cells treated with Ad-RGD-SART-1 (Figure 5A). In MCF7 cells, Cyclin B, Rb and Rb2 increased or stayed at the same level, and Kip1/p27, Mad2 and p53 decreased (Figure 5B). Among apoptosis-related proteins, Bax, BRAUCE, Fas Ligand, hILP, PARP, p53, RIP and TRADD decreased in A549 cells treated with Ad-RGD-SART-1 (Figure 5C). In MCF7 cells, Bax, Bcl-2, hILP, PARP, p53, RIP and TRADD decreased (Figure 5D).

Discussion

Although numerous clinical trials of cancer gene therapy have been performed, there have been no reports of their clinical usefulness to date. The reasons that gene therapy is currently unable to yield clinical benefits are: i) insufficient gene transduction efficiency of vectors; and ii) insufficient therapeutic potency of the transduced gene products. For example, gene therapy using cell cycle-associated proteins, such as p53, and gene therapy using prodrugs, such as herpes simplex virus thymidine kinase, require high transduction efficiency to induce their therapeutic effects. There are no vector systems that exhibit sufficient transduction efficiency to provide therapeutic benefits. Although these strategies may result in local control of the tumor, there is no therapeutic benefit against metastatic lesions. Another cancer gene therapy strategy is immuno-gene therapy, using gene-modified cytotoxic T lymphocytes or administration vaccines in the form of gene-modified tumor cells or

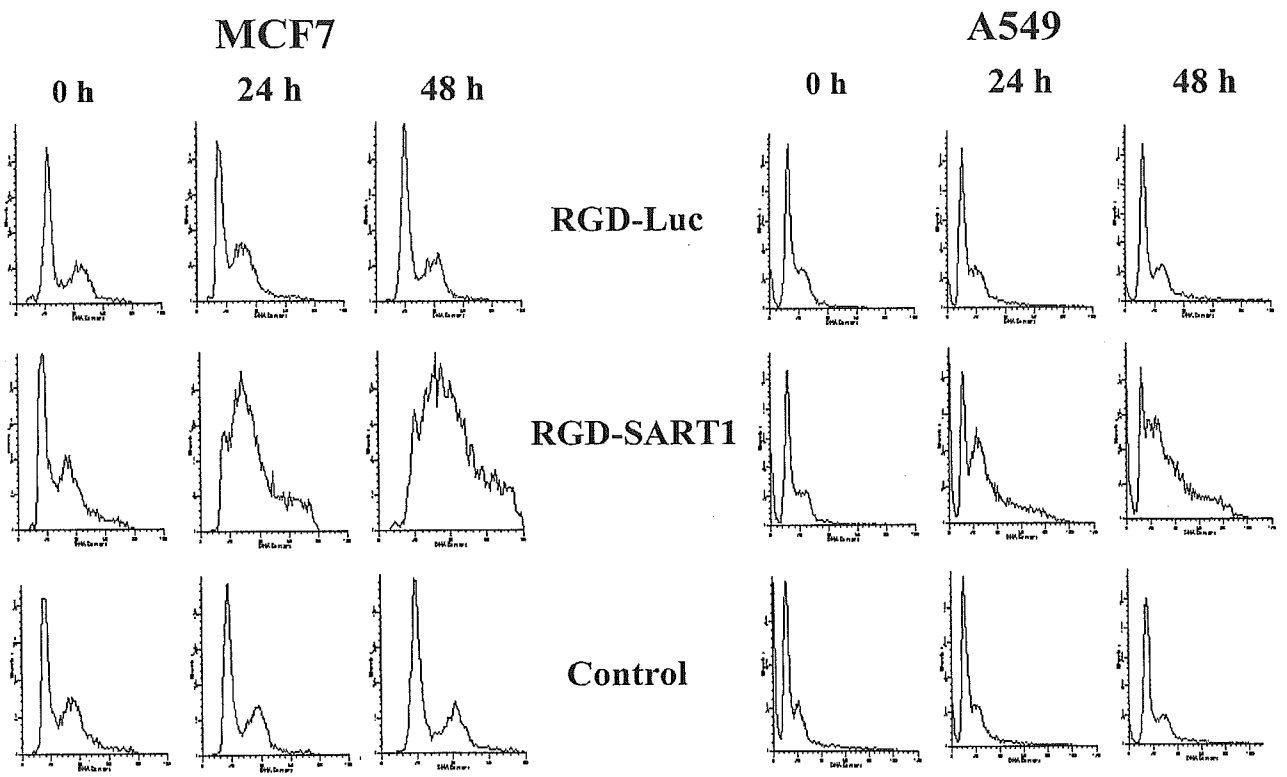

Figure 3. *Analysis of cell cycle by flow cytometry. The histogram shows the cell cycle and cell death status after treatment with Ad-RGD-SART-1 and Ad-RGD-Luc. At 24 and 48 h after infection, both the floating cells and adherent cells were collected in a single tube. For determination of DNA content, propidium iodide (PI) solution was added to the sample, as described in Materials and Methods.*

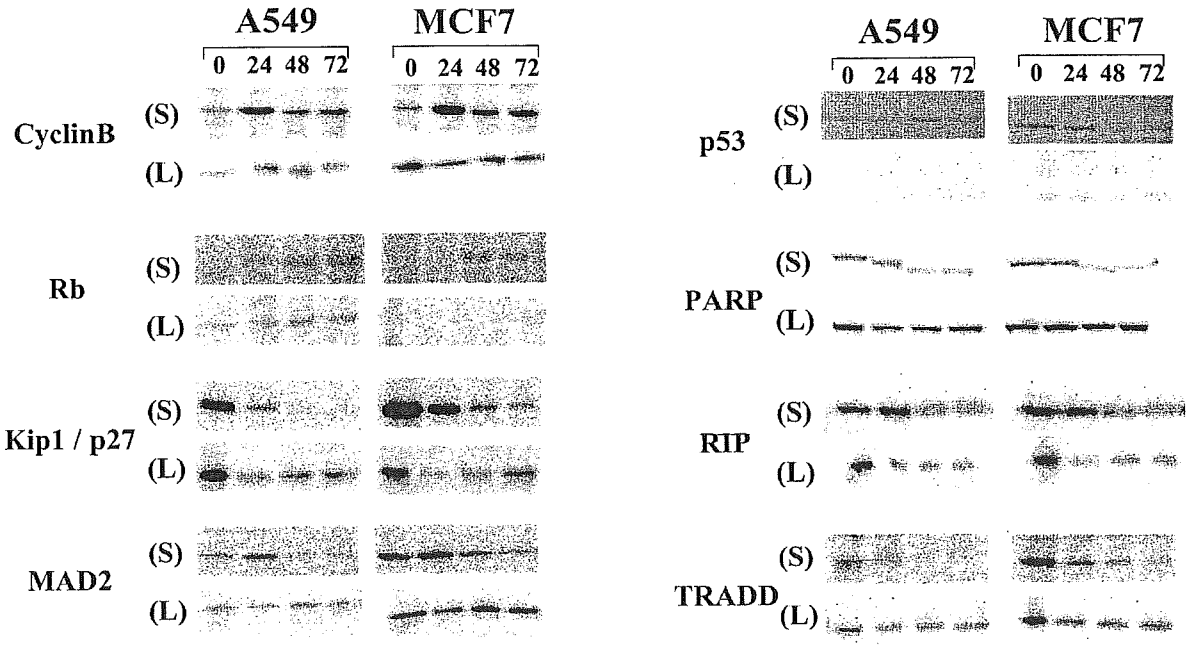

Figure 4. *Analysis of proteins associated with cell cycle and apoptosis by Western blotting. Cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were treated with 24 anti-cell cycle-related protein antibodies and with 22 anti-apoptosis-related protein antibodies. Proteins that were increased or decreased after Ad-RGD-SART-1 treatment in A549 and MCF7 cells are shown. (S); SART-1, (L); Luciferase. M.O.I: 30.*