tus before transplantation, disease characteristics (disease type and disease lineage), donor characteristics (age, sex, sex compatibility, and ABO compatibility), transplant characteristics (total body irradiation-containing regimen, tacrolimus versus cyclosporine, and total nucleated cell count harvested per recipient weight). The median was used as the cut-off point for continuous variables. The χ^2 test and Mann-Whitney test were used to compare results of two groups. The Hardy-Weinberg equilibrium for the *NKG2D* gene polymorphism was tested using the Haploview program.[32] Multivariate Cox models were used to evaluate the hazard ratio associated with the NKG2D polymorphism. Co-variates found to be statistically significant in univariate analyses ($p \leq 0.10$) were included in the models. For both the univariate and multivariate analyses, p values were two-sided and outcomes were considered to be statistically significant with $p \leq 0.05$.

Results

Frequencies of NKG2D haplotype

The *NKG2D* gene polymorphism was analyzed in 145 pairs of unrelated donors-recipients of bone marrow following myeloablative conditioning (Table 1). The haplotype frequencies of *LNK1/LNK1*, *HNK1/LNK1* and *HNK1/HNK1* were 43%, 42% and 15%, respectively in donors and 35%, 45% and 20%, respectively in recipients. These frequencies were similar to those reported in previous studies in Japanese populations[21,24] and were in accordance with the Hardy-Weinberg equilibrium (p=0.80).

Transplant outcomes according to NKG2D haplotype

With a median follow-up of 115 months among survivors (range, 74 to 140 months), 30 recipients (21%) had relapsed or progressed and 62 (47%) had died. Three patients (2%) died before engraftment. The analysis of the influence of the *NKG2D* genotype on clinical outcomes after transplantation was stratified according to whether the recipients had standard-risk disease or high-risk disease to account for the already recognized prognostic difference. The overall survival at 5 years in patients with standard-risk disease was 63% while that of patients with high-risk disease was 44% (p=0.06). The 5-year cumulative incidences of trasplant-related mortality were 32% and 27%, respectively (p=0.33) and those of disease relapse were 10% and 31%, respectively (p=0.0006).

The transplant outcomes according to *NKG2D* genotype are summarized in Table 2. Patients with standard-risk disease receiving transplants from donors with the *HNK1* haplotype had a significantly better 5-year overall survival (73% *vs.* 49%, p=0.01; Figure 1A) and lower transplant-related mortality rate (22% *vs.* 45%, p=0.02; Figure 1B) than those receiving transplants from donors without the *HNK1* haplotype. No difference was noted in disease relapse in relation to the donors' polymorphism (9% *vs.* 11%, p=0.81; Figure 1C) or in the development of grades II to IV acute GVHD (28% *vs.* 41%, p=0.25) or chronic GVHD (37% *vs.* 41%, p=0.83). When patients with acute myeloid leukemia or myelodysplastic syndrome were separately analyzed, there was still no difference in disease relapse in relation to NKG2D polymorphisms (*data not shown*). In patients with high-risk disease, the donor *HNK1* haplotype had no significant effects on transplant outcomes (Table 2).

Multivariate analysis

Any factors found to be significant in univariate analyses were included in the multivariate analysis. When patients with standard-risk disease were analyzed, the *HNK1* haplotype in donors remained statistically significant in multivariate analyses for both overall survival and transplant-related mortality (Table 3). The presence of the *HNK1* haplotype in the donor resulted in better overall survival (hazard ratio, 0.44; 95% confidence interval, 0.23 to 0.85; p=0.01) and transplant-related mortality (hazard ratio, 0.42; 95% confidence interval, 0.21 to 0.86; p=0.02).

Table 2. **Univariate analysis of the association of *NKG2D* polymorphisms with clinical outcomes after transplantation.**

	N.	5-year OS	p	5-year TRM	p	5-year relapse	p	Grade II-IV acute GVHD	p	Chronic GVHD	p
Standard-risk disease											
Donor *NKG2D* haplotype			0.01		0.02		0.81		0.25		0.83
HNK1-positive	55	73%		22%		9%		28%		37%	
HNK1-negative	38	49%		45%		11%		41%		41%	
Recipient *NKG2D* haplotype			0.39		0.31		0.93		0.48		0.98
HNK1-positive	61	62%		33%		10%		37%		39%	
HNK1-negative	32	66%		28%		9%		25%		38%	
High-risk disease											
Donor *NKG2D* haplotype			0.91		0.77		0.93		0.08		0.47
HNK1-positive	28	43%		26%		33%		54%		44%	
HNK1-negative	24	46%		29%		29%		30%		35%	
Recipient *NKG2D* haplotype			0.41		0.43		0.10		0.40		0.68
HNK1-positive	33	42%		23%		39%		39%		37%	
HNK1-negative	19	47%		35%		18%		50%		47%	

OS: overall survival; TRM: transplant-related mortality.

The donor and recipient *HNK1* haplotype did not significantly influence the transplant outcomes in patients with high-risk disease.

Main causes of death

The main causes of death according to the *HNK1* haplotype of the donors and recipients are illustrated in Figure 2A for patients with standard-risk disease, and in Figure 2B for those with high-risk disease. In patients with standard-risk disease receiving transplants from *HNK1*-negative donors, the most frequent cause of death was acute GVHD, followed by interstitial pneumonia. Transplants from *HNK1*-positive donors resulted in a statistically significantly reduced incidence of death attributed to acute GVHD (Figure 3A; p=0.006) as well as a trend toward a lower incidence of death attributed to interstitial pneumonia (Figure 3B; p=0.09). Other causes of death did not differ according to the *HNK1* haplotype.

Discussion

The current study showed an association between the *NKG2D-HNK1* haplotype in unrelated donors of HLA-matched myeloablative bone marrow transplants (haplotype frequency, 61%) and a significantly reduced transplant-related mortality and better overall survival for their recipients with standard-risk disease. The polymorphism of the donor *NKG2D* gene did not influence disease relapse or the development of grades II to IV acute GVHD or chronic GVHD in the patients. One possible explanation for the absence of the beneficial effects of the *HNK1* haplotype in patients with high-risk disease may be that the number of cases in the study was insufficient for a meaningful assessment of the effect. Alternatively, disease progression may precede the emergence of the potential advantageous effects of the *HNK1* donor haplotype that could protect the recipient from severe transplant-related complications. There was a larger difference in disease relapse between patients with standard-risk disease and those with high-risk disease: 10% and 31% at 3 years after transplantation, respectively.

NKG2D plays important roles in immunity to microbial infections and is especially prominent in controlling viral and bacterial infections.[16] Therefore, the reduced transplant-related mortality in patients with standard-risk disease receiving grafts from donors with the *HNK1* haplotype in this study might be a consequence of increased resistance to infections in the recipients. However, the hypothesis is too speculative because of the unavailability of data on causes of infections in this cohort. Further studies will be needed to clarify whether the *HNK1* haplotype in donors can effectively protect patients against infections.

Several studies have shown that NK cell activity has an important role in the outcomes of patients undergoing allogeneic transplantation.[33,34] Alloreactive NK cells reduced the risk of relapse of acute myeloid leukemia without increasing the incidence of GVHD, resulting in a marked improvement of event-free survival in a series of haploidentical transplant recipients.[35,36] In HLA-identical sibling transplants, the absence of HLA-C and HLA-B ligand for donor-inhibitory killer immunoglobulin-like receptors (KIR) provided benefits in terms of survival and relapse of patients with acute myeloid leukemia and myelodysplastic syndrome in recipients of T-cell-depleted SCT.[37] On the other hand, the JMDP found that KIR ligand mismatch was unfavorably correlated with relapse of leukemia and survival in patients undergoing T-cell-replete unrelated bone marrow transplants.[38] All patients in the present study received grafts from an HLA-A, -B, and -C allele-matched donor, implying KIR ligand match between each patient and donor. It is an open question whether the *NKG2D* polymorphism could affect the outcomes of patients undergoing transplantation with KIR-mismatched grafts.

In this study, major and minor ABO incompatibilities between the donor and recipient tended to be associated with poorer transplant outcomes, regardless of the risk

Table 3. **Multivariate analysis of the association of *NKG2D* polymorphisms with clinical outcomes after transplantation.**

Variable	Overall survival			Transplant-related mortality			Relapse			Grades II-IV acute GVHD			Chronic GVHD		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Standard-risk disease															
HNK1-positive donor	0.44	0.23-0.85	0.01	0.42	0.21-0.86	0.02	0.71	0.19-2.67	0.61	0.83	0.39-1.75	0.63	0.83	0.39-1.75	0.62
HNK1-positive recipient	1.22	0.60-2.50	0.58	1.32	0.61-2.87	0.48	1.11	0.28-4.48	0.88	1.54	0.66-3.57	0.32	1.06	0.49-2.31	0.88
Donor age, >31 years	-	-	-	-	-	-	-	-	-	2.17	0.95-4.96	0.07	-	-	-
Major ABO incompatibility	-	-	-	-	-	-	-	-	-	3.12	1.49-6.56	0.003	0.50	0.17-1.45	0.20
Minor ABO incompatibility	2.42	1.17-5.03	0.02	-	-	-	-	-	-	-	-	-	0.29	0.07-1.24	0.10
High-risk disease															
HNK1-positive donor	0.68	0.30-1.51	0.34	0.62	0.20-1.91	0.40	1.25	0.41-3.80	0.69	1.87	0.69-5.07	0.22	1.55	0.60-4.01	0.37
HNK1-positive recipient	1.41	0.65-3.07	0.39	0.76	0.25-2.29	0.63	2.35	0.66-8.44	0.19	0.47	0.18-1.22	0.12	0.92	0.35-2.38	0.86
Age, >26 years	1.95	0.93-4.09	0.08	6.30	1.86-21.32	0.003	-	-	-	-	-	-	-	-	-
Donor age, >31 years	-	-	-	-	-	-	0.53	0.17-1.65	0.27	-	-	-	-	-	-
Minor ABO incompatibility	2.94	1.19-7.25	0.02	-	-	-	-	-	-	5.10	2.08-12.52	0.004	-	-	-

category of the disease. These findings are compatible with those of a previous study by the JMDP,[39] although the impact of ABO incompatibilities on SCT outcomes is controversial.

This study also identified age as a significant predictive factor for transplant-related mortality in the patients with standard-risk disease. This is consistent with the results of a previous study[40] showing that age over 35 years increased the risk of transplant-related mortality after allogeneic myeloablative SCT in high-risk patients.

A possible limitation of this study is the fact that no direct evidence is yet available regarding the ability of *NKG2D* polymorphisms to protect against microbial infections. The association observed between the *NKG2D* haplotype and transplant outcome might be due to another genetic polymorphism in linkage disequilibrium responsible for a better transplant outcome. One candidate gene is *NKG2F* (*KLRC4*), which is located in the NK complex region adjacent to the *NKG2D* gene, because an intrinsic SNP (rs2617171) in the gene has been reported to be in complete linkage with the *NKG2D* genotype.[24] Alternatively, polymorphisms may not be directly associated with controlling infection, but rather may be associated with other factors, such as sensitivity to treatment against GVHD or protection against organ toxicities related to transplants, which also influence the transplant outcome. These hypotheses have yet to be verified give the insufficient evidence.

Polymorphisms in genes encoding for nucleotide-binding oligomerization domain 2 (NOD2)/caspase recruitment domain 15 (CARD15),[9] heme oxygenase-1 (HO-1) promoter,[6] the Toll-like receptor 4,[4] CC chemokine ligand (CCL) 5 promoter,[32] transforming growth factor (TGF) β1,[11] interleukin (IL) 12, tumor necrosis factor (TNF) α,[15] IL-23,[5] mannose-binding lectin (MBL),[10] Fcγ receptor IIa (FcγRIIa), myeloperoxidase (MPO), FcγRIIIb, IL-1Ra, IL-10,[12] Fc receptor-like 3 (FCRL3), peptidylarginine deimi-

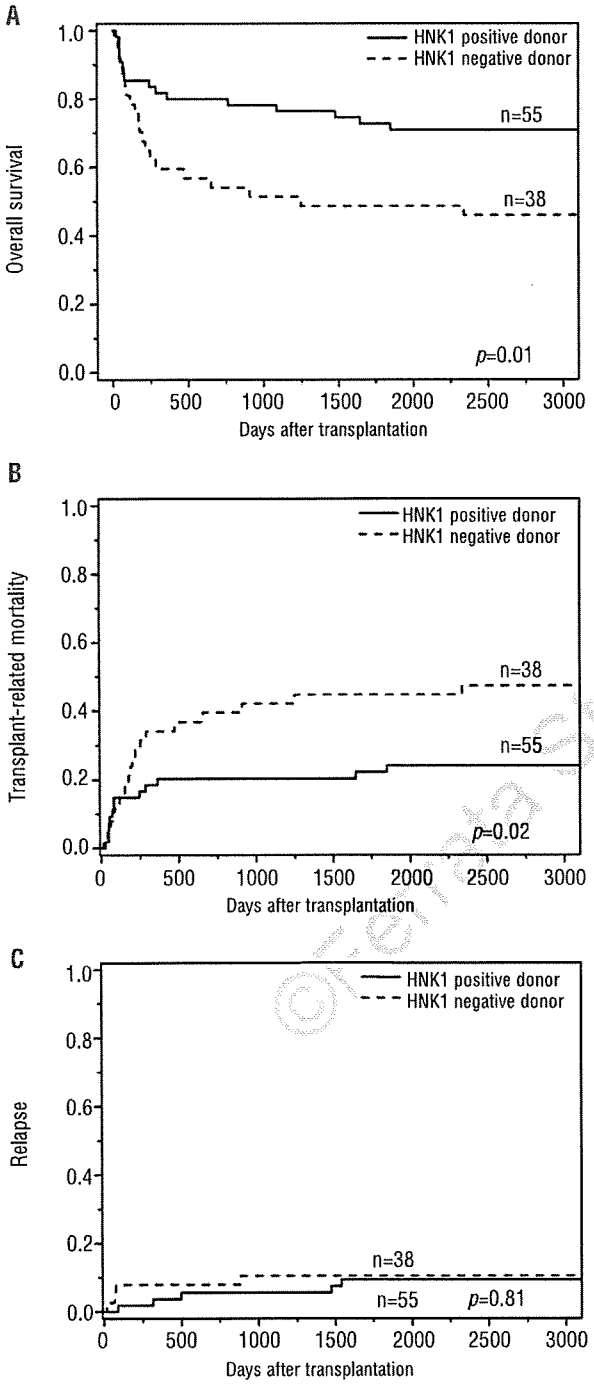

Figure 1. **Kaplan-Meier analysis of (A) overall survival, (B) cumulative incidence of transplant-related mortality and (C) disease relapse after transplantation according to the donor *NKG2D* polymorphism in patients with standard-risk disease. Patients with donors with the *HNK1* haplotype had better overall survival and lower transplant-related mortality. Donor haplotype had no significant impact on disease relapse.**

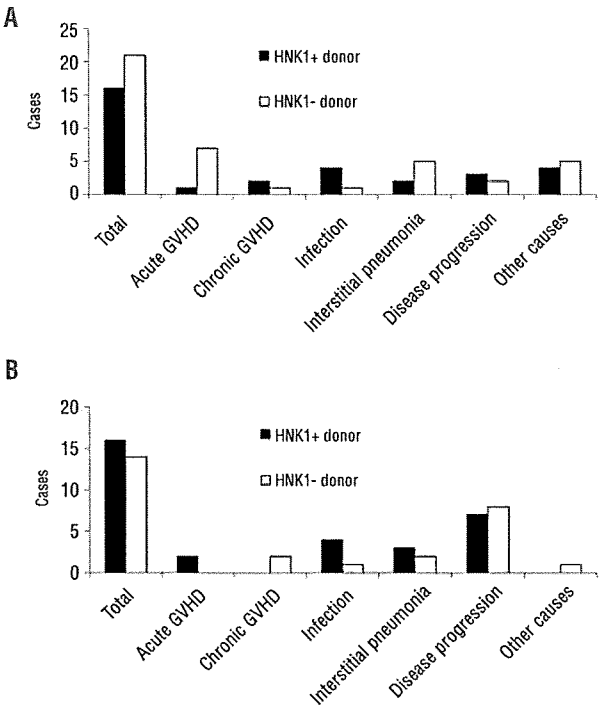

Figure 2. **Main causes of death after transplantation according to the *NKG2D* polymorphism in patients with (A) standard-risk disease (B) high-risk disease.**

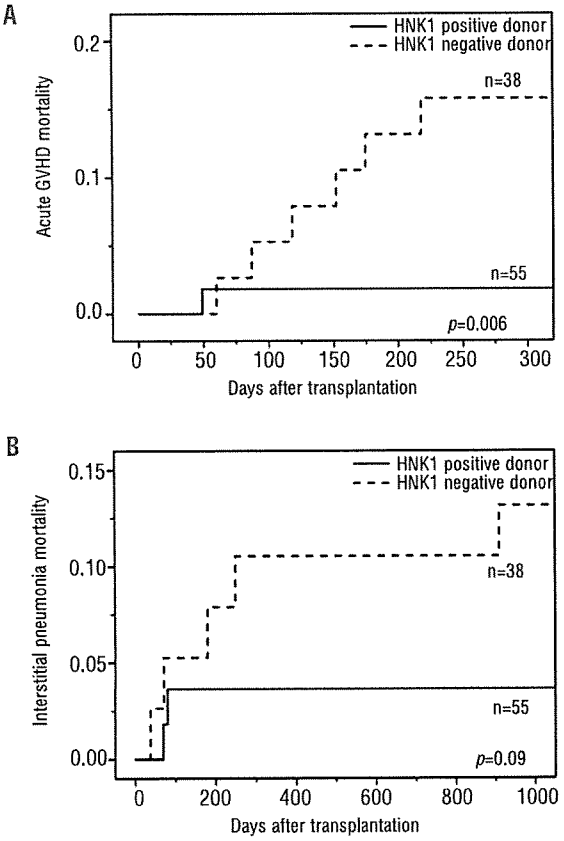

Figure 3. **Cumulative incidence of deaths due to (A) acute GVHD and (B) interstitial pneumonia after transplantation in patients with standard-risk disease. The *HNK1* haplotype in donors was associated with a significantly lower incidence of deaths due to acute GVHD (p=0.006) as well as a trend toward a lower incidence of deaths due to interstitial pneumonia (p=0.09).**

nase citullinating enzymes 4 (PADI4)[13] and methylenetetrahydrofolate reductase (MTHFR)[14] have been shown to influence the outcome after allogeneic SCT. Most of them are associated with the development of GVHD. Only the *NOD2/CARD*15 and *HO-1* promoter polymorphisms have a significant impact on overall survival after SCT. Furthermore, the impact of the *HO-1* promoter polymorphisms depends on donor cells but not on recipient cells, as observed with the *NKG2D* polymorphism which, in the donor, was shown to be significantly associated with overall survival in the present study. This may prompt the determination of the donor *NKG2D* polymorphism prior to SCT in order to choose the best donor, expected to minimize transplant-related mortality after SCT, when multiple donors for a patient are available. Otherwise, prior information on the donor *NKG2D* polymorphism may be helpful in selecting risk-specific appropriate precautions following transplantation.

In conclusion, the present data suggest that the *NKG2D* polymorphism, in addition to HLA disparity between recipients and donors, affects prognosis after a bone marrow transplant from an unrelated donor. However, care should be made in drawing conclusions because the number of patients in the present study was small. The finding of a gene polymorphism may not be equivalent to differences in gene expression, which may be influenced by multiple factors because the NKG2D receptor is found on many tissues and cells.[11] Experimental evidence is required to substantiate the effect of the *NKG2D* polymorphism on immune function. We next plan to conduct a prospective study to confirm these results and to extend this investigation to other transplantation settings, such as related donor SCT, reduced-intensity SCT, HLA-mismatched SCT and SCT for patients with non-hematologic malignancies.

Authorship and Disclosures

JLE and AT designed and performed the research, and contributed to the same aspects of the work; AT, JLE and SN wrote the paper; AT, YKa, and SOh performed the statistical analyses; MO, HS, HA, KM, SOk, MI, TF, YM, and YKo contributed to data collection.

The authors reported no potential conflicts of interest.

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CASE REPORT

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

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

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

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1 Introduction

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

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

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

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

2 Patients

2.1 Patient 1

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

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

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

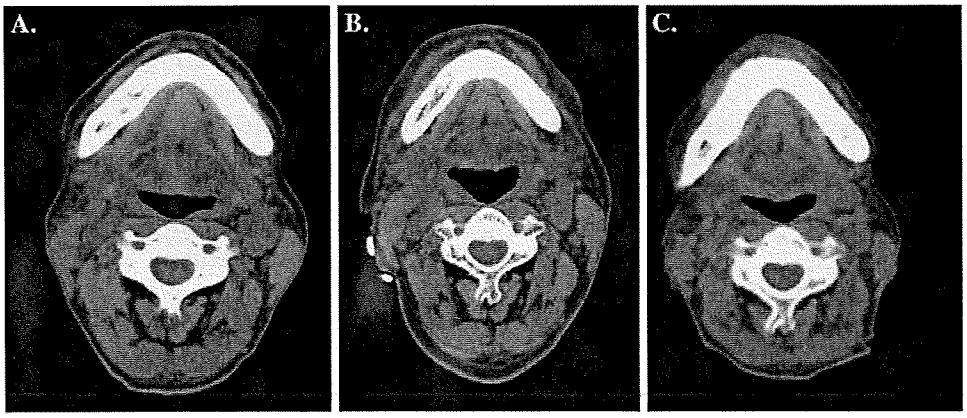

Fig. 1 Changes in the cervical lymphoma lesions after CBT in patient 1. CT scan on 23 months after the second CBT showed a marked reduction in size of the cervical lymph nodes in comparison to those before the first and the second CBT

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

2.2 Patient 2

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

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

3 Discussion

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

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

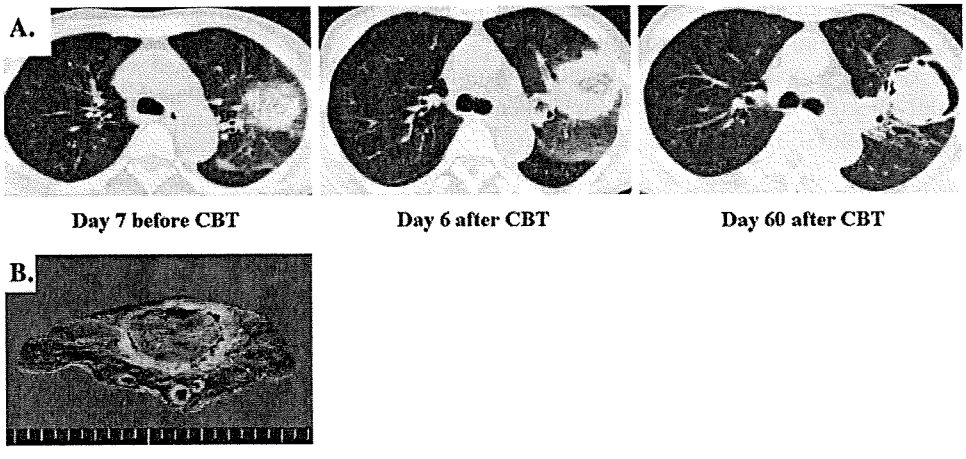

Fig. 2 Pulmonary aspergillosis lesion of patient 2. **a** Changes in the CT findings before and after CBT. **b** *Left upper* lung resected on day113 after CBT

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

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

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Hydroxyurea upregulates NKG2D ligand expression in myeloid leukemia cells synergistically with valproic acid and potentially enhances susceptibility of leukemic cells to natural killer cell-mediated cytolysis

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Valproic acid (VPA), a histone deacetylase inhibitor, upregulates NKG2D ligands (NKG2DLs) on some monocytic and lymphoid leukemic cells. However, its effect on myeloid leukemia cells and synergistic agents that can augment the effect of VPA remains unknown. Of the various myeloid cell lines examined, OUN-1, a chronic myelogenous leukemia cell line, showed the most prominent upregulation of MICA/B and ULBP2 in response to VPA. The NKG2DL upregulation was observed only in leukemic cells without apoptosis and the effect was abrogated by pretreatment of cells with caffeine, an inhibitor of ATM/ATR. Several activators of ATM/ATR were screened for their effect on NKG2DL expression, but only hydroxyurea (HU) efficiently upregulated both MICA/B and ULBP2 expression on the cell line. VPA and HU synergistically upregulated the NKG2DLs on OUN-1 cells as well as primary leukemic cells from some patients with acute myeloid leukemia. The upregulation of NKG2DLs by VPA and/or HU was associated with increased transcription of each NKG2DL gene. OUN-1 cells treated with VPA + HU were more susceptible to killing by natural killer (NK) cells than untreated cells and the enhanced cytotoxicity of NK cells was blocked by the treatment of NK cells with anti-NKG2D monoclonal antibodies. The same concentrations of VPA and HU did not affect the cytotoxicity of NK cells against OUN-1 cells. These data suggest that VPA and HU might enhance the NK cell-mediated antileukemia effect by increasing the susceptibility of myeloid leukemic cells to NK cells. (*Cancer Sci* 2010; 101: 609–615)

Natural killer (NK) cells play an essential role in the eradication of myeloid leukemia cells after allogeneic stem cell transplantation.[1–3] Several lines of evidence indicate that the expression level of NKG2D ligand (NKG2DL) on leukemia cells affects the sensitivity of the leukemic cells to killing by NK cells.[4–11] Various agents have been evaluated for their inducibility of NKG2DLs on leukemic cells, to augment the NK cell-mediated antileukemia effect.[4,8,9,12–14] Valproic acid (VPA), a histone deacetylase inhibitor, is a potent inducer of NKG2DLs such as MICA/B and ULBPs on malignant cells.[9,12,14] VPA augments the expression of MICA and ULBP2 on several monocytic and lymphoid leukemia cell lines and primary acute myeloid leukemia (AML) cells *in vitro* and *in vivo*.[9,12] However, the mechanisms for the upregulation of NKG2DL on AML cells by VPA has not been studied extensively due to the lack of myeloid leukemia cell lines that display an upregulation of NKG2DLs in response to VPA. Clarifying the mechanisms associated with upregulation could identify other reagents that synergize with VPA to augment the expression of NKG2DL by myeloid leukemia cells and thereby enhance the susceptibility of leukemic cells to NK cells.

The expression of NKG2DL is mediated by the activation of ATR and ATM.[10,15,16] Several reagents, including hydroxyurea (HU) and cisplastin, as well as physical stress, such as ionizing irradiation, activate ATM/ATR.[17,18] Some of the ATR activators might not only induce apoptosis of myeloid leukemia cells[19] but might also synergize with VPA in the induction of NKG2DL expression in leukemic cells, and thereby enhance the sensitivity of the leukemic cells to NK cell-mediated cytotoxicity if the agents do not impair the NK cell function.

This study screened various myeloid leukemia cell lines for the upregulation of NKG2DL expression induced by VPA and investigated mechanisms for the upregulation. This report describes the synergistic effect of HU with VPA in the induction of NKG2DL on myeloid leukemia cells that were not susceptible to apoptosis.

Materials and Methods

Leukemia cells. Myeloid cell lines derived from chronic myeloid leukemia, including KH88, SAS413, and OUN-1, and an AML cell line, NB4, were kindly provided by Dr. Masaki Yasukawa of Ehime University (Matsuyama, Japan). K562 was purchased from the Health Science Research Resources Bank (Osaka, Japan). A Burkitt's lymphoma cell line Daudi, T-cell leukemia cell lines Molt-4 and Jurkat, an AML cell line HL60, a chronic myeloid leukemia cell line KU812, and monocytic leukemia cell lines U937 and THP-1 were purchased from RIKEN BRC (Ibaraki, Japan). These cell lines were maintained in RPMI-1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. Primary leukemic cells were isolated using the density gradient method from either the peripheral blood or bone marrow aspirates containing >90% leukemic cells of five patients with AML aged between 25 and 52 years. The leukemic cells were cryopreserved until use. All patients provided their informed consent for the use of their samples and this study protocol was approved by the institutional ethical committee.

Monoclonal antibodies and reagents. Valproic acid, HU, cisplatin, CPT-11, and doxorubicin were purchased from Sigma (Kyowa Hakko Kogyo, Tokyo, Japan). Caffeine, cyclosporine, FK506, SB202190, BAPTA-AM, PD98059, and JNK-1were purchased from Sigma. mAbs specific to MICA/B, ULBP1, ULBP2, ULBP3, and anti-NKG2D were purchased from R&D Systems (Minneapolis, MN, USA). FITC-conjugated goat antimouse IgG, annexin V–phycoerythrin (PE), anti-CD3-PE,

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anti-CD56-PE-Cy5, and anti-CD107a-FITC were all purchased from BD PharMingen (San Diego, CA, USA).

Flow cytometry. The cells were stained with the appropriate Abs or the respective isotype control Abs, followed by incubation with FITC-conjugated goat antimouse IgG. An annexin V–PE apoptosis detection kit I from BD PharMingen was used according to the instructions of the manufacturer. Data acquisition and a flow cytometric analysis were carried out on a BD FACSCalibur using the CellQuest software package (BD Biosciences, Franklin Lakes, NJ, USA).

RNA extraction and real-time PCR. RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The reverse transcription of 1 μg RNA into cDNA was carried out using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and stored at −20°C until use. The quantification of NKG2DL gene expression was carried out using a LightCycler (Roche Diagnostics, Tokyo, Japan) with specific primers. The LightCycler with a GAPDH primer kit (Search-LC, Heidelberg, Germany) was used for quantification of mRNA for *GAPDH*, a housekeeping gene, in the same samples. The relative amount of NKG2DL mRNA to GAPDH mRNA (NKG2DL/GAPDH) was used to represent expression levels of the *NKG2D-L* gene.[20] PCR was carried out using standard conditions. The PCR used the sense primer 5′-GCCATGAACGTCAGGAATTT-3′ and antisense primer 5′-GACGCCAGCTCAGTGTGATA-3′ for MICA, and the sense primer 5′-TTACTTCTCAATGGGAGACTGT-3′ and antisense primer 5′-TGTGCCTGAGGACATGGCGA-3′ for ULBP2. The housekeeping gene *GAPDH* was used as a loading control sense primer 5′-CTATTCGATGCCGTGTATGC-3′, and antisense primer 5′-GCCTGGTCCAGACTTCTTTC-3′.

Natural killer cell preparation. Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy individual and two patients with AML using density gradient centrifugation. Then 10^6 PBMCs were cultured with 2×10^5 irradiated (45 Gy) K562 cells transfected with the membrane-bound form of interleukin-15 and human 4-1BBL (K562-mb15-41BBL) in RPMI-1640 containing 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 100 IU/mL interleukin-2 for 14 d.[21] The cultured PBMCs contained >90% $CD3^-CD56^+CD16^+$ NK cells. In some experiments, the cultured NK cells were incubated in the presence of VPA or VPA + HU, washed once with PBS, then used as effector cells.

Drug treatment of leukemic cells. Leukemic cell lines were cultured for 24 h in 24-well tissue culture plates at 37°C and 5% CO_2 in the absence or presence of different drug concentrations. The following therapeutic drugs were tested: 200 μg/mL VPA; 20 μg/mL HU; 10 μM cisplatin; 100 μM CPT-11; and 0.05 μM doxorubicin. Primary AML cells were cultured for 48 h in the complete medium containing growth factors including Flt3 ligand at 100 ng/mL, stem cell factor at 100 ng/mL, and granulocyte macrophage colony stimulating factor at 20 ng/mL (all from Amgen, Thousand Oaks, CA, USA)[22] with or without VPA and/or HU. In some experiments, leukemic cells were incubated for 1 h at 37°C in the presence of 5 mM caffeine, 1 μM cyclosporine, 1 μM FK506, 10 μM SB202190, 5 μM BAPTA-AM, 10 μM PD98059, or 5 μM JNK-1 prior to culture with VPA.

Cytotoxicity assay. NK cell cytotoxicity against leukemic cell lines was assessed using the standard chromium release assay, as described previously.[23] In blocking experiments, anti-NKG2D Abs were added to the NK cell suspension at 10 μg/mL and incubated at 37°C for 30 min before the addition of target cells. The percentage of specific lysis was calculated using the formula: 100 × (count per minute [cpm] released from test sample − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release).

CD107a mobilization assay. Two microliters of FITC-CD107a mAb was added to the suspension of effector and target cell mixtures in 96-well round microplates and incubated for 3 h at 37°C. One microliter of 2 mM monencin (Sigma) in 100% ethanol was included in the cell suspension to prevent the acidification of the endosomal compartment, which could alter the fluorescence of internalized CD107a:FITC-CD107a mAb complexes. After the incubation, the plate was centrifuged to the pellet cells and the supernatant was removed. Cell–cell conjugate was disrupted by washing the cell with PBS supplemented with 0.02% azide and 0.5 mM EDTA. Samples were then mixed vigorously then stained with mAbs specific for CD3 and CD56, followed by flow cytometric analysis.[24]

Statistical method. Differences in the expression levels of NKG2DL in leukemia cell lines were assessed using Student's *t*-test.

Results

Upregulation of NKG2DL on leukemic cell lines induced by VPA. Several myeloid and lymphoid leukemic cells were examined for the expression of MICA/B and ULBP1–3 before and after incubation in the presence of VPA in order to identify a cell line suitable for the screening of the agents that can augment VPA-induced NKG2DL expression. Three myeloid cell lines, as well as Molt-4 and Jurkat, showed apparent upregulation of both MICA/B and ULBP2 after incubation with VPA (Fig. 1a,b). Time-course experiments revealed 24 h of incubation in the presence of VPA to be optimal for the maximum induction of MICA/B and ULBP2 in OUN-1 cells. The upregulation shown in the histogram was most evident in OUN-1 cells (Fig. 1a), thus, this cell line was chosen for the further analyses.

Agents that upregulate NKG2DLs on leukemic cells induce apoptosis[19,25–27] and examination of the total cell population after VPA treatment might underestimate the inducibility of NKG2DL by the agents due to the presence of apoptotic cells. Indeed, when apoptotic and non-apoptotic cells were examined separately after VPA treatment, the apparent upregulation of MICA/B and ULBP2 was only observed in annexin V-negative non-apoptotic cells (Fig. 1c). The mean fluorescence intensity levels of MICA/B on VPA treated or untreated OUN-1 cells were 10.4 ± 2.3 and 4.8 ± 1.5 ($P < 0.05$), respectively; the mean fluorescence intensity levels of ULBP2 on VPA treated or untreated OUN-1 cells were 12.9 ± 2.9 and 6.1 ± 1.7 ($P < 0.05$), respectively. Therefore, only the annexin V-negative cell population was observed in the subsequent analyses.

Although little is known about the mechanisms for the upregulation of NKG2DL by VPA, the expression of NKG2DL is controlled by ATM/ATR kinase and various signaling pathways affect the expression of NKG2DL by T cells.[16,28] Various inhibitors were tested for their ability to block NKG2DL expression to determine which pathway mediates the upregulation of NKG2DL in OUN-1 cells (Fig. 1d). Only caffeine, an ATM/ATR inhibitor, abrogated the upregulation of NKG2DL induced by VPA, suggesting ATM/ATR kinases are involved in NKG2DL induction.

Effect of ATM/ATR activators on OUN-1. Several reagents capable of stimulating ATM/ATR were examined for the inducibility of MICA/B and ULBP2 on OUN-1 cells. HU induced expression of both MICA and ULBP2 on OUN-1 cells, whereas cisplatin and CPT-11 failed to show such stimulatory effects (Fig. 2a). Doxorubicin, an agent capable of upregulating NKG2DLs on multiple myeloma cells,[10] upregulated MICA/B alone. In addition, the upregulation of the NKG2DL by HU was only evident in non-apoptotic cells (Fig. 2b).

Synergism of HU and VPA in the upregulation of NKG2DL expression. The treatment of OUN-1 cells with HU and VPA upregulated NKG2DL expression to a greater degree than HU or VPA alone (Fig. 3a,b). This synergistic effect was also observed in leukemic cells from two of three patients with AML from

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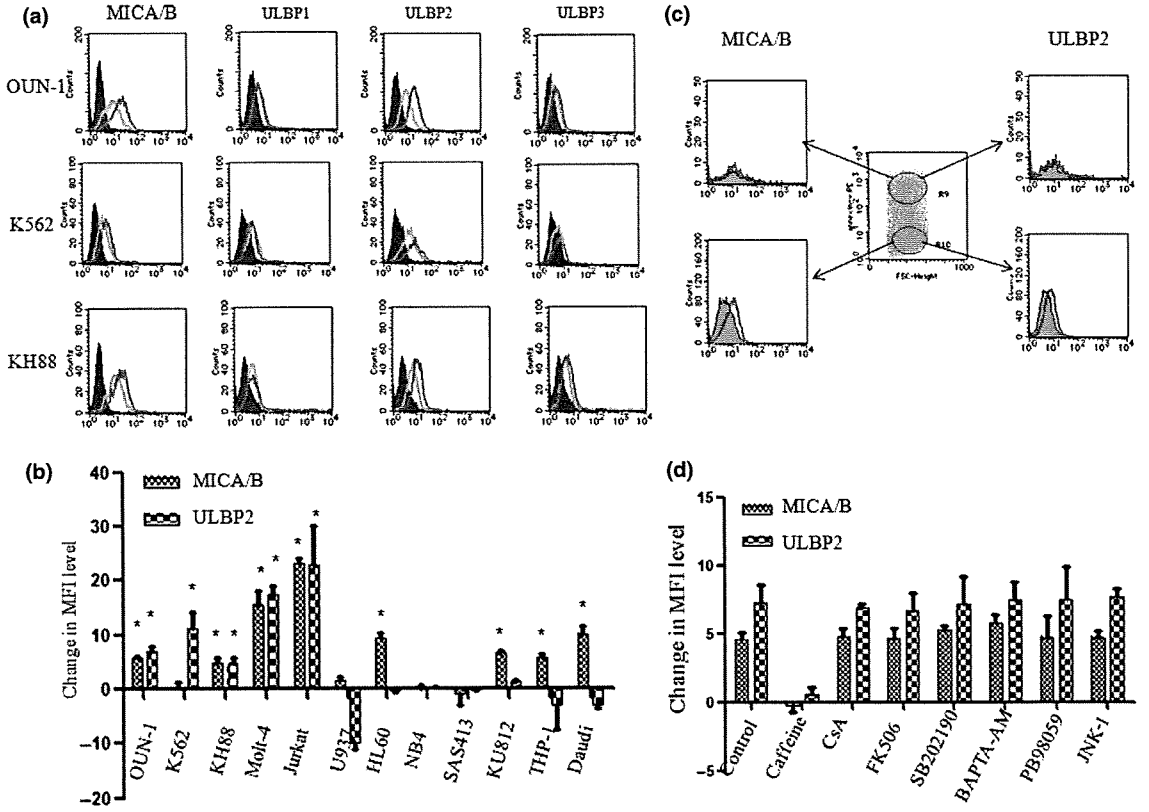

Fig. 1. Upregulation of NKG2D ligand (NKG2DL) expression on leukemic cell lines by treatment with valproic acid (VPA). (a) Histograms of NKG2DL expression on three myeloid leukemia cell lines are shown. Green lines, untreated cells; purple lines, isotype control; red lines, VPA treated cells. (b) Changes in the mean fluorescence intensity (MFI) level of MICA/B and ULBP2 in various leukemic cell lines. Each column represents the difference in the MFI level calculated by subtracting the MFI level of untreated cells from that of VPA treated cells. The differences in the MFI levels are indicated as the mean + SD from three independent experiments. $*P < 0.05$. (c) Selective upregulation of NKG2DLs by VPA treatment on non-apoptotic OUN-1 cells. Apoptotic and non-apoptotic cells defined by the expression of annexin V were separately assessed for the expression of NKG2DLs. Green lines, untreated cells; red lines, VPA treated cells. (d) Effect of various reagents on VPA-induced MICA/B and ULBP2 expression by OUN-1 cells. OUN-1 cells were pretreated with the indicated reagent for 1 h and were incubated in the presence of VPA. Each column represents the difference in the MFI level calculated by subtracting the MFI level of untreated cells from that of VPA treated cells. BAPTA-AM, calcium chelator; CsA, cyclosporine; FK506, tacrolimus; JNK-1, an inhibitor of JNK; PD98059, an inhibitor of ERK1/2; SB202190, an inhibitor of p38MAPK.

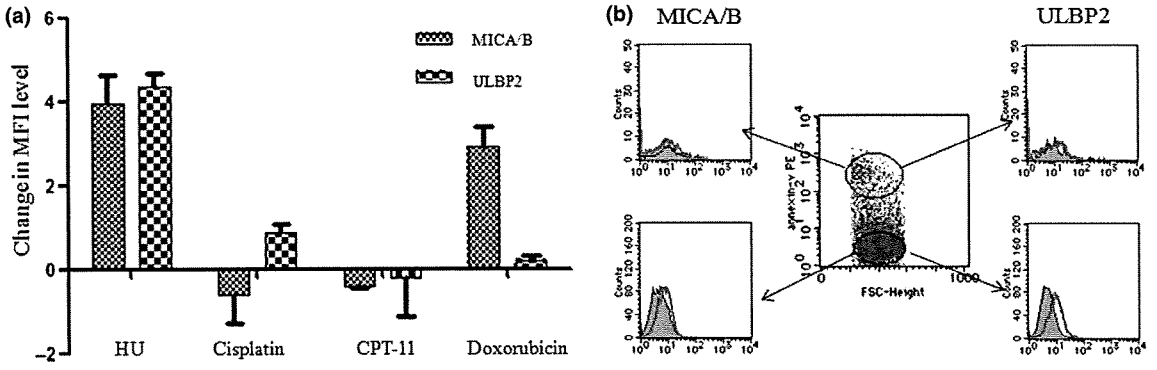

Fig. 2. Effect of ATM/ATR activators on the expression of NKG2D ligand (NKG2DL) by chronic myelogenous leukemia cell line OUN-1. (a) OUN-1 cells were incubated in the presence of 20 μg/mL hydroxyurea (HU), 10 μM cisplatin, 100 μM CPT-11, or 0.05 μM doxorubicin for 24 h. Each column represents the difference in the mean fluorescence intensity (MFI) level calculated by subtracting the MFI level of untreated cells from that of the cells treated with the indicated reagent. (b) Selective upregulation of NKG2DLs by HU treatment on non-apoptotic OUN-1 cells. Apoptotic and non-apoptotic cells defined by the expression of annexin V were separately assessed for the expression of NKG2DLs. Green lines, untreated cells; red lines, HU treated cells.

whom a sufficient number of leukemic cells could be obtained. The synergistic effect on OUN-1 cells was abolished by pre-treatment of the cells with caffeine (Fig. 3c). When the mRNA levels of the NKG2DLs were compared between untreated and treated cells using real-time PCR, both VPA and HU treated cells showed 2.5-fold higher MICA/B RNA and ULBP2 mRNA levels than untreated cells. The combined use of VPA and HU further increased the mRNA levels to 3.5-fold of the control

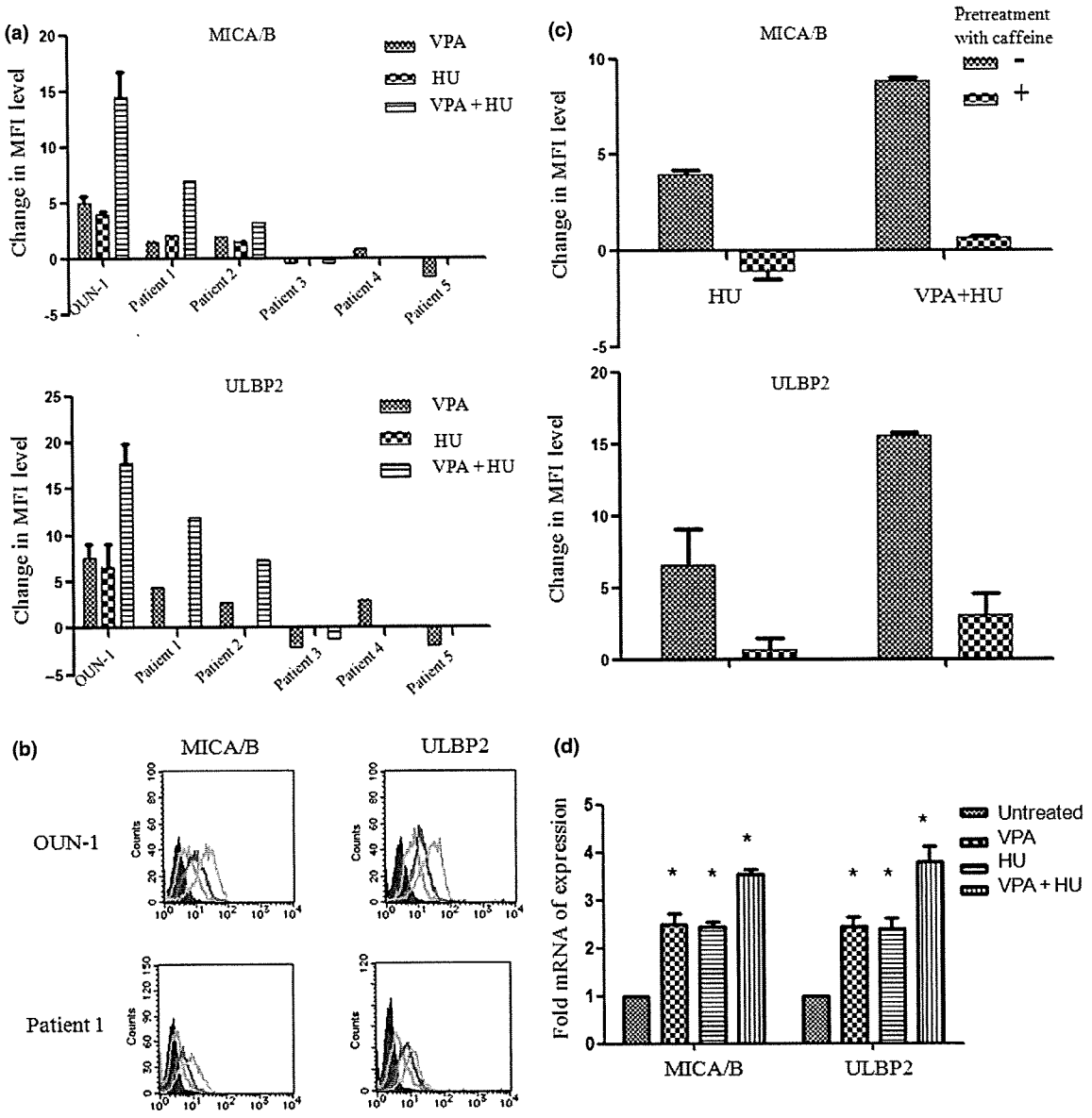

Fig. 3. Synergistic effect of valproic acid (VPA) and hydroxyurea (HU) on NKG2D ligand (NKG2DL) expression by OUN-1 chronic myelogenous leukemia cells. (a) OUN-1 cells and primary leukemic cells from five patients with acute myeloid leukemia were treated with VPA, HU, or VPA + HU for either 24 h (OUN-1) or 48 h (primary leukemic cells). Each column represents the difference in the mean fluorescence intensity (MFI) level calculated by subtracting the MFI level of untreated cells from that of the cells treated with the indicated condition. Leukemic cells from Patients 3–5 were only examined for the inducibility of MICA/B and ULBP2 by VPA or HU alone. (b) Representative histograms of OUN-1 and Patient 1's leukemic cells treated with VPA and HU. Green lines, untreated cells; green lines, cells treated with VPA + HU; purple lines, isotype control; red lines, VPA treated cells. (c) Effect of caffeine on the expression of NKG2DLs induced by VPA + HU. OUN-1 cells were pretreated with 5 mM caffeine then incubated with either HU or VPA + HU for 24 h. (d) Effect of VPA and HU on the expression of MICA/B and ULBP2 genes. The gene expression levels relative to GAPDH were determined using real-time PCR. Each column and error bar represents the mean ± SD of the ratios of the NKG2DL mRNA level to the GAPDH mRNA level from three independent experiments. $*P < 0.01$.

(Fig. 3d). These findings indicate the transcriptional upregulation of each gene to underlie the augmentation of the NKG2DL expression induced by these reagents.

Effect of leukemic cell pretreatment with VPA and HU on NK cell-mediated cytotoxicity. The cytotoxicity of NK cells isolated from healthy individuals was examined against OUN-1 cells treated with or without NKG2DL inducers. Treatment of OUN-1 cells with VPA or VPA + HU significantly increased the cytotoxicity, although the enhancing effect by VPA + HU was comparable to that by VPA alone (Fig. 4A). The enhancing effect of VPA was blocked by the pretreatment of OUN-1 cells with anti-NKG2D mAbs (Fig. 4B). VPA treatment of leukemic cells obtained from two AML patients also increased the cytotoxicity by autologous NK cells and the enhancing effect of VPA was blocked by the pretreatment of leukemic cells with anti-NKG2D mAbs (Fig. 4C). A ^{51}Cr-release assay and degranulation assay indicated that the treatment of NK cells with VPA or VPA + HU did not affect their cytotoxicity against OUN-1 cells (Fig. 4D,E), thus suggesting that NK cells can exert cytotoxicity against leukemic cells in the presence of these agents.

Discussion

NKG2DL expression on leukemic cells plays a key role in the antileukemia effect by NK cells because NKG2D can mediates cytotoxicity depending on the NKG2DL expression levels on

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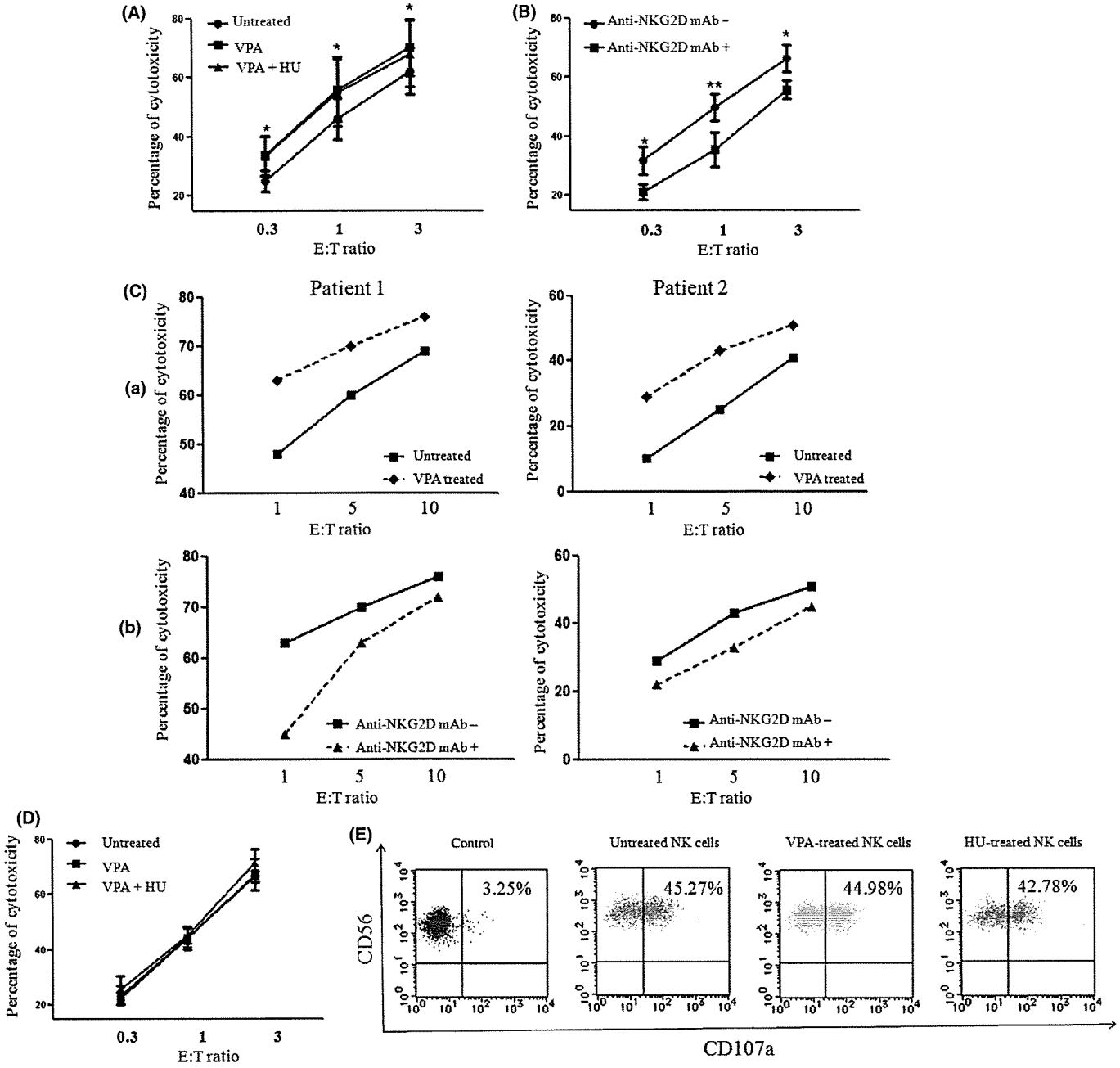

Fig. 4. Enhanced natural killer (NK) cell-mediated lysis of OUN-1 chronic myelogenous leukemia cells following treatment with valproic acid (VPA) and hydroxyurea (HU). (A) OUN-1 cells were incubated with or without 200 μg/mL VPA or in combination with 20 μg/mL HU for 24 h then examined for their sensitivity to killing by cultured NK cells from a healthy individual. *$P < 0.05$. (B) OUN-1 cells treated with VPA + HU were incubated in the presence or absence of anti-NKG2D mAb, then tested for their sensitivity to NK cell-mediated lysis. *$P < 0.05$; **$P < 0.01$. (C) (a) Acute myeloid leukemia cells from two patients were incubated with or without 200 μg/mL VPA for 48 h, and the cytotoxicity of cultured NK cells from the patients against autologous leukemic cells was assessed by chromium release assay. (b) Cultured NK cells were incubated in the presence of anti-NKG2D Abs or medium alone for 30 min then used for chromium release assay against VPA treated leukemic cells. (D) NK cells were incubated in the presence or absence of VPA or VPA + HU for 24 h, and their cytotoxicity against VPA-treated OUN-1 cells was compared. (E) Degranulation of NK cells that were treated with VPA or HU for 24 h in response to OUN-1 cells. The scattergrams represent the CD107a expression by $CD3^{-}CD56^{+}$ NK cells after 3 h of incubation with OUN-1 cells. E:T, Effector: Target.

leukemic cells, even in the presence of inhibitory signals through a KIR-KIR-L interaction.[5] A previous AML patient relapsed after allogeneic stem cell transplantation in association with the loss of ULBP2 expression by AML cells.[21] If the agents non-toxic to NK cells are capable of upregulating NKG2DLs on leukemic cells, then those agents might potentiate NK cell-mediated leukemic cell killing.

Of the various agents that have been tested for their inducibility of NKG2DL expression, this study focused on the effect of VPA because it has an antileukemia effect by itself, and its low toxicity allows the use of other agents in combination.[9,19,29] The screening of various myeloid leukemia cell lines identified OUN-1 as a suitable cell line for the analysis of mechanisms underlying the NKG2DL upregulation by VPA. The successful

blocking of the NKG2DL-inducing effect by caffeine led to screening ATM/ATR activators, and HU was found, for the first time, to be a potent NKG2DL inducer on myeloid leukemia cells. This antileukemic agent, with low toxicity, upregulated NKGD2L expression on OUN-1 cells as well as on primary leukemic cells from some patients with AML, synergistically with VPA.

The effect of VPA on various cancer cells has been extensively studied both *in vitro* and *in vivo*.[9,12,14,16] VPA increases the transcription of MICA/B without affecting ULBPs in hepatoma cells.[14] Diermayr *et al.* showed the upregulation of ULBP1 and MICA/B on primary leukemic cells, thus resulting in their increased sensitivity to NK cell-mediated killing.[5] A recent clinical trial showed VPA increases the expression level of MICA, ULBP2, and ULBP3 by AML cells *in vivo*.[9] However, despite the large body of evidence for the upregulation of NKG2DLs on leukemic cells, the mechanisms for NKG2DL induction remained unknown, possibly due to the lack of suitable cell lines for *in vitro* studies. The identification of OUN-1 as an NKG2DL inducible myeloid leukemia cell line allowed the determination that the ATR/ATM pathway is involved in NKG2DL induction. Genotoxic stress and stalled DNA replication induce NKG2DL expression by activating a DNA damage checkpoint pathway initiated by ATM or ATR protein kinase[15] and caffeine, an inhibitor of ATM/ATR catalytic activity, interferes with the induction of MICA expression on activated T cells.[16] In addition to HU identified in the present study, the identification of other ATM/ATR activators could further help to upregulate NKG2DL expression by myeloid leukemia cells.

The effects of various agents on the expression of NKG2DLS have been assessed by analyzing the total cell populations exposed to the agents with flow cytometry.[4,12,14,22] The present study revealed that this method underestimates the upregulation of NKG2DL expression by test agents because many of the agents that upregulate NKG2DLs induce apoptosis of the target cells and the apoptotic cells might fail to express NKG2DLs. The understanding of this phenomenon should be particularly important when primary leukemic cells are screened for their response to NKG2DL inducers, because sensitive screening methods are required to identify the optimal combination of such inducers in AML cells of individual patients.

Hydroxyurea is often used to control the blood cell count in patients with myeloproliferative disorders and AML as a palliative treatment.[30–32] A recent study showed that VPA and HU can modulate the cell cycle and cooperatively induce apoptosis of cancer cells.[19] Several clinical trials of VPA and all-*trans* retinoic acid for patients with myeloid malignancies used HU to control leukocytosis.[9,29] HU might synergize with VPA in patients responsive to the combination therapy, not only by augmenting apoptosis of leukemic cells but also by increasing the susceptibility of leukemic cells to NK cell-mediated cytotoxicity. It is plausible that giving VPA and HU simultaneously might further augment the NK cell-mediated killing of myeloid leukemia cells by upregulating NKG2DLs. Clinical trials using VPA in combination with HU are therefore warranted for the palliative treatments of elderly patients with either AML or myeloproliferative disorders.

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ORIGINAL ARTICLE

Oral valganciclovir as preemptive therapy is effective for cytomegalovirus infection in allogeneic hematopoietic stem cell transplant recipients

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Abstract Between March 2007 and January 2008, the safety and efficacy of oral valganciclovir (VGC) preemptive therapy for cytomegalovirus (CMV) infection was evaluated in ten consecutive patients who received allogeneic hematopoietic stem cell transplantation (HSCT). Patients were screened once or twice per week after engraftment using CMV pp65 antigenemia assay. When more than 2 CMV antigen-positive cells per 50,000 leukocytes were detected, preemptive therapy with oral VGC was initiated at a dose of 900 mg twice daily for 3 weeks. Nine patients (90%) completed the 3-week VGC treatment except for one patient who developed febrile neutropenia. There was no other significant toxicity. CMV antigen-positive cells were rapidly decreased in all nine patients and became undetectable by the end of the VGC treatment. None of the patients developed CMV disease. CMV infection relapsed in four of the ten patients (40%) after the VGC treatment. These observations suggest that preemptive therapy with VGC is effective for preventing CMV disease in allogeneic HSCT patients. Further studies with a large number of patients will be necessary to determine the optimal initial- and maintenance-dose of VGC.

Keywords Allogeneic hematopoietic stem cell transplantation · Cytomegalovirus infection · Preemptive therapy · Valganciclovir

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1 Introduction

Despite improvement in the treatment of cytomegalovirus (CMV) infection and CMV disease with ganciclovir (GCV) and/or foscarnet, CMV disease is still a major cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT) [1–4]. Major risk factors for CMV disease include CMV seropositivity before transplantation, development of graft-versus-host disease (GVHD), unrelated donor transplantation, and T cell depleted transplantation [3, 5–7]. In addition, new transplantation modalities such as nonmyeloablative conditioning regimens consisting of intensive immunosuppression increase the risk of late-onset CMV infection and CMV disease [2, 8]. Therefore, extended prevention of CMV disease may be required, especially for high-risk recipients, not only those within 100 days after HSCT but also those in the later period after HSCT [8–10]. Currently, the prevention of CMV disease involves general prophylaxis and preemptive therapy. Preemptive therapy is based on the early detection of CMV infection by virus surveillance, by monitoring with either CMV antigenemia assay or PCR techniques and followed by immediate treatment with anti-CMV drugs

[4, 11–13]. Intravenous GCV (IV-GCV) and/or foscarnet are commonly used for preemptive therapy and are effective for decreasing the incidence of early CMV disease [11, 13, 14]. However, these antiviral treatments are given intravenously and often require hospitalization, as well as high costs and IV-related complications.

Valganciclovir hydrochloride (VGC) is an oral valine-ester GCV prodrug with a tenfold higher bioavailability than oral GCV, and it is rapidly hydrolyzed to GCV after oral administration. VGC and IV-GCV have similar efficacy in the treatment of CMV retinitis in HIV-infected patients and in preemptive CMV treatment in solid organ (heart, renal, and renal-pancreas) transplant patients [15–19]. Recently, several studies have shown the efficacy of VGC for preemptive therapy in allogeneic HSCT patients [20–23]. We evaluated the safety and efficacy of oral VGC as preemptive therapy for CMV reactivation in ten allogeneic HSCT patients.

2 Patients and methods

2.1 Patients

This was a prospective multicenter study with VGC. The study patients were adults who had received an allogeneic bone marrow or peripheral blood stem cell transplant. Patients were eligible when they screened for CMV infection using CMV pp65 antigenemia assay and more than two CMV antigen-positive cells were detected. Patients unable to take oral medication, and those who impaired renal function (serum creatinine level >2.0 mg/dL) were ineligible. Patients, who developed CMV disease, had received antiviral agents other than acyclovir and who developed more than stage 2 gastrointestinal GVHD were also ineligible. Ten consecutive patients who received allogeneic HSCT at Kyushu University Hospital and Hamanomachi General Hospital between March 2007 and January 2008 were included in the study (Table 1). This study was approved by Institutional Review Board of each institute and a written informed consent was obtained from each participating patient.

Eight patients had acute myeloid leukemia, one had myelodysplastic syndrome, and one had non-Hodgkin's lymphoma. The median age of the patients at the time of transplantation was 56 years (range 33–63). They received bone marrow grafts from an HLA-matched sibling donor ($n = 1$), a matched unrelated donor ($n = 8$), or an HLA-1 locus mismatched unrelated donor ($n = 1$). All of the patients were CMV seropositive before transplantation. Nine patients received myeloablative preparative regimens including total body irradiation/cyclophosphamide (Cy) in five patients and busulfan (BU)/Cy in four patients.

Table 1 Patient characteristics

Number of patients	10
Median age, years (range)	56 (33–65)
Diagnosis	
Acute myeloid leukemia	8
Myelodysplastic syndrome	1
Non-Hodgkin's lymphoma	1
Stem cell source	
HLA-identical sibling bone marrow	1
HLA-matched unrelated bone marrow	8
HLA-mismatched unrelated bone marrow	1
CMV serologic status	
Donor + /Recipient +	9
Donor −/Recipient +	1
Preparative regimens	
TBI/Cy	5
Bu/Cy	4
Flu/Bu/TBI	1
GVHD prophylaxis	
Taclorimus + MTX	9
CSP + MTX	1
Acute GVHD prior to CMV reactivation	
Grade I	1
Grade II	7
Grade III	2
PSL treatment at the time of starting VCG	8

Bu busulfan, *CMV* cytomegalovirus, *CSP* cyclosporine, *Cy* cyclophosphamide, *Flu* fludarabine, *GVHD* graft-versus-host disease, *TBI* total body irradiation, *MTX* methotrexate, *PSL* prednisolone, *VGC* valganciclovir

The remaining patient received a fludarabine-based reduced-intensity conditioning regimen. GVHD prophylaxis consisted of taclorimus/short-term methotrexate (MTX) ($n = 9$) or cyclosporine/short-term MTX ($n = 1$). Patients who developed grade II–IV acute GVHD were given methylprednisolone (mPSL) or prednisolone (PSL) at a dose of 1 or 2 mg/kg. Acyclovir was administered orally (1,000 mg/day) or intravenously (500 mg/day) from days −7 to 35 as a prophylaxis against herpes simplex infection.

2.2 CMV antigenemia assay

CMV antigenemia assay was determined as previously described [7, 24]. In brief, peripheral blood leukocytes isolated from 3 mL of EDTA-treated blood were applied to slides by centrifugation and fixed with cold acetone. The slides were stained using a direct immunoperoxidase technique that employed the peroxidase-conjugated monoclonal antibody HRP-C7 (Teijin, Tokyo, Japan) against the CMV pp65 antigen. CMV antigen-positive cells were counted under a light microscope and the results were

expressed as the number of CMV antigen-positive cells per 50,000 leukocytes.

2.3 Definition of CMV infection and CMV disease

A positive test for CMV antigenemia was defined as the presence of one or more CMV antigen-positive cells per 50,000 leukocytes. CMV infection was considered in patients with a positive test for CMV antigenemia. CMV disease was diagnosed according to published recommendations [25]. Patients with clinical manifestations of CMV disease, such as interstitial pneumonia and gastroenteritis in the presence of CMV infection, were examined histopathologically and immunochemically from biopsy specimens.

2.4 Preemptive therapy with VGC for CMV infection

Monitoring with CMV antigenemia assay was performed at least once per week after engraftment until day 100 after HSCT and once every other week thereafter. Preemptive therapy with VGC for CMV infection was initiated at the time of the first detection of more than two CMV antigen-positive cells per 50,000 leukocytes. VGC was administered orally at a dose of 900 mg twice daily for 3 weeks. The dose was adjusted for patients with impaired renal function according to the manufacturer's recommendation. Acyclovir for the prophylaxis against herpes simplex infection was discontinued when VGC treatment was started. Supplemental immunoglobulin was administered only when a total IgG level was less than 400 mg/dL.

2.5 Endpoints and definitions

The primary endpoint was the rate of complete response of the VGC preemptive therapy to the CMV infection. The efficacy of VGC was monitored weekly using a CMV antigenemia assay. A complete response was defined as the conversion from positive to negative CMV antigenemia test results at the completion of the treatment. Patients who persistently showed positive test results for CMV antigenemia after 3 weeks of preemptive therapy or developed CMV disease during the period of preemptive therapy were considered a treatment failure.

The secondary endpoints included the safety of preemptive therapy, the incidence of CMV disease during VGC treatment, and the incidence of a recurrent CMV reactivation after the completion of VGC treatment. The patients were monitored with the CMV antigenemia assay for 5 weeks after the completion of the VGC treatment. At least once per week, a safety analysis was conducted. The analysis included the monitoring of blood counts, liver and renal function tests, and documenting other unexpected side effects. The incidence of CMV disease was evaluated for the entire period of the study. The incidence of recurrent reactivation of CMV infection after the VGC preemptive therapy was based on the conversion from negative CMV antigenemia to positive CMV antigenemia test results with more than two CMV antigen-positive cells per 50,000 leukocytes during the 5-week follow-up period.

3 Results

3.1 CMV infection and VGC preemptive therapy

Forty-seven patients received allogeneic bone marrow/peripheral blood stem cell transplants at these two institutes during the study period. Thirty-one patients showed positive CMV antigenemia test results after transplantation. Ten patients were enrolled into this study, but the remaining 21 patients were not enrolled mostly by their inability to take oral medication. Ten enrolled patients were given preemptive therapy with VGC for CMV infection (Table 1). All patients were CMV seropositive before transplantation, and nine donors were also CMV seropositive. In these patients, more than 2 CMV antigen-positive cells per 50,000 leukocytes were detected after a median of 69 days (range 22–252) following transplantation. The median number of CMV antigen-positive cells at the initiation of VGC therapy was 5 per 50,000 leukocytes (range 3–59). All of the patients developed acute GVHD prior to CMV infection after a median of 23 days (range 11–135). The severity of acute GVHD was grade I in one patient, grade II in seven, and grade III in two. Eight patients received mPSL or PSL for the treatment of acute GVHD. Preemptive therapy with VGC was started within five days after the detection of CMV antigen-positive cells. Nine patients completed 21 days of VGC treatment, whereas one patient failed to complete the therapy because of the development of grade 4 neutropenia and subsequent febrile neutropenia. Patients were followed at least 5 weeks after the completion of VGC preemptive therapy. The median follow-up was day 122 (range 41–355).

3.2 Response to VGC preemptive therapy

All patients showed negative test results for CMV antigenemia within 3 weeks after the initiation of the VGC treatment. In nine patients, CMV antigen-positive cells became negative within 2 weeks (Fig. 1). The remaining patient, who had 60/50,000 CMV antigen-positive cells at the time of initiation of VGC treatment, took 3 weeks to clear CMV antigen-positive cells. None of the patients required other anti-CMV agents. None of the patients developed CMV disease during the preemptive therapy or