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Appendix

The following transplant centers in Japan participated in this study: Kitakyushu Municipal Hospital, Hamanomachi Hospital, Toyama Prefectural Central Hospital, Harasanshin General Hospital, National Cancer Center Hospital, Osaka University Hospital, Kyushu University Hospital, Institute of Medical Science at the University of Tokyo, Japanese Red Cross Nagoya First Hospital, Kokura Memorial Hospital, Chiba University Hospital, Kyoto Prefectural University of Medicine Hospital, Meitetsu Hospital, Toranomon Hospital, Kashiwa Hospital at Jikei University, Kyoto First Red Cross Hospital, Osaka City University Hospital, Tokai University Hospital, Kagawa Medical University Hospital, Beppu National Hospital, Kameda General Hospital, University of Tokyo Hospital, Kansai University of Medicine Hospital, Osaka National Hospital, Social Insurance Kyoto Hospital, Matsushita Memorial Hospital, Osaka Red Cross Hospital, Nagoya Daini Red Cross Hospital, Kanazawa University Hospital, Kurobe City Hospital, Kumamoto National Hospital and Ryukyu University Hospital.

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Cidofovir for treating adenoviral hemorrhagic cystitis in hematopoietic stem cell transplant recipients

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Summary:

Adenovirus (AdV) infection is an important cause of morbidity and mortality in hematopoietic stem cell transplant (HSCT) recipients. We treated 16 patients with AdV hemorrhagic cystitis (HC) following HSCT with cidofovir (CDV; 1 mg/kg/day, three times weekly for 3 weeks). Patients included 10 males and six females with a median age of 50 years (range 10–62). Two of the 16 patients were unevaluable because of early death from nonadenoviral causes. CDV therapy cleared AdV from urine in 12 of 14 patients (86%). Of 14 patients, 10 (71%) showed clinical improvements in HC. Among 14 patients, seven (50%) had avoided renal damage, the most important CDV toxicity. One patient previously treated with foscarnet for cytomegalovirus (CMV) required hemodialysis, and CDV treatment was discontinued. In another patient, CDV treatment was discontinued because of grade 2 nephrotoxicity. Four patients became positive for CMV antigenemia while being treated with CDV, and two developed herpes simplex virus (HSV) stomatitis while being treated with CDV. CDV proved effective in treating AdV HC in transplant patients. However, CDV at 1 mg/kg/day given three times weekly failed to prevent breakthrough infection with CMV and HSV in some patients.
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Adenovirus (AdV) infections including hemorrhagic cystitis (HC)[1–3] are emerging as life-threatening complications in hematopoietic stem cell transplant patients.[4] Reported occurrence rates of AdV infection complicating allogeneic hematopoietic stem cell transplantation (HSCT) vary from 5 to 21%,[1,5–8] and reported mortality rates have ranged from 7.7 to 38%.[6,9–11]

For treatment of AdV infection, reduction of immunosuppression[8] or infusion of donor lymphocytes[12] have been proposed. However, since AdV infections often occur in the presence of severe graft-versus-host disease (GVHD), immunotherapy may not be feasible. While specific anti-AdV therapy is therefore needed, no presently available drug has been proven to be effective, although some treatment success with ganciclovir (GCV),[13] vidarabine (AraA)[14] and ribavirin[15,16] have been reported. Unfortunately, these results could not be reproduced.[17] Cidofovir (CDV), a monophosphate nucleotide analogue of cytosine that inhibits viral DNA polymerase, demonstrates *in vitro* and *in vivo* activity against several viruses including herpesviruses, AdV, papilloma viruses, polyoma viruses, and poxvirus.[18] Several reports have described the effectiveness of CDV in post-transplant AdV disease.[19,20] The dose-limiting toxicity of intravenous CDV, when given at the recommended dose of 5 mg/kg once weekly, is nephrotoxicity.[18] Recently, a smaller, more frequent dose of CDV, 1 mg/kg/day three times weekly, demonstrated efficacy for treatment of post-transplant AdV infection.[20] In the present study, we have prospectively evaluated both toxicity and efficacy of CDV treatment for AdV HC in transplant patients. In all, 16 transplant patients were treated with CDV at a dose of 1 mg/kg, three times weekly for 3 weeks.

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Materials and methods

Diagnosis of AdV HC

To exclude regimen-related HC, only patients who developed macroscopic hematuria with clinical signs of cystitis newly appearing *de novo* at least 10 days after HSCT and also had no tendency toward generalized bleeding or bacteriuria were considered to have HC.[21] According to previously reported criteria with minor modifications,[22] the severity of HC was graded as mild, sustained microscopic

hematuria; moderate, gross hematuria and dysuria without clots; severe, gross hematuria and dysuria with clots. At the onset of HC, a urine specimen was obtained for viral culture and polymerase chain reaction (PCR). For rapid diagnosis, immunochromatography was performed (Adenocheck; Santen, Osaka, Japan). All patients underwent all the three diagnostic modalities (viral culture, PCR, and immunochromatography). When AdV was detected by one or more of these methods, a diagnosis of AdV HC was made.

Viral culture from urine

A 2 ml volume of urine was centrifuged overnight at 20 000 *g* and the sediment was added to culture of Hep-2 cells for up to 4 weeks. When a cytopathic effect of viral infection was observed, viral species were identified using monoclonal antibodies against AdV. Viral culture was carried out before the initiation of CDV treatment and 1 week after the last dose of CDV. The clearance of AdV was defined as the negative viral culture after treatment.

PCR of urine samples

After 2 ml of a urine sample was centrifuged at 15 000 *g* for 1 h at 4°C, the sediment was resuspended in 100 μl of PBS. DNA was purified using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Next, 5 μl of purified DNA was subjected to PCR assay using a GeneAmp Kit and a GeneAmp PCR System 9600 (Perkin-Elmer, Boston, MA, USA). Primers used to screen for AdV infection were AD185S (5′-tccagcaacttcatgtccatgg-3′) and AD 185A (5′-tcgatgacgccgcggt-3). The size of the final products was confirmed by 3% agarose gel electrophoresis.[21]

Patient characteristics

In total, 16 patients were treated with CDV (10 males and six females with a median age of 50 years, ranging from 10 to 62). All patients had AdV HC. In all, 14 patients underwent allogeneic HSCT for acute myelogenous leukemia (AML) ($n=1$), acute lymphoblastic leukemia (ALL) ($n=2$), adult T-cell leukemia/lymphoma (ATL) ($n=2$), chronic myelogenous leukemia ($n=1$), myelodysplastic syndrome (MDS) ($n=1$), multiple myeloma (MM) ($n=2$), malignant lymphoma (ML) ($n=1$), and severe aplastic anemia (SAA) ($n=3$). Two other patients with systemic sclerosis underwent CD34+ cell autologous HSCT. Among allogeneic transplants, sources of stem cells were as follows: three from HLA-identical family donors, one from a DR-mismatch family donor, two from haplo-identical family donors, five from unrelated donors, and three from unrelated cord bloods. Patients No. 3 and no. 12 received antithymocyte globulin (ATG) as part of conditioning (Table 1).

Patient No. 2 died of cerebral infarction 5 days after initiation of CDV treatment, while patient No. 13 died from fungal pneumonia 11 days after initiation of CDV treatment. As these two patients with early death unrelated to AdV were excluded from analysis, 14 patients were evaluable. All patients received immunosuppressive therapy including cyclosporine, tacrolimus, and a steroid, as shown in Table 2. Serotypes of AdV isolated from urine were type 11 (11 patients), type 35 (one patient), or not determined (two patients). Onset of AdV HC ranged from 17 to 142 days after post transplantation (median, 37). Intervals between the onset of AdV HC and CDV treatment ranged from 0 to 56 days (median, 3). Two patients received AraA for treatment of AdV HC (Table 2).

CDV treatment

All of the patients gave their written informed consent in accordance with the requirements of the Institutional Review Board. The treatment regimen consisted of CDV, 1 mg/kg per day three times weekly for 3 weeks. Oral Probenecid (2 g) was given 3 h before CDV administration,

Table 1 Characteristics of patients

Patient no.	*Sex/age*	*Diagnosis*	*Transplant*	*Stem cell source*	*Use of ATG*	*Recipient CMV Ab*	*Donor CMV Ab*	*Recipient HSV Ab*
1	F/20	ALL/2CR	UBMT	DR mismatch	No	Positive	Negative	Positive
2	F/51	ATL/CR	CBT	B, DR mismatch	No	Positive	NE	Positive
3	M/32	SAA	UBMT	Match	Yes	Positive	Positive	Positive
4	F/54	SSc	autoPBSCT	CD34	No	Positive	NE	Positive
5	M/51	ML	alloPBSCT	Haplo-identical	No	Positive	Positive	Positive
6	F/41	ATL/Ref	alloPBSCT	Identical	No	Positive	Positive	Positive
7	F/52	MM	alloPBSCT	DR mismatch	No	Positive	Positive	Positive
8	M/47	ATL	UBMT	Match	No	Positive	Positive	Positive
9	M/17	SAA	BMT	Haplo	No	Positive	Positive	Positive
10	M/10	ALL/Ref	CBT	Three-loci mismatch	No	Positive	NE	Positive
11	M/36	CML/BC	alloPBSCT	Identical	No	Positive	Positive	Positive
12	M/45	SAA	UBMT	DR mismatch	Yes	Positive	Negative	Positive
13	M/62	MM	UBMT	B, DR mismatch	No	Positive	Negative	Positive
14	M/61	MDS	alloPBSCT	Identical	No	Positive	Positive	NE
15	M/50	AML/Ref	CBT	A, B mismatch	No	Positive	NE	NE
16	F/49	SSc	autoPBSCT	CD34	No	Positive	NE	Positive

ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; ATL = adult T-cell leukemia/lymphoma; SAA = severe aplastic anemia; ML = malignant lymphoma; CML = chronic myelogenous leukemia; CR = complete remission; Ref = refractory; BC = blastic crisis; MM = multiple myeloma; MDS = myelodysplastic syndrome; SSc = systemic sclerosis; BMT = bone marrow transplantation; UBMT = unrelated BMT; CBT = cord blood transplantation; PBSCT = peripheral blood stem cell transplantation; auto = autologous; allo = allogeneic; ATG = anti-thymocyte globulin; CMV = cytomegalovirus; HSV = herpes simplex virus; Ab = antibody; NE = not evaluated.

Table 2 characteristics of AdV disease

Patient no.	*GVHD*	*Immunosuppressive at HC onset*	*Serotype of AdV*	*Viral study at the onset of HC culture/PCR/IC*	*Onset of HC (days from transplant)*	*Start of CDV administration (days from transplant)*	*Interval from onset to CDV treatment (days)*	*Prior therapy for HC*
1	Grade II	FK506/PSL	11	+/+/+	40	59	19	AraA
3	No	Cs	11	+/+/+	29	30	1	No
4	NE	PSL	11	+/+/+	63	66	3	No
5	No	FK506/PSL	35	+/+/+	17	17	0	No
6	Grade II	Cs/PSL-FK506/mPSL	ND	+/+/+	18	19	1	No
7	Grade II	Cs/PSL	ND	+/+/+	80	83	3	No
8	Grade II	PSL	11	+/+/+	53	109	56	AraA
9	No	FK506/PSL	11	+/+/+	25	25	0	No
10	Grade III	FK506	11	+/+/+	26	39	13	No
11	Chronic lung	FK506/PSL	11	+/+/+	142	149	7	No
12	No	FK506	11	+/+/+	34	34	0	No
14	Grade II	FK506/PSL	11	+/+/+	126	129	3	No
15	Grade II	Cs/PSL	11	+/+/+	50	64	14	No
16	NE	PSL	11	+/+/+	31	31	0	No

AdV = adenovirus; HC = hemorrhagic cystitis; CDV = cidofovir; IC = immunochromatography; NE = not evaluable; ND = not determined; GVHD = graft-versus-host disease; FK506 = tacrolimus; Cs = cyclosporine; PSL = prednisolone; AraA = vidaravin.

Table 3 Outcome of CDV treatment

Patient no.	*Improvement of HC*	*Onset of effect (days)*	*Eradication of ADV from the urine**	*Initial creat (mg/dl)*	*Max creat (mg/dl)*	*Final creat (mg/dl)*	*Renal toxicity (NCI-CTC)*	*Previous PFA treatment*	*Activation of herpesviruses during CDV treatment*
1	Effective	6	Effective	1.38	1.38	1.02	1→1	No	None
3	No	—	Effective	0.54	0.83	1.02	0→0	No	None
4	Effective	7	Effective	0.76	1.02	0.59	0→1	No	None
5	No	—	Effective	1.2	2.38	1.79	1→2	No	CMV antigenemia, HSV stomatitis
6	Effective	13	Effective	0.54	0.59	0.59	0→0	No	CMV antigenemia
7	Effective	12	Effective	0.89	0.97	0.59	0→0	No	None
8	No	—	No	1.2	5.3	5.3	1→3	Yes	None
9	Effective	9	Effective	0.56	1.21	0.83	0→1	No	None
10	Effective	9	Effective	0.35	0.41	0.41	0→0	No	None
11	No	—	No	1.3	2.8	2.8	1→2	No	None
12	Effective	14	Effective	1	1.38	1.03	0→1	Yes	CMV antigenemia, HSV stomatitis
14	Effective	14	Effective	0.8	1.2	1	0→1	No	None
15	Effective	10	Effective	1.4	1.4	0.9	1→1	No	None
16	Effective	8	Effective	1.05	1.34	0.67	1→1	No	CMV antigenemia

*The eradication of AdV was defined by negative culture for AdV 1 week after the last dose of CDV.
Initial creat = serum creatine when starting CDV treatment; Max creat = maximal serum creatine during CDV treatment; Final creat = serum creatine upon completing CDV treatment; CDV = cidofovir; PFA = foscarnet; HC = hemorrhagic cystitis; AdV = adenovirus; CMV = cytomegalovirus; HSV = herpes simplex virus.

while 1 g was given 1 and 8 h afterward. Intravenous hydration with normal saline also was given. Patients were followed up for 2 months after the completion of CDV treatment.

Results

Outcome of CDV therapy

CDV therapy was successful in clearing AdV from the urine in 12 of 14 patients (86%), as defined by negative culture for AdV 1 week after the last dose of CDV. Of 14 patients, 10 (71%) showed clinical improvement in HC (Table 3). Median time to improvement of HC grade after CDV therapy was 9.5 days (range, 6–14; Table 3 and Figure 1). Patients No. 3 and No. 5 had persistent symptoms of HC despite eradication of AdV in the urine.

Toxicity

Serum creatinine concentration for all patients, at the time of initiation and termination of CDV treatment, as well as the maximum serum creatinine concentration during CDV treatment, are shown in Table 3. Renal toxicity was graded according to the Common Toxcity Criteria of National Cancer Institute (NCI-CTC Version 2.0; April 30, 1999). Among 14 patients, seven (50%) had no renal toxicity.

Patient No.
Week 1 2 3 4
Day 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Grade of HC

1 3 4 5 6 7 8 9 10 11 12 14 15 16

Severe
Moderate
Mild
Occult blood negative

Figure 1 Clinical courses of 14 patients with adenoviral HC, who received cidofovir treatment.

Patient No. 8, who had been treated with foscarnet for CMV antigenemia, required hemodialysis and discontinuation of CDV treatment. Patient No. 11 had grade 2 renal toxicity and CDV treatment was terminated. In contrast, patient No. 5 had grade 2 renal toxicity, but could continue CDV treatment. Patient No. 10 developed veno-occlusive disease (VOD) during CDV treatment.

Virally associated findings during the treatment with CDV

Table 1 showed donor/recipient CMV and recipient HSV serostatus. As CDV has been reported to have significant anti-CMV and anti-HSV activity, concurrent use of acyclovir (ACV) or GCV was avoided to reduce renal toxicity. Patient No. 5 developed CMV antigenemia when CDV treatment was started, and CMV antigenemia persisted during CDV treatment. After completion of CDV treatment, he was treated with GCV, which abolished CMV antigenemia. Before CDV treatment, patient No. 6 was treated with GCV for CMV antigenemia that persisted throughout CDV therapy. Patient No. 12 developed CMV antigenemia during CDV treatment. After completion of CDV treatment, CMV antigenemia was abolished by treatment with foscarnet. Patient No. 16 developed CMV antigenemia during CDV treatment, and because of an increase in CMV antigenemia GCV was added. During CDV treatment, patients No. 5 and No. 12 developed HSV-1 stomatitis, which was treated successfully with ACV (Table 3).

Discussion

The present study reports the outcome in AdV HC treated with CDV. As expected, the main toxicity of CDV treatment was renal. Among 14 evaluable patients, two developed severe renal toxicity, resulting in discontinuation of CDV treatment. One of these patients who required hemodialysis had a history of foscarnet treatment. Previous treatment with foscarnet has been reported to exacerbate CDV renal toxicity,[23] which was proved for this patient. CDV renal toxicity complicating treatment of AdV HC is difficult to evaluate. Many other nephrotoxic agents, including cyclosporine, tacrolimus, and amphotericin B, are frequently administered to HSCT patients; furthermore, AdV infection itself can cause renal damage such as nephritis[16] and obstructive nephropathy.[24] In this study, six patients (Nos. 5, 9, 12, 14, 15, and 16) experienced increased level of serum creatinine concentrations, but continued CDV treatment, with improvement in terms of both AdV HC and renal function (Table 3). Thus, AdV HC itself may have contributed to the increase in serum creatinine during CDV treatment. Use of CDV before emergence of renal damage from AdV infection would be desirable. Patient No. 10 developed VOD, which has not been reported previously as a form of CDV toxicity. More information is necessary to determine whether or not VOD is among CDV toxicities.

Among 14 evaluable patients, 10 (71%) showed clinical improvement of AdV HC, which is similar to a success rate of 63% reported in patients with definite AdV disease reported by the European Group for Blood and Marrow Transplantation.[25] A long delay between AdV infection and treatment has been linked to a greater risk of treatment failure.[17] For rapid diagnosis, we used immunochromatography. At the onset of HC, all patients in the study were positive for AdV by this method. Positivity was confirmed later both by PCR result and by isolation of AdV from urine. Thus, immunochromatography appears reliable for rapid diagnosis of AdV HC. Since post-transplant AdV infection causes significant mortality[6,9–11] and HC causes considerable patient discomfort, CDV would appear to be beneficial treatment while maintaining an acceptable toxicity profile.

At a dose of 5 mg/kg/week, CDV has been reported to have significant anti-CMV and anti-HSV activity.[26] Indeed, CDV is considered a second-line treatment for GCV-refractory CMV disease.[27] Among our patients, two had persistent CMV antigenemia and two developed CMV antigenemia during treatment with CDV. In addition, two patients developed HSV-1 stomatitis. Thus, CDV at a dose of 1 mg/kg/day three times weekly may be insufficient to prevent or treat CMV or HSV disease. Alternatively, patients who develop AdV HC might be immune compromised to the extent that for them CDV treatment may not be effective against CMV or HSV. Vigilance against infection by and/or additional prophylaxis agents for herpesviruses, therefore, is important during CDV treatment with 1 mg/kg three times weekly.

In conclusion, CDV at a dose of 1 mg/kg/day, three times weekly could be administered with acceptable toxicity for effective treatment of AdV HC. Prospective randomized trials are necessary to further study the use of CDV for AdV HC.

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Cytolytic activity and regulatory functions of inhibitory NK cell receptor–expressing T cells expanded from granulocyte colony-stimulating factor–mobilized peripheral blood mononuclear cells

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Inhibitory natural killer cell receptor (NKR)–expressing cells may induce a graft-versus-leukemia/tumor (GVL/T) effect against leukemic cells and tumor cells that have mismatched or decreased expression of HLA class I molecules and may not cause graft-versus-host disease (GVHD) against host cells that have normal expression of HLA class I molecules. In our study, we were able to expand inhibitory NKR (CD94/NKG2A)–expressing CD8+ T cells from granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cells (G-PBMCs) by more than 500-fold using stimulation by an anti-CD3 monoclonal antibody with interleukin 15 (IL-15). These expanded and purified CD94-expressing cells attacked various malignant cell lines, including solid cancer cell lines, as well as the patients' leukemic cells but not autologous and allogeneic phytohemagglutinin (PHA) blasts in vitro. Also, these CD94-expressing cells prevented the growth of K562 leukemic cells and CW2 colon cancer cells in NOD/SCID mice in vivo. On the other hand, the CD94-expressing cells have low responsiveness to alloantigen in mixed lymphocyte culture (MLC) and have high transforming growth factor (TGF)–β1– but low IL-2–producing capacity. Therefore, CD94-expressing cells with cytolytic activity against the recipient's leukemic and tumor cells without enhancement of alloresponse might be able to be expanded from donor G-PBMCs. (Blood. 2004;104:768-774)

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Introduction

The regulation of graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) effect is the most important issue in allogeneic stem cell transplantation (allo SCT).

It recently has been shown that inhibitory natural killer cell receptors (NKRs) on NK cells negatively regulate NK cell functions through their binding to major histocompatibility complex (MHC) class I molecules.[1-3] It also has been revealed that T cells, especially memory CD8+ T cells, expressed NKRs. NK-like activity and T-cell receptor (TCR)–mediated killing activity of cytotoxic T lymphocytes (CTLs) expressing inhibitory NKRs were suppressed by HLA class I recognition by the NKRs. Inhibitory NKR-positive cells could attack class I–negative target cells but not the same class I–positive cells.[4-7]

Partially HLA-matched bone marrow transplantation (BMT) resulted in a large expansion of donor-derived CTLs expressing CD158b inhibitory NKRs, which did not cause GVHD but allowed a discriminatory GVL reaction.[8] Also, based on the rule of NKR incompatibility, the GVL effectors may be operational in patients who have undergone HLA-mismatched hematopoietic cell transplantation.[9] Mixed lymphocyte reaction and anti-CD3 mAb–redirected cytotoxicity were inhibited by engagement of transgenic CD158b molecules in CD158b transgenic mice.[10,11] Ruggeri et al[12] reported surprisingly good clinical results that indicated no relapse, no rejection, and no acute GVHD after HLA-haplotype–mismatched transplantations with NKR ligand incompatibility in the GVH direction for acute myeloid leukemia (AML) patients. They also reported that donor allogeneic NK cells attacked host antigen-presenting cells (APCs), resulting in the suppression of GVHD. With regard to the clinical advantage of NKR ligand incompatibility in allo SCT from an unrelated donor, Davies et al[13] showed negative data without using antithymocyte globulin (ATG), while Giebel et al[14] showed positive data using ATG as part of GVHD prophylaxis.

In our previous studies, the proportion of CD158b, which is a specific receptor for HLA-C, on CD8+ T cells was found to be increased in patients with chronic GVHD (cGVHD). Also, the proportion of CD94/NKG2A, which is a specific receptor for HLA-E,[15] on T cells was larger in cGVHD patients with good prognosis than in cGVHD patients with poor prognosis. Furthermore, the addition of CD94-enriched fractions to CD94-depleted fractions suppressed the proliferation of T cells in MLCs.[16-19] Therefore, NKR-expressing cells might be involved in the regulation of allogeneic response after allo SCT. That is, inhibitory NKR induction on alloreactive CTLs may prevent GVHD and mismatch

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of NKRs, and ligands may be useful for induction of the GVL effect during allo SCT.[20-22]

Although granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cell (G-PBMC) grafts contain at least 10 times more T cells than do standard bone marrow grafts, the incidence and severity of acute graft-versus-host disease (aGVHD) after allogeneic peripheral blood stem cell transplantation (PBSCT) are not higher than those observed with allogeneic marrow. Also, there is a possibility that allogeneic PBSCT prevents leukemia relapse by induction of the GVL effect.[23-25] It was previously reported that G-PBMC leukapheresis products contain large numbers of $CD14^+$ cells, which suppress donor T-cell proliferation in a dose-dependent fashion.[26,27] Also, we have shown that the induction of a costimulatory molecule, CD28 responsive complex, in $CD4^+$ cells appears to be suppressed by the presence of $CD14^+$ cells in G-PBMCs.[28] Therefore, it seems useful to use G-PBMCs as a source of lymphocytes in order to manipulate cells for cell therapy to modulate GVHD and GVL. In this study, we expanded NKR-expressing T cells from donor G-PBMCs and investigated their cytolytic characteristics and regulatory functions.

Materials and methods

Donors and G-CSF mobilization

Peripheral blood stem cell donors were administered rhG-CSF (Lenograstim, 1.2 million units (MU)/10 μg, Chugai or Filgrastim, 1 MU/10 μg, Kirin-Sankyo, Japan) by subcutaneous injection at a dose of 10 μg/kg once daily for 4 to 5 days. Leukapheresis was performed from day 4 of rhG-CSF administration, and G-PBMCs were obtained from the first leukapheresis. PBMCs before administration of G-CSF (PreG-PBMC) and G-PBMC samples were cryopreserved to enable simultaneous testing.

Immunofluorescent staining for flow cytometric analysis and monoclonal antibodies

The phycoerythrin (PE)–conjugated monoclonal antibody (mAb) HP-3D9 (anti-CD94) was obtained from Ancell (Bayport, MN), and Z199 (anti-NKG2A), ON72 (anti-NKG2D), Z231 (anti-NKp44), and C1.7 (anti-CD244) were obtained from Immunotec (Marseilles, France). Fluorescein isothiocyanate (FITC)–conjugated anti-CD3, anti-CD8 mAb, and anti–HLA-A, -B, -C mAb (G46-2.6) were purchased from Pharmingen (San Diego, CA). Anti-CD56 mAb and anti–granzyme A mAb were obtained from Becton Dickinson (BD, San Jose, CA). Anti–HLA class I mAb BRA-23/9 and W6/32 were obtained from NeoMarkers (Fremont, CA), and rat anti–HLA class I mAb (YTH862.2) was obtained from Serotec (Oxford, England). Anti-CD3 mAb OKT3 was obtained from Ortho Biotech (Raritan, NJ). Anti-NKG2C and anti-NKG2D mAb were obtained from R&D Systems (Minneapolis, MN). Intracellular granzyme A was stained using cytofix/cytoperm reagent according to the manufacturer's instructions (Becton Dickinson). The fluorescence intensity of the cells was analyzed using a FACS Calibur (Becton Dickinson). Statistical analysis was performed using Student *t* test.

Immunomagnetic cell sorting

Purified $CD14^+$ cells (> 95% $CD14^+$, as determined by flow cytometric analysis), $CD8^+$ cells (> 90% $CD8^+$), and $CD94^+$ cells (> 90% $CD94^+$) were obtained by magnetic cell sorting (MACS) using magnetic microbeads according to the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Induction of CD94/NKG2A on $CD8^+$ T cells by stimulation with immobilized anti-CD3 monoclonal antibody and IL-15

For coating with anti-CD3 mAb, 24-well flat-bottom plates or tissue culture flasks were preincubated with OKT3 (1 μg/mL) in 100 mM Tris [tris(hydroxymethyl)aminomethane]-HCl buffer (pH 9.5) for 16 hours at 4°C. Seven paired PreG-PBMCs and G-PBMCs (1×10^6/mL) were cultured on 24-well plates in RPMI 1640 supplemented with 10% fetal calf serum with 5 ng/mL of recombinant human IL-15 (R&D Systems) at 37°C for 7 days. $CD8^+$ cells (500×10^3/mL) purified from G-PBMC cultures were established on 24-well plates with or without the use of 0.45-μm micropore membranes (Falcon, Becton Dickinson, Franklin Lakes, NJ). Purified $CD14^+$ cells (300×10^3) derived from the same G-PBMCs were added to the culture directly or through the membrane.

Expansion of CD94-expressing cells from G-PBMCs

Six paired PreG-PBMCs and G-PBMCs (2.5×10^6) were cultured with immobilized anti-CD3 mAb (1 μg/mL) and IL-15 (5 ng/mL) in RPMI 1640 with 5% human AB serum in T25 culture flasks for 7 days. After 5 days of culture, 5 mL of fresh medium was added. Absolute numbers of $CD94^+$/$CD3^+$, $CD94^+$/$CD8^+$, $NKG2A^+$/$CD3^+$, and $NKG2A^+$/$CD8^+$ cells on day 7 were calculated from multiplication of total number of expanded cells and the proportion of these cells in expanded cells.

PCR reaction and TCR spectratyping

First-strand cDNA synthesis was performed with 60 ng RNA, 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates (dNTPs), 2.5 μM Random 9 mer, and 0.25U/μL avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa RNA PCR Kit, Japan). Then polymerase chain reaction (PCR) amplification of the cDNA was carried out using a sense primer (5′-CAGCATGAGGGGCTACCCG-3′) and an antisense primer (5′-GTGTGAGGAAGGGGGTCATG-3′) for exon 4 of HLA-E.[29] A sense primer (5′-TTCGAGCAAGAGATGGCCACGGCT-3′) and an antisense primer (5′-ATACTCCTGCTTGCTGATCCACAT-3′) for β-actin were used as an internal standard.

For analysis of the TCR-VB repertoire, PCR amplification of the cDNA was carried out using corresponding primers to the variable regions of TCR-VB and CB.[30] Samples consisting of 1 μL of PCR product with a size standard (labeled ROX) and paraformamide were heated at 95°C for 2 minutes and then placed for a moment on ice. TCR spectratyping was performed using a capillary electrophoresis system (PRISM310 Genetic Analyzer, ABI, Foster City, CA).

Evaluation of cytolytic activity using 4-hour ^{51}Cr release assay

After 7 days of stimulation by immobilized anti-CD3 mAb with IL-15 in a T25 flask, CD94-expressing cells were purified by MACS. The cytolytic activities of purified CD94-expressing cells were tested against ^{51}Cr-labeled human malignant cell lines, patients' own leukemic cells, autologous PHA blasts, and allogeneic PHA blasts (5×10^3). K562 cells, an erythroleukemic cell line, were cultured with interferon-γ (IFN-γ) (0.2 μg/mL) for 2 days to induce HLA class I expression. An HLA-Cw3 signal peptide (VMAPRTLIL), which can bind to HLA-E, and an irrelevant B15 peptide (VTAPRTVLL)[31] were synthesized by Kurabo (Osaka, Japan) (purity, 95%). Several leukemic cell lines and solid cancer cell lines were obtained from Riken (Tsukuba, Japan).

Mixed lymphocyte culture

Responder CD94-expressing cells and CD94-depleted cells (50×10^3) were cultured with 50×10^3 irradiated (30 Gy) allogeneic, third-party PBMC stimulators in 200 μL of RPMI 1640 supplemented with 10% fetal calf serum in round-bottom 96-well plates (Corning, Corning, New York). After 2 days of incubation at 37°C in 5% CO_2, cultures were pulsed with ^3H-thymidine (1.0 μCi [0.037 MBq]/well) for the final 16 hours. The cells were then harvested, and ^3H-thymidine incorporation was measured in triplicate using a 196 gas flow counter (Packard Instrument, Downers Grove, IL). Anti–TGF-β1 mAb (25 μg/mL, R&D Systems) was added to the MLC medium using CD94-expressing cells as responders.

Measurement of cytokine concentrations

Cytokine concentrations in MLC (50×10^3 responders and 50×10^3 irradiated stimulators) after 2 days and in culture medium (50×10^3)

stimulated by phorbol myristate acetate (PMA) (10 ng/mL) and ionomycin (500 ng/mL) after 1 day were estimated. TGF-β1 was measured by using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), and other cytokines were measured by using a LiquiChip Human Cytokine System (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

NOD/SCID mice

Female 5- to 8-week-old NOD/SCID mice were purchased from Clea (Tokyo, Japan). Breeding and maintenance were performed in a micro-isolator under sterile conditions. K562 cells or CW2 colon cancer cells with or without purified CD94-expressing cells expanded from G-PBMCs were suspended in 0.5 mL phosphate-buffered saline (PBS) and injected subcutaneously into the right flanks of the NOD/SCID mice. NOD/SCID mice did not receive irradiation or anti-ASGM1 antibody.

Results

Induction of CD94/NKG2A expression on $CD8^+$ T cells in G-PBMCs by immobilized anti-CD3 monoclonal antibody stimulation with IL-15

We found that the proportion of CD94/NKG2A-expressing $CD3^+$/$CD8^+$ T cells in G-PBMCs was increased after immobilized anti-CD3 mAb stimulation (Table 1). Although there was no difference between the proportions of CD94/NKG2A-expressing T cells in PBMCs obtained from 7 donors before administration of G-CSF (PreG-PBMCs) and after administration of G-CSF (G-PBMCs) without stimulation, the proportions of CD94/NKG2A-expressing T cells derived from G-PBMCs after 7 days of stimulation with immobilized anti-CD3 mAb both with and without IL-15 were significantly higher than the proportions of CD94/NKG2A-expressing T cells derived from PreG-PBMCs (Table 1). We also found that the proportions of CD94/NKG2A-expressing $CD8^+$ T cells that had been purified from G-PBMCs before culture were increased by immobilized anti-CD3 mAb stimulation with IL-15 (Table 2). The addition of 3×10^5 purified $CD14^+$ cells derived from the same G-PBMCs to purified $CD8^+$ T cells induced much more CD94/NKG2A expression on those purified $CD8^+$ T cells. This effect of purified $CD14^+$ cells tended to be inhibited by the use of a membrane (Table 2). These results suggest that $CD14^+$ cells play an important role in the induction of CD94/NKG2A expression on T cells and that this effect might require at least partial contact between responder cells and $CD14^+$ cells. Furthermore, it was revealed that CD94/NKG2A expression on purified $CD8^+$ T cells from G-PBMCs could be induced in our culture system. TCR engagement has been reported to play an important role in the induction of inhibitory NKRs on $CD8^+$ T cells.[6] Several cytokines, such as IL-12 and IL-15, are known to be CD94/NKG2A-inducible cytokines.[7,32] It is possible that IL-15 induces inhibitory NKRs on $CD8^+$ T cells derived from G-PBMCs during T-cell activation by the immobilized anti-CD3 mAb.

Expansion of CD94-expressing cells from G-PBMCs

PreG-PBMCs and G-PBMCs contained almost equal numbers of CD94/NKG2A-expressing T cells before stimulation. CD94/NKG2A-expressing $CD8^+$ T cells from both PreG-PBMCs and G-PBMCs were expanded by more than 100-fold after 7 days of culture. Moreover, a significantly greater number of CD94/NKG2A-expressing T cells was obtained from G-PBMCs than from PreG-PBMCs (Table 3). $CD94^+$ cells (> 90% $CD94^+$) were obtained by MACS using magnetic microbeads, and more than 80% of CD94-expressing cells coexpressed CD8 (data not shown). The CD94-depleted cells contained only low CD94-expressing $CD8^+$ cells (mean fluorescence intensity [MFI], CD94-depleted cells vs CD94-expressing cells, 24.7 ± 7.2 vs 234.1 ± 30.5, n = 7). In contrast, most of these $CD94^+$ cells did not express CD56. Furthermore, these $CD94^+$ cells contained granzyme A, which is an important enzyme for induction of apoptosis of target cells in the cytoplasm.[33] Also, CD94-expressing cells expanded from G-PBMCs had a large repertoire of TCR-Vβ, as revealed by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis using 26 kinds of TCR-Vβ primer pairs (data not shown). These expanded $CD8^+$ T cells expressed NKG2D and CD244 but did not express CD158a, CD158b, NKB1, CD161, nor NKp44 (data not shown).

Characteristics of cytolytic activities of CD94-expressing cells expanded from G-PBMCs against K562 leukemic cells

We investigated the characteristics of cytolytic activity of CD94-expressing cells expanded from donor G-PBMCs. The cytolytic activity level of purified CD94-expressing cells detected by a standard 4-hour ^{51}Cr release assay against HLA class I–deficient K562 cells was found to be always higher than that of CD94-depleted cells and also higher than that against autologous PHA blasts (Figure 1A). Furthermore, the cytolytic activity level of CD94-expressing cells against allogeneic PHA blasts was as low as that against auto PHA blasts (Figure 1B). HLA class I expression is inducible on K562 cells by IFN-γ. The MFIs of HLA class I molecules on untreated K562 and IFN-γ–treated K562 cells were 21.8 ± 14.6 (n = 6) and 90.1 ± 25.5 (n = 6), respectively. Although we did not show the surface expression level of HLA-E on HLA class I–expressing cells, we could show HLA-E mRNA induction in HLA class I–expressing K562 cells in an RT-PCR experiment (data not shown). Therefore, the leader peptide of HLA class I stabilizes HLA-E expression and subsequently may be able to induce a higher level of HLA-E expression on HLA class I–expressing cells. The cytolytic activity of CD94-expressing cells against IFN-γ–treated K562 cells was attenuated compared with

Table 1. Proportion of CD94/NKG2A-expressing cells in paired PreG- and G-PBMC before and after stimulation by anti-CD3 monoclonal antibody

	Before		After anti-CD3 stimulation		Anti-CD3 and IL-15	
Surface marker	PreG	G-PBMC	PreG	G-PBMC	PreG	G-PBMC
$CD94^+/CD3^+$	5.7 ± 3.1	4.6 ± 1.9	8.4 ± 4.4	17.2 ± 8.7*	24.2 ± 10.4	32.8 ± 8.2†
$CD94^+/CD8^+$	2.6 ± 1.6	2.7 ± 1.2	7.3 ± 4.0	15.2 ± 8.4*	17.6 ± 10.0	24.2 ± 5.6‡
$NKG2A^+/CD3^+$	1.8 ± 1.4	1.8 ± 0.7	3.0 ± 1.9	6.3 ± 5.0	11.3 ± 7.1	16.9 ± 7.7‡
$NKG2A^+/CD8^+$	0.5 ± 0.3	0.4 ± 0.5	2.3 ± 1.3	5.1 ± 4.3	8.6 ± 3.1	13.6 ± 6.3*

Values indicate the percentage of CD94 or NKG2A-expressing cells (means ± SDs, n = 7). Significant difference were noted when comparing the value of PreG and G-PBMC after stimulation with and without IL-15 (*$P < .01$; ‡$P < .05$, †$P < .1$).

Table 2. Induction of CD94/NKG2A expression on purified CD8+ cells from G-PBMC

			Addition of CD14+ cells	
Surface marker	Before	After	Without membrane	With membrane
CD94+/CD8+	1.7 ± 0.8	9.1 ± 5.8*	19.7 ± 9.3†	11.7 ± 6.0‡
NKG2A+/CD8+	0.4 ± 0.3	3.0 ± 2.7†	10.1 ± 6.8†	5.0 ± 3.5‡

Values indicate the percentage of CD94 or NKG2A-expressing cells after anti-CD3 stimulation in the presence of IL-15 (means ± SDs, n = 7). Significant differences were noted when comparing the value of before and after stimulation; CD8+ cell only and addition of purified 3 × 10⁵ CD14+ cells; without membrane; and the contact inhibition by the membrane.

*$P < .01$.

†$P < .05$.

‡$P < .1$.

that against untreated K562 cells. Furthermore, HLA-Cw3 peptide (0.3 mM), which is a signal sequence of HLA-C and makes a complex with HLA-E as a ligand for CD94/NKG2A, suppressed the cytolytic activity of CD94-expressing cells against HLA class I–expressing K562 cells. The suppressive effect of Cw3 peptide was higher than that of an irrelevant B15 peptide. In contrast, anti-NKG2A mAb (10 μg/mL) restored the HLA class I protective effect against IFN-γ–treated K562 cells (Figure 2A). Also, anti–HLA class I mAbs (G46-2.6 and W6/32, 20 μg/mL) restored the cytolytic activity of CD94-expressing cells against IFN-γ–treated K562 cells that had increased mRNA of HLA-E, while other anti–HLA class I mAbs (YTH862.2 and BRA-23/9, 20 μg/mL) did not have any effect (Figure 2B). Furthermore, anti-NKG2D mAb suppressed the cytolytic activity of CD94-expressing cells against K562 cells, while anti-NKG2C mAb and anti-CD244 mAb did not have any effect (20 μg/mL) (Figure 2C).

Cytolytic activities of CD94-expressing cells against various leukemic cell lines, solid cancer cell lines, and the patient's leukemic cells

We analyzed the cytolytic activities of CD94-expressing cells against 7 human leukemic cell lines and 3 solid cancer cell lines. Cytolytic activities against HLA class I^{low} cells (MFI < 50; K562 and CW2 colon cancer cells) were more than 30% (effector-to-target, 10:1). On the other hand, cytolytic activities against HLA class $I^{intermediate}$ cells (50 < MFI < 150; HL60, KCL22, HEL, and U937 leukemic cells) were 20% to 30%, and cytolytic activities against HLA class I^{high} cells (MFI > 200; J111 and BALL-1 leukemic cells and RC2 and 3TKB renal cancer cells) were less than 10% (data not shown).

We then investigated the cytolytic activities of HLA-matched donor CD94-expressing cells against the patient's chronic myelogenous leukemia (CML) cells derived from bone marrow cells before allo SCT in the chronic phase and against the patient's leukemic cells of CML myeloid blastic crisis (CML-BC), acute myelogenous leukemia (AML, M2), acute lymphocytic leukemia (ALL, L2), Ph1-positive ALL, adult T-cell leukemia (ATL), another AML (M2), and myelodysplastic syndrome (MDS) overt leukemia. Expanded and purified CD94-expressing cells derived from each donor attacked K562 cells and the patients' own leukemic cells to varying degrees, depending on the type of tumor (the tumors having different expression levels of HLA class I and probably having different expression levels of adhesion molecules and stimulatory NKR ligands) but did not attack auto PHA blasts (Figure 3A). Also, allogeneic third-party CD94-expressing cells attacked the patients' primary AML (M0, M2, M4) and CML (CP and BC) leukemic cells. However, these cells did not attack ALL (L1) cells, ATL cells, lymphoblastic leukemia lymphoma (LBL) cells, auto PHA blasts, or allo PHA blasts. Anti–HLA class I mAb partially restored this killing activity against ALL (Ph1) leukemic cells, auto PHA blasts, and allo PHA blasts (Figure 3B-C). These expanded donor and allogeneic CD94-expressing cells attacked HLA class I molecule–lacking K562 leukemic cells and also the patient's leukemic cells that were not HLA class I^{high} cells (MFI < 200) as PHA blasts.

Proliferation in MLC and cytokine productive capacity of CD94-expressing cells

Proliferation of T cells detected by ³H-thymidine incorporation was suppressed in MLC using CD94-expressing cells as responders compared with that in the case of using CD94-depleted cells as responders (2697 ± 1124 vs 6586 ± 2283 cpm, $P < .01$, n = 6). TGF-β1 concentration in MLC medium using CD94-expressing cells as responders was significantly higher than that in MLC medium using CD94-depleted cells as responders (383.2 ± 144.3 vs 236.4 ± 89.6 pg/mL, $P < .05$, n = 8). IL-2 and IFN-γ concentrations in MLC medium were not significantly different. On the other hand, IL-2 concentration in culture medium stimulated by PMA and ionomycin using CD94-depleted cells was significantly higher than that in culture medium using CD94-expressing cells (13 485.9 ± 10 640.4 vs 1767.7 ± 223.8 pg/mL, $P < .05$, n = 8). Furthermore, it was revealed that anti–TGF-β1 mAb could restore T-cell proliferation in MLCs using CD94-expressing cells as responders (2697 ± 1124 vs 3470 ± 1262 cpm, $P < .01$, n = 6).

Inhibition of growth of K562 leukemic cells and CW2 colon cancer cells by CD94-expressing cells in NOD/SCID mice

NOD/SCID mice were coinjected subcutaneously with K562 cells and purified CD94-expressing cells expanded from G-PBMCs. CD94-expressing cells inhibited the growth of K562 cells completely (ratio of CD94-expressing cells to K562 cells: 1×10^7 to 2×10^7) in NOD/SCID mice (Figure 4A). CD94-expressing cells also inhibited the growth of CW2 colon cancer cells completely (ratio of CD94-expressing cells to CW2 cells: 0.5×10^7 to 1×10^7) in NOD/SCID mice (Figure 4B).

Table 3. Expansion of CD94/NKG2A-expressing cells from paired PreG- and G-PBMC

	Before, mean absolute no. cells ± ×10⁶		After stimulation, mean absolute no. cells ± ×10⁶ (mean fold expression)	
Surface marker	PreG	G-PBMC	PreG	G-PBMC
CD94+/CD3+	0.048 ± 0.026	0.041 ± 0.012	2.87 ± 1.38 (59.8)	5.51 ± 2.62* (134.4)
CD94+/CD8+	0.020 ± 0.007	0.010 ± 0.004	2.63 ± 1.25 (131.5)	5.20 ± 2.36† (520.0)
NKG2A+/CD3+	0.021 ± 0.012	0.018 ± 0.010	2.09 ± 1.10 (99.5)	4.20 ± 2.44* (233.3)
NKG2A+/CD8+	0.0052 ± 0.0036	0.0036 ± 0.0012	1.90 ± 1.02 (365.4)	3.98 ± 2.21* (1105.6)

Cultures were started from 2.5 × 10⁶ mononuclear cells. Values indicate absolute number of cells before and after culture. Significant differences were noted when comparing the value of PreG and G-PBMC after stimulation (*$P < .05$, †$P < .01$).

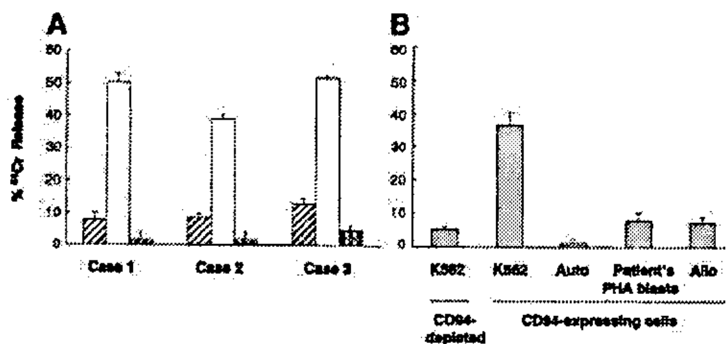

Figure 1. Cytolytic activities of CD94-expressing cells against K562 leukemic cells and PHA blasts. (A) Cytolytic activities of CD94-depleted cells (▨) and CD94-expressing cells expanded from G-PBMCs against K562 cells (□) and autologous PHA blasts (▩). (B) Cytolytic activities of CD94-depleted cells and CD94-expressing cells expanded from G-PBMCs against K562 cells, autologous PHA blasts, patient's PHA blasts, and allogeneic third-party PHA blasts. The data represented are the means ± SDs (effector-to-target ratio is 10:1).

Discussion

In this study, we found that the proportion of CD94/NKG2A-expressing $CD3^+/CD8^+$ T cells in G-PBMCs was increased after immobilized anti-CD3 mAb stimulation with IL-15. We also found that $CD14^+$ cells derived from G-PBMCs play an important role in the induction of CD94/NKG2A expression on purified $CD8^+$ T cells. Therefore, $CD8^+$ T cells derived from G-PBMCs could express CD94/NKG2A after stimulation. Also, we were able to expand CD94-expressing $CD8^+$ T cells from donor G-PBMCs by more than 500-fold. The absolute number of CD94-expressing T cells after stimulation from G-PBMCs was significantly higher than that from PreG-PBMCs. It is possible that a greater number of $CD14^+$ cells in G-PBMCs than in PreG-PBMCs can stimulate the first signal through TCR. This TCR engagement has been reported to play an important role in the induction of inhibitory NKRs on $CD8^+$ T cells.[6] Although we showed a CD94-inducing effect of $CD14^+$ cells in a contact-dependent manner, other factors such as cytokines may be implicated in this effect. Also, it is not clear enough whether G-CSF has an effect on progenitor cells of CD94/NKG2A-expressing cells. Nevertheless, G-PBMCs, which are easy to obtain and store at the time of PBSC collection from the donor, may be a useful source for the expansion of inhibitory NKR-expressing cells.

These expanded and purified CD94-expressing cells had CD8 expression but not CD56 expression on their surfaces. Also, these CD94-expressing cells contained granzyme A in the cytoplasm and had a large repertoire of TCR-Vβ as revealed by RT-PCR analysis using 26 kinds of TCR-Vβ primer pairs. Furthermore, these expanded CD8 T cells did not express other killer cell immunoglobulin-like receptors (KIRs) such as CD158a, CD158b, or NKB1 or NK cell–activating markers such as CD161 or NKp44, but they did express NK cell–activating receptors NKG2D and CD244. Therefore, these cells have both inhibitory receptors (CD94/NKG2A) and activating receptors (NKG2D). The cytolytic activity of CD94-expressing cells depends at least partially on NKG2D-activating NKR, because anti-NKG2D mAb suppressed this activity. However, it is possible that other receptors that were not analyzed in this study are involved in the killing activity.

HLA-E, a CD94/NKG2A ligand, preferably bound to a peptide derived from the signal sequences of most HLA-A, -B, -C, and -G and was also up-regulated by these peptides.[31] We investigated the characteristics of cytolytic activities of CD94-expressing cells using IFN-γ–induced HLA class I molecule–expressing K562 cells that had increased mRNA of HLA-E. HLA-C signal peptide was found to suppress the cytolytic activity of CD94-expressing cells against IFN-γ–induced HLA class I molecule–expressing K562 cells. Also, anti-NKG2A mAb and some anti–HLA class I mAbs partially restored the cytolytic activity of CD94-expressing cells against HLA class I molecule–protected K562 cells. In addition, results of analysis of the cytolytic activities of CD94-expressing cells against 10 malignant cell lines, including 3 solid cancer cell lines, indicated that this killing activity roughly depended on the expression of HLA class I molecules on the cell surface. However, the cytolytic activity of CD94-expressing cells does not depend entirely on the expression of HLA class I molecules. The cytolytic activity of CD94-expressing cells may be regulated by the balance among the expression levels of HLA class I, HLA-E itself, and certain molecules on target cells.

We also investigated the cytolytic activities of CD94-expressing cells against 17 patients' primary leukemic cells. Donor and allogeneic CD94-expressing cells could attack patients' CML cells and AML cells but could not attack some patients' leukemic cells such as ATL cells, which had high expression levels of HLA class I molecules. Also, the addition of anti–HLA class I mAb induced

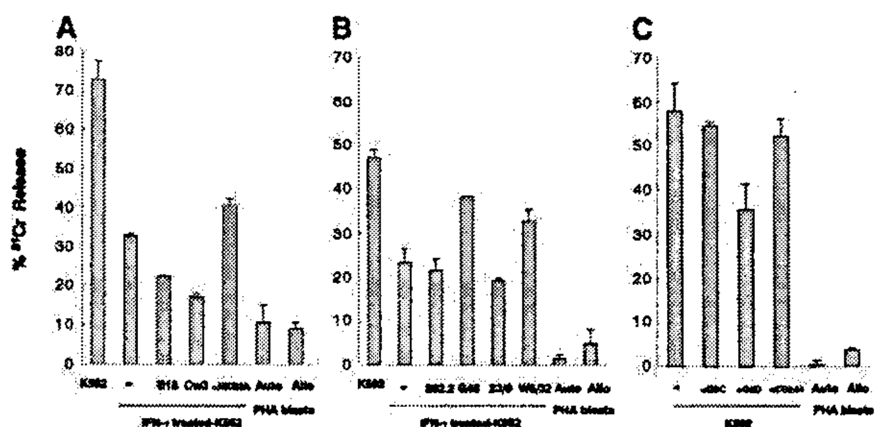

Figure 2. Characteristics of cytolytic activities of CD94-expressing cells against K562 leukemic cells. (A) Cytolytic activities of CD94-expressing cells against untreated K562 cells, IFN-γ–treated K562 cells, IFN-γ–treated K562 cells with HLA-B15 peptide (0.3 mM), with HLA-Cw3 peptide (0.3 mM), and with anti-NKG2A mAb (10 μg/mL) against autologous PHA blasts and allogeneic PHA blasts. (B) Cytolytic activities of CD94-expressing cells against untreated K562 cells, IFN-γ–treated K562 cells, and IFN-γ–treated K562 cells with anti–HLA class I mAbs (YTH862.2, G46-2.6, BRA-23/9, and W6/32, 20 μg/mL) and against autologous PHA blasts and allogeneic PHA blasts. (C) The cytolytic activities of CD94-expressing cells against K562 cells, against K562 cells with anti-NKG2C, anti-NKG2D, and anti-CD244 mAbs (20 μg/mL), against autologous PHA blasts and allogeneic PHA blasts. The data represented are the means ± SDs (effector-to-target ratio is 10:1).

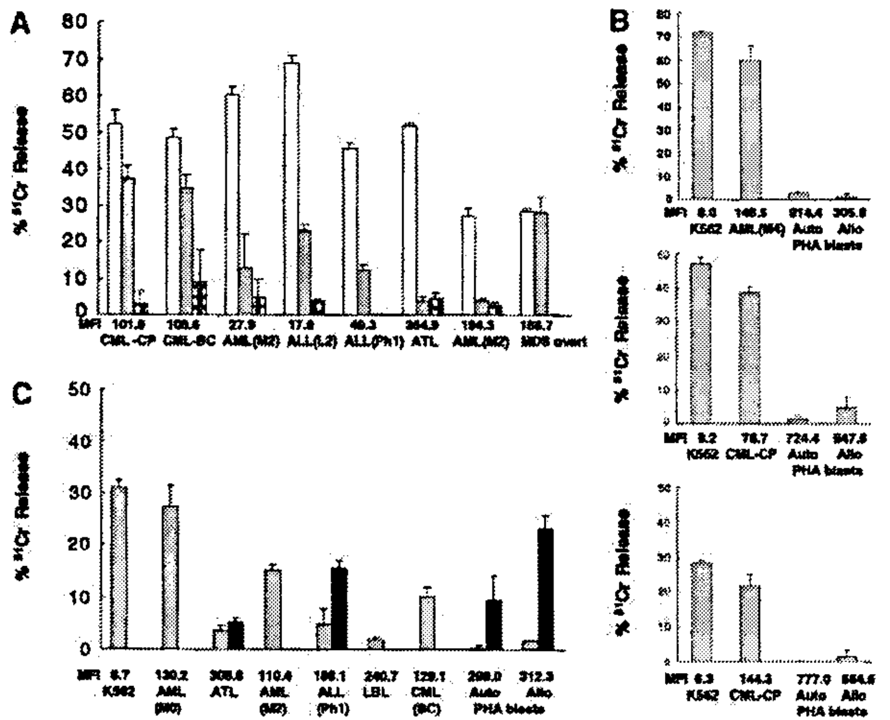

Figure 3. Cytolytic activities of CD94-expressing cells expanded from G-PBMCs against the patients' leukemic cells. (A) Cytolytic activities of HLA-matched donor CD94-expressing cells expanded from 8 different donor G-PBMCs against K562 cells (□), the patients' own leukemic cells (▨), and autologous PHA blasts (▩). (B, C) Cytolytic activities of allogeneic third-party CD94-expressing cells expanded from 4 different donor G-PBMCs against K562 cells, the patients' primary leukemic cells, autologous PHA blasts, and allogeneic PHA blasts. Addition of anti–HLA class I mAbs (W6/32, 20 μg/mL) (■). MFIs of HLA class I on target cells are indicated. The data represented are the means ± SDs (effector-to-target ratio is 10:1).

restoration of the cytolytic activity of CD94-expressing cells against PHA blasts and ALL (Ph1) but not ATL cells. Although these CD94-expressing cells attacked HLA class I$^{\text{low-intermediate}}$ patients' leukemic cells, the killing activity varied, depending on the type of leukemia. Patients' leukemic cells have different expression levels of HLA class I, and they may have different expression levels of other regulatory molecules for the killing activities of CD94-expressing cells. Therefore, not only HLA class I molecules on leukemic cells but also other molecules such as adhesion molecules and stimulatory NKR ligands such as MHC class I chain–related protein (MIC[34]) and activating molecules on effector cells might be important for the regulation of these killing activities.

In vivo analysis revealed that CD94-expressing cells prevented the growth of K562 leukemic cells and also CW2 colon cancer cells in NOD/SCID mice. These models suggest that CD94-expressing cells may therefore have a graft-versus-leukemia/tumor effect.

In addition, the CD94-expressing cells exhibited low proliferative capacity in MLC and high TGF-β1 productivity with attenuated IL-2 productivity. These cells therefore have low responsiveness to alloantigens and may also have a suppressive effect on HLA class I–induced alloresponse.

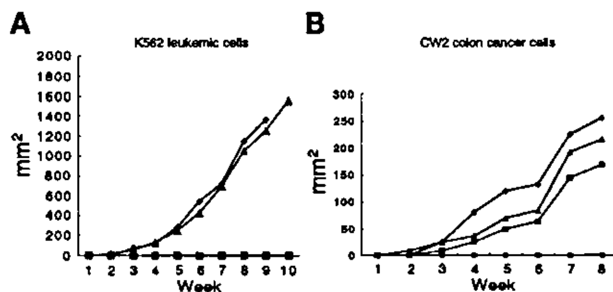

Figure 4. CD94-expressing cells expanded from G-PBMCs prevent growth of K562 leukemic cells and CW2 colon cancer cells in NOD/SCID mice. (A) Mice were subcutaneously injected with 2 × 10^7^ K562 cells only (♦ and ▲; died after 9 and 13 weeks, respectively) or with 1 × 10^7^ CD94-expressing cells (2 mice, ■, survived more than 30 weeks). (B) Mice were subcutaneously injected with 1 × 10^7^ CW2 colon cancer cells only (♦, ▲, and ■; died after 9, 9, and 14 weeks, respectively) or with 0.5 × 10^7^ CD94-expressing cells (3 mice, ●, survived more than 30 weeks).

We previously reported increased expression of CD158 and CD94/NKG2A on T cells in chronic GVHD patients with good prognosis and showed that these inhibitory NKR-expressing cells have a suppressive effect on allogeneic response in MLC.[16-19] Therefore, inhibitory NKR expression during allogeneic stimulation after allo SCT may play an important role in modulation of GVHD. Based on clinical and experimental data, we speculate that these inhibitory NKR-expressing cells have a GVL/T effect against leukemic cells and tumor cells that have decreased expression levels of HLA class I molecules and do not enhance GVHD against host cells that have normal expression levels of HLA class I molecules.

Elucidation of cytolytic characteristics, proliferative characteristics, and cytokine productivity of inhibitory NKR-expressing cells might provide clues about how to control the delicate balance between GVHD and GVL. It may be possible to use expanded CD94-expressing cells from donor G-PBMCs, which contain a large number of T cells, for allogeneic cell therapy instead of naive donor lymphocyte infusion to induce the GVL effect without enhancing GVHD. Donor G-PBMCs, which are an alternative stem cell source for allogeneic stem cell transplantation, might also be a useful source of lymphocytes for expanding NKR-expressing cells for cell therapy for some patients whose leukemic cells and tumor cells have escaped from allogeneic recognition by usual cytotoxic T cells because of the low expression level of HLA class I molecules.

Acknowledgments

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Monitoring the Expression Profiles of Doxorubicin-Resistant K562 Human Leukemia Cells by Serial Analysis of Gene Expression

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Abstract

We examined the expression profiles of doxorubicin-resistant K562 cells by serial analysis of gene expression (SAGE) to identify novel and/or partially characterized genes that might be related to drug resistance in human leukemia. SAGE complementary DNA (cDNA) libraries were constructed from K562 and doxorubicin-resistant K562 (K562/ADM) cells, and concatamer sequences were analyzed with SAGE 2000 software. We used 9792 tags in the identification of 1076 different transcripts, 296 of which were similarly expressed in K562 and K562/ADM cells. There were 343 genes more actively expressed in K562/ADM than in parental K562 cells and 437 genes expressed less often in K562/ADM cells. K562/ADM cells showed increased expression of well-known genes, including the genes for spectrin β, eukaryotic translation initiation factor 1A (*EIF1A*), RAD23 homolog B, laminin receptor 1, and polyA-, RAN-, and PAI-1 messenger RNA-binding proteins. K562/ADM cells showed decreased expression of the genes for fatty acid desaturase 1 (*FADS1*), hemoglobin ε 1, *N*-myristoyltransferase 1, hemoglobin α 2, NADH dehydrogenase Fe-S protein 6, heat shock 90-kDa protein, and karyopherin β1. Quantitative reverse transcription–polymerase chain reaction analysis confirmed the increased expression of *EIF1A* and the decreased expression of *FADS1* in K562/ADM cells. Prior to this investigation, such differences in the expression of these genes in doxorubicin-resistant leukemia cells were unknown. Although we do not provide any evidence in the present report for the potential roles of these genes in drug resistance, SAGE may provide a perspective into our understanding of drug resistance in human leukemia that is different from that provided by cDNA microarray analysis.

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Key words: SAGE; Expression profile; K562; Doxorubicin; Resistance

1. Introduction

Drug resistance is a major obstacle to successful chemotherapy in cancer patients. Because doxorubicin (Adriamycin) and other anthracyclines remain key drugs in treating hematologic malignancies, including leukemias, lymphomas, and multiple myeloma, elucidating the mechanisms of anthracycline resistance is one of the most critical goals in hematologic oncology. Several mechanisms of drug resistance in tumors are known and include altered drug metabolism in cancer cells conferred through the P-glycoprotein encoded by the multidrug resistance 1 (*MDR1*) gene, the redox detoxifying action of glutathione, enhanced hepatic P-450 activity, mutation of topoisomerase II, and resistance to apoptosis [1-4]. Therefore, the development of drug resistance in cancer cannot be explained with a single mechanism, and the altered expression of a diverse array of genes influencing various biochemical pathways may be involved.

The complementary DNA (cDNA) microarray technique has enabled expression profile analyses of thousands of genes and has contributed to our understanding of the development of drug resistance and chemosensitivity in breast [5,6], ovarian [7] and other cancers [8,9], even though cDNA microarray analysis requires known probes. Serial analysis of gene expression (SAGE) does not require preexisting clones and has enabled us to identify and quantitate the expression of both new and previously known genes [10]. SAGE analysis may therefore be useful for investigating the development of drug resistance in cancer. In this study, we used SAGE to monitor messenger RNA (mRNA) expression in doxorubicin-resistant K562

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leukemia cells and observed previously unknown differences in the expression of several genes in the doxorubicin-resistant cells.

2. Materials and Methods

2.1. Cell Lines

K562 (chronic myelogenous leukemia), K562/ADM (doxorubicin-resistant variant K562), and K/eto (etoposide-resistant K562) cell lines [11-14] were kindly provided by Dr. H. Fujii (Nihon Kayaku, Tokyo, Japan). K562/ADM cells overexpress P-glycoprotein, which is encoded by the MDR1 gene [12]. Cells were maintained in RPMI 1640 medium supplemented with 10% mycoplasma-free heat-inactivated fetal calf serum (Hyclone, Logan, UT, USA), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell characteristics were the same as previously described [11-13]. Exponentially growing cells were used in this study.

2.2. SAGE Protocol

SAGE libraries of K562 and K562/ADM cells were generated according to the method described by Velculescu et al [10]. Total RNA was prepared with Trizol reagent (Gibco BRL, Gaithersburg, MD, USA). PolyA+ RNA was obtained with a MessageMaker kit (Gibco BRL) according to the manufacturer's instructions and converted to cDNA with a SuperScript Choice System (Gibco BRL) with a 5′-biotinylated oligo(dT). Biotinylated double-stranded cDNA was cleaved with the restriction enzyme *Nla*III (New England Biolabs, Beverly, MA, USA), and the 3′-terminal fragments were bound to streptavidin-coated magnetic beads (Dynal, Oslo, Norway). Captured 3′ cDNA fragments were divided into 2 pools, and each pool was ligated to one of the linkers containing recognition sites for *Bsm*FI. Nucleotide sequences of the linkers were as follows: Linker 1, 5′-TTTGGATTTGCTGGTGCAGTACAACTAGGCTTA ATAGGGACATG-3′, 5′-TCCCTATTAAGCCTAGTT GTACTGCACCAGCAAATCC[amino modifier C5]-3′; Linker 2, 5′-TTTCTGCTCGAATTCAAGCTTCTAACG ATGTACGGGGACATG-3′, 5′-TCCCCGTACATCGTT AGAAGCTTGAATTCGAGCAG[amino modifier C5]-3′. Linkered cDNA was released from the beads by digestion with *Bsm*FI. After digestion, linker tags were blunted with Klenow fragment and ligated to generate ditags (tags ligated tail-to-tail). Ditags were amplified by the polymerase chain reaction (PCR) for 32 cycles with 5′-GGATTTGCT GGTGCAGTACA-3′ and 5′-CTGCTCGAATTCAAGCT TCT-3′ as primers. PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE), and the band containing the ditags was excised. After digestion with *Nla*III, ditags were ligated to produce concatamer. Concatamers were separated by PAGE, and products between 400 base pairs (bp) and 1200 bp were excised. These products were cloned into pZero-1 vector (Invitrogen, Carlsbad, CA, USA) digested with *Sph*I (New England Biolabs). PCR for insert screening of colonies was performed with M13 forward and reverse sequences as primers. PCR products larger than 600 bp were sequenced with the BigDye terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed with an ABI377 automated DNA sequencer (Applied Biosystems).

2.3. Data Analysis

Concatamer sequences were analyzed with SAGE 2000 software version 4.12, which was kindly provided by Dr. K. Kinzler (Johns Hopkins University). Expressed genes were searched at the Serial Analysis of Gene Expression Tag to Gene Mapping home page (http://www.ncbi.nlm.nih.gov/SAGE) to identify individual genes. Detailed SAGE protocols can be seen at the SAGE home page (http://www.sagenet.org).

2.4. Quantitative Real-Time Reverse Transcription–PCR

Total RNA was prepared with an RNeasy kit (Qiagen, Hilden, Germany) and used for cDNA synthesis with an oligo(dT) primer (Amersham Biosciences, Piscataway, NJ, USA). For detection and quantification of mRNA levels for eukaryotic translation initiation factor 1A (*EIF1A*) and fatty acid desaturase 1 (*FADS1*), the LightCycler (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions. For relative quantification, the mRNA expression of the glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as an external control. Reverse transcription–PCR (RT-PCR) reactions were performed with the QuantiTect SYBR Green PCR Kit (Qiagen). PCR was performed in a LightCycler with a 900-second preincubation at 95°C followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C (*EIF1A*) or 60°C (GAPDH and FADS1), and 20 seconds at 72°C. The following primers were used: GAPDH-specific forward primer, 5′-GAAGGTGAAGGTCGGAGTC-3′; GAPDH-specific reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′; FADS1-specific forward primer, 5′-GTCTTCTTCCTG CTGTACCTG-3′; FADS1-specific reverse primer, 5′-GGT TCCACTTTGAGGTGCTGA-3′; EIF1A-specific forward primer, 5′-ATGGACGGCTAGAAGCAATGT-3′; and EIF1A-specific reverse primer, 5′-CTTCTAGCTTCGTCT GCATTG-3′. Analyses of the quantitative real-time PCR curves were determined by LightCycler 3.5 software.

3. Results

3.1. Generation and Analysis of SAGE Tags

The doxorubicin (Adriamycin)-resistant variant of K562 (K562/ADM) was stable for several months in medium without doxorubicin [11]. K562/ADM cells also showed resistance to daunorubicin, although there was no difference in the proliferative activities of K562 and K562/ADM cells in the absence of anthracyclines (data not shown).

SAGE libraries were constructed from K562 and K562/ADM cells. Using 9792 tags (5055 from K562 cells and 4737 from K562/ADM cells), we identified 1076 different tran-

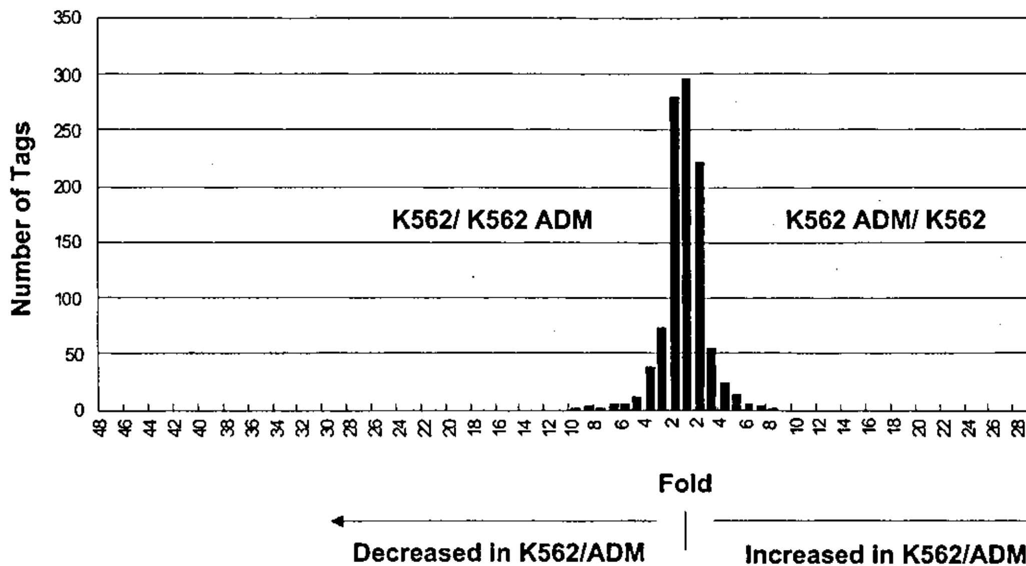

Figure 1. Comparison of gene expression frequency in K562 and K562/ADM cells. Relative expression of each transcript was determined by dividing the number of tags observed in K562 or K562/ADM cells as indicated. To avoid division by zero, we used a tag value of 1 for any tag that was not detected in a given sample. Ratios are plotted on the x-axis, and the number of genes displaying each ratio is plotted on the y-axis.

scripts appearing more than twice. Of these transcripts, 780 genes exhibited altered levels of expression in K562/ADM cells compared with K562 parental cells (Figure 1). Tables 1 and 2 show the 30 most actively expressed transcripts in K562 and K562/ADM cells, respectively. The authors will provide the other transcript profiles if they are requested. The most frequently expressed genes in K562 were the enhancer of invasion 10 gene (*HEI10*) with an expression frequency of 2.08%, followed by the genes for ribosomal protein L41 (1.42%) and ribosomal protein L35 (1.27%) (Table 1). On the other hand, the most actively expressed genes in K562/ADM were the SET translocation gene (2.28%), followed by *HEI10* (1.90%) and the gene for heterogeneous nuclear ribonucleoprotein A1 (1.56%) (Table 2). Both K562 and K562/ADM cells showed high expression levels of numerous genes encoding ribosomal proteins.

3.2. Identification of Transcripts Differentially Expressed in K562/ADM Cells

Tables 3 and 4 list the tags differentially expressed in K562/ADM cells at levels more than 8-fold higher and lower, respectively, than in K562 cells. The most highly differentially expressed gene in K562/ADM cells was the functionally unknown gene similar to the hypothetical protein KIAA0324 (Table 3). K562/ADM cells also showed increased expression of well-characterized genes, including those for spectrin β, eukaryotic translation initiation factor 1A, RAD23 homolog B, laminin receptor 1, and polyA-, RAN-, and PAI-1 mRNA-binding proteins. K562/ADM cells showed decreased expression of the genes for fatty acid desaturase 1, hemoglobin ε 1, *N*-myristoyltransferase 1, hemoglobin α 2, NADH dehydrogenase Fe-S protein 6, heat shock 90-kDa protein, and karyopherin β1.

3.3. Confirmation of Differential Gene Expression by Quantitative Real-Time RT-PCR

To confirm the differential gene expression profiles obtained by SAGE, we performed quantitative real-time RT-PCR analyses for *EIF1A* and *FADS1*. We also compared the expression of these 2 genes in K562/ADM cells and in another drug-resistant K562 subline (K/eto), which is resistant to etoposide [14]. As was anticipated by the SAGE data, quantitative real-time RT-PCR analysis revealed that K562/ADM cells expressed *EIF1A* more and *FADS1* less than K562 cells (Figure 2). Etoposide-resistant K/eto cells also showed decreased expression of *FADS1* compared with the parental K562 cells, whereas the expression of *EIF1A* in K/eto cells was lower than in K562 cells, in contrast to the expression of this gene in K562/ADM cells.

4. Discussion

Techniques that identify differentially expressed genes can provide insights into cell biology. Several methods developed for identifying differentially expressed genes in mammalian cells, including subtractive hybridization, differential display, and cDNA microarray analysis, have made great contributions to our understanding of the mechanisms of drug resistance in cancer. However, global quantitative analysis of gene expression is difficult with these methods. In contrast to these methods, SAGE allows us to quantitatively analyze large numbers of transcripts without requiring known probes [1].

With regard to the distinct gene expression patterns of K562 and K562/ADM cells, unexpected gene profiles were observed. Among the genes differentially expressed in K562

Table 1.
Transcript Profiles in K562 Cells*

Tag	Abundance, %	Unigene Cluster, *Hs* No.	Matched Gene
CCACTGCACT	2.08	107003	Enhancer of invasion 10
TTGGTCCTCT	1.42	520738	Ribosomal protein L41
CGCCGCCGGC	1.27	182825	Ribosomal protein L35
CCTGTAATCC	1.11	181874	Interferon-induced protein with tetratricopeptide repeats 4
CCGGGCGTGG	0.95	503564	Fatty acid desaturase 1
GTGAAACCCC	0.81	323949	Kangai 1; CD82 antigen
CCAAACGTGT	0.61	181307	H3 histone, family 3A
AGCACCTCCA	0.59	75309	Eukaryotic translation elongation factor 2
CTGTTGATTG	0.59	356721	Heterogeneous nuclear ribonucleoprotein A1
CCAGAACAGA	0.57	400295	Ribosomal protein L30
GTGAAACCCT	0.53	25204	Carbohydrate sulfotransferase 12
		291000	DKFZp761G058 hypothetical protein
		511820	Carboxypeptidase M
CGCTGGTTCC	0.44	388664	Ribosomal protein L11
		289019	Latent transforming growth factor β-binding protein 3
GGGACTGGGC	0.4	117848	Hemoglobin, ε 1
CCATTGCACT	0.38	194382	Solute carrier family 2 (facilitated glucose transporter), member 6
		348603	UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 7
GAGGGAGTTT	0.38	356342	Ribosomal protein L27a
TTACCATATC	0.38	300141	Ribosomal protein L39
CTGTTGGTGA	0.34	386384	Ribosomal protein S23
GGGCTGGGGT	0.34	430207	Ribosomal protein L29
TGCACGTTTT	0.34	265174	Ribosomal protein L32
AGCTCTCCCT	0.32	374588	Ribosomal protein L17
ATGCAGAGCT	0.32	302145	Hemoglobin, γ G
TTCATACACC	0.32		No match
AGGCTACGGA	0.3	449070	Ribosomal protein L13a
		23270	DKFZP566F2124 protein
GTGGCGGGCG	0.3	22926	KIAA0795 protein
		269347	Sialic acid-binding Ig-like lectin 11
		436680	Potassium voltage-gated channel, subfamily H (eag-related), member 6
		512794	Hypothetical protein FLJ14457
TATCTGTCTA	0.3	436687	SET translocation (myeloid leukemia associated)
AACCCGGGAG	0.28	418291	Interleukin 10 receptor, β
		335157	KIAA0408 gene product
ACATCATCGA	0.28	408054	Ribosomal protein L12
CCACTGTACT	0.28	287515	Hypothetical protein FLJ12331
		138453	Chromosome Y open-reading frame 14
TTGAGCCAGC	0.28	91142	KH-type splicing regulatory protein
AGGAAAGCTG	0.26	408018	Ribosomal protein L36

*The top 30 transcripts expressed in K562 cells are listed. The tag sequence represents the 10–base pair SAGE tag. Probable Unigene cluster matches are listed with the numbers for *Homo sapiens* (*Hs*).

and K562/ADM cells, the most actively expressed gene in K562/ADM cells (29-fold higher than in K562 cells) is a functionally unknown gene with a weak similarity to the hypothetical protein KIAA0324. Increased expression of *EIF1A*, *PABPC1* (polyA-binding protein cytoplasmic 1), and *PAI-RBP1* (PAI-1 mRNA–binding protein) suggests that protein synthesis may be active in doxorubicin-resistant K562 cells. RAD23 homolog B is a component of the XPC-hHR23B complex in humans that rapidly binds to damaged DNA and thus confers strong discrimination to the damaged DNA site [15]. The XPC-RAD23B complex serves as the earliest damage detector to initiate nucleotide excision repair [16,17]. The high expression of this gene may be the result of a process of selection against doxorubicin-induced DNA damage [18]. The laminin receptor 1 (*LAMR1*) gene encodes a receptor for laminin, and high expression of this gene is correlated with the invasive phenotype of human colorectal cancer [19]. Damiano et al recently reported the role of cell adhesion molecules in mediated drug resistance [20]. Moreover, the human 37-kDa laminin receptor precursor has also been reported to be involved in multidrug resistance [21].

FADS1 was the gene exhibiting the largest decrease in expression in K562/ADM cells. Fatty acid desaturase 1 is identical to human Δ5-desaturase [22], and its activity in hematopoietic and lymphoid cells is increased by the induction of differentiation [23]. The expression levels of several globin genes, including those for hemoglobin ε 1 and hemoglobin α 2, were also decreased in K562/ADM cells, suggesting that a dedifferentiation change may occur in the process of acquiring drug resistance. Decreased expression of the genes related to mitochondrial electron transport and protein metabolism, including the genes for NADH dehydroge-

Table 2.
Transcript Profiles in K562/ADM Cells*

Tag	Abundance, %	Unigene Cluster, *Hs* No.	Matched Gene
TATCTGTCTA	2.28	436687	SET translocation (myeloid leukemia associated)
CCACTGCACT	1.90	107003	Enhancer of invasion 10
CTGTTGATTG	1.56	356721	Heterogeneous nuclear ribonucleoprotein A1
GGGTGCAAAA	1.27	356721	Heterogeneous nuclear ribonucleoprotein A1
GAGGGAGTTT	1.06	356342	Ribosomal protein L27a
GCTTTCTCAC	1.03	131059	Gene trap ankyrin repeat
CCAAACGTGT	0.91	181307	H3 histone, family 3A
GGATTTGGCC	0.74	437594	Ribosomal protein, large P2
		9711	HSNOV1 novel protein
		259326	Cell cycle progression 8 protein
GATTTTGTAG	0.63	459987	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B
GGTTGAAAAA	0.61	446545	*Homo sapiens* transcribed sequence with weak similarity to protein pir:T02345 T02345 hypothetical protein KIAA0324
TGCACGTTTT	0.59	265174	Ribosomal protein L32
CAATAAATGT	0.57	80545	Ribosomal protein L37
GTGAAACCCC	0.55	323949	Kangai 1; CD82 antigen
AGGCTACGGA	0.53	449070	Ribosomal protein L13a
		23270	DKFZP566F2124 protein
CTGGGTTAAT	0.53	381184	Ribosomal protein S19
ACTGGGGAAT	0.51	24763	RAN-binding protein 1
CCTGTAATCC	0.44	181874	Interferon-induced protein with tetratricopeptide repeats 4
CCAGAACAGA	0.42	400295	Ribosomal protein L30
TCTCCATACC	0.42	374491	Proliferation-associated 2G4, 38 kDa
		387474	*Homo sapiens* transcribed sequences
AATAGGTCCA	0.4	512676	Ribosomal protein S25
AGGCGAGATC	0.4	233952	Proteasome (prosome, macropain) subunit, α type, 7 (PSMA7)
		195464	Filamin A, α (actin-binding protein 280)
CCATTGCACT	0.4	244378	Solute carrier family 2 (facilitated glucose transporter), member 6 (SLC2A6)
		411308	UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 7
GGCAGCACAA	0.4	371181	*Homo sapiens* cDNA FLJ43979 fis, clone TESTI4018751
		386834	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide
		388584	Serologically defined colon cancer antigen 1
GTATTCCCCT	0.4	117176	PolyA-binding protein, nuclear 1
GTAAGTGTAC	0.38	205401	Spectrin, β, nonerythrocytic 1
TGGGCTTCCT	0.38	443711	Ankyrin 1, erythrocytic
TACCAGCACA	0.36	119908	Nucleolar protein NOP5/NOP58
TTCATACACC	0.36		No match
TTGGTCCTCT	0.36	520738	Ribosomal protein L41
GAGAGCTCCC	0.34	14927	Hypothetical protein FLJ39582

*The top 30 transcripts expressed in K562/ADM cells are listed. The tag sequence represents the 10–base pair SAGE tag. Probable Unigene cluster matches are listed with the numbers for *Homo sapiens* (*Hs*).

Table 3.
Transcripts Increased in K562/ADM Cells*

Tag	Abundance, K562	Abundance, K562/ADM	Fold Increase	Unigene Cluster	Matched Gene
GGTTGAAAAA	1	29	29	446545	*Homo sapiens* transcribed sequence with weak similarity to protein pir:T02345 T02345 hypothetical protein KIAA0324
GGCAGCACAA	0	19	19	371181	*Homo sapiens* cDNA FLJ43979 fis, clone TESTI4018751
				386834	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase
				388584	Activation protein, ζ polypeptide, serologically defined colon cancer antigen 1
GTAAGTGTAC	0	18	18	205401	Spectrin, β, nonerythrocytic 1
TTCTTTTCAT	1	15	15	147824	Eukaryotic translation initiation factor 1A
CCCGGCTCTT	0	14	14	93354	Platelet-activating factor acetylhydrolase, isoform Ib, β subunit, 30 kDa
ATTTGAGAAG	0	11	11	159087	RAD23 homolog B
AAAAGAAACT	1	9	9	172182	PolyA-binding protein, cytoplasmic 1
ACTGGGGAAT	3	24	8	24763	RAN-binding protein 1
CTTAAGGATT	1	8	8	356427	PAI-1 mRNA–binding protein
GAAAAATGGT	1	8	8	374553	Laminin receptor 1

*The tag sequence represents the 10–base pair SAGE tag. Unigene cluster matches are listed.