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LETTER TO THE EDITOR
Neonatal Onset Hemophagocytic Lymphohistiocytosis in a Premature Infant

To The Editor: We read with interest the report by Tanoshima et al. [1] on familial haemophagocytic lymphohistiocytosis (HLH). We saw HLH in a male infant born with a weight of 1,728 g at gestational age 34 weeks. Enlarged liver (2 cm), spleen (5 cm), and fever (38.0°C) developed 2 weeks after birth; the WBC was 4.300×10^9/L (neutrophils 1,840/μl, lymphocytes 480/μl), Hb 7.7g/dl, platelets 34×10^9/L, and C-reactive protein concentration 2.0 mg/dl. Perinatal infections, including cytomegalovirus, Epstein-Barr virus, and herpes simplex virus, were excluded. There was no evidence indicating an inborn error of metabolism. At age 3 weeks, serum ferritin (63,900 mg/dl), AST (701 IU/L), and ALT (239 IU/L) values increased. Bone marrow studies showed no hemophagocytosis, while massive pleural effusion (100 ml) contained hemophagocytosing macrophages. Flow cytometric analysis of peripheral blood lymphocytes showed 96.7% $CD3^+$ cells, 0.5% $CD56^+$ cells, 2.8% $CD19^+$ cells and with a CD4/CD8 ratio of 12:1.The absolute T cell count was 505/μl, including 466/μl $CD4^+CD8^-$ cells and 39/μl $CD4^-CD8^+$ cells. γδT cells (0.0%) or $CD4^-CD8^-\alpha\beta$T cells (0.6%) were not increased in the peripheral blood. Circulating $CD8^+$T cells (7.6%) or $CD56^+$ cells (0.5%) were few but positive for intracellular perforin staining. There were no mutations in the coding sequences of *PRF1*. When the Western blotting was performed using platelets of the patient because of the insufficient number of collected mononuclear cells, Munc13-4, syntaxin 11 and Rab27 were normally expressed [2]. Peripheral blood lymphocyte counts increased to 1,100/μl after high dose steroid and cyclosporine A (CsA) therapy. The patient then suffered from clonic convulsions due to cerebral hemorrhages. Head MRI indicated intraparenchymal bleeding, but no specific lesions in the white matter or basal ganglia. The bloody cerebrospinal fluids were not evaluable for the presence of pleocytosis or hemophagocytosis. During the following year, the patient experienced six episodes of HLH. High dose steroid and CsA therapy led to partial remission and reduced pleural effusion. Etoposide was not used for the poor condition of premature infant. The dose escalation of CsA became necessary for disease control and generalized seizures frequently occurred at 16 months of age. Despite the intensive treatment with antiviral agents, γ-globulin, steroids and CsA, the patient died of multiple organ failure at 24 months after birth.

Herpes virus infections are the major cause of HLH in infants less than 1 year of age [3]. HLH is occasionally associated with primary immunodeficiency diseases or inherited metabolic diseases, although the pathological mechanism remains elusive [4]. FHL is mostly diagnosed within 2 years of age, but onset in the newborn period is very rare [5,6]. This observation may be in part explained by less frequent infections during the neonatal period because viral infection triggers uncontrolled activation of $CD8^+$T cells and hypercytokinemia in FHL patients. The current patient might carry an unidentified genetic defect of FHL [7]. Alternatively, the decreased number of circulating cytotoxic lymphocytes in this patient could have reflected an underlying primary immunodeficiency disease associated with HLH.

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Characteristics of Hemophagocytic Lymphohistiocytosis in Neonates: A Nationwide Survey in Japan

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Objective To assess the etiology, prognosis, and appropriate treatment of hemophagocytic lymphohistiocytosis (HLH) in neonates.

Study design We collected information on neonates in whom HLH was diagnosed between 1997 and 2007 from participating members of the Japanese Society of Pediatric Hematology.

Results HLH was diagnosed in 20 patients within 4 weeks after birth. Of the diagnostic criteria for HLH-2004, the incidence of fever was quite low in preterm infants, and hypertriglyceridemia and neutropenia were uncommon. Familial HLH (n = 6) or severe combined immunodeficiency-associated HLH (n = 1) was diagnosed in 7 patients, and 2 of them have survived. Herpes simplex virus-associated HLH was diagnosed in 6 patients, and 2 of them have survived. The overall survival rate for the 20 patients was 40%.

Conclusions HLH is rare in neonates and has a poor prognosis. Early diagnosis and immediate treatment are required when considering the possibility of herpes simplex virus-associated or familial HLH. *(J Pediatr 2009;155:235-8).*

Hemophagocytic lymphohistiocytosis (HLH) comprises a heterogeneous class of often fatal disorders characterized by activation and disregulation of T-cells and macrophages.[1] The central role played by CD8 T cell activation, interferon overproduction, and macrophage activation in the pathophysiology of the disease was demonstrated in perforin-deficient mice.[2] HLH encompasses several entities, including a primary form that may be familial HLH, with an estimated incidence of 1 in 50 000 births,[3] and a secondary form associated with infections, malignancies, and rheumatologic disorders.[4]

Neonatal HLH with an onset within 4 weeks after birth is rare, and the diagnosis is frequently delayed, made only at autopsy or missed completely. Speculating that neonatal HLH might differ from HLH in older children in etiology, manifestations, or laboratory findings, we conducted this nationwide Japanese survey of cases of neonatal HLH to clarify the etiology and prognosis. On the basis of the findings, we propose an appropriate strategy for diagnosis and treatment.

Methods

Clinical and laboratory findings at the onset, treatment, and outcome were analyzed in neonates in whom HLH was diagnosed at ≤4 weeks after birth between 1997 and 2007. Data were collected from participating members of the Japanese Society of Pediatric Hematology. The diagnosis of HLH was mainly based on the HLH-2004 criteria (**Table I**).[5] Patients whose laboratory data did not fulfill the HLH-2004 criteria were also included when they had a family history of HLH or hemophagocytosis in the peripheral blood.[6] All aspects of this investigation were approved by the appropriate institutional review boards.

Results

HLH was diagnosed in 20 neonates (10 male, 10 female; **Table II**). The day of onset was from 0 to 28 days after birth (median, 6.5 days); HLH was diagnosed in 6 patients at birth. Eight patients were preterm, <37 weeks gestational age.

Clinical and biological findings are summarized in **Figure 1**. Fever was noted in only 1 of the 8 preterm infants (12.5%), compared with 10 of the 12 term infants (83.3%). Hypertriglyceridemia and neutropenia were

β2-MG	beta-2-microglobulin
CSF	Cerebrospinal fluid
HLH	Hemophagocytic lymphohistiocytosis
HSV	Herpes simplex virus
LDH	Lactate dehydrogenase
SCID	Severe combined immunodeficiency
sIL-2R	Soluble interleukin-2 receptor

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*A list of hospitals that enrolled patient(s) in this study appears in the **Appendix** (available at www.jpeds.com).

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Table I. Diagnostic guidelines for hemophagocytic lymphohistiocytosis

The diagnosis of HLH can be established when 1 of either 1 or 2 below is fulfilled:
1) A molecular diagnosis consistent with HLH
2) Diagnostic criteria for HLH fulfilled (5 of the 8 criteria below)
Fever
Splenomegaly
Cytopenias (affecting ≥ 2 of 3 lineages in the peripheral blood):
  Hemoglobin <100 g/L
  Platelets <100 x 10^9/L
  Neutrophils <1.0 x 10^9/L
Hypertriglyceridemia and/or hypofibrinogenemia:
  Fasting triglycerides ≥3.0 mmol/L (ie, ≥265 mg/dL)
  Fibrinogen ≤1.5 g/L
Hemophagocytosis in bone marrow or spleen or lymph nodes.
  No evidence of malignancy
Low or absent NK-cell activity (according to local laboratory reference)
Ferritin ≥500 microgram/L
Soluble CD25 (ie, soluble IL-2 receptor ≥ 2400 U/mL)

infrequent. Respiratory distress was noted in 15 patients at birth, including all 8 preterm infants. Nineteen patients had an elevated lactate dehydrogenase (LDH) level. The urinary beta-2-microglobulin (β2-MG) level was elevated to >1 000 μg/L (range, 3.33-96 630 μg/L; median, 3 900 μg/L) in 8 of the 11 patients examined (72.7%). Of the 6 patients who had the disease at birth, fetal distress had been diagnosed in 4 before delivery. Hydrops fetalis was present in 1 patient (patient 8). Two patients had skin manifestations, erythema in patient 4 and a dusky-red papular rash in patient 19. Abnormal results on magnetic resonance imaging of the brain were recorded in 3 patients with clinical manifestations of impaired consciousness (patient 6), seizure (patient 11), or ocular deviation (patient 9). The cerebrospinal fluid (CSF) was examined in 10 patients, and results were abnormal in 2 cases, with a polymorphonuclear cell dominant pleocytosis in patient 6 and a raised protein level but a normal white blood cell count and impaired consciousness in patient 8.

Six patients (30%) were considered to have familial HLH. Severe combined immunodeficiency (SCID)-associated HLH was diagnosed in 1 patient. Herpes simplex virus (HSV)-associated HLH was noted in 6 patients (30%). The other 7 patients had HLH associated with cytomegalovirus, coxsackievirus, methicillin-resistant *Staphylococcus aureus*, or cause unknown.

Patients were treated with corticosteroid (n = 17, 85%), intravenous gamma globulin (n = 15, 75%), exchange transfusion/plasma exchange (n = 12, 60%), and cyclosporine, etoposide, or acyclovir (n = 8-9, 40%-45%; **Table II**). Three patients with familial HLH-3 (UNC13D deficiency), SCID-HLH, and unknown cause-HLH underwent subsequent hematopoetic stem cell transplantation.

Eight of the 20 patients survived (40%). Of the 7 patients with familial HLH or SCID-HLH, only 2 (28.6%) have survived (**Figure 2**), and of 6 patients with HSV-HLH, only 2 (33.3%) survived, despite receiving acyclovir. No significant statistical differences were found between the 8 survivors and the 12 non-survivors in their laboratory findings, including aspartate aminotransferase, LDH, ferritin, soluble IL-2 receptor (sIL-2R), and fibrinogen (data not shown).

Table II. Patient characteristics

n	Sex	GA (weeks)	Onset (days after birth)	Matched numbers of HLH-2004 diagnostic criteria	Type of HLH	Therapy	Outcome
1[21]	M	23	17	5/6	CMV	γ, S	Dead 73d
2	F	27	0	4/5	FHL*	γ, S	Dead 2 m 18d
3	F	29	7	2/5	HSV	γ	Dead 10d
4	M	30	0	6/7	FHL*	S, C, Et	Alive 7m+ (with disease)
5[22]	M	31	0	6/8	FHL*	S	Dead 42d
6	F	33	11	5/8	COX	E	Alive 1y 9m+
7	F	36	0	5/7	unknown	γ, S, E, HSCT	Alive 9y 4 m
8[23]	M	36	0	6/8	FHL-3	γ, S, C, Et, P, CA	Dead 114d
9	F	37	14	7/8	unknown	γ, S, Et, ACV	Alive 5y 11m+
10	F	38	5	7/8	HSV	γ, S, C, Et, ACV	Dead 27d
11	M	39	3	6/8	HSV	γ, S, C, Et, P, ACV	Dead 61d
12	F	39	0	6/8	unknown	γ, S, C, Et, E	Dead 35d
13[24]	M	39	4	5/7	HSV	γ, S, C, E, ACV	Alive 1y 10m+
14	F	39	28	6/7	unknown	S, Et, E	Dead 38d
15	M	39	8	5/7	HSV	γ, S, C, E, ACV	Alive 2y 6m+
16	M	39	24	7/7	FHL-3	γ, S, C, HSCT	Alive 1y 6m+
17	M	39	17	5/6	FHL-2	γ, S, E, ACV	Dead 29d
18	F	40	6	6/6	HSV	γ, S, E, ACV	Dead 29d
19	F	41	19	5/8	SCID	γ, S, C, Et, E, ACV, HSCT	Dead 2 m 27d
20	M	41	17	5/8	MRSA	antibiotics	Alive 7y 7m+

M, Male; *F*, female; *CMV*, cytomegalovirus; *FHL*, Familial hemophagocytic lymphohistiocytosis; *HSV*, herpes simplex virus; *COX*, coxsackievirus; *SCID*, severe combined immunodeficiency disease; *MRSA*, methicillin-resistant *Staphylococcus aureus*; γ, gamma globulin; *S*, corticosteroid; *C*, cyclosporine; *Et*, etoposide; *E*, exchange transfusion; *HSCT*, hematopoietic stem cell transplantation; *P*, plasma exchange; *CA*, cytosine arabinoside; *ACV*, acyclovir; *d*, days; *m*, month(s); *y*, year(s).
*FHL of unknown genetic defects with positive family history.

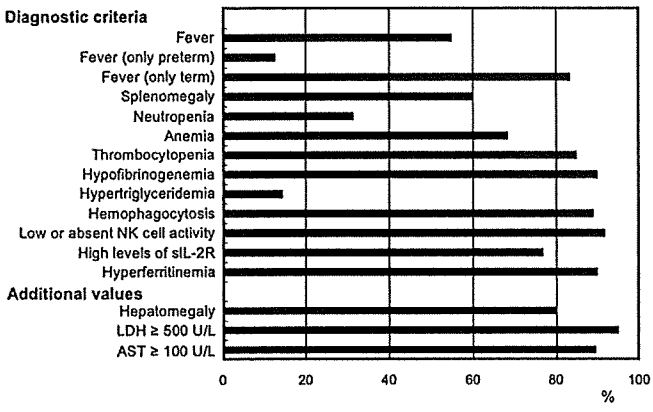

Figure 1. Incidence of clinical and biochemical findings of 20 patients with neonatal hemophagocytic lymphohistiocytosis. Of the usual diagnostic features, neutropenia and hypertriglyceridemia were low, and the incidence of fever was low in the preterm neonates.

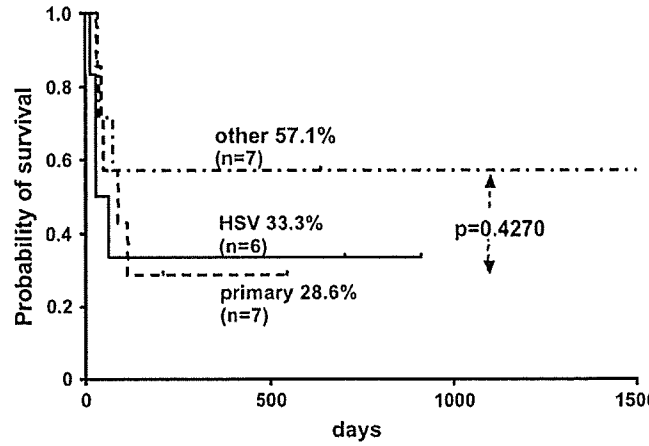

Figure 2. Outcome of patients with HLH according to the etiology. The 5-year overall survival rate of primary HLH (familial and severe combined immunodeficiency-associated HLH) and HSV-associated HLH was poor compared with that of other HLH, although no significant difference was observed in the 3 groups.

Discussion

Most reports of HLH in neonates have been included in childhood HLH studies.[7,8] Consequently, the characteristics of neonatal HLH have not been well defined. In a recent study of infantile HLH, the 9 cases of neonatal HLH had a poor prognosis, 2 of the 9 cases with familial HLH developed disease at birth and 7 patients with HSV-HLH or enterovirus-associated HLH presented between 4 and 13 days after birth.[9] Although HSV and enterovirus are uncommon pathogens in HLH in older children, they have been reported as causes of severe HLH in neonates.[6,10-12] In this study, 30% of the cases were diagnosed as HSV-HLH, with a poor prognosis.

The onset of familial HLH occurs at <1 year of age in 70% to 80% of the cases, and only approximately 10% of patients had symptoms within the neonatal period.[13] However, our study and an earlier report revealed that at least 30% of neonatal HLH could be classified as familial HLH.[9] In most cases of familial HLH, immunochemotherapy can temporarily control the disease, but the disease is eventually fatal unless hematopoetic stem cell transplantation is performed.[14] Considering that at least 60% of neonatal HLH is HSV-HLH or familial HLH, the appropriate treatment is high-dose acyclovir[11] followed by immunotherapy started immediately after making the diagnosis and surveying for pathogens.

Because fever seldom developed in the preterm infants in this study, HLH enters the differential diagnosis in preterm infants even when fever is absent. Most older children with HLH have hypertriglyceridemia,[15] which was found in only 14.3% of our patients. This difference may reflect the different metabolism of lipids in neonates compared with older patients.[16]

Neonatal hemochromatosis is sometimes indistinguishable from neonatal HLH. A ferritin level exceeding 10 000 mg/L has a high specificity and sensitivity for HLH,[17] and fever, cytopenias, and hypertriglyceridemia are not observed in patients with neonatal hemochromatosis.[18,19] However, these findings are not always useful for differentiating the 2 conditions in neonates. Evaluation of hemophagocytosis, natural killer cell activation, and sIL-2R may help. Urinary β2-MG is also a useful marker for HLH.[20]

In conclusion, the incidence of clinical and laboratory findings of neonatal HLH differ from those of childhood HLH. Neonates with hepatomegaly, thrombocytopenia, and elevated LDH levels should be further examined to exclude HLH. Once HLH is suspected, viral studies, levels of urinary β2-MG, sIL-2R, and natural killer cell activity, and an examination for hemophagocytosis in the bone marrow should be performed. Simultaneously, prompt treatment with combined high-dose acyclovir, gamma globulin, and cyclosporine with or without corticosteroid should be instituted. Etoposide should be used immediately after recognizing inadequate control of the disease by the initial immunotherapy. In familial cases, subsequent pregnancies and offspring should be closely monitored. ■

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Appendix

List of hospitals that enrolled patient(s) in this study: Asahikawa-Kosei general Hospital; Chiba University Hospital; Dokkyo Medical University Hospital; Ehime University Hospital; Gifu Prefectural General Medical Center; Gunma Children's Medical Center; Hiroshima University Hospital; Jichi Medical University Hospital; Kanagawa Children's Medical Center; University Hospital, Kyoto Prefectural University of Medicine; Nippon Medical School Tama Nagayama Hospital; Sapporo Medical University Hospital; Tsukuba University Hospital; Wakayama Medical University Hospital; Yamagata University Hospital.

Aurora-A kinase: a novel target of cellular immunotherapy for leukemia

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Aurora-A kinase (Aur-A) is a member of the serine/threonine kinase family that regulates the cell division process, and has recently been implicated in tumorigenesis. In this study, we identified an antigenic 9–amino-acid epitope (Aur-$A_{207-215}$: YLILEYAPL) derived from Aur-A capable of generating leukemia-reactive cytotoxic T lymphocytes (CTLs) in the context of HLA-A*0201. The synthetic peptide of this epitope appeared to be capable of binding to HLA-A*2402 as well as HLA-A*0201 molecules. Leukemia cell lines and freshly isolated leukemia cells, particularly chronic myelogenous leukemia (CML) cells, appeared to express Aur-A abundantly. Aur-A–specific CTLs were able to lyse human leukemia cell lines and freshly isolated leukemia cells, but not normal cells, in an HLA-A*0201–restricted manner. Importantly, Aur-A–specific CTLs were able to lyse CD34+ CML progenitor cells but did not show any cytotoxicity against normal CD34+ hematopoietic stem cells. The tetramer assay revealed that the Aur-$A_{207-215}$ epitope–specific CTL precursors are present in peripheral blood of HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia, but not in healthy individuals. Our results indicate that cellular immunotherapy targeting Aur-A is a promising strategy for treatment of leukemia. (Blood. 2009;113: 66-74)

Introduction

Cellular immunotherapy for malignancies targeting various tumor-associated antigens has been developed.[1,2] Recently, some attractive target antigens recognized by leukemia-reactive cytotoxic T lymphocytes (CTLs), such as WT1 and PR1, have been discovered and phase 1/2 clinical studies of cancer immunotherapy targeting these antigens have been conducted; however, the clinical response against hematologic malignancies remains unsatisfactory.[3,4] To establish effective cancer immunotherapy, identification of target antigens that are recognized efficiently by tumor-specific CTLs is necessary. Antigens that can serve as ideal targets recognizable by tumor-specific CTLs need to have several essential characteristics. First, their expression should be limited to, or abundant in, tumor cells rather than normal cells. Second, the antigens should be efficiently processed in tumor cells and expressed on the cell surface in context with common HLA molecules. Third, target antigens should play an important role in tumorigenesis and/or progression of malignancies, because their expression is essential for tumor survival.

Aurora-A kinase (Aur-A) is a member of the serine/threonine kinase family, and the *Aur-A* gene is located at chromosome 20q13, a region frequently amplified in breast cancer.[5] Aur-A is mainly expressed in the G_2/M phase of the cell cycle and regulates mitotic cell division in normal cells.[6-8] Among normal tissues, Aur-A is expressed exclusively in testis, but in various kinds of cancer it is aberrantly overexpressed, and associated with poor prognosis.[9-14] Overexpression of Aur-A determined by amplification of *Aur-A* mRNA has also been widely observed in hematologic malignancies.[15-19] Aur-A overexpression has been linked with centrosome amplification, aneuploidy, and chromosome instability.[20,21] Furthermore, ectopic overexpression of Aur-A efficiently transforms immortalized rodent fibroblasts.[9,20] These data strongly suggest that *Aur-A* is one of the fundamental cancer-associated genes and a potential target for cancer treatment. In addition, previous reports have demonstrated that silencing of the gene encoding Aur-A in cancer cells results in inhibition of their growth and enhancement of the cytotoxic effect of anticancer agents.[22] Therefore the development of small molecules with an Aur-A–inhibitory function may make it possible to reduce or block the oncogenic activity of Aur-A. On the basis of this concept, clinical studies using Aur-A inhibitors for cancer treatment are now under way; however, their clinical efficacy is still unknown.[23-27] The biologic characteristics of Aur-A mentioned above suggest that it is an ideal target for tumor-specific CTLs, and that cancer immunotherapy targeting Aur-A could be feasible. In this study, therefore, we attempted to verify the feasibility of cellular immunotherapy for leukemia targeting Aur-A.

Methods

Synthetic peptides

Candidate peptides derived from Aur-A with high binding affinity for the HLA-A*0201 or HLA-A*2402 molecule were predicted algorithmically by the BIMAS program (http://www-bimas.cit.nih.gov/molbio/hla_bind/). On the basis of these data, peptides with favorable binding affinity for the HLA-A*0201 or HLA-A*2402 molecule were selected and synthesized (Thermo Electron; Greiner Bio-One, Tokyo, Japan). Amino acid sequences

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Table 1. Binding affinities of synthetic peptides

HLA	Position	Length, mer	Sequence	Score	Fluorescence index
A*0201	Aur-$A_{271-279}$	9	KIADFGWSV	3911	0.93
A*0201	Aur-A_{63-71}	9	KLVSSHKPV	243	0.23
A*0201	Aur-$A_{207-215}$	9	YLILEYAPL	147	1.47
A*0201	$WT1_{7-15}$	9	DLNALLPAV	12	0.06
A*0201	$CMVpp65_{495-503}$	9	NLVPMVATV	160	1.71
A*2402	Aur-$A_{207-215}$	9	YLILEYAPL	6	0.99
A*2402	$WT1_{7-15}$	9	DLNALLPAV	0.18	0.02
A*2402	$WT1_{235-243}Y$	9	CYTWNQMNL	200	4.5

The binding affinities of synthetic peptides for HLA molecules were predicted by computer algorithms available on the National Institutes of Health BIMAS website (http://www-bimas.cit.nih.gov/molbio/hla_bind). The binding affinities of synthetic peptides for HLA molecules were evaluated by MHC stabilization assay as detailed in "HLA peptide–binding assay."

of the peptides used in this study are listed in Table 1. All the peptides were synthesized with a purity exceeding 80%.

HLA peptide–binding assay

Binding affinity of peptides for the HLA-A*0201 or HLA-A*2402 molecule was assessed by an HLA-A*0201 or HLA-A*2402 stabilization assay as described previously.[28,29] Briefly, the HLA-A*0201–positive cell line (T2) or the *HLA-A*2402* gene–transfected T2 cell line (T2-A24) was plated in 24-well plates at 10^6 cells per well and incubated overnight with the candidate peptides at a concentration of 10 μM in serum-free RPMI 1640 medium. The T2 and T2-A24 cells were washed twice with phosphate-buffered saline (PBS), and then incubated with fluorescein isothiocyanate (FITC)–conjugated anti–HLA-A2 or HLA-A24 monoclonal antibody (MoAb; One Lambda, Canoga Park, CA) at 4°C for 20 minutes. The cells were washed and suspended in 1 mL PBS and analyzed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). Measurement of mean fluorescence intensity and analysis of data were done with CellQuest Software (Becton Dickinson). The fluorescence index (FI) was calculated as FI = (sample mean − background mean) / background mean.

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki.

B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus. LCLs, T2, T2-A24, and leukemia cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The *HLA-A*0201* gene–transfected C1R cell line (C1R-A*0201; kindly provided by Dr A. John Barrett, National Heart, Lung, and Blood Institute [NHLBI], Bethesda, MD) was cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) from leukemia patients and healthy volunteers, and cord blood mononuclear cells (CBMCs) from healthy donors were isolated and stored in liquid nitrogen until use. All leukemia samples contained more than 95% leukemia cells. $CD34^+$ cells from BMMCs and CBMCs were isolated using $CD34^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec, Auburn, CA). In some experiments, BMMCs and CBMCs were stained with FITC-conjugated anti-CD34 MoAb and phycoerythrin (PE)–conjugated anti-CD38 MoAb, and $CD34^+CD38^{high}$ cells and $CD34^+CD38^{low}$ cells were sorted with an EPICS ALTRA cell sorter (Beckman-Coulter, Fullerton, CA).

Generation of Aur-A peptide–specific CTL lines

Aur-A peptide–specific CTLs were generated as described previously.[30] Briefly, monocytes ($CD14^+$ mononuclear cells) were isolated from PBMCs of HLA-A*0201–positive individuals using $CD14^+$ cell-isolating MACS beads. Monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, 75 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor, 10 ng/mL recombinant human interleukin 4 (IL-4; R&D Systems, Minneapolis, MN), and 100 U/mL recombinant human tumor necrosis factor-α (Dainippon Pharmaceutical, Osaka, Japan) to generate mature dendritic cells (DCs). $CD8^+$ T lymphocytes isolated from PBMCs using $CD8^+$ cell-isolating MACS beads were plated in 96-well round-bottomed plates at 10^5 cells per well and stimulated with 10^4 autologous DCs pulsed with synthetic peptide derived from Aur-A at a concentration of 10 μM. The cells were cultured in RPMI 1640 medium supplemented with 10% human AB serum. After 7 days, the cells were restimulated with 10^4 autologous DCs pulsed with Aur-A peptide, and 10 U/mL IL-2 (Boehringer Mannheim, Mannheim, Germany) was added 4 days later. After culturing for a further 3 days (day 15 of culture), the cells were stimulated with 10^5 autologous PBMCs treated with mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan) pulsed with Aur-A peptide. Thereafter, the cells were restimulated weekly by MMC-treated autologous PBMCs pulsed with Aur-A peptide. The Aur-A peptide–specific cytotoxic activity of growing cells was examined by standard ^{51}Cr-release assay.

Cytotoxicity assays

The standard ^{51}Cr-release assays were performed as described previously.[31] Briefly, 10^4 ^{51}Cr-labeled ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, MA) target cells and various numbers of effector cells in 200 μL RPMI 1640 medium supplemented with 10% FCS were seeded into 96-well round-bottom plates. The target cells were incubated with or without synthetic peptide for 2 hours before adding the effector cells. To assess the HLA class I restriction of cytotoxicity, target cells were incubated with an anti–HLA class I framework MoAb (w6/32; ATCC, Manassas, VA) or an anti–HLA-DR MoAb (L243; ATCC) at an optimal concentration (10 μg/mL) for 1 hour before adding the effector cells. Aur-A peptide specificity of cytotoxicity was examined by cold target inhibition assay as follows. ^{51}Cr-labeled target cells (hot targets) were mixed with various numbers of ^{51}Cr-unlabeled Aur-A peptide–loaded HLA-A*0201–positive LCLs or with ^{51}Cr-unlabeled Aur-A peptide–loaded HLA-A*0201–negative LCLs (cold targets). After incubation with the effector cells for 5 hours, 100 μL supernatant was collected from each well. The percentage of specific lysis was calculated as: (experimental release cpm − spontaneous release cpm) / (maximal release cpm − spontaneous release cpm) × 100 (%).

Quantitative analysis of *Aur-A* mRNA expression

Total RNA was extracted from each sample with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. Quantitative real-time polymerase chain reaction (QRT-PCR) of *Aur-A* mRNA (Hs00269212_m1) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA (4326317E) as an internal control was performed using the TaqMan Gene Expression assay (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The expression level of *Aur-A* mRNA was corrected by reference to that of *GAPDH* mRNA, and the relative amount of *Aur-A* mRNA in each sample was calculated by the comparative ΔCt method.

Western blotting of Aur-A protein

Western blotting was performed as follows. Briefly, 10^6 cells were lysed in lysis buffer (25 mM HEPES, pH 7.5, 1% NP-40, 50 mM NaCl, 5 mM EDTA) with a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and then incubated on ice, sonicated, frozen, and thawed. After centrifugation at 12 000*g* for 15 minutes at 4°C, the supernatant was collected as the lysate. After addition of sodium dodecyl sulfate (SDS) buffer, the total cell lysates were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE), and blotted onto nitrocellulose membranes. The blots were reacted with anti–Aur-A mouse MoAb (Abcam, Cambridge, United Kingdom) followed by incubation with horseradish peroxidase–conjugated anti–mouse IgG antibody (GE Healthcare, Little Chalfont, United Kingdom). The probed proteins were visualized using an enhanced chemiluminescence system (GE Healthcare). The blotted membranes were also examined with anti–β-actin mouse MoAb (Sigma-Aldrich, St Louis, MO) to confirm that samples of equal volume had been loaded.

Detection of Aur-$A_{207\text{-}215}$–specific CTL precursors in leukemia patients and healthy individuals by tetramer assays and enzyme-linked immunospot assays

HLA-A*0201/Aur-$A_{207\text{-}215}$ peptide and HLA-A*2402/Aur-$A_{207\text{-}215}$ peptide tetramers were produced as described previously.[32,33] Briefly, recombinant HLA-A*0201 or HLA-A*2402 and the β_2-microglobulin molecule were generated by the gene-transfer method. Expression of the HLA heavy chain was limited to the extracellular domain, and the C terminus of the domain was modified by addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA-peptide complexes were folded in vitro by adding the HLA protein to β_2-microglobulin in the presence of Aur-$A_{207\text{-}215}$ (YLILEYAPL), HIV-1 p17 $Gag_{77\text{-}85}$ (SLYNTVATL), or HIV-1 $Env_{584\text{-}592}$ (RYLRDQQLL) peptide. After gel purification, the HLA complex was biotinylated using recombinant BirA enzyme (Avidity, Denver, CO), and HLA-peptide tetramers were made by mixing the biotinylated HLA with PE-labeled streptavidin (Molecular Probes, Eugene, OR) at a molar ratio of 4:1.

PBMCs from HLA-A*0201– or HLA-A*2402–positive leukemia patients and healthy individuals were seeded in 24-well plates at 1.5×10^6 per well in the presence of the Aur-$A_{207\text{-}215}$ peptide at a concentration of 10 μM in RPMI 1640 medium supplemented with 10% human AB serum and 10 U/mL IL-2. After culturing for 14 days, Aur-$A_{207\text{-}215}$–specific CTL frequencies in cultured cells were examined by tetramer staining. Cultured PBMCs were stained with FITC-conjugated anti-CD8 MoAb and the tetramer at a concentration of 20 μg/mL at 4°C for 20 minutes. After washing twice, stained cells were analyzed using a FACSCalibur and Cell Quest Software.

Enzyme-linked immunospot (ELISPOT) assays were carried out as described previously.[33] Briefly, 96-well flat-bottom MultiScreen-HA plates with a nitrocellulose base (Millipore, Bedford, MA) were coated with 10 μg/mL anti–interferon-γ (IFN-γ) MoAb (R&D Systems) and incubated overnight at 4°C. After being washed with PBS, the plates were blocked with the assay medium for 1 hour at 37°C. T2-A24 cells (5.0×10^4/well) were pulsed with Aur-$A_{207\text{-}215}$ peptide at a concentration of 10 μM or with PBS alone, and incubated in RPMI 1640 medium supplemented with 10% FCS for 1 hour at 37°C. Then, the responder cells generated were seeded into each well to mix with the target peptide–loaded T2-A24 cells, and the plates were incubated in a 5% CO_2 incubator at 37°C for 20 hours. After incubation, plates were washed vigorously with PBS containing 0.1% Tween 20. A polyclonal rabbit anti–IFN-γ antibody (Endgen, Woburn, MA) was added to each well and the plates was left for 90 minutes at room temperature, followed by exposure to peroxidase-conjugated goat anti–rabbit IgG (Zymed, San Francisco, CA) for an additional 90 minutes. To reveal IFN-γ–specific spots, 100 μL 0.1 M sodium acetate buffer (pH 5.0) containing 3-amino-9-ethylcarbazole (Sigma-Aldrich) and 0.015% H_2O_2 were added to each well. After 40 minutes, the color reaction was interrupted by washing with water, and the plates were dried. Diffuse large spots were counted under a dissecting microscope (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Results

Binding activities of Aur-A peptides for HLA-A*0201 and HLA-A*2402 molecules

The BIMAS-predicted binding scores and results of the binding assay for the HLA-A*0201 and HLA-A*2402 molecules with the 3 candidate Aur-A peptides and the positive and negative control peptides are summarized in Table 1. Among the 3 candidate Aur-A peptides, Aur-$A_{207\text{-}215}$ showed high binding affinity for HLA-A*0201 in comparison with the others. Interestingly, Aur-$A_{207\text{-}215}$ peptide appeared to be capable of binding to HLA-A*2402 as well as HLA-A*0201. These data suggest that Aur-$A_{207\text{-}215}$ peptide can elicit Aur-A–specific CTLs.

Establishment of an Aur-$A_{207\text{-}215}$ peptide–specific CTL line

By repeated stimulation of $CD8^+$ T lymphocytes with Aur-A peptide–loaded autologous DCs, as detailed in "Methods," an Aur-$A_{207\text{-}215}$ peptide–specific CTL line, designated AUR-1, was established from an HLA-A*0201–positive individual. It was possible to generate Aur-$A_{207\text{-}215}$ peptide–specific CTLs from 2 other HLA-A*0201–positive individuals; however, long-term maintenance of these CTL lines was unsuccessful. Therefore, detailed studies of the functional characteristics of Aur-A–specific CTLs were performed using AUR-1. Establishment of Aur-$A_{63\text{-}71}$–specific or Aur-$A_{271\text{-}279}$–specific stable CTL lines was unsuccessful. As shown in Figure 1, AUR-1 showed strong cytotoxicity against Aur-$A_{207\text{-}215}$ peptide–loaded autologous and allogeneic HLA-A*0201–positive LCLs but not Aur-$A_{207\text{-}215}$ peptide–unloaded HLA-A*0201–positive LCLs. AUR-1 did not show any cytotoxicity against Aur-$A_{207\text{-}215}$ peptide–loaded HLA-A*0201–negative allogeneic LCLs. Autologous LCLs loaded with other HLA-A*0201–binding peptides were not lysed by AUR-1 (data not shown). To confirm HLA-A*0201 restriction of Aur-$A_{207\text{-}215}$ peptide–specific cytotoxicity mediated by AUR-1, cytotoxic activity against the *HLA-A*0201* gene–transfectant cell line C1R-A*0201 was examined. AUR-1 was cytotoxic to C1R-A*0201 cells only in the presence of Aur-$A_{207\text{-}215}$ peptide, and this cytotoxicity was significantly attenuated by anti–HLA class I MoAb but not by anti–HLA-DR MoAb (data not shown). These data indicate that Aur-$A_{207\text{-}215}$–specific cytotoxicity of AUR-1 is restricted by HLA-A*0201.

Aur-$A_{207\text{-}215}$–specific and HLA-A*0201–restricted lysis of Aur-A–expressing leukemia cell lines by AUR-1

Aur-A mRNA expression levels in leukemia cell lines and PBMCs of healthy people as a control were assessed by the QRT-PCR method. The amount of *Aur-A* mRNA in each cell line relative to that in the chronic myelogenous leukemia (CML) cell line K562 was calculated. Similarly, Aur-A protein expression levels in leukemia cell lines and normal PBMCs were examined by Western blotting. As shown in Figure 2A, Aur-A appeared to be expressed abundantly in all the leukemia cell lines examined, including acute myelogenous leukemia (AML) and CML cell lines. In contrast, expression of Aur-A in normal PBMCs was undetectable.

AUR-1 exerted cytotoxicity against the HLA-A*0201–positive leukemia cell lines GANMO-1 and CMK11-5, but not against the HLA-A*0201–negative cell lines MEG01, KAZZ, OUN-1, and K562 (Figure 2B). As shown in Figure 2C, cytotoxicity against leukemia cell lines mediated by AUR-1 was inhibited by addition of anti–HLA class I framework MoAb but not anti–HLA-DR MoAb. The cold target inhibition assay showed that the cytotoxicity of AUR-1 against the HLA-A*0201–positive leukemia cell line was significantly abrogated

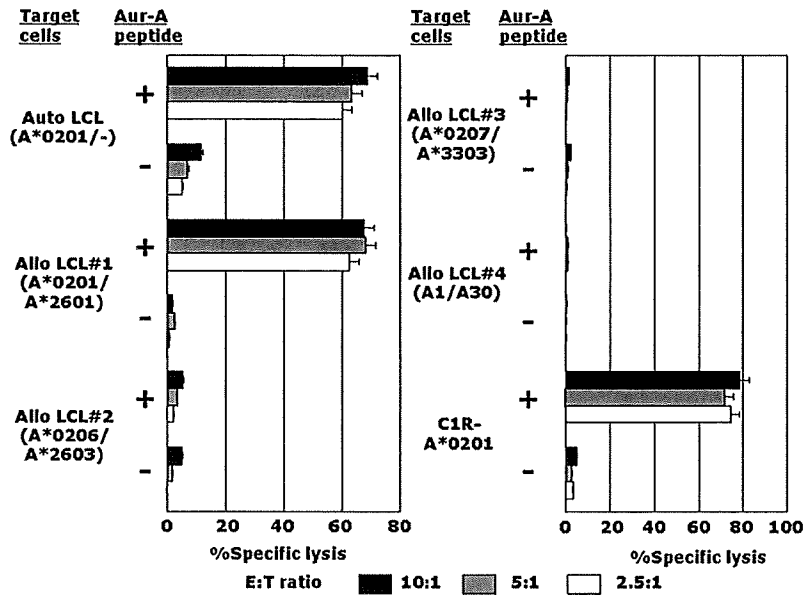

Figure 1. Establishment of an HLA-A*0201–restricted and Aur-$A_{207-215}$ peptide–specific CTL line, AUR-1. The cytotoxicity of the CTL line designated AUR-1 against various LCLs and *HLA-A*0201* gene–transfected cells (C1R-A*0201), which were loaded or unloaded with Aur-$A_{207-215}$ peptide, was determined by 4-hour ^{51}Cr-release assays at effector-to-target (E:T) ratios of 10:1, 5:1, and 2.5:1.

by adding ^{51}Cr-unlabeled Aur-$A_{207-215}$ peptide–loaded autologous LCL, but not HLA-A*0201–negative allogeneic LCL, indicating that the cytotoxicity of AUR-1 against leukemia cells is Aur-A specific (Figure 2D). These results show that AUR-1 can exert cytotoxicity against leukemia cell lines in an HLA-A*0201–restricted manner through recognition of the Aur-$A_{207-215}$ epitope that is naturally processed from Aur-A protein in leukemia cells and presented on the cell surface in the context of HLA class I molecules.

Freshly isolated leukemia cells, but not normal PBMCs or normal mitotic cells, express Aur-A abundantly and are lysed by AUR-1

Next, we examined whether Aur-A–specific CTLs can discriminate freshly isolated leukemia cells from normal cells and whether AUR-1 can lyse freshly isolated leukemia cells as well as leukemia cell lines. As shown in Figure 3A, Aur-A appeared to be overexpressed in a wide spectrum of leukemia, including acute lymphoblastic leukemia (ALL), AML, and CML, as reported previously.[17-19] Among the various kinds of leukemia, CML cells express a very high level of *Aur-A* mRNA. In contrast, expression levels of *Aur-A* mRNA in normal PBMCs and phytohemagglutinin (PHA)–stimulated peripheral blood T lymphocytes (normal mitotic cells) were extremely low in comparison with those in freshly isolated leukemia cells.

The cytotoxicity of AUR-1 against freshly isolated leukemia cells was examined by standard ^{51}Cr-release assay. Because the frequency of HLA-A*0201 in the Japanese population is less than 10%, only 3 HLA-A*0201–positive leukemia samples were available. As expected, all HLA-A*0201–positive freshly isolated leukemia cells were lysed by AUR-1; however, HLA-A*0201–negative freshly isolated leukemia cells, HLA-A*0201–positive normal PBMCs, and HLA-A*0201–positive PHA lymphoblasts were resistant to AUR-1–mediated cytotoxicity (Figure 3B). Taken together, Aur-A–specific CTLs appeared to be capable of discriminating leukemia cells from normal cells in an HLA-restricted manner.

CD34$^+$ leukemia progenitor cells, but not CD34$^+$ normal hematopoietic progenitor cells, express Aur-A abundantly and are susceptible to AUR-1–mediated cytotoxicity

Because it is important to determine whether Aur-A–specific CTLs can specifically recognize and lyse leukemia progenitors, we further examined Aur-A expression and susceptibility to AUR-1–mediated cytotoxicity of CD34$^+$ fractions in leukemia cells and normal hematopoietic cells. The CD34$^+$ cells were sorted from BMMCs of patients with CML and CBMCs, and their *Aur-A* mRNA expression was examined by QRT-PCR. As shown in Figure 4A, the expression levels of *Aur-A* mRNA in CML CD34$^+$ progenitor cells appeared to be significantly higher than in normal CD34$^+$ hematopoietic progenitor cells. Because it is suggested that leukemia stem cells and normal hematopoietic stem cells are present in the CD34$^+$CD38^{low} fraction, we further examined Aur-A expression in CD34$^+$CD38^{low} cells of CML BMMCs and CBMCs. Consequently, it appeared that *Aur-A* mRNA was abundantly expressed in the CD34$^+$CD38^{low} fraction of CML (Figure 4B); however, the expression level of *Aur-A* mRNA in the CD34$^+$CD38^{low} fraction of normal hematopoietic progenitors was significantly low (Figure 4C).

Since a sufficient number of CD34$^+$CD38^{low} cells could not be obtained, whole CD34$^+$ cells were used as target cells for cytotoxicity assays (Figure 4D). As expected, AUR-1 exerted strong cytotoxicity against CD34$^+$ cells isolated from CML BMMCs of 2 patients. In contrast, AUR-1 did not show any cytotoxicity against normal CD34$^+$ hematopoietic progenitor cells. These data strongly suggest that Aur-A–specific CTLs can discriminate leukemia progenitor cells from normal hematopoietic stem cells and selectively inhibit the growth of leukemia stem cells, and that immunotherapy targeting Aur-A is effective and safe.

Presence of Aur-$A_{207-215}$–specific CTL precursors in peripheral blood of leukemia patients

When considering the feasibility of cellular immunotherapy for leukemia targeting Aur-A, it seems important to clarify whether Aur-A–specific CTL precursors are present in patients with leukemia. Aur-$A_{207-215}$–specific CTL precursors in HLA-A*0201–positive patients with leukemia including AML in complete remission (CR) after allogeneic hematopoietic stem cell transplantation, ALL in CR after chemotherapy, and CML in the chronic phase before imatinib therapy, and 8 healthy subjects were analyzed by tetramer assay. Because Aur-$A_{207-215}$ peptide can bind to HLA-A*2402 as well as HLA-A*0201, we also examined Aur-$A_{207-215}$–specific CTL precursors in 2 HLA-A*2402–positive

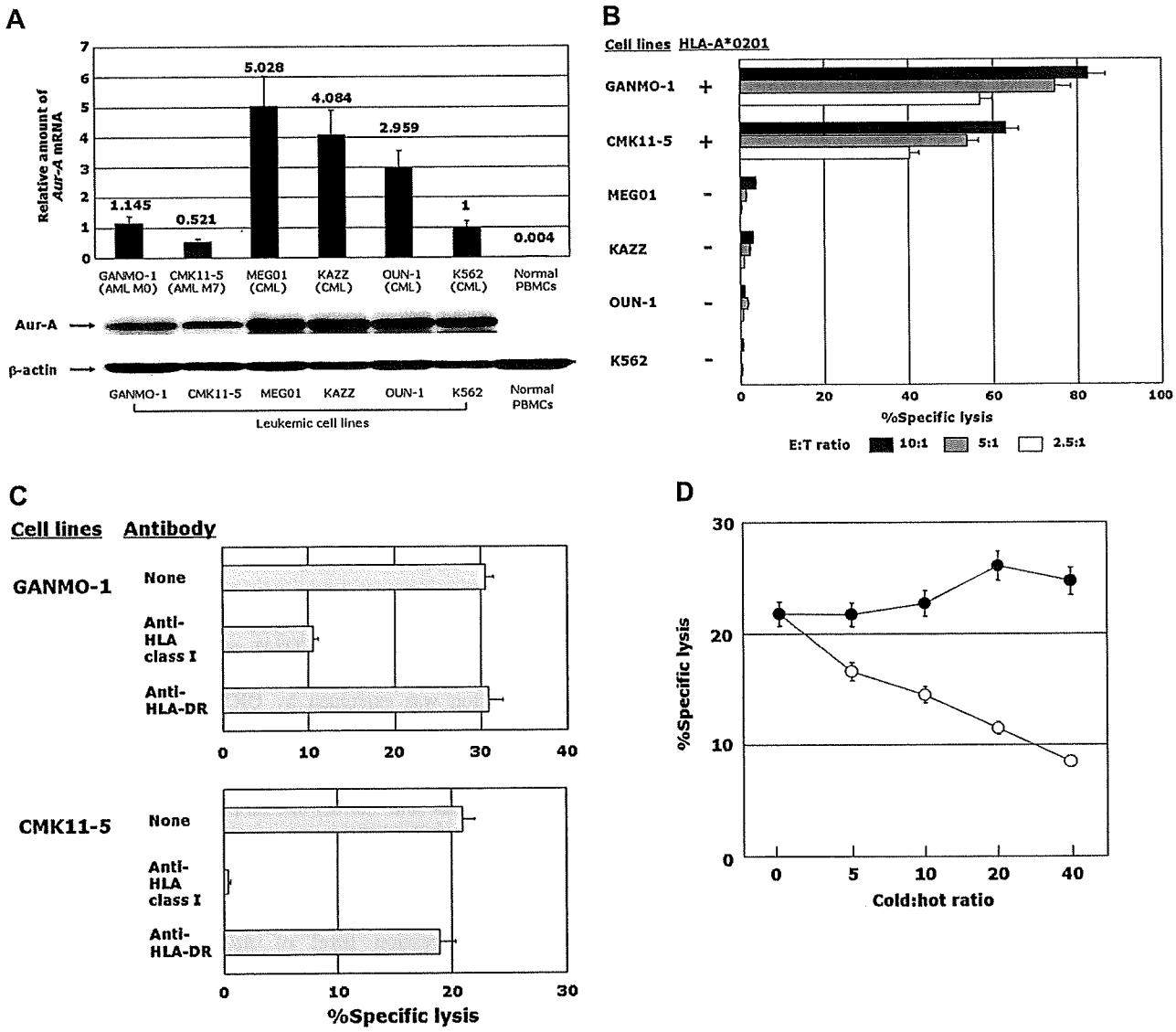

Figure 2. Expression of Aur-A in leukemia cell lines and the cytotoxicity of AUR-1 against leukemia cell lines. (A) Expression of *Aur-A* mRNA and protein in leukemia cell lines and normal PBMCs. Expression levels of *Aur-A* mRNA in the cells were determined by QRT-PCR as detailed in "Methods." The level of *Aur-A* mRNA expression in the K562 leukemia cell line, which strongly expresses Aur-A, is shown as 1.0 and the expression levels in the cells were calculated relative to this value. Aur-A protein expression was examined by Western blotting using anti–Aur-A antibody and anti–β-actin antibody as the control. (B) Cytotoxicity of the Aur-$A_{207\text{-}215}$–specific CTL line AUR-1 against leukemia cell lines. The cytotoxicity of AUR-1 to HLA-A*0201–positive and HLA-A*0201–negative leukemia cell lines was determined by 4-hour ^{51}Cr-release assays at E/T ratios of 10:1, 5:1, and 2.5:1. (C) HLA class I restriction of cytotoxicity mediated by AUR-1 against leukemia cells. The cytotoxicity of AUR-1 against leukemia cell lines (GANMO-1 and CMK11-5) was determined by 4-hour ^{51}Cr-release assays at an E/T ratio of 2.5:1 in the presence or absence of anti–HLA class I MoAb or anti–HLA-DR MoAb. (D) Cold target inhibition assays. ^{51}Cr-labeled GANMO-1 cells (5×10^3 cells) were mixed with various numbers of ^{51}Cr-unlabeled Aur-$A_{207\text{-}215}$ peptide–loaded autologous LCL cells (○) or with ^{51}Cr-unlabeled Aur-$A_{207\text{-}215}$ peptide–loaded HLA-A*0201–negative allogeneic LCL cells (●). The cytotoxicity of AUR-1 to the mixture of ^{51}Cr-labeled and unlabeled target cells was determined by 4-hour ^{51}Cr-release assays at an effector-to-^{51}Cr-labeled target cell ratio of 10:1.

patients with CML in chronic phase after therapy with interferon or imatinib and 2 healthy individuals. Since we were unable to detect Aur-A–specific CTL precursors when freshly isolated lymphocytes were used for assays, PBMCs were stimulated with Aur-$A_{207\text{-}215}$ peptide and then analyzed. Representative data of tetramer assays for HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia are shown in Figure 5A. The frequencies of Aur-$A_{207\text{-}215}$–specific CTL precursors in HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia and healthy individuals are summarized in Figure 5B. Consequently, Aur-$A_{207\text{-}215}$–specific CTL precursors were apparently detected in both HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia. The frequency of Aur-$A_{207\text{-}215}$–specific CTL precursors in leukemia patients appeared to be significantly higher than that in healthy individuals (0.25% ± 0.1% for leukemia patients, and 0.05% ± 0.03% for healthy individuals; $P < .001$). These data strongly suggest that Aur-A–specific CTL precursors are primed in patients with leukemia, and that vaccination with Aur-A peptide may efficiently induce an Aur-A–specific immune response in leukemia patients. To determine whether Aur-$A_{207\text{-}215}$–specific CTL precursors detected by tetramer assays are indeed functional, we performed tetramer assays and ELISPOT assays using the same samples simultaneously, and determined the correlation between the 2 sets of data. PBMCs isolated from 7 HLA-A*0201– or HLA-A*2402–positive individuals were used for tetramer assays and ELISPOT assays. Consequently, the frequencies of Aur-$A_{207\text{-}215}$ peptide–specific CTL precursors detected by these 2 different assay systems appeared to be closely correlated

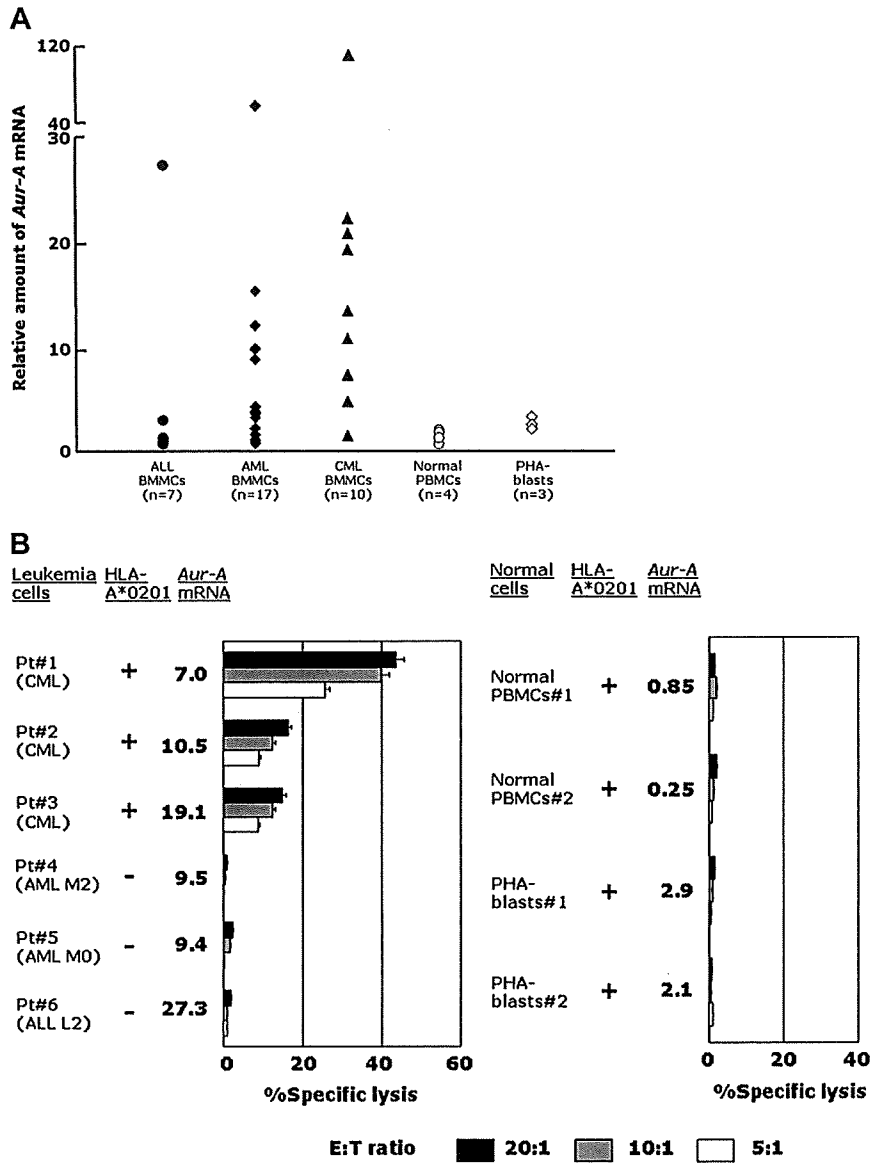

Figure 3. Expression of Aur-A in freshly isolated leukemia cells and the cytotoxicity of AUR-1 against freshly isolated leukemia cells. (A) Expression of *Aur-A* mRNA in freshly isolated leukemia cells, normal PBMCs, and PHA-stimulated T lymphocytes. Expression levels of *Aur-A* mRNA in freshly isolated leukemia cells and normal cells were determined using samples obtained from 7 patients with ALL, 17 patients with AML, 10 patients with CML in chronic phase, 4 healthy individuals, and PHA-stimulated T lymphoblasts obtained from 3 healthy individuals. To prepare PHA-stimulated T lymphoblasts, PBMCs were cultured in RPMI 1640 medium supplemented with 10% FCS and PHA at an appropriate concentration for 4 days. The level of *Aur-A* mRNA in normal PBMCs is shown as 1.0 and the expression levels in samples were calculated relative to this value. (B) Cytotoxicity of AUR-1 against freshly isolated leukemia cells and normal cells. The cytotoxicity of AUR-1 against HLA-A*0201–positive and HLA-A*0201–negative freshly isolated leukemia cells, HLA-A*0201–positive normal PBMCs, and HLA-A*0201–positive normal PHA-stimulated T lymphoblasts was determined by ^{51}Cr-release assays at E:T ratios of 20:1, 10:1, and 5:1. Expression levels of *Aur-A* mRNA in samples are also shown.

(r = 0.817; Figure S1B). These data strongly suggest that Aur-A tetramer–positive cells certainly have a functional response to stimulation with Aur-A.

Discussion

In the present study, we demonstrated that Aur-A is an ideal target antigen of cellular immunotherapy for leukemia, based on the following findings. First, Aur-A is broadly overexpressed in various types of leukemia but not in normal tissues except for testis, which is negative for HLA expression. Second, an Aur-A–derived peptide, Aur-$A_{207-215}$, can bind to HLA-A*0201 and HLA*2402 molecules and elicit Aur-A–specific CTLs. Third, Aur-A is efficiently processed in leukemia cells, and leukemia cell lines and freshly isolated leukemia cells, but not normal cells, are lysed by Aur-A–specific CTLs in an HLA class I–restricted manner. Fourth, Aur-A–specific CTL precursors are certainly present in the peripheral blood of patients with leukemia.

One of the important characteristics of proteins that could be used as ideal tumor-associated antigens for cancer immunotherapy is an essential role in tumorigenesis and/or tumor progression. Aur-A is localized mainly at spindle poles and the mitotic spindle during mitosis, where it regulates the functions of centrosomes, spindles, and kinetochores required for proper mitotic progression. Recent studies have revealed that Aur-A is frequently overexpressed in various cancer cells, indicating its involvement in tumorigenesis.[9-14] Overexpression of Aur-A contributes to genetic instability and tumorigenesis by disrupting the proper assembly of the mitotic checkpoint complex at the level of the Cdc20-BubR1 interaction.[34] Its overexpression also causes resistance to apoptosis induced by taxol in human cancer cell lines.[35,36] Moreover, Aur-A is a key regulatory component of the p53 pathway, as its overexpression leads to increased p53 degradation, thus facilitating oncogenic transformation.[37] In addition, Aur-A expression in tumors is often associated with poor histologic differentiation and poor prognosis.[12-14] These characteristics indicate that Aur-A is an ideal target antigen for cancer immunotherapy. Although Aur-A is also expressed in normal cells during mitosis, its expression level in normal tissue is quite low; therefore, normal mitotic cells are resistant to Aur-A–specific CTL-mediated cytotoxicity, as shown in the present study.

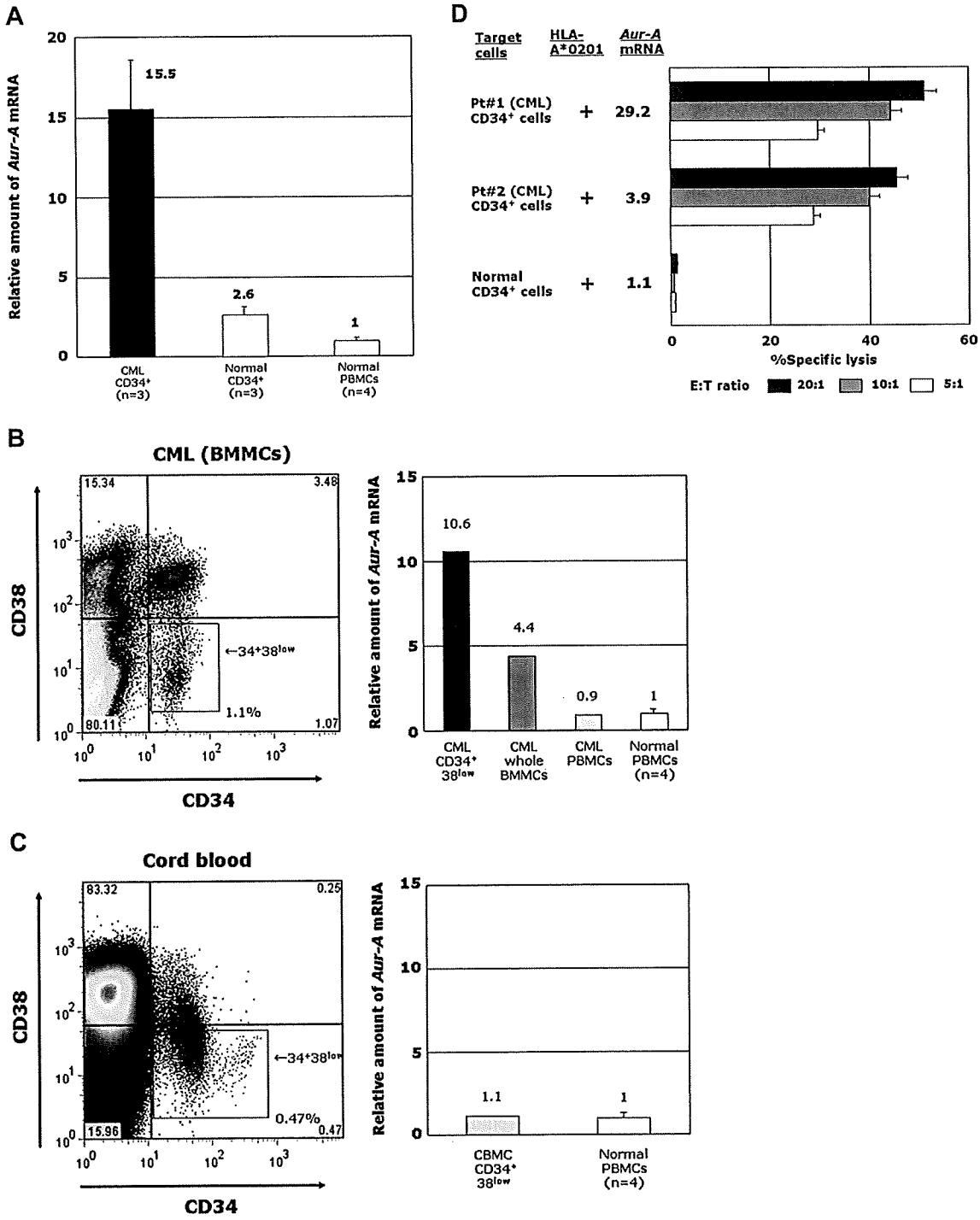

Figure 4. Expression of Aur-A in CD34$^+$CD38^{low} fractions of CML cells and normal hematopoietic progenitor cells, and cytotoxicity of AUR-1 against CD34$^+$ CML cells and CD34$^+$ normal hematopoietic stem cells. (A) Expression levels of *Aur-A* mRNA in CD34$^+$ cells isolated from BMMCs of patients with CML, CD34$^+$ cells isolated from normal BMMCs and CBMCs, and normal PBMCs. Expression levels of *Aur-A* mRNA in leukemic CD34$^+$ cells, normal hematopoietic stem cells, and normal PBMCs were determined using 3 samples of CML BMMCs, 1 sample of normal BMMCs, 2 samples of CBMCs, and 4 samples of normal PBMCs. The level of *Aur-A* mRNA in normal PBMCs is shown as 1.0 and the expression levels in samples were calculated relative to this value. (B) Representative data of *Aur-A* mRNA expression in the CD34$^+$CD38^{low} fraction of BMMCs, whole BMMCs, and PBMCs isolated from a patient with CML in chronic phase and PBMCs isolated from 4 healthy individuals. The CD34$^+$CD38^{low} cells were collected using a cell sorter. (C) Representative data of *Aur-A* mRNA expression in the CD34$^+$CD38^{low} fraction of CBMCs isolated from a normal donor and PBMCs isolated from 4 healthy individuals. The CD34$^+$CD38^{low} cells were collected using a cell sorter. (D) Cytotoxicity of AUR-1 against CD34$^+$ leukemia progenitor cells and normal CD34$^+$ hematopoietic progenitor cells. The cytotoxicity of AUR-1 against CD34$^+$ leukemia cells isolated from 2 HLA-A*0201–positive patients with CML and normal CD34$^+$ hematopoietic progenitor cells isolated from an HLA-A*0201–positive cord blood donor was determined by ^{51}Cr-release assays at E/T ratios of 20:1, 10:1, and 5:1. Expression levels of *Aur-A* mRNA in samples are also shown.

As reported previously,[17-19] the present study demonstrated that Aur-A is overexpressed widely in various types of leukemia including AML, ALL, and CML. Among the leukemias, CML cells appeared to express a large amount of Aur-A. It was also found that Aur-A is abundantly expressed in the CD34$^+$CD38^{low} fraction of CML cells. Previous gene expression profiling analysis has also shown that mitogen-activated protein kinases, which activate mitotic kinases including Aurora kinases, are overexpressed in

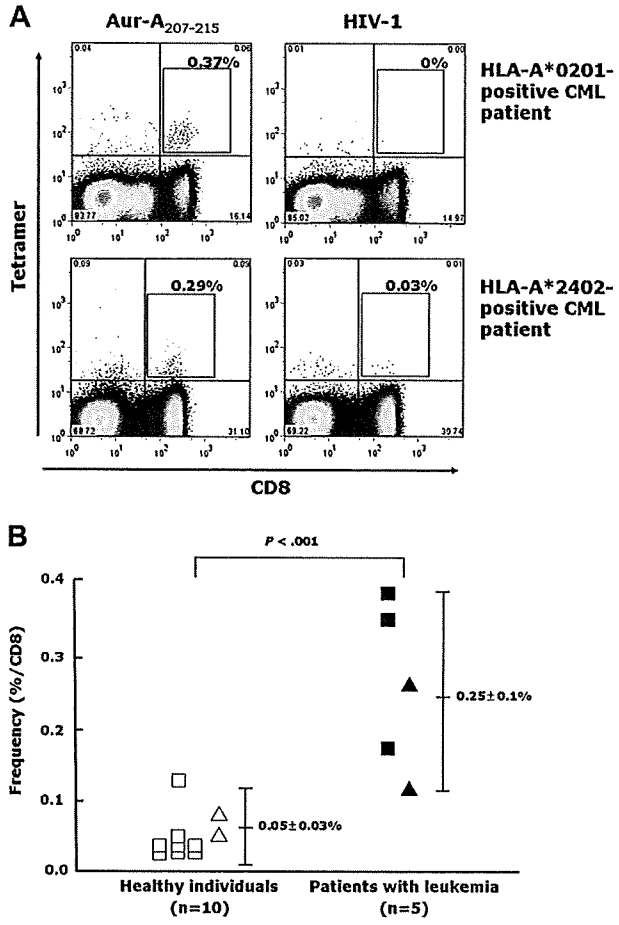

Figure 5. Detection of Aur-$A_{207-215}$–specific CTL precursors in patients with leukemia. (A) Representative data of the tetramer assay for Aur-$A_{207-215}$–specific CTL precursors. PBMCs isolated from HLA-A*0201–positive and HLA-A*2402–positive patients with CML in chronic phase were stimulated with Aur-$A_{207-215}$ peptide and then stained with HLA-A*0201/Aur-$A_{207-215}$ tetramer and HLA-A*2402/Aur-$A_{207-215}$ tetramer, respectively. HLA-A*0201/HIV-1 p17 Gag_{77-85} (SLYNTVATL) tetramer and HLA-A*2402/HIV-1 $Env_{584-592}$ (RYLRDQQLL) tetramer were used as negative controls. (B) Summary of tetramer assays for Aur-$A_{207-215}$–specific CTL precursors. PBMCs isolated from 3 HLA-A*0201–positive patients with leukemia (a patient with AML in complete remission after allogeneic stem cell transplantation, a patient with ALL in complete remission after chemotherapy, and a patient with untreated CML in chronic phase; ■), 2 HLA-A*2402–positive patients with leukemia (2 patients with CML in chronic phase after therapy with interferon or imatinib; ▲), 8 HLA-A*0201–positive healthy individuals (□), and 2 HLA-A*2402–positive healthy individuals (△) were stained with HLA-A*0201/Aur-$A_{207-215}$ or HLA-A*2402/Aur-$A_{207-215}$ tetramer. The frequency of Aur-$A_{207-215}$–specific CTL precursors in the patients with leukemia was significantly higher than that in healthy individuals (Student *t* test; $P < .001$).

$CD34^+$ progenitor cells in CML.[38] In contrast to overexpression of Aur-A in the $CD34^+CD38^{low}$ fraction of CML cells, the expression level of Aur-A in the $CD34^+CD38^{low}$ fraction of normal hematopoietic progenitors appeared to be markedly lower than that in leukemic cells. We therefore addressed the question of whether Aur-A–specific CTLs can lyse leukemic progenitors. Because a sufficient number of $CD34^+CD38^{low}$ cells could not be obtained, $CD34^+$ cells were used as target cells. Consequently, in parallel with the expression levels of Aur-A, $CD34^+$ CML cells but not $CD34^+$ normal hematopoietic progenitor cells were efficiently lysed by Aur-A–specific CTLs. Although the detailed characteristics of leukemic stem cells are still obscure, they are considered to be present in the $CD34^+CD38^{low}$ fraction.[39-41] Taken together, targeting of Aur-A may be effective for eradicating leukemic stem cells.

Another interesting finding of this study was that Aur-$A_{207-215}$ peptide is able to bind to HLA-A*2402 as well as to HLA-A*0201. Although AUR-1 could not recognize the complex of Aur-$A_{207-215}$ peptide and HLA-A*0206 or HLA-A*0207, this peptide can bind to the HLA-A*0206 molecule (data not shown; written communication from Dr K. Udaka, Kochi University, Nangoku, Japan, August 5, 2007). Binding of a single peptide to both HLA-A*0201 and HLA-A*2402 has also been reported previously for a WT1-derived peptide ($WT1_{235-243}$; CMTWNQMNL),[30,42] which is now used as a cancer peptide vaccine. Since CTLs recognize a tumor-associated epitope in the context of HLA class I molecules, identification of a peptide that can bind to common HLA types is essential for development of a universal cancer peptide vaccine. Because HLA-A*2402 is the most common HLA type in the Japanese population, Aur-$A_{207-215}$ is a promiscuous peptide and therefore likely useful for development of a cancer vaccine for Asian as well as white patients.

To date, 3 Aurora kinases, Aur-A, Aur-B, and Aur-C, have been identified in mammals. The Aurora kinases show different subcellular localization patterns and perform distinct tasks during cell division.[43] These molecules show a similar domain organization: a N-terminal domain of 39-129 residues, a protein kinase domain, and a short C-terminal domain of 15-20 residues. The N-terminal domain of Aurora kinases shows low sequence conservation, and this determines selectivity during protein-protein interactions.[44] In contrast, the catalytic domain is more highly conserved. Importantly, Aur-$A_{207-215}$ is located in the catalytic domain and the conserved residues of Aur-A, Aur-B, and Aur-C (Figure S2). Interestingly, the Aur-$B_{149-157}$, Aur-$B_{151-159}$, and Aur-C_{83-91} peptides, which are derived from the catalytic domain of Aur-B and Aur-C, can bind to HLA-A*0201 and HLA*2402 molecules (Figure S3), suggesting that these residues could be a universal target epitope for cancer immunotherapy.

In summary, we have demonstrated for the first time that Aur-A is a potentially ideal target of cellular immunotherapy for leukemia. When considering the evidence that Aur-A is overexpressed widely in various kinds of cancer, Aur-A–targeting cancer immunotherapy may be universally applicable. On the basis of our present data, we are now planning a clinical trial of Aur-A peptide vaccination for cancer patients.

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Authorship

Contribution: T.O. and H.F. designed and performed the research and wrote the paper; K.S., T.A., Y.Y., and T.H. discussed and interpreted the experimental results and provided clinical materials; K.K. made and supplied the tetramer; and M.Y. designed the research, wrote and edited the paper, and provided financial support.

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The Role of Zinc Finger Protein 521/Early Hematopoietic Zinc Finger Protein in Erythroid Cell Differentiation*[S]

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ZNF521 (zinc finger protein 521) is a transcription factor with an N-terminal transcriptional repressor motif and 30 zinc finger domains. Although a high expression level of ZNF521 in human $CD34^+$ progenitors and hematopoietic malignancies has been demonstrated, the functional role of ZNF521 in hematopoietic cell differentiation has not been clarified. In this study, we analyzed the role of ZNF521 in erythroid cell differentiation using the short hairpin RNA (shRNA)-mediated gene silencing method. Down-regulation of ZNF521 mediated by transient expression of shRNA for *ZNF521* resulted in increased synthesis of hemoglobin in K562 and HEL cell lines as compared with control cells. K562-derived clones in which *ZNF521* was constitutively silenced by shRNA also showed marked synthesis of hemoglobin and an increased expression level of glycophorin A. Since GATA-1 is the key regulator of erythroid differentiation, the effect of ZNF521 on transcription activity of GATA-1 was analyzed using a luciferase assay. GATA-1 activity was markedly inhibited by ZNF521 in a dose-dependent manner. Deletion analysis of ZNF521 showed that the repressive effect requires an N-terminal repression motif. Furthermore, the direct interaction of ZNF521 with GATA-1 was demonstrated. These results indicate that ZNF521 modulates erythroid cell differentiation through direct binding with GATA-1.

ZNF521 (zinc finger protein 521), formerly designated as EHZF (early hematopoietic zinc finger protein), the human homolog of mouse EVI3/Zfp521, is a transcription factor with 30 Krüppel-like zinc finger (ZF)[3] domains (1). The gene encoding EVI3 was initially identified as a common site of retroviral integration in murine AKXD B-cell lymphomas (2). The integration site of *Evi3* is located upstream of the first translated exon and results in up-regulation of *Evi3* by a promoter insertion mechanism. Although EVI3 expression in normal B cells is ontogenetically and developmentally regulated, alteration of EVI3 expression induced by retrovirus insertion might be involved in the genesis of murine B-cell lymphoma.

ZNF423/OAZ (olfactory-associated zinc finger protein), another 30-ZF protein that displays highly homology with ZNF521, was identified as a protein interacting with EBF1 (early B-cell factor 1) in a yeast two-hybrid screen (3). EBF1, previously designated as OLF-1, is a basic helix-loop-helix transcription factor required for development of the olfactory epithelium and B-cell lineage commitment (4, 5). ZNF423 inhibits EBF1-mediated *trans*-activation. Although ZNF423 is not expressed in normal B cells, ZNF423 expressed ectopically at a high level following retroviral integration has been detected in murine AKXD B-cell lymphomas (6). Although direct interaction between ZNF521 and EBF1 has not been demonstrated, ZNF521 also inhibits the transcriptional activity of EBF1. These findings indicate that aberrant expression of ZNF521 and/or ZNF423 results in inhibition of B-cell differentiation and induction of B-cell malignancies by modulating the transcriptional activities of EBF1 (1, 7). Genome-wide analysis has detected frequent monoallelic deletion of *EBF1* or *PAX5*, which is downstream of EBF1, in B-progenitor acute lymphoblastic leukemia (8). In addition, fusion of *PAX5* to *ZNF521* resulting in formation of PAX5-ZNF521 fusion protein has been found in B-cell acute lymphoblastic leukemia (8).

Both ZNF521 and ZNF423 contain an N-terminal 12-amino acid motif, which is associated with transcriptional repression and conserved among other ZF transcriptional repressors, including FOG-1, FOG-2, BCL11A, and SALL family members (9–11). ZNF423 has also been identified as a cofactor of Smad protein in bone morphogenetic protein (BMP) signaling. ZNF423 binds to Smad1 and Smad4 in response to BMP2 and activates transcription of BMP target genes, such as *Xenopus Vent-2* (12) and murine *Smad6* (13). On the basis of structural and sequential homology, ZNF521 is speculated to have func-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Fig. 1.

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[3] The abbreviations used are: ZF, zinc finger; BMP, bone morphogenic protein; CF, C-terminal finger; GST, glutathione *S*-transferase; NF, N-terminal finger; NuRD, nucleosome remodeling and deacetylase corepressor complex; shRNA, short hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation.

tions similar to those of ZNF423. Bond *et al.* (1) showed that ZNF521 interacts with Smad1 and Smad4 and increases BMP-responsive element-dependent transcriptional activity following treatment with BMP2 or BMP4.

Both ZNF521 and ZNF423 are expressed in brain, heart, and skeletal muscle. In addition to these organs, ZNF521 is expressed at relatively high levels in spleen, lymph nodes, and fetal liver, whereas ZNF423 is not expressed in these organs. In hematopoietic cells, *ZNF521* mRNA is expressed abundantly in $CD34^+$ early hematopoietic progenitors, and its expression level declines rapidly during their differentiation (1). The *ZNF521* transcript is not detected in peripheral blood leukocytes. On the other hand, a significant expression level of *ZNF521* mRNA is detected in most cases of acute myelogenous leukemia (1, 14). *ZNF521* mRNA is also expressed in several hematopoietic cell lines derived from acute myelogenous leukemia and chronic myelogenous leukemia at blast crisis. Among them, a large amount of *ZNF521* transcript is detected in K562 and HEL cell lines (1), both of which have potential for differentiation to erythroid lineage. These findings strongly suggest that ZNF521 plays an important role in hematopoietic differentiation and leukemogenesis. On the basis of this concept, we investigated the function of ZNF521 in hematopoietic differentiation by using shRNA-mediated silencing of *ZNF521* in these cell lines. The data we obtained implicate ZNF521 in the suppression of erythroid differentiation of hematopoietic progenitors via inhibition of the transcriptional activity of GATA-1. Furthermore, it was suggested that ZNF521 interacts directly with GATA-1 via the C-terminal ZF domains and suppresses the transcriptional activity of GATA-1 through the N-terminal suppression domain.

EXPERIMENTAL PROCEDURES

Cells and Cell Lines—The K562 and HEL cell lines were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, and the 293T and NIH3T3 cell lines were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 5% CO_2 incubator at 37 °C.

Plasmids—Two segments of *ZNF521* cDNA (start codon to the XhoI site and the XhoI site to the terminal codon) were generated by reverse transcription-PCR. Total RNA was isolated from 2×10^7 K562 cells using an RNeasy minikit (Qiagen, Valencia, CA), and cDNA was generated using an Omniscript reverse transcriptase kit (Qiagen). Reverse transcription-PCR was performed with PrimeSTAR HS DNA polymerase (TaKaRa, Tokyo, Japan) for 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. The following primers were used for PCR: *ZNF521* 5′ fragment, forward (5′-GTCGACGCAGGATGTCTCGCCGCAAGCAAG-3′) and reverse (5′-TTGCTTGTTTACTCGAGGGACCAGTCATG-3′); *ZNF521* 3′ fragment, forward (5′-CTGGTCCCTCGAGTAAACAAGCAAAAG-3′) and reverse (5′-GCGGCCGCCTAACTGCTGTGTTGGGTCATTG-3′). The PCR products were cloned into the pUC 118 vector using a Mighty Cloning Kit (TaKaRa) and sequenced. The full-length *ZNF521* cDNA was constructed by ligation of the cDNA fragments and cloned into the pcDNA3 vector (Invitrogen) or p3×FLAG-CMV-7.1 expression vector (Sigma). The pcDNA3 3×FLAG/*ZNF521* was constructed by PCR using the primers to generate a 5′-cDNA fragment encoding the 3×FLAG epitope fused to the N terminus of ZNF521 protein.

pGL3-MαP-Luc, a plasmid in which the 3×GATA-1 binding site is inserted just upstream of the JunB promoter in JunB-MP-Luc (15), was kindly provided by Dr. I. Matsumura. Human *GATA-1* cDNA was kindly provided by Dr. S. Takahashi. The pcDNA3 *myc/GATA-1* was constructed by PCR using the primers to generate a 5′ cDNA fragment encoding the Myc epitope fused to the N terminus of GATA-1 protein.

For the establishment of stable cell lines, K562 cells were transferred to culture medium containing G418 (Invitrogen) at a concentration of 2 mg/ml 48 h after transfection. Resistant clones were then isolated and expanded over several weeks. The levels of GATA-1 expression in established clone cells were examined by Western blotting.

shRNA Plasmids and Transfection—Two kinds of shRNA for *ZNF521*, GGGACAAGAAGTACCACTG and GGAAGAGCATATTAGACAG, were designed, and their expression vectors were constructed using pcPURU6β vector (TaKaRa). These vectors were transfected into K562 cells using a Nucleofector device (Amaxa Biosystems GmbH, Cologne, Germany).

For the establishment of stable cell lines, K562 cells were transferred to culture medium containing puromycin (Invitrogen) at a concentration of 5 μg/ml 48 h after transfection. Resistant clones were then isolated and expanded over several weeks. The expression levels of *ZNF521* mRNA were examined by reverse transcription-PCR and confirmed by Northern blot analysis and real time PCR.

Quantitative Real Time PCR—Quantitative real time PCR of *ZNF521* mRNA (Hs01031125_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (4326317E) as an internal control was performed using the TaqMan Gene Expression assay (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions using an Applied Biosystems 7500 real time PCR system (Applied Biosystems). The expression level of *ZNF521* mRNA was corrected by reference to that of GAPDH mRNA, and the relative amount of *ZNF521* mRNA in each sample was calculated by comparative ΔCt method.

Benzidine Staining—The hemoglobinization of K562 cells was analyzed by benzidine staining 2, 4, 6, and 8 days after transient transfection with shRNA for *ZNF521*. In brief, cells were collected and washed twice with cold phosphate-buffered saline and then stained with benzidine solution containing 3% H_2O_2 for 5 min, and 200 cells were counted in each analysis under a light microscope. Cells with blue-brown-stained cytoplasm were counted as benzidine-staining-positive cells. The experiments were repeated three times. Statistical analysis between the two groups was performed using a Tukey-Kramer test.

Northern Blot Analysis—Northern blotting was performed by using a ^{32}P-random-labeled *ZNF521* cDNA fragment generated by PCR using the primers 5′-AGACAGGTTCATTGTGACCT-3′ and 5′-TGGAAGCACAGTGTCGAGAT-3′. A β-actin control probe was also labeled by random labeling.

Flow Cytometric Analysis—Expression of cell surface glycophorin A was examined by flow cytometric analysis using PE-

conjugated anti-glycophorin A monoclonal antibody (BD Biosciences). Cells to be used as unstained controls for glycophorin A were incubated with control PE-conjugated mouse monoclonal IgG (Pharmingen).

Luciferase Assays—Four micrograms of the plasmid DNAs (cDNA for *GATA-1*, *ZNF521*, and/or empty vector), 1.1 μg of the *GATA-1* reporter gene pGL3-MαP-Luc (15), and 0.1 μg of the sea pansy luciferase expression plasmid pRL-SV40 (Toyo B-Net, Tokyo, Japan) as an internal control were transduced into NIH3T3 cells (2×10^5 cells seeded in a 60-mm dish) using FuGENE 6 (Roche Applied Science). After 16 h, the cells were harvested, and luciferase activity and sea pansy luciferase activity were assayed using the Pica Gene dual sea pansy luminescence kit (Toyo B-Net) and a luminometer in accordance with the manufacturer's instructions. Results are expressed as the ratios of luciferase activity to sea pansy luciferase activity. Statistical analysis of data between the two groups was performed using a paired sample *t* test. Five micrograms of the *GATA-1* reporter gene pGL3-MαP-Luc and 0.05 μg of the sea pansy luciferase expression plasmid pRL-SV40 were transfected into K562 cells (1×10^6 cells) by electroporation. After 36 h, the cells were lysed, and dual luciferase assays were performed.

Glutathione S-Transferase (GST) Fusion Protein Pull-down Assay—Five *ZNF521* cDNA fragments were constructed by PCR and sequenced. They were then cloned into the pEUE01-His vector (CellFree Sciences Co. Ltd., Matsuyama, Japan) containing the His_6 tag region and SP6 promoter. The full-length *GATA-1* cDNA and ZF parts of GATA-1 generated by PCR were cloned into the pEUE01-GST vector (CellFree Sciences Co. Ltd.) containing a GST tag region and SP6 promoter. The His_6-ZNF521 proteins and GST-GATA-1 fusion protein were synthesized by use of the wheat cell-free protein synthesis system (16). His_6-ZNF521 fusion proteins were purified using nickel-Sepharose high performance (GE Healthcare). GST-GATA-1 fusion protein was purified using glutathione-Sepharose 4B (GE Healthcare). Expression of the fusion proteins was confirmed by SDS-PAGE following Coomassie Blue staining and by Western blotting analysis. GST-GATA-1 fusion protein immobilized on beads and various amounts of His_6-ZNF521 proteins were mixed in 1 ml of lysis buffer (20 mM sodium phosphate, pH 7.0, 250 mM NaCl, 30 mM sodium pyrophosphate, 0.1% Nonidet P-40, 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride) and incubated for 4 h at 4 °C. The beads were washed five times with lysis buffer and analyzed by SDS-PAGE followed by Western blotting using anti-His antibody (TaKaRa).

Immunoprecipitation and Western Blot Analysis—The p3×FLAG-*ZNF521* CMV-7.1 plasmid and the pcDNA3 *Myc/GATA-1* plasmid were transfected into 293T cells using FuGENE HD (Roche Applied Science). After 48 h, the cells were lysed at 4 °C in 1 ml of lysis buffer. After centrifugation, the supernatants were mixed with EZview red anti-FLAG M2 affinity gel or anti-Myc affinity gel (Sigma) and incubated for 2 h at 4 °C. The beads were then washed five times with lysis buffer. The bound protein was released from the beads by boiling for 5 min in 10 μl of sample buffer and analyzed by SDS-PAGE followed by Western blot analysis using anti-Myc antibody (Sigma) and anti-FLAG antibody (Sigma). Total cellular lysate was isolated from K562/3×FLAG-ZNF521 cell lines, and immunoprecipitation was performed with anti-FLAG M2 affinity gel. The protein was analyzed by SDS-PAGE followed by Western blot analysis using anti-GATA-1 antibody (N6; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) .

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed with a Quik ChIP kit (IMGENEX, San Diego, CA). K562/3×FLAG-ZNF521 cells (5×10^6 cells) were fixed with 1% of formaldehyde for 10 min at 37 °C and the reactions were stopped by adding 125 mM glycine. Chromatin was sonicated with 20 pulses, 30-s pulses followed by 20 s of rest on ice, using Bioruptor (Cosmo Bio, Tokyo, Japan). After sonication, immunoprecipitation was performed with EZview red anti-FLAG M2 affinity gel (Sigma) and normal mouse IgG (Santa Cruz Biotechnology). The immunoprecipitated DNA samples and input samples were eluted and reverse cross-linked and purified using phenol/chloroform extraction. The primers used were as follows. GATA-1-binding sites on the β-globin locus were 5′-GGAAGATACTGATAAGTTGAC-3′/5′-TGGAAATCTGGCTTATTGGAG-3′. PCR was performed with HotStarTaq DNA polymerase (Qiagen) for 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were electrophoresed on 1.5% agarose gel and visualized by UV illumination following ethidium bromide staining.

RESULTS

Inhibition of ZNF521 Expression Promotes Erythroid Maturation in K562 and HEL Cells—It is well known that K562 and HEL cells are able to differentiate toward erythroid lineage when they are exposed to chemical inducers (17–19). Because both K562 and HEL cells constitutively express ZNF521, we investigated whether down-regulation of this ZF protein influences the differentiation of these cells. To silence *ZNF521* gene expression, two kinds of shRNA corresponding to a sequence of the *ZNF521* coding region were cloned into the expression vector and transfected transiently into K562 and HEL cells by electroporation. The erythroid differentiation was monitored every other day by benzidine staining. A green fluorescent protein-expressing plasmid was used to quantify the transduction efficiency after electroporation. Flow cytometric analysis revealed that more than 60% of both K562 and HEL cells appeared to be green fluorescent protein-positive after 24 h (data not shown). The expression levels of *ZNF521* mRNA were decreased in shRNA-mediated cell lines as compared with those in control cell lines. As shown in Fig. 1, *A* and *B*, from day 4 to day 8 after transfection of shRNA plasmids for *ZNF521*, the number of benzidine staining-positive cells increased significantly as compared with control cells in both the K562 and HEL cell lines.

We further examined the effect of ZNF521 down-regulation on erythroid differentiation of hematopoietic cells by establishing K562-derived clones in which *ZNF521* mRNA is stably disrupted. After transfection of two kinds of *ZNF521* shRNA expression vectors and an empty vector into K562 cells, stable clones were isolated by limiting dilution following selection with puromycin. We also established K562 clones overexpressing *GATA-1* to compare the phenotype of the *ZNF521*-silenced K562 clones. As shown in Fig. 1*C*, the expression levels of *ZNF521* mRNA in these clone cells determined by Northern

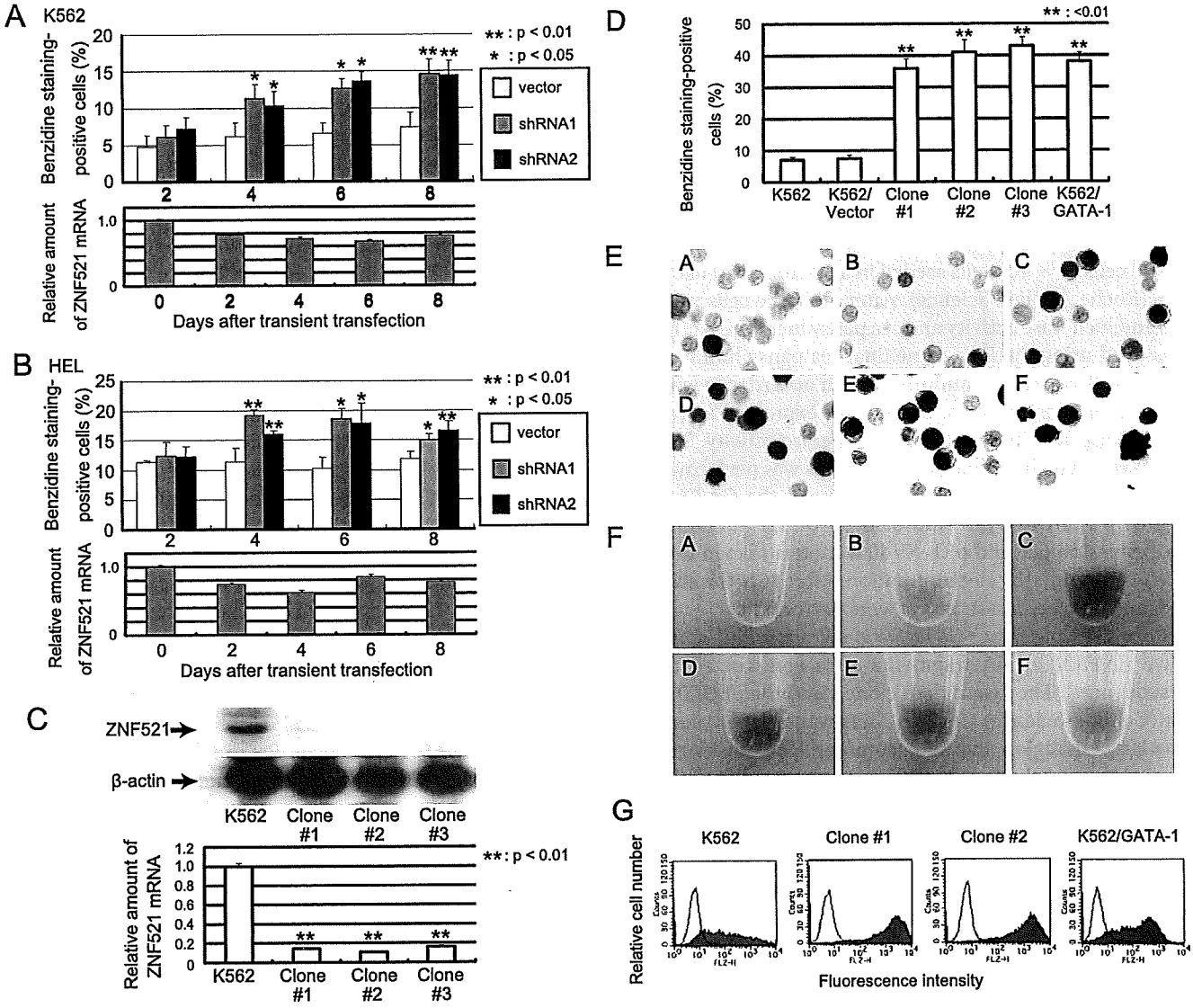

FIGURE 1. **Inhibition of ZNF521 expression by *ZNF521* shRNA induces erythroid differentiation in K562 and HEL cell lines.** *A*, K562 cells were transfected with either one of the expression plasmids for *ZNF521* shRNA (*gray bars*, shRNA1; *black bars*, shRNA2) or a noncoding vector (*white bars*) by electroporation. Benzidine staining-positive cells were counted every other day. Each value represents the mean ± S.E. of two independent experiments conducted in triplicate. Quantitative real time PCR of *ZNF521* mRNA and GAPDH mRNA was performed. The expression levels of *ZNF521* mRNA were corrected by reference to that of GAPDH mRNA. The amounts of *ZNF521* mRNA in shRNA1-mediated lines relative to that in control lines were indicated. *B*, similarly, HEL cells were transfected with either one of the *ZNF521* shRNA expression plasmids (*gray bars*, shRNA1; *black bars*, shRNA2) or a noncoding vector (*white bars*) by electroporation. Benzidine staining-positive cells were counted every other day. *C*, Northern blotting for expression of ZNF521 and β-actin mRNAs was performed. Total RNAs were isolated from a control clone and *ZNF521*-silenced clones and *GATA-1*-overexpressing clones. Twenty micrograms of total RNAs was analyzed by Northern blot hybridization with a probe for *ZNF521* or β-actin. Quantitative real time PCR of *ZNF521* mRNA and GAPDH mRNA was performed. The expression level of *ZNF521* mRNA was corrected by reference to that of GAPDH mRNA. *D*, the percentages of benzidine staining-positive cells among parental K562 cells, the control clone, *ZNF521*-silenced clones, and *GATA-1*-overexpressing clones are shown. *E*, microscopic observations of benzidine staining in parental K562 cells (*A*, K562), a control clone (*B*, K562/vector), *ZNF521*-silenced clones (*C*, clone 1; *D*, clone 2 and clone 3), and *GATA-1*-overexpressing clone (*F*, K562/GATA-1). *F*, appearance of the pellets of parental K562 cells (*A*, K562), a control clone (*B*, K562/vector), *ZNF521*-silenced clones (*C*, clone 1; *D*, clone 2 and clone 3), and *GATA-1*-overexpressing clone (*F*, K562/GATA-1). *G*, expression of glycophorin A on the cell surface of parent K562 cells, *ZNF521*-silenced clones, and the *GATA-1*-overexpressing clone examined by flow cytometric analysis.

blot analysis, and real-time PCR appeared to be dramatically decreased. We compared the erythroid differentiation of *ZNF521*-silenced clones and *GATA-1*-overexpressing clones with the control clones by benzidine staining. As shown in Fig. 1, *D* and *E*, *ZNF521*-silenced clones showed a marked increase of benzidine staining-positive cells. The increased hemoglobinization in the *ZNF521*-silenced clones and *GATA-1*-overexpressing clones was apparently visible (Fig. 1*F*). Next, we examined the expression of glycophorin A, an erythroid lineage-associated cell surface marker, using flow cytometric analysis. As shown in Fig. 1*G*, the expression level of glycophorin A on the cell surface was significantly increased in *ZNF521*-silenced clones as compared with the parental K562 cells. Similarly, an increased level of glycophorin A expression was observed in the *GATA-1*-overexpressing clones. We also compared the gene expression patterns in a *ZNF521*-silenced clone and a control clone comprehensively using Human Genome Survey Microarray version 2.0, covering more than 30000 genes (Applied Biosystems, Foster City, CA). Consequently, expression levels of erythroid lineage-related genes, including globin