than 30 days, 1 patients developed IFI after day 30. None of 63 patients who survived longer than 100 days developed IFI after day 100. Our results contrast with the previous reports on BMT and PBSC transplantation where improvement in fungal management decreased early IFI and late IFI became the majority of IFI [8,19,22,29]. In myeloablative CBT, late infection is considered a significant complication [4], whereas study results focused on fungal infection have not been published. Some hypotheses can explain the low incidence of late IFI after RICBT. First, cGVHD after RICBT is uncommon and mild. There is minimal effect of cGVHD on delay in immune recovery following RICBT. Second, steroids are not frequently administered late after RICBT for the treatment of complications such as GVHD. The incidence of cGVHD was 28% in the present study, and none of them required steroid treatments. Further studies are awaited for the clinical features of late IFI after RICBT.

The present study demonstrated clinical features of fungal infections after RICBT, leaving several issues to be investigated. First, the present study is a small-sized retrospective 1 . Unrecognized bias might affect the study results, and we obtained little information on rare fungal infections such as Fusarium and Zygomycetes. Large-sized prospective studies are awaited. Second, the diagnostic yields of IFI need to be addressed. Most of the diagnoses in our study were made based on EORTC/MSG criteria [16] using clinical, laboratory, and imaging findings. Although the clinical usefulness of the diagnostic criteria has been established, pathologic diagnosis of IFI was not confirmed in many patients and the diagnostic yields remain unclear. Underestimation of IFI incidence also remains possible, because postmortem examinations were not obtained in most patients who died without diagnosis of IFI. Because such limitations cannot be avoided in studying deep fungal infections [30], clinicians need to be aware of the limitations.

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Cytomegalovirus Infections following Umbilical Cord Blood Transplantation Using Reduced Intensity Conditioning Regimens for Adult Patients

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ABSTRACT

Cytomegalovirus (CMV) infection is a major complication after allogeneic hematopoietic stem cell transplantation (Allo-HSCT); however, we have little information on the clinical features of CMV reactivation after cord blood transplantation using reduced-intensity regimens (RI-CBT) for adults. We reviewed medical records of 140 patients who underwent RI-CBT at Toranomon Hospital between January 2002 and March 2005. All the patients were monitored for CMV-antigenemia weekly, and, if turned positive, received preemptive foscarnet or ganciclovir. Seventy-seven patients developed positive antigenemia at a median onset of day 35 (range, 4-92) after transplant. Median of the maximal number of CMV pp65-positive cells per 50,000 cells was 22 (range, 1-1806). CMV disease developed in 22 patients on a median of day 35 (range, 15-106); 21 had enterocolitis and 1 had adrenalitis. CMV antigenemia had not been detected in 2 patients, when CMV disease was diagnosed. CMV disease was successfully treated using ganciclovir or foscarnet in 14 patients. The other 8 patients died without improvement of CMV disease. In multivariate analysis, grade II-IV acute graft-versus-host disease was a risk factor of CMV disease (relative risk 3.48, 95% confidential interval 1.47-8.23). CMV reactivation and disease develop early after RI-CBT. CMV enterocolitis may be a common complication after RI-CBT.

KEY WORDS

Graft-versus-host disease • Ganciclovir • Foscarnet • Cytomegalovirus antigenemia • CD34-positive cells

INTRODUCTION

Cord blood transplantation (CBT) is an attractive alternative for patients with hematologic diseases who lack a matched related or unrelated donor. The value of CBT using myeloablative preparative regimens has been confirmed for pediatric patients [1,2]. Myeloablative CBT for adult patients achieves engraftment in 90% of the patients, but carries a 50% risk of transplant-related mortality (TRM), mostly resulting from infection [3,4]. We and other groups have reported the feasibility of CBT using reduced-intensity regimens (RI-CBT) for adult patients with advanced hematologic diseases [5,6].

Because of delayed immune recovery and graft-versus-host disease (GVHD), infection is the leading cause of TRM after CBT using myeloablative preparative regimens [2-4,7]. However, studies on immune recovery following RI-CBT gave us hope that RI-CBT recipients may less frequently experience GVHD and infectious complications. Cytomegalovirus (CMV) has been 1 of the most feared infectious complications in CBT [8], as well as in conventional allogeneic marrow or peripheral blood stem cell trans-

plantation (PBSCT) [9,10], although we have little information on CMV infection following RI-CBT. We investigated its frequency and clinical features in patients who underwent RI-CBT for advanced hematologic diseases.

PATIENTS AND METHODS

Study Patients and Donors

We reviewed medical records of 140 patients who underwent RI-CBT at Toranomon Hospital between January 2002 and March 2005. All the patients had diseases that were incurable with conventional treatments, and were considered inappropriate for conventional allogeneic stem cell transplantation (allo-SCT) because of the lack of an human leukocyte antigen (HLA)-identical sibling or a suitable related/unrelated donor, aged >50 years old and/or organ dysfunction (generally attributable to previous intense chemotherapy and/or radiotherapy). All the patients provided written informed consent in accordance with the requirements of the institutional review board.

HLA Typing and Donor Matching

An unrelated cord blood donor was searched through the Japan Cord Blood Bank Network [11] for patients without an HLA-identical sibling donor or a suitable related/unrelated donor.

Preparative Regimen

All the patients received purine analog-based preparative regimens (Table 1).

Engraftment

Engraftment was defined as white blood cell counts $>1.0 \times 10^9$/L or absolute neutrophil counts $>0.5 \times 10^9$/L for 2 consecutive days. Granulocyte-colony stimulating factor (G-CSF) was administered i.v. from day 1 until engraftment.

Supportive Care and Management of Preengraftment Fever and GVHD

All the patients were managed in reverse isolation in laminar airflow-equipped rooms, and received trimethoprim/sulfamethoxazole for *Pneumocystis jiroveci* prophylaxis. Fluoroquinolone and fluconazole or itraconazole were administered for prophylaxis of bacterial and fungal infections, respectively. Prophylaxis of herpes virus infection with acyclovir 600 mg/day was also given [12]. Neutropenic fever was managed according to the guidelines [13].

Diagnosis and management of preengraftment immune reaction were reported previously [14]. GVHD was clinically diagnosed in combination with skin or gut biopsies after engraftment or attainment of 100% donor chimerism. Acute (aGVHD) and chronic GVHD (cGVHD) were graded according to the established criteria [15,16]. GVHD prophylaxis was a continuous infusion of cyclosporine 3 mg/kg or tacrolimus 0.03 mg/kg from day −1 until the patients tolerated oral administration. It was tapered off from day 60 until day 150 or depending on the status of GVHD. If grade II-IV aGVHD developed, 0.5-1.0 mg/kg/day of prednisolone was added to cyclosporine or tacrolimus, and tapered from the beginning of clinical response.

Table 1. *Patient Characteristics*

Variable	Number
Age (median [range])	55 (17-79)
Sex (men/women)	81/59
Primary diseases	
Acute lymphoblastic leukemia	19
Acute myeloid leukemia	44
Chronic myelogenous leukemia	5
Adult T cell leukemia	19
Myelodysplastic syndrome	14
Malignant lymphoma	29
Multiple myeloma	4
Aplastic anemia	6
Risk of underlying diseases (high/low)*	99/41
Preparative regimens	
Flud 125 mg/m^2 + L-PAM (80 mg/m^2) + TBI (2-8 Gy)	121
Flud 125 mg/m^2 + L-PAM (140 mg/m^2) + TBI (4-8 Gy)	5
Flud 125 mg/m^2 + L-PAM (100 mg/m^2) + TBI (4-8 Gy)	2
Flud 150 mg/m^2 + BU 8 mg/kg + TBI (4-8 Gy)	8
Flud 125 mg/m^2 + L-PAM (80-140 mg/m^2)	2
Flud 150 mg/m^2 + BU 8 mg/kg	1
L-PAM 140 mg/m^2	1
Number of infused mononuclear cells ×10E7/kg (median [range])	2.7 (0.4-5.7)
Number of infused CD34$^+$ cells ×10E5/kg (median [range])	0.73 (0.01-5.7)
HLA antigen disparity 0/1/2/3	3/21/114/2
GVHD prophylaxis	
Cyclosporine	85
Tacrolimus	55

Flud indicates fludarabine; L-PAM, melphalan; BU, busulfan; TBI, total body irradiation; GVHD, graft-versus-host disease.

*Acute leukemia in complete remission, chronic myelocytic leukemia in the chronic phase, malignant lymphoma in complete remission, multiple myeloma in complete remission, myelodysplastic syndrome in refractory anemia (RA), and aplastic anemia were defined as low risk. All others were considered high risk.

Management of CMV Infection

CMV-specific IgM antibodies in the cord blood units were not examined. Because most patients had been heavily treated and received multiple transfusions, anti-CMV antibodies were not examined before transplantation. Anti-CMV high-titer i.v. immunoglobulin was not regularly administered. All packed

red blood cells and platelets were transfused using leukocyte-depleting filters.

CMV infection was defined as isolation of CMV or detection of viral proteins or nucleic acid in any body fluid or tissue specimen. CMV disease was diagnosed as follows: CMV enterocolitis was diagnosed by gastrointestinal symptoms with histologic demonstration of CMV on biopsy materials obtained by endoscopy; CMV pneumonia was diagnosed when either a bronchoalveolar lavage or a lung biopsy was positive for CMV in a patient with characteristic signs, symptoms, and chest radiographic findings; CMV retinitis was diagnosed by characteristic retinal opacities without other likely explanations for the retinal findings. CMV pp65 antigenemia was monitored weekly after engraftment or when patients died before engraftment. Briefly, 1.5×10^5 peripheral blood leukocytes were attached to slides using a cytocentrifuge and fixed with cold acetone. From 1/3 to 1/2 of the centrifuged cells were fixed on the slides. The cells were incubated with monoclonal antibody HRP-C7 (Teijin, Tokyo, Japan) raised against immediate-early antigen, and stained by the direct immunoperoxidase method. These cells were analyzed under a light microscope and results were presented as the number of positive cells per 50,000 cells [17].

CMV antigenemia was managed according to the report by Kanda et al [17]. If CMV pp65-positive cells exceeded 10/50,000, patients preemptively received either ganciclovir 5 mg/kg once daily or foscarnet 30 mg/kg twice daily. Initiation of ganciclovir or foscarnet with <10 positive cells was optional in the patients who received more than 0.5 mg/kg of prednisolone. The doses were adjusted for renal function [18]. Ganciclovir or foscarnet was discontinued when 2 consecutive results of CMV antigen were negative. When CMV disease was diagnosed during preemptive therapy, we increased the dose of ganciclovir to 5 mg/kg twice a day, or foscarnet to 60 mg/kg twice or 3 times daily.

Endpoints and Statistical Analysis

The aims of this study were (1) to determine the incidence of CMV infection after RI-CBT, (2) to investigate its clinical features, and (3) to identify its risk factors. The cumulative incidences of CMV disease and CMV reactivation defined by the detection of CMV pp65 were evaluated using Gray's method [19], considering death without CMV reactivation as a competing risk. Potential confounding factors considered in the analysis were patient's age, sex, stem cell doses, HLA disparity, GVHD prophylaxis, conditioning regimens, and aGVHD. The influence of these factors on the incidence of CMV disease and CMV reactivation was evaluated with the proportional hazard modeling treating the development of aGVHD and the use of corticosteroids as time-dependent covariates. Factors associated with at least borderline significance ($P < .10$) in the univariate analyses were subjected to a multivariate analysis using backward stepwise proportional-hazard modeling. P-values of <.05 were considered statistically significant.

RESULTS

Patient's Characteristics and Clinical Outcomes

Patient's characteristics are shown in Table 1. Of the 140 RI-CBT recipients, 112 patients (80%) achieved primary engraftment on a median of day 20 (range, 10-57). Sixty (43%) and 8 (6%) patients died of transplant-related causes and disease progression, respectively, within 100 days of RI-CBT. Preengraftment immune reaction [20] was diagnosed in 67 patients. Of the 112 patients who achieved engraftment, 47 (42%) developed grade II-IV aGVHD at a median onset of day 25 (range, 13-94). Sixty-one patients received prednisolone or methylprednisolone >0.5 mg/kg/day within 100 days of RI-CBT because of preengraftment immune reaction (n = 26), engraftment syndrome (n = 3), aGVHD (n = 16), and others (n = 16). As of November 2005, the median follow-up of the surviving patients was 13.0 months (range, 1.0-40.7). Overall survival rates were 85% (95% confidence interval [CI]; 79-91%) and 53% (95% CI; 45-62%) at days 30 and 100, respectively.

Clinical Features of CMV Reactivation and Diseases

Clinical features of CMV reactivation and diseases are summarized in Table 2. CMV antigenemia was found in 77 patients (55%, 95% CI; 51-59%) on a median of day 35 (range, 4 to 92). Twenty-eight of those patients received prednisolone or methylprednisolone >0.5 mg/kg/day before development of

Table 2. *Clinical Features of CMV Reactivation and Disease*

Variable
CMV reactivation
Number of patients
Onset (median [range])
Maximal levels of CMV antigenemia (range)
Preemptive therapy (ganciclovir/foscarnet/none)
CMV disease
Number of patients
Diagnose of CMV disease (median, [range])
Organ involvement
enterocolitis
pneumonia
retinitis
adrenalitis
Use of anti-CMV agents at the onset of CMV disease (ganciclovir/foscarnet/none)

CMV indicates cytomegalovirus.

Table 3. *Univariate and Multivariate Analyses for the Incidence of CMV Reactivation*

Factor	Relative risk (95% CI)	P value
Univariate		
Age	1.005 (0.989-1.021)	.53
Sex	1.10 (0.71-1.72)	.66
Disease status	0.68 (0.44-1.07)	.098
Number of HLA mismatch	1.37 (0.80-2.36)	.25
Number of infused mononuclear cells	0.99 (0.68-1.45)	.95
Number of infused $CD34^+$ cells	1.55 (1.28-1.87)	5.8×10^{-6}
GVHD prophylaxis (cyclosporine vs. tacrolimus)	0.59 (0.37-0.94)	.025
Preengraftment immune reaction	1.14 (0.74-1.78)	.55
Acute GVHD (grade II-IV)	1.21 (0.70-2.10)	.49
Use of steroid*	1.64 (1.02-2.64)	.042
Multivariate		
Number of infused $CD34^+$ cells	1.55 (1.28-1.87)	5.8×10^{-6}

CMV indicates cytomegalovirus; CI, confidence index; GVHD, graft-versus-host disease.
*Use of prednisolone or methyl-prednisolone >0.5 mg/kg/day.

CMV antigenemia. Forty-nine patients received foscarnet (n = 41) or ganciclovir (n = 8) preemptively. Initial dose of ganciclovir was 5 mg/kg once daily. The remaining 28 patients had not received foscarnet or ganciclovir according to our preemptive strategy, because of <10/50,000 of CMV pp65-positive cells.

Diagnosis of CMV disease was established in 22 patients (16%, 95% CI; 13-19%) on a median of day 33 (range 15-106); the diagnosis comprised enterocolitis (n = 21) and adrenalitis (n = 1). Of the 22 patients, 9 patients had received preemptive therapy before developing CMV disease. The remaining 13 patients had not received foscarnet or ganciclovir according to our preemptive strategy, mostly because of <10/50,000 of CMV pp65-positive cells.

Diagnosis of CMV disease was established at postmortem examination in 1 patient. The other 21 patients were treated with either foscarnet or ganciclovir. CMV disease was successfully treated in 14 patients. The remaining 8 patients died without improvement of CMV disease, although CMV disease was not the primary cause of death in any of these 8 patients.

Risk Factors of CMV Antigenemia and CMV Disease

Risk factors of CMV reactivation and CMV disease were shown in Table 3 and Table 4, respectively. CD34-positive cell dose was significantly associated with CMV reactivation on multivariate analysis (relative risk, 1.55; 95% CI 1.28-1.87; $P = 5.8 \times 10^{-6}$). Grade II-IV aGVHD was a risk factor of CMV disease on multivariate analysis (relative risk, 3.48; 95% CI 1.47-8.23; $P = .0045$).

DISCUSSION

The present study demonstrated that CMV infection is a significant complication of RI-CBT. The incidence of CMV reactivation was 55% in our study, which was comparable with previous reports on RIST [21-23] and myeloablative bone marrow transplantation (BMT) and PBSCT [24,25]. In contrast, previously reported incidence of CMV reactivation after CBT (79%) [8] was higher than that of ours, although it is the only previous report on CMV reactivation after CBT. The differences in preparative regimens and patient characteristics between the study [8] and ours may have affected the incidence of CMV reactivation. One of the unique findings in the present study was that the timing of CMV reactivation after RI-CBT was earlier than that after RIST without in vivo or ex vivo T cell depletion [21]. Another unique finding was the high incidence of CMV disease compared with transplantation of other stem cell sources [21,24,25]. Of the 77 patients with CMV antigenemia, 22 developed CMV disease in our study. The risk of progression from CMV reactivation to CMV disease may be high in CBT because of the intense immunosuppression [26].

The present study suggests that CMV infection is more likely to reactivate and to progress in RI-CBT than in transplantation using other stem cell sources. Several reasons can explain this hypothesis. First, the preparative regimens including total body irradiation (TBI) in our study might have damaged recipient-

Table 4. *Univariate and Multivariate Analyses for the Incidence of CMV Disease*

Factor	Relative risk (95% CI)	P value
Univariate		
Age	1.021 (0.991-1.052)	.16
Sex	1.15 (0.50-2.64)	.74
Disease status	1.92 (0.65-5.64)	.24
Number of HLA mismatch	2.54 (0.42-15.22)	.31
Number of infused mononuclear cells	0.59 (0.36-0.98)	.041
Number of infused $CD34^+$ cells	1.34 (0.93-1.93)	.11
GVHD prophylaxis (cyclosporine vs. tacrolimus)	0.85 (0.36-1.99)	.70
Preengraftment immune reaction	0.76 (0.33-1.76)	.52
Acute GVHD (grade II-IV)	3.48 (1.47-8.23)	.0045
Use of steroid*	1.36 (0.53-3.48)	.53
Multivariate		
Acute GVHD (grade II-IV)	3.48 (1.47-8.23)	.0045

CMV indicates cytomegalovirus; CI, confidence index; GVHD, graft-versus-host disease.
*Use of prednisolone or methyl-prednisolone >0.5 mg/kg/day.

derived anti-CMV immune cells. That contrasts with the report that recipient-derived T cells are associated with immune reaction against CMV early after transplantation following preparative regimens without TBI [27]. The issue needs to be considered in determining preparative regimens for RI-CBT. Second, transplanted cord blood stem cells are immunologically naïve. Although anti-CMV cytotoxic T-lymphocytes in transplant grafts are considered to suppress CMV proliferation early after transplantation in CMV seropositive recipients [28], passive immunity via grafts against CMV cannot be expected in CBT, and thus the risk of reactivation may be high. Third, post-transplant immune recovery is delayed in CBT. Little is known about post-CBT immune recovery with only few reports. Although the numbers of T cells, B cells, and NK cells, and their in vitro reactivity after CBT are comparable with those after BMT [29,30], post-CBT incidence of infections including CMV is high [2,7,8,31-35], and immune recovery is probably delayed compared with BMT and PBSCT. Intense reactivation itself can reportedly delay the recovery of cellular immunity [36], which might be associated with the high incidence of CMV disease in the present study. Finally, immunosuppression was intensified to control post-CBT immune reaction. In our study, GVHD prophylaxis was cyclosporine or tacrolimus alone, which was mild compared with conventional transplantation. Immune reaction occasionally occurs before and at engraftment, requiring steroid treatments [6,14,37]. In the present study, 62 patients received steroids within 100 days of CBT. Steroids might have suppressed the recovery of anti-CMV cytotoxic T-lymphocytes [38].

Some challenges remain to improve the management of CMV reactivation after RI-CBT. First, optimal methods need to be established to monitor CMV reactivation. We have introduced preemptive therapy based on the results of CMV antigenemia in our hospital. The efficacy of this method in PBSCT has been reported [17], although it might not be applicable to CBT. Of the 22 patients with CMV disease, 9 progressed from CMV antigenemia to disease despite preemptive therapy. CMV disease developed with less than 10/50,000 pp65-positive cells in 13 patients. These observations suggest that antiviral therapy might be necessary immediately after CMV antigenemia is detected in CBT. More sensitive diagnostic tests such as genetic examinations [39] are also helpful in early detection of CMV reactivation. Second, the optimal preemptive strategy that is applicable to CBT has to be established. Because the disease rate in the untreated CMV positives was 46%, preemptive administration of anti-CMV agents might be required for patients with <10/50,000 pp65-positive cells. Alternatively, universal prophylaxis of CMV might be worth investigating. Optimal dose of preemptive ganciclovir and foscarnet must be also investigated. We reduced doses of preemptive foscarnet and ganciclovir mostly because of concerns of its renal toxicity and myelotoxicity, respectively. However, the failure rate of preemptive ganciclovir or foscarnet was 18%, and it was higher than that in the studies in which those were not reduced [40,41]. Clinical impact of the dose of ganciclovir or foscarnet on preemptive therapy should be investigated in future clinical studies. Finally, identification of high-risk group for CMV reactivation is necessary. The reported risk factors in conventional HSCT include GVHD, steroid administration, CMV serostatus of recipients and donors, and age [22,28,42-44]. The high dose of transfused CD34-positive cells was an independent risk factor for CMV reactivation in our analysis (Table 3). The association between the number of CD34-positive cells and CMV infection has not been reported in previous studies on BMT and PBSCT. It remains unknown and awaits further investigations. aGVHD was an independent risk factor for CMV disease. This is comparable with the report on CMV disease after allo-SCT [25].

Most of the patients with CMV disease had CMV enterocolitis in the present study. None developed CMV pneumonia or retinitis. Although the reason for the high incidence of gastrointestinal CMV disease after RI-CBT remains unclear, the use of TBI and melphalan in the preparative regimens that have significant gastrointestinal mucous toxicity [45] and complications of gut GVHD and thrombotic microangiopathy [14] may be related. Although there are different opinions on the usefulness of antigenemia in diagnosing CMV enterocolitis [46-48], the present study demonstrated that monitoring CMV antigenemia can play a certain role in early diagnosis of CMV enterocolitis after RI-CBT. Further studies are necessary to demonstrate the pathogenesis of gastrointestinal CMV disease after RI-CBT and to develop diagnostic methods for its early detection.

Although the present study provided novel information on CMV infection after RI-CBT, some issues remain to be investigated. First, the present study was retrospective and small sized. Prospective, large-sized studies are awaited. Second, RI-CBT recipients are likely to have potential organ dysfunction because most of them are at advanced ages and have been heavy treated with chemotherapies. Such characteristics of patients may affect the treatment of CMV infection. Pharmacokinetics of antiviral agents in older patients has not been well investigated, requiring further studies. Third, recipient pretransplant CMV serostatus was reported to correlate with mortality after CBT [49]. However, anti-CMV antibodies were not examined before transplantation in this study because most patients had been heavily treated and received multiple transfusions. Pretransplant CMV serostatus needs to be investigated in future studies.

Fourth, day 100 mortality was 49% in the present study. It is higher than that in the previous study reported by the Minnesota group [5]. The exact reason of these differences remains unknown; however, it might be partly from the difference in patient's backgrounds between these studies. This high mortality rate and patient's backgrounds in the present study might have affected the results. Fifth, the management of CMV infection in the present study might have affected the incidence of CMV disease; we used the reduced dose of foscarnet or ganciclovir and anti-CMV high-titer i.v. immunoglobulin was not regularly administered. Finally, late CMV infection remains to be investigated. Because CMV antigenemia-guided pre-emptive strategy has been established [50], the prognosis of CMV infection following BMT and PBSCT improved; however, late CMV disease remains a significant issue [51]. The observation period was short in the present study, and could not provide enough information on late CMV disease.

The present study demonstrated that CMV infection is a significant complication of RI-CBT. Although RI-CBT is an attractive alternative, physicians should be alert to the fact that this transplant procedure is associated with a high risk of CMV infection.

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Alternative splicing due to an intronic SNP in *HMSD* generates a novel minor histocompatibility antigen

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Here we report the identification of a novel human leukocyte antigen (HLA)-B44–restricted minor histocompatibility antigen (mHA) with expression limited to hematopoietic cells. cDNA expression cloning studies demonstrated that the cytotoxic T lymphocyte (CTL) epitope of interest was encoded by a novel allelic splice variant of *HMSD*, hereafter designated as *HMSD-v*. The immunogenicity of the epitope was generated by differential protein expression due to alternative splicing, which was completely controlled by 1 intronic single-nucleotide polymorphism located in the consensus 5′ splice site adjacent to an exon. Both *HMSD-v* and *HMSD* transcripts were selectively expressed at higher levels in mature dendritic cells and primary leukemia cells, especially those of myeloid lineage. Engraftment of mHA$^+$ myeloid leukemia stem cells in nonobese diabetic/severe combined immunodeficient (NOD/SCID)/γc^{null} mice was completely inhibited by in vitro preincubation with the mHA-specific CTL clone, suggesting that this mHA is expressed on leukemic stem cells. The patient from whom the CTL clone was isolated demonstrated a significant increase of the mHA-specific T cells in posttransplantation peripheral blood, whereas mHA-specific T cells were undetectable in pretransplantation peripheral blood and in peripheral blood from his donor. These findings suggest that the *HMSD-v*–encoded mHA (designated ACC-6) could serve as a target antigen for immunotherapy against hematologic malignancies. (Blood. 2007; 110:1055-1063)

Introduction

Minor histocompatibility antigens (mHAs) are major histocompatibility complex (MHC)-bound peptides derived from cellular proteins encoded by polymorphic genes. Following human leukocyte antigen (HLA)-matched allogeneic hematopoietic cell transplantation (HCT), donor-recipient disparities in mHAs can induce a favorable graft-versus-leukemia (GVL) effect that is often associated with graft-versus-host disease (GVHD).[1-3] Significant efforts have been made to identify mHAs, particularly those specific for hematopoietic cells, since such mHAs are speculated to contribute to the GVL effect. The first report on the identification of a hematopoietic lineage-specific mHA, HA-1, was generated by the Goulmy group in 1998 (den Haan et al[4]) as a result of biochemical analysis of peptides eluted from HLA-A*0201 molecules. The only other mHAs with selective expression in hematopoietic cells described to date are HA-2[5]; ACC-1 and ACC-2[6]; and DRN-7,[7] HB-1,[8,9] and PANE1,[10] the latter 2 of which are B-cell lineage-specific. Thus, identification of more mHAs should facilitate a better understanding of the biology of GVL and the development of effective immunotherapy to induce GVL reactions.

Immunogenicity of most autosomal mHAs identified to date results from single-nucleotide polymorphisms (SNPs) that cause amino-acid substitutions within epitopes, leading to the differential display/recognition of peptides between HCT donor and recipient via several mechanisms: peptide binding to MHC observed in HA-1/A2-,[4] HA-2-,[5] and *CTSH*-encoded mHAs[11]; proteasomal cleavage in HA-3[12]; peptide transport in HA-8[13]; and altered recognition of MHC-peptide complex by cognate T cells in HB-1,[8,9] HA-1/B60,[14] ECGF1/B7,[15] and SP110/A3.[7] Other examples of mechanisms of mHA generation include differential protein expression due to a nonsense mutation in *PANE1*[10] and a frame-shift mutation in *P2X5*.[16] *UGT2B17*[17] is the sole example of differential protein expression due to gene deletion instead of an SNP. Because SNPs are scattered throughout the genome, it has been speculated that mHAs caused by those other than coding SNPs should be present.

In this study, we report the identification of a novel gene encoding an HLA-B44–restricted mHA that is recognized by the 2A12 cytotoxic T lymphocyte (CTL) clone and selectively expressed in primary hematologic malignant cells, especially those of myeloid lineage, multiple myeloma (MM) cells, and normal mature dendritic cells (DCs). The antigenic peptide recognized by 2A12-CTL was encoded by a novel allelic splice variant of *HMSD*,

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hereafter designated as *HMSD-v*, due to an intronic SNP located in the consensus 5′ splice site adjacent to an exon. The leukemic stem cell (LSC) engraftment assay using severely immunodeficient mice demonstrated that the engraftment of primary acute myeloid leukemia (AML) cells was completely abolished by coincubation with the CTL clone before injection. These findings suggest that this novel mHA epitope may be an attractive therapeutic target for immunotherapy.

Patients, materials, and methods

Cell isolation and cell cultures

This study was approved by the Institutional Review Board of Aichi Cancer Center. All blood or tissue samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. B-lymphoid cell lines (B-LCLs) were derived from donors, recipients, and healthy volunteers. B-LCLs and all cell lines of hematologic malignancy were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate (referred to as complete medium). CD40 ligand-activated B (CD40-B) cells were generated as previously described.[18]

Immature DCs were generated by culturing $CD14^+$ cells isolated from peripheral-blood mononuclear cells (PBMCs) with 500 U/mL GM-CSF and 500 U/mL interleukin 4 (IL-4) in AIM-V medium (Invitrogen, Carlsbad, CA) for 2 days, and then DCs were matured by cultivating the immature DCs for 2 additional days with 10 ng/mL IL-1β, 20 ng/mL IL-6, 10 ng/mL tissue necrosis factor α (TNF-α; all cytokines were from R&D Systems, Minneapolis, MN), and 1 μg/mL PGE2 (Cayman Chemical, Ann Arbor, MI). When necessary, cells were retrovirally transduced with restricting HLA cDNA by a method described previously.[18,19]

Generation of CTL lines and clones

CTL lines were generated from PBMCs ($\sim 10^6$) obtained at day 197 after HCT by primary stimulation with irradiated (33 Gy) pre-HCT recipient PBMCs ($\sim 10^6$), thereafter stimulated weekly with irradiated (33 Gy) recipient CD40-B cells (2×10^6) twice in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine (referred to as CTL medium).[11] IL-2 was added on days 1 and 5 after the second and third stimulation. CTL clones were isolated by standard limiting dilution and expanded in CTL medium as previously described.[11,20]

Chromium release assay

Target cells were labeled with 3.7 MBq of ^{51}Cr for 2 hours, and 10^3 target cells/well were mixed with CTLs at the effector-target (E/T) ratio indicated in a standard 4-hour cytotoxicity. All assays were performed at least in duplicate. Some target cells were pretreated with interferon γ (IFN-γ; 500 U/mL) and TNF-α (10 ng/mL; both from R&D Systems) for 48 hours as indicated. Percent specific lysis was calculated as follows: ([experimental cpm − spontaneous cpm]/[maximum cpm − spontaneous cpm]) × 100, where cpm indicates counts per minute.

cDNA library construction

The cDNA library used in the present study was the same one that had been used to identify HLA-A31- and HLA-A33-restricted *cathepsin H*-encoded mHAs (ACC-4 and ACC-5) previously.[11] The cDNA library was constructed from mRNA of a B-LCL derived from an AML patient (UPN-027) using the SuperScript Plasmid System (Invitrogen). The library contained 1.5×10^6 cDNA clones with an average insert size of approximately 2500 bp. cDNA pools, each consisting of approximately 120 and 5 clones for initial and second screens, respectively, were expanded for 24 hours in 96 deep-well plates, and plasmid DNA was extracted with the QIAprep 96 Turbo Miniprep kit (Qiagen, Valencia, CA).

Transfection of 293T cells and ELISA

Twenty thousand 293T cells retrovirally transduced with HLA-B*4403 were plated in each well of 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0.12 μg of plasmid containing a pool of the cDNA library using Trans IT-293 (Mirus, Madison, WI). Ten thousand CTL-2A12 cells were added to each well 20 hours after transfection. After overnight incubation at 37°C, 50 μL of supernatant was collected and IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA).

Genotyping of polymorphisms

Genomic DNA was isolated from each B-LCL with a QIAamp DNA blood kit (Qiagen). Total RNA was extracted using an RNeasy Mini Kit (Qiagen), and cDNA was synthesized by standard methods. Genomic DNA or cDNA was amplified using KOD-plus-DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The polymerase chain reaction (PCR) temperature profile was 30 cycles of 94°C for 15 seconds, 58°C for 20 seconds, and 68°C for 40 seconds on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

The primer sequences used to amplify from exon 1 to exon 4 of *HMSD* cDNA were as follows: sense, 5′-CCTCTCCGACCCGGTCTC-3′; antisense, 5′-GGGAAAAGCTAAAGCTAGAGAAAA-3′. Exonic sequence and intronic sequence adjacent to *HMSD* exon 1 and 2 were amplified with primers as follows: exon 1 sense, 5′-GACTGAAAACTCCCGGACAG-3′; exon 1 antisense, 5′-GAAAGGTCTGGAGCAACAGG-3′; exon 2 sense, 5′-GCAGACATTCACTCACAGCA-3′; exon 2 antisense, 5′-AAGCACCCACATGAGTGACC-3′. PCR products were purified and directly sequenced with the same primer.

Construction of minigenes and truncated genes for *HMSD-v*

Mammalian expression plasmids containing the full-length or truncated forms of the *HMSD-v* cDNA were constructed by reverse transcriptase (RT)-PCR using the isolated cDNA clone as a template. The constructs all encoded a Kozak sequence and initiator methionine (CCACC-ATG) and a stop codon (TAA). All products were ligated into *Hin*dIII-*Not*I-cut pEAK10 vector (Edge Bio Systems, Gaithersburg, MD) and verified by sequencing.

Epitope reconstitution assay

The candidate *HMSD*-encoded epitopes were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled donor B-LCLs were incubated for 30 minutes in complete medium containing 10-fold serial dilutions of the peptides and then used as targets in standard cytotoxicity assays.

Real-time PCR assay for *HMSD* and *HMSD-v* expression

cDNAs were prepared from various hematologic malignant cell lines, primary cell cultures, freshly isolated $CD34^+$ bone marrow (BM) and peripheral-blood hematopoietic cells and their subpopulations, immature and mature DCs, activated B and T cells, $CD34^+$ subsets of primary leukemic cells, and $CD138^+$ subsets of primary MM cells. Cell sorting was performed using magnetic-activated cell separation (MACS) immunomagnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany). A panel of cDNA made from different human adult and fetal tissues was purchased (MTC panels human I and II; BD Biosciences, San Diego, CA). Real-time PCR analysis was performed using the TaqMan assay as described previously.[11] Because of uncertainty of which allele(s) were included in each cDNA pool from the MTC panels, quantitative PCR primers and a probe were designed to detect the exon 3-4 boundary, which is shared by both alleles. The following sequences spanning the exon 3-4 boundary were used as primers with TaqMan probe to detect both *HMSD* and *HMSD-v* transcripts simultaneously: sense, 5′-AGAACTGCCAACGGGCTCTT-3′; antisense, 5′-TTGGTAGAATTTGCCACAGGAAT-3′; probe, 5′-(FAM)-CTTATGATTTCCTCACAGGTT-(MGB)-3′. To selectively detect *HMSD-v* transcripts, the following oligonucleotides specific for the exon 1-3 boundary were used: sense, 5′-CTCCGACCCGGTCTCACTT-3′; antisense, 5′-TCTCCATCTTCACCTCCGATTT-3′; probe, 5′-(FAM)-CAAAGTGCCCCAGTTC-(MGB)-3′.

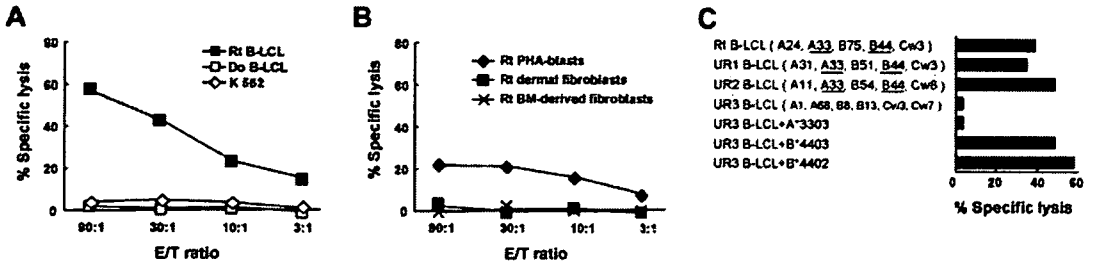

Figure 1. Specificity of the HLA-B44–restricted CTL clone 2A12. The cytolytic activity of CTL-2A12 was evaluated in a standard 4-hour ^{51}Cr release assay at the indicated E/T ratios. (A) CTL-2A12 recognition of target cells derived from recipient (Rt) but not donor (Do) B-LCLs. NK-sensitive K562 cells were used to determine nonspecific lysis. (B) CTL-2A12 recognition of Rt PHA-stimulated T cells (PHA blasts) but not of Rt dermal fibroblasts and bone marrow (BM)-derived fibroblasts pretreated with 500 U/mL IFN-γ and 10 ng/mL TNF-α for 48 hours before ^{51}Cr labeling. (C) CTL-2A12 recognition of an HLA-B*4403- and -B*4402-restricted mHA epitope. The following target cells were tested: Rt B-LCL, B-LCLs of 2 unrelated individuals (UR1 and UR2) sharing an HLA-A33, B44 haplotype with the recipient, and B-LCLs of an HLA class I-mismatched individual (UR3) that were transduced with either HLA-A*3303, B*4403, or B*4402 (E/T ratio, 30:1).

CD45 mRNA expression was detected as described previously.[21] A primer and probe set for human *GAPDH* (Applied Biosystems) was used as an internal control. PCR was performed according to the manufacturer's instructions in the ABI PRISM 7700HT Sequence Detector System (Applied Biosystems). Samples were quantified using relative standard curves for each experiment. All results were normalized with respect to the internal control and are expressed relative to the levels found in recipient B-LCLs.

LSC engraftment assay of AML cells in immunodeficient NOG mice

BM cells were obtained from patients with AML at diagnosis and then positively selected for $CD34^+$ subsets using MACS immunomagnetic beads (Miltenyi). NOD/Shi-scid, IL-2Rγc^{null} (NOG) mice[22] were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). All mice were maintained under specific pathogen-free conditions in the Aichi Cancer Center Research Institute. The Ethical Review Committee of the Institute approved the experimental protocol. The ACC-2^D mHA-specific CTL clone 3B5[6] restricted by the same HLA-B*4403 allele as CTL-2A12 was used as a control CTL clone for this assay. AML cells (7.0×10^6) were preincubated for 16 hours in CTL medium supplemented with 25 units/mL recombinant human IL-2 at 37°C with 5% CO_2 either alone or in the presence of CTL-2A12 or CTL-3B5 at a T-cell/AML cell ratio of 5:1. Thereafter, the cultures were harvested and resuspended in a total volume of 300 μL and were inoculated via the tail vein of 8- to 12-week-old NOG mice (3 mice per group). Five weeks after inoculation, mice were killed, peripheral blood was aspirated from the heart, and BM cells were obtained by flushing the femora with complete medium. Nucleated cells were prepared for flow cytometry by incubation at 4°C for 20 minutes in PBS and 2% FCS with antihuman CD45 and CD34 (all from BD Biosciences) and were analyzed with a FACSCalibur flow cytometer and CellQuest 3.3 software (BD Biosciences). Percentage of engraftment was examined by 1-way analysis of variance (ANOVA) test.

Real-time PCR assay for detecting CTLs specific for ACC-6, a newly identified mHA

Complementary DNAs for a standard curve were prepared from mixtures of ACC-6–specific CTL clone (CTL-2A12) at various ratios with $CD3^+$ cells from healthy donors, and cDNAs of peripheral blood $CD3^+$ cells from the donor and patient before and after HCT were prepared from the AML patient (UPN-027). Real-time PCR analysis was performed using a TaqMan assay as described in "Real-time PCR assay for *HMSD* and *HMSD-v* expression." The primers and fluorogenic probe sequences spanning the CTL-2A12 complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3 sequences identical to that of CTL-2A12. Samples were quantified with the comparative cycle threshold (C_T) method. The delta C_T value was determined by subtracting the average GAPDH C_T value from the average CTL-2A12 CDR3 C_T value. The standard curve for the proportion of CTL-2A12 among $CD3^+$ cells (Figure 7A) was composed by plotting mean delta C_T values for each ratio, and the percentages of T cells carrying the CDR3 sequence identical to CTL-2A12A were calculated by using this standard curve.

Results

Characterization of a CTL clone

The $CD8^+$ CTL clone 2A12 (CTL-2A12) was 1 of 24 putative CTL clones isolated from day-197 post-HCT PBMCs of a male with refractory AML with multilineage dysplasia (UPN-027) receiving an HLA-identical HCT from his brother (A*2402, A*3303, B75, B*4403, Cw3, DR4, DR6).[11] The patient developed grade 1 acute GVHD in the first 2 years after transplantation and then suffered from glomerular IgG deposition and mild bronchiolitis obliterans organizing pneumonia. He is alive and in good condition and has been disease free for more than 3 years.

Cytotoxicity assays revealed that CTL-2A12 lysed the recipient B-LCL and less efficiently phytohemagglutinin (PHA)-stimulated T-cell blasts but not donor B-LCL or natural killer (NK)-sensitive K562 cells (Figure 1A,B). No cytotoxicity was observed against the recipient's dermal fibroblasts and BM-derived fibroblasts even after treatment with IFN-γ and TNF-α (Figure 1B). Cytotoxicity against recipient B-LCL was blocked by anti-HLA class I antibody (Ab) but not by anti-HLA-DR Ab, suggesting HLA class I-restricted recognition of mHA (data not shown). Based on the screening results of a panel of B-LCLs derived from individuals partially sharing HLA class I alleles with the recipient (Figure 1C UR1 and UR2; data not shown), those from HLA-mismatched individuals that were transduced with either HLA-A*3303 or -B*4403 were further tested. CTL-2A12 lysed UR3 B-LCLs when transduced with HLA-B*4403. In addition, UR3 B-LCLs transduced with HLA-B*4402 were also recognized, indicating that the mHA peptide can be presented by both HLA-B*4403 and -B*4402 (Figure 1C).

Identification of the gene encoding the mHA and elucidation of the mechanism of antigenicity

cDNA expression cloning using a cDNA library was conducted as described in "Patients, materials, and methods, cDNA library construction." In the first round of screening, 1 of 96 plasmid pools induced IFN-γ production by CTL-2A12. Two-step subclonings (~5 cDNAs and 1 cDNA) of this pool finally resulted in the isolation of a cDNA clone (data not shown).

The cDNA clone was sequenced and a BLAST search[23] revealed that this cDNA clone was previously unreported, but partially identical to XM_209104. XM_209104 was designated histocompatibility (minor) serpin domain containing (*HMSD*) by

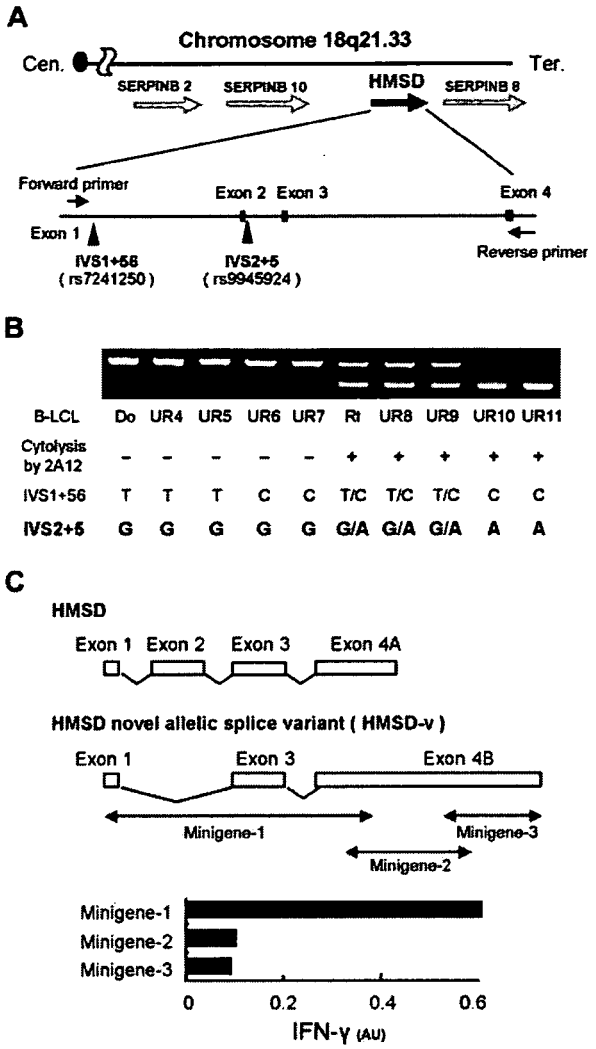

Figure 2. Identification of a novel splice variant transcript of *HMSD* encoding the mHA. (A) Summary of genome mapping around chromosome 18q21.33 showing relative positions of *HMSD*. Two identical cDNA clones were homologous to exons 1 and 3 plus exon 4 but lacked exon 2. This novel allelic splice variant of *HMSD* was designated *HMSD-v* (panel C). Search for potential SNPs responsible for the alternative splicing revealed 2 potential SNPs at IVS1+56 and IVS2+5 (arrowheads). Cen indicates centromere, Tel, telomere. (B) The correlation between sequence polymorphisms of the 2 SNPs and susceptibility of B-LCLs to CTL-2A12. Detection of allelic polymorphisms in B-LCLs was conducted by RT-PCR. Primers were set in exon 1 and the 5′ part of exon 4 of *HMSD* (horizontal arrows in panel A). Due to the lack of exon 2, the mHA$^+$ allele produced a smaller PCR product. Genotyping of the 2 SNPs mentioned above and cytolysis of B-LCLs by CTL-2A12 are summarized below the results of electrophoresis. The correlation between the genotyping results of SNPs at IVS2+5, CTL-2A12 cytolysis, and the bands of electrophoresis produced by mHA$^+$ and mHA$^-$ allele showed complete concordance. (C) Schematic representation of *HMSD* and *HMSD-v* and mapping of the region encoding the CTL-2A12 mHA epitope by minigenes. The *HMSD-v* cDNA was divided into 3 minigenes, and mammalian expression plasmids containing individual minigenes were constructed. 293T/B*4403 cells were transfected with individual plasmids and cocultured with CTL-2A12. Supernatants were then harvested and assayed for IFN-γ production by ELISA. Release of IFN-γ is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm.

the Human Genome organization Nomenclature Committee (Figure 2A). *HMSD* is a gene predicted by RefSeq[24] based on previously reported expressed sequence tags (ESTs). We speculated that this novel cDNA clone was a splice variant of *HMSD* (Figure 2C) because it had exons 1 and 3 plus exon 4B but lacked exon 2. The first third of exon 4B was identical to exon 4A of *HMSD*. Primers were set in exon 1 and the 5′ part of exon 4 (Figure 2A), and RT-PCR was carried out using cDNA from B-LCLs typed by CTL-2A12. Interestingly, these PCR products from mHA$^-$ samples consisted of 1 longer band (674 bp), whereas those from mHA$^+$ samples consisted of the longer band and a shorter band (500 bp) or a single shorter band. This association was concordant with all 34 samples we examined (Figure 2B; data not shown), which revealed that differential expression of *HMSD* and its splice variant is responsible for antigenicity. Exon 1, exon 2, and introns adjacent to exons 1 and 2 were sequenced to account for the alternative splicing, and we found 2 sequence polymorphisms of intronic SNPs, the intervening sequence 1+56 (IVS1+56; rs7241250) and IVS2+5 (rs9945924), in our samples. The correlation between these 2 SNPs and susceptibility to CTL-2A12 was studied, which demonstrated that IVS2+5G>A, but not the SNP at IVS1+56, was completely concordant with cytolysis by CTL-2A12 (Figure 2B). Because the alternatively spliced cDNA clone isolated was generated as an allelic splice variant due to SNP, it was designated *HMSD-v*.

Identification of an HLA-B*4403-restricted epitope of *HMSD-v* and epitope reconstitution assay

To identify the epitope recognized by CTL-2A12, *HMSD-v* cDNA was divided into 3 minigenes overlapping each other by around 100 bp (Figure 2C) and then transfected into 293T/B*4403 cells. CTL-2A12 recognized 293T/B*4403 transfected with minigene-1, which expressed the first 809 bp of *HMSD-v* (Figure 2C). After searching all frames, 2 reading frames in the *HMSD-v* transcript were found to be able to encode polypeptides starting with an ATG codon, which was at least 9 amino acids (aa's) long (Figure 3A). The longest 53-mer polypeptide was divided into 16- or 17-aa peptides with 9 aa's overlapping each other, and downstream 3 peptides were expressed as minigenes starting with ATG (methionine) in 293T/B*4403 cells and tested. The construct encoding the first polypeptide, MEIFIEVFSHFLLQLT, was clearly recognized by CTL-2A12 (Figure 3B). To determine the mHA epitope, the minigene was serially deleted from its C-terminus and tested. An undecameric peptide was sufficient to induce IFN-γ production from CTL-2A12 (Figure 3A underlined; Table 1).

Subsequently, a peptide reconstitution assay was conducted. Undecameric peptide (MEIFIEVFSHF), its C-terminal deleted decameric peptide (MEIFIEVFSH), and N-terminal deleted decameric peptide (EIFIEVFSHF) were synthesized and titrated by adding to the mHA$^-$ donor B-LCL, and among these, only undecameric peptide showed dose-dependent cytolysis with a half-maximal lysis at 20 nM (Figure 4A). This undecameric peptide contains the HLA-B*4403 anchor motif—a glutamic acid at position 2 and a phenylalanine at the C-terminus[25,26]—although undecameric peptide is not common as a T-cell epitope. We designated the mHA as ACC-6 (Aichi Cancer Center No. 6).

HMSD and *HMSD-v* mRNA expression in various hematopoietic and nonhematopoietic cells

To determine the expression of *HMSD* and *HMSD-v* mRNA in a more comprehensive manner, real-time PCR was performed. Individual real-time PCR analysis specific for the *HMSD-v* transcript and for both *HMSD* and *HMSD-v* transcripts revealed that both were equally present in cDNA samples from B-LCLs heterozygous for the defined mHA (data not shown). Thus, further real-time PCR analysis was performed to quantify the total expression of both transcripts partly because mHA allelic status of commercial

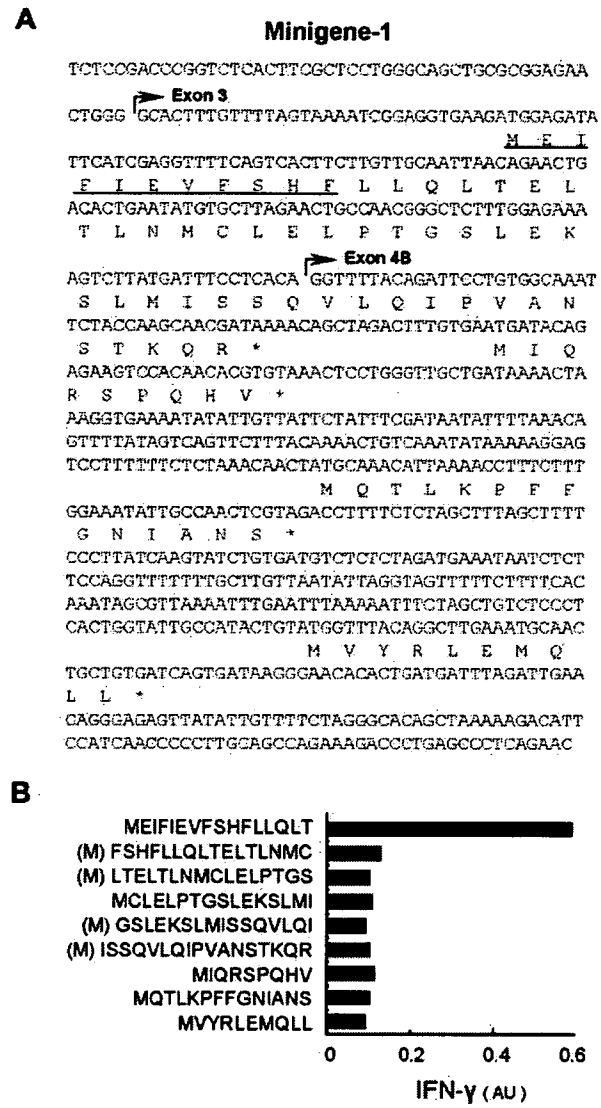

Figure 3. The nucleotide and deduced amino-acid sequences of minigene-1 encoding the CTL-2A12 mHA epitope. (A) Exon 2 encoding the original start codon in *HMSD* was deleted. After searching all frames, 2 reading frames in the *HMSD-v* transcript shown here were found to be able to encode polypeptides longer than 9 aa's starting with an ATG codon. Polypeptides longer than 9 aa's are all indicated. Asterisks indicate a stop codon. The start of exon 3 and exon 4B are indicated with horizontal arrows. The epitope recognized by CTL-2A12 is underlined (see Figure 4). (B) Six small minigenes with 9 aa's overlapping derived from the longest 53-mer polypeptide and downstream 3 minigenes (shown in panel A) were expressed in 293T/B*4403 cells and cocultured with CTL-2A12. Production of IFN-γ was similarly measured by ELISA. Release of IFN-γ is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm. (M) indicates an artificially added methionine as a start codon.

tissue cDNAs was unknown. High levels of expression were observed in primary AML and MM cells, mature DCs, CD40-B cells and PHA blasts (Figure 5A top panel), and malignant hematopoietic cell lines (especially those of myeloid lineage; Figure 5A bottom panel). In contrast, most normal tissues (Figure 5B top panel), including resting primary hematopoietic cells (Figure 5A top panel), showed lower or no expression, except for testis, which expressed a moderate amount of transcript. Weak expression observed in commercial cDNA from nonhematopoietic tissues including brain, lung, and placenta could be caused at least in part by contaminating hematopoietic cells or resident cells of

Table 1. Fine epitope mapping with minigenes

Minigene sequence	Length, bp	CTL response
M E I F I E V F S H F L L Q L T	16	+
M E I F I E V F S H F L L Q L	15	+
M E I F I E V F S H F L L Q	14	+
M E I F I E V F S H F L L	13	+
M E I F I E V F S H F L	12	+
M E I F I E V F S H F	11	+
M E I F I E V F S H	10	–
M E I F I E V F S	9	–

To determine the mHA epitope, a minigene encoding 16 amino acids, which stimulated CTL-2A12, was serially deleted from its C terminus and then tested by ELISA. An undecameric but not decameric peptide was sufficient to induce IFN-γ production from the CTL-2A12.

hematopoietic origin such as pulmonary macrophages, because relatively high levels of *CD45* transcript were detected in those tissues (Figure 5B bottom panel).

It is possible that *HMSD-v* is differentially expressed from *HMSD* in cell types other than B-LCLs, where both transcripts were generated at similar levels. Thus, we examined both total *HMSD* and *HMSD-v* transcripts in various primary cells that were heterozygous for the ACC-6 allele. As shown in Figure 5C, the *HMSD-v* levels were approximately half of total *HMSD* levels in all cell types tested.

Inhibition of human AML-cell engraftment in severely immunodeficient NOG mice by CTL-2A12

We first confirmed that the positively selected CD34$^+$ fraction of primary AML cells positive for HLA-B*4403 and the ACC-6$^+$ allele (all heterozygous) by genotyping was efficiently lysed by

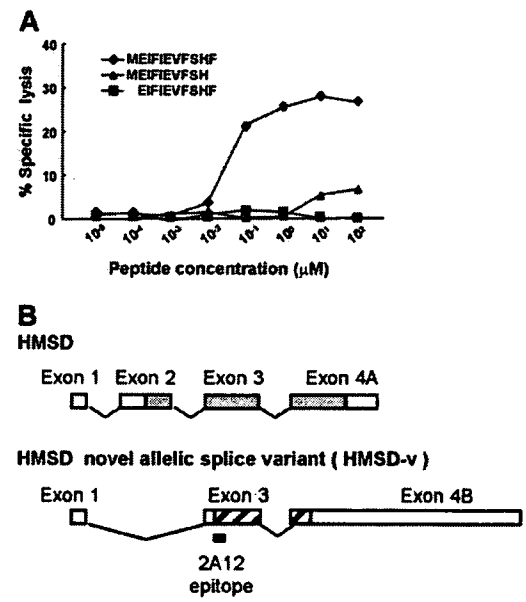

Figure 4. Identification of the CTL-2A12 minimal mHA epitope. (A) A peptide reconstitution assay was conducted to determine the concentration of peptides needed to stimulate CTL-2A12. Undecameric peptide (MEIFIEVFSHF), its C-terminal deleted decameric peptide (MEIFIEVFSH), and N-terminal deleted decameric peptide (EIFIEVFSHF) were synthesized and titrated by adding to the antigen-negative donor B-LCL. (B) Transcript of *HMSD* (encoding a 139-mer polypeptide) predicted by computer algorithm is indicated with ▭. ▨ indicates the presumed *HMSD-v* transcript region encoding a 53-mer polypeptide starting with an ATG codon and including the CTL-2A12 epitope. The location of the identified 2A12 epitope is shown below the *HMSD-v* cDNA. These 2 polypeptides have no homology because they are translated from different reading frames.

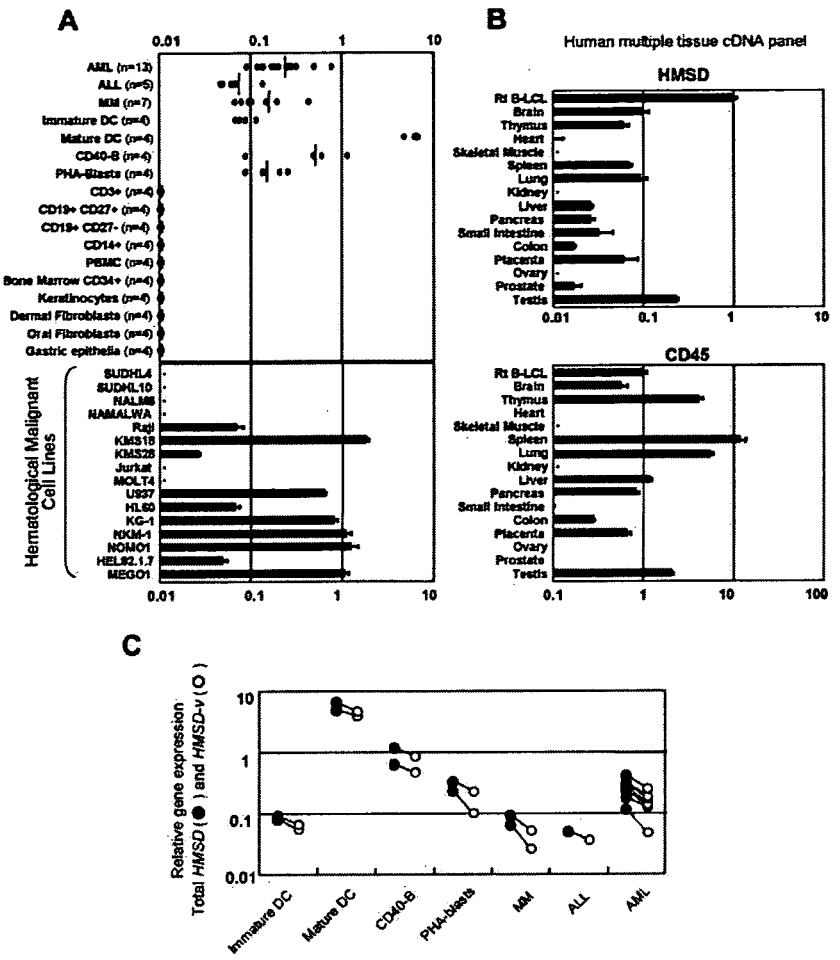

Figure 5. Selective mRNA expression of *HMSD* and *HMSD-v*. (A) Total *HMSD* expression was determined by real-time quantitative PCR in various normal tissues and malignant hematopoietic cell lines using a primer-probe set that detects the exon 3-4 boundary. Targeted mRNA expression in the recipient B-LCL is set as 1.0. In the top dotted plot graph, cDNAs prepared from CD34$^+$ subsets of primary leukemic cells and CD138$^+$ subsets of primary MM cells, freshly isolated hematopoietic cells, their subpopulations, immature and mature DCs, activated B and T cells, freshly isolated CD34$^+$ bone marrow cells, and primary cell cultures were similarly analyzed. Values in the parentheses indicate the number of the individuals tested. In the bottom and middle panels, cDNAs prepared from 16 hematologic malignant cell lines are shown. SUDHL4 and SUDHL10 are derived from B-cell non-Hodgkin lymphoma; NALM6 from acute B-lymphocyte leukemia; NAMALWA and Raji from Burkitt lymphoma; KMS18 and KMS28 from multiple myeloma (MM); Jurkat and MOLT4 from acute T-lymphocyte leukemia; U937 from histiocytic lymphoma; HL60, KG-1, NKM-1, NOMO1, and HEL92.1.7 from acute myeloid leukemia; and MEGO1 from chronic myeloid leukemia (blast crisis). (B) cDNAs of 15 normal tissue samples purchased from Clontech (MTC panels human I and II) were analyzed for total *HMSD* expression (top panel) and CD45 mRNA expression (bottom panel). Messenger RNA expression in the recipient B-LCL is set as 1.0. (C) *HMSD-v* expression levels (○) were compared with total *HMSD* expression levels (●) using a primer-probe set that detects the exon 1-3 boundary specific for *HMSD-v* mRNA. Among primary hematopoietic cells shown in the top of panel A, cells that were found to be heterozygous for ACC-6 allele were further selected and tested. Paired samples are linked.

CTL-2A12 (Figure 6A). The mRNA expression level of total *HMSD* in these AML cells was 47% (AML-1), 28% (AML-2), and 24% (AML-3) of that in the ACC-6-heterozygous recipient B-LCL, respectively.

Next, to determine whether the ACC-6 mHA recognized by CTL-2A12 is indeed expressed on LSCs and thus might have been involved in a GVL effect in AML patient UPN-027, we performed the LSC engraftment assay as previously reported[27] but substituted the significantly immunodeficient NOG mice because the absence of NK activity in NOG mice has been shown to facilitate the engraftment level of xenogenic human hematopoietic cells.[22] The CD34$^+$ fractions of primary AML cells that were lysed by CTL-2A12 (AML-2 in Figure 6A) were selected for this assay, since it was found to be negative for the HLA-B*4403-restricted mHA ACC-2^{D,6} and not lysed by the ACC-2^D-specific clone CTL-3B5 (data not shown), which was used as an irrelevant control. These AML CD34$^+$ cells were incubated in vitro for 16 hours either alone or in the presence of CTL-2A12 or control CTL-3B5 at a T-cell/AML cell ratio of 5:1. Subsequently the mixtures were inoculated into NOG mice. After 5 weeks, flow cytometric analysis of BM and PBMCs was conducted to study the expression of human CD45, CD34, and CD8. Representative flow cytometric profiles are shown in Figure 6B. BM cells of control mice receiving AML-2 cells cultured in medium alone or with control CTL-3B5 before inoculation were found to contain 2.79% to 25.44% (mean, 20.29%) human CD45$^+$ CD34$^+$ cells, whereas PBMCs of the same 2 groups of mice contained 2.97% to 9.69% human cells. In contrast, human cells were not detectable in either BM or PBMCs of the mice inoculated with AML cells precultured with CTL-2A12. Percentage AML engraftment at 5 weeks after inoculation under these conditions is summarized in Figure 6C, indicating that CTL-2A12 eradicated AML stem cells with repopulating capacity ($P = .015$ for BM).

Follow-up of ACC-6–specific CTLs in peripheral blood from an AML patient (UPN-027)

To detect ACC-6–specific CTLs in peripheral blood from AML patient UPN-027 and from his donor, we performed real-time quantitative PCR (Figure 7A) using a set of primers and a fluorogenic probe specific for the unique CDR3 sequence of the CTL-2A12 TCR β chain at several time points. Although ACC-6-specific CTLs were not detected in blood samples from the donor and the patient before HCT, they became detectable in patient samples after HCT at frequencies of 0.11%, 0.23%, 0.83%, and 0.16% among CD3$^+$ cells at days 29, 91, 197, and 548, respectively (Figure 7B). During this period of time, there were no documented clinical manifestations of recurrent disease, and only grade 1 acute GVHD was noted.

Discussion

Antigenicity of the majority of previously identified human mHAs is generated by differences in amino-acid sequence between donor

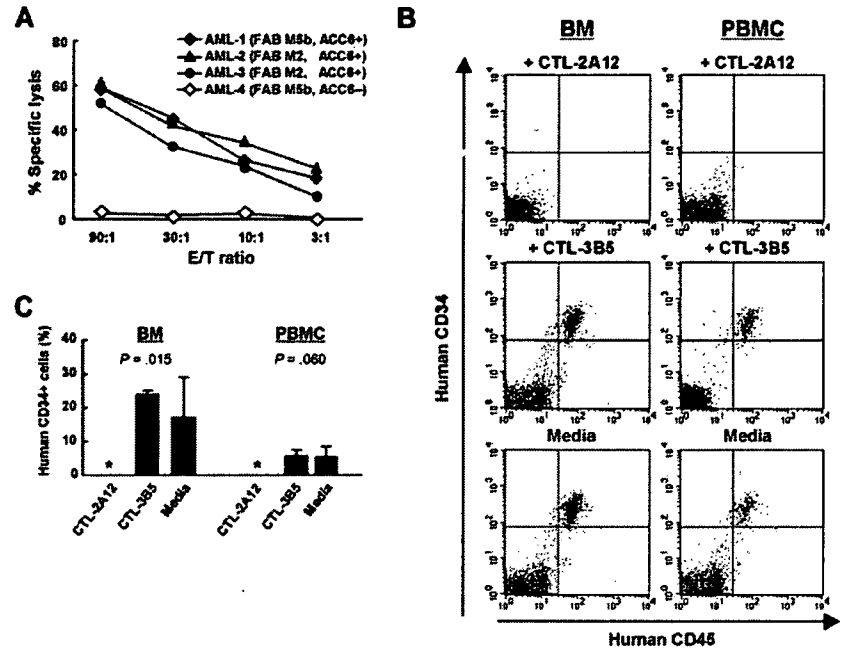

Figure 6. Inhibition of human AML stem cell engraftment in severely immunodeficient NOG mice by CTL-2A12. (A) Specific lysis by CTL-2A12 of primary leukemia cells. A standard 4-hour ^{51}Cr release assay was conducted at the indicated E/T ratios. The $CD34^+$ fraction of 3 primary AML cells positive for HLA-B*4403 and the $ACC\text{-}6^+$ allele by genotyping (AML-1, -2 and -3; the expression level of *HMSD* was 47%, 28%, and 24% of that in the recipient B-LCL, respectively) and 1 $HLA\text{-}B^*4403^+$, ACC-6 allele-negative (AML-4) were tested. FAB denotes French-American-British classification. (B) Representative flow cytometric profiles of peripheral blood and BM cells from AML-inoculated NOG mice for the expression of human CD45 and CD34. Peripheral blood and BM cells were obtained 5 weeks after inoculation from mice receiving 7.0×10^6 AML-2 $CD34^+$ cells (negative for $ACC\text{-}2^D$ mHA) that had been incubated with either CTL-2A12 (top), control CTL-3B5 (middle; HLA-B*4403-restricted, $ACC\text{-}2^D$ mHA-specific CTL), or culture medium alone (bottom) at a T-cell/AML cell ratio of 5:1. (C) Summary of results from engraftment experiments. Mean (± SD) percentage of CD45 and CD34 double-positive cells of 3 mice in each group at 5 weeks after inoculation and the *P* values examined by 1-way ANOVA test are shown. Asterisk indicates that CD45 and CD34 double-positive cells were not detectable in NOG mice inoculated with AML-2 cells preincubated with CTL-2A12.

and recipient due to nonsynonymous SNPs. In this study, we identified a novel HLA-B44–restricted mHA epitope (ACC-6) encoded by an allelic splice variant of *HMSD* (*HMSD-v*) in which exclusion of exon 2 due to alternative splicing was completely controlled by an intronic SNP at IVS2+5. Indeed, by RT-PCR, the novel *HMSD-v* was not detected in cDNA samples from mHA^- B-LCLs, whereas it was detectable in mHA^+ B-LCLs. An interesting question is why the splicing of exon 2 was completely controlled by the intronic SNP. In general, during intron splicing reactions, U1snRNA first binds the 5′ splice site of an intron, spliceosome assembly starts, lariat formation is made with several other factors, and thereafter the intron is spliced out (reviewed in Valadkhan[28]). Here U1snRNA is an important initiator of the cascade. It has been shown that aberrant splicing can result from mutations that either destroy or create splice-site consensus sequences at the 5′ splice site such that approximately half of the observed aberrant splicing is exon skipping while intron retention is rarely observed.[29] In this case, we speculate that the G-to-A substitution of the intronic SNP at nucleotide 5 in intron 2 (IVS2+5G>A, 5′-GUACAU-3′), in addition to the presence of nonconsensus IVS2+4C (underlined), which is commonly observed in both mHA^+ and mHA^- alleles and thus is likely to be permissive, completely disrupts the consensus alignment sequence critical for U1snRNA binding (5′-GUAAGU-3′) such that U1snRNA cannot stably bind the 5′ end of intron 2 in the precursor mRNA from the mHA^+ allele. A similar mutation (IVS3+5G>C, 5′-GUAACU-3′) and resultant exon 3 skipping was reported as a disease-causing mutation in the *NF1* gene.[30] Accordingly, intron 2 cannot be spliced out; a large lariat consisting of intron 1, exon 2, and intron 2 is formed; and then the large lariat is spliced out. In the latter case, 1 nucleotide (IVS1+4) does not match the U1snRNA sequence, but this mismatch is again likely to be permissive. Indeed, it has been shown that a mismatch at nucleotide 3, 4, or 6 of the 5′ splice site is not critical compared with others.[31,32] To our knowledge, this is the first demonstration of an mHA whose antigenicity is controlled by alternative splicing due to an intronic SNP, which may represent an important mechanism for the generation of mHAs.

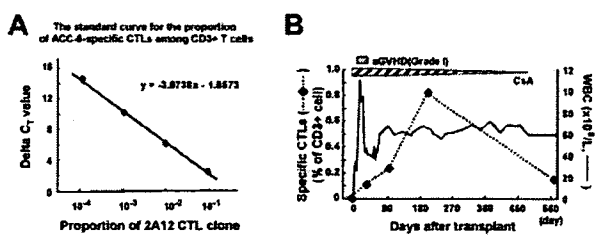

Figure 7. Detection of ACC-6–specific CTLs in peripheral blood from the AML patient (UPN-027) by real-time quantitative PCR using a set of primers and fluorogenic probe specific for the CTL-2A12 CDR3 sequence. (A) The standard curve for the proportion of ACC-6–specific CTL-2A12 serially diluted into $CD3^+$ cells from healthy donors using the comparative C_T (threshold cycle) method. The y-axis is delta C_T value. The x-axis is the log proportion of ACC-6–specific CTLs among $CD3^+$ T cells. (B) The frequency of T cells carrying the CDR3 sequence of CTL-2A12 over a period of 1.5 years after HCT. The percentages of such T cells among $CD3^+$ T cells (left y-axis) were estimated by using a standard curve in panel A and are indicated before HCT and after HCT at day 29, day 91, day 197, and day 548, respectively (diamonds with dotted line). Also noted are white blood cell (WBC) counts (right y-axis), acute GVHD (gray bar), and immunosuppressive therapy with cyclosporine A (CsA; hatched bar) during the same time period.

The novel epitope was located on exon 3 and was transcribed from a reading frame different from the *HMSD* transcripts (Figure 4B). Although exon 3 is shared by *HMSD* and *HMSD-v*, it is speculated that polypeptide including the epitope was not being translated from *HMSD*, because donor B-LCL was not lysed by CTL-2A12. In general, ribosomes initiate translation from the first AUG start codon, but sometimes second or other AUG codons downstream can serve as start codons due to "leaky scanning."[33] However, it seems this is not the case for *HMSD* because the donor B-LCL homozygous for this allele was not lysed at all. This identification of an mHA unexpectedly generated from a previously unknown alternative transcript due to SNP has important implications for the identification of other new mHAs.

LSCs, which are present at very low frequencies, have a particularly strong capacity for proliferation, differentiation, and self-renewal[34] and likely play an important role in disease refractoriness or relapse after chemotherapy and transplantation. Thus, complete eradication of such stem cells is critical for cure in any treatment modalities. The LSC engraftment assay of AML cells in

immunodeficient mice has been shown to be a powerful method for testing the effect of treatment, here mHA-specific CTLs, on LSCs. In addition, preliminary analysis has shown that CTL-2A12 lysed the $CD34^+CD38^-$ fraction of AML cells (Figure S1, available on the *Blood* website; see the Supplemental Figures link at the top of the online article), which is considered to contain leukemic stem-like cells.[35] These data clearly demonstrate that ACC-6 mHA is expressed on such stem cells and may serve as target for cognate CTL-2A12 in vivo.

We performed quantitative RT-PCR analyses for *HMSD* transcripts in various tissues with great interest because cytotoxicity assays suggested its limited expression in hematopoietic cells. Notably, *HMSD* showed selective expression in several hematopoietic primary tumor cells (especially those of myeloid lineage), mature DCs, and activated B and T cells. Since high expression was observed in mature DCs as in the case of *HMHA1* encoding HA-1 mHA,[36] immune responses to *HMSD*-derived mHAs may induce not only a GVL effect[37] against hematopoietic tumor cells but also GVHD,[38] since recipient DCs are responsible for initiating GVHD after HCT. Collectively, our data suggest that this novel mHA, ACC-6, might be a good target for immunotherapy inducing GVL if potential GVHD induction can be managed until recipient DCs have been eliminated early after HCT. Finally, relatively high expression of *HMSD* in the $CD138^+$ fraction of MM cells and their susceptibility to 2A12-CTL (Figure S2) suggest that ACC-6 may serve as a potential target for immunotherapy of multiple myeloma.

It is of interest to correlate clinical outcomes with ACC-6-specific T-cell kinetics after HCT using reagents such as tetramers. The preparation of HLA-B44 tetramer, however, is known to be very difficult,[39] so we used real-time quantitative RT-PCR using CTL-2A12 CDR3 sequence-specific primers/probe, because Yee et al[40] have previously shown strong concordance between semiquantitative RT-PCR analysis of a clone-specific CDR3 region and tetramer analysis used to monitor the fate of adoptively infused CTL clones for the treatment of melanoma. The highest frequency of 0.83% among $CD3^+$ cells was obtained at day 197 after HCT, concordant with the fact that CTL-2A12 was generated from the PBMCs collected at that time. This magnitude is somewhat lower than that observed in the case of LRH-1-specific T cells (1.6% of $CD8^+$ T cells) at the peak level after donor lymphocyte infusion (DLI)[16] but similar to that observed in the case of HA-1-specific T cells (1000 to 6000 tetramer-positive cells per mL blood, corresponding to 0.2% to 1.0% among $CD3^+$ cells).[41] The possibility that the ACC-6 mHA might preferentially induce GVL is supported by the fact that ACC-6–specific CTLs were detectable in the recipient's peripheral blood at a relatively high level after resolution of mild acute GVHD and that LSCs could be eradicated as shown in the NOG mice model. Whether or not ACC-6 mismatching in donor-recipient pairs may be associated with an increased risk of GVHD or morbidity would need to be studied using a large cohort of patients.

The therapeutic applicability of particular mHAs, calculated from the disparity rate and restricting HLA allele frequency, is an issue of interest.[42] The observed frequency of this $ACC\text{-}6^+$ phenotype was approximately 35% (n = 48/135) in healthy Japanese donors (data not shown) and HLA-B*4403 is present in around 20% of Japanese populations, so that ACC-6 incompatibility is expected to occur in approximately 4.6% of HCT recipient-donor pairs. Because CTL-2A12 lysed $HLA\text{-}B^*4402^+$ B-LCLs possessing the $ACC\text{-}6^+$ phenotype derived from white individuals, this novel epitope peptide can also bind to HLA-B*4402, which is a relatively common allele (around 20%) in white populations. Actually, data from the HapMap Project[43] demonstrate that the genotype frequency of carrying at least one IVS2+5A ($ACC\text{-}6^+$) allele is 0.381 for individuals registered in the Centre d'Etude du Polymorphisme Humain (CEPH) cell bank,[44] thus this mHA should also be applicable to white patients. These results together suggest that *HMSD*-derived products could be attractive targets for immunotherapy and that given the possible role of intronic SNPs, a mechanism of alternative splicing should be also taken into consideration when searching for novel mHAs.

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Authorship

Contribution: T.K., Y.A., and T.T. designed research; T.K., Y.A., and H.T. performed research; T.K., Y.A., S.O., and S.M. analyzed data; A.O., M.M., A.T., K.M., H.I., Y.M., and Y.K. contributed vital reagents or analytical tools; and T.K., Y.A., K.T., K.K., and T.T. wrote the paper.

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ORIGINAL ARTICLE

Characterization of a reference material for *BCR-ABL* (M-BCR) mRNA quantitation by real-time amplification assays: towards new standards for gene expression measurements

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Monitoring of *BCR-ABL* transcripts has become established practice in the management of chronic myeloid leukemia. However, nucleic acid amplification techniques are prone to variations which limit the reliability of real-time quantitative PCR (RQ-PCR) for clinical decision making, highlighting the need for standardization of assays and reporting of minimal residual disease (MRD) data. We evaluated a lyophilized preparation of a leukemic cell line (K562) as a potential quality control reagent. This was found to be relatively stable, yielding comparable respective levels of *ABL*, *GUS* and *BCR-ABL* transcripts as determined by RQ-PCR before and after accelerated degradation experiments as well as following 5 years storage at −20°C. Vials of freeze-dried cells were sent at ambient temperature to 22 laboratories on four continents, with RQ-PCR analyses detecting *BCR-ABL* transcripts at levels comparable to those observed in primary patient samples. Our results suggest that freeze-dried cells can be used as quality control reagents with a range of analytical instrumentations and could enable the development of urgently needed international standards simulating clinically relevant levels of MRD.

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Introduction

Detection of minimal residual disease (MRD) has prognostic value in many hematological malignancies, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), non-Hodgkin's lymphoma and multiple myeloma. Quantitative MRD data can be obtained with real-time quantitative PCR (RQ-PCR) analysis of immunoglobulin and T-cell receptor gene rearrangements, chromosomal breakpoint fusion region aberrations, fusion gene transcripts, or aberrantly expressed genes, depending on the type of disease.[1] A typical example of the use of RQ-PCR in onco-hematology is the monitoring of *BCR-ABL* transcripts in CML. In a recent international study, 70% of patients treated with imatinib achieved complete cytogenetic remission.[2] However, to evaluate the effectiveness of such treatment in more detail, a more sensitive test, such as RQ-PCR, is required. Indeed, recent studies have shown that RQ-PCR is better able to monitor the response to treatment compared to the classical cytogenetic method.[3–5] In addition, RQ-PCR-based detection of *BCR-ABL* in CML has become an important tool in predicting relapse after allogeneic transplant, allowing for early intervention strategies such as donor lymphocyte infusion.[6–9]

However, the reverse transcription RT-PCR reaction is fraught with difficulties[10] and is characterized by significant variation and lack of reproducibility between different laboratories.[11,12] Thus, standardization and validation are necessary before routine use of an assay can occur, in order to control for inter- and intra-laboratory variations in results, to reduce laboratory errors and to detect critical loss of assay sensitivity.[13] Standardization can be achieved through the use of calibrated reference reagents that should be included in every assay run. Reference reagents and World Health Organization (WHO) International Standards have been established for nucleic acid amplification technique (NAT) assays for the detection of several

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viruses. These reference materials have been invaluable in the validation and standardization of assays for the detection of viral contamination of blood and blood products[14,15] and therapeutic adaptation based on the blood viral load.[16] For a material to be suitable to act as an international biological reference standard, it should have reproducible performance in assays at the international level and be stable over time. Furthermore, this material needs to be suitable whatever method is used (commercial or 'in-house' reagents, using the various instruments available). In the past, efforts to standardize RQ-PCR analyses for leukemia in terms of assay design and reaction conditions have been made through the Europe Against Cancer (EAC) Program, offering a better platform to compare results between laboratories.[17,18]

The aims of the present international collaborative study, involving 22 laboratories from four continents, were to prepare an appropriate reference material for RQ-PCR testing of leukemia-associated fusion genes and to assess the suitability of such a material for standardization and validation, irrespective of the RQ-PCR machine and protocol used. The material chosen for this study was the human leukemic cell line K562, which expresses the fusion gene *BCR-ABL* (M-BCR, b3a2), with the majority of the laboratories using the EAC protocol. This international study was completed using data obtained in one institution (Marseille, France).

Materials and methods

Cells

CML-derived K562 cells were grown in RPMI1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Invitrogen, Cergy, France). Cells were pelleted, reformulated and lyophilized at a concentration of 3×10^6 cells/ml in 5 ml glass vials (batch identified as 01/604). Each vial was filled with 1 ml cell suspension and approximately 1500 vials were filled. Vials were stored at −20°C and dispatched at ambient temperature.

Plasmid DNA samples

Plasmids, containing the inserted sequences of cDNAs corresponding to control (*ABL*) and fusion (*BCR-ABL*) genes, were sent to each laboratory (kindly provided by Ipsogen, Marseille, France). These plasmids cover exons 2 and 3 of *ABL* and the breakpoint region of *BCR-ABL* M-BCR, respectively. Thus, they can be used with primers and probe sets from EAC, commercially available through Ipsogen; however, for in-house protocols or commercial kits, depending on the position of primers, the provided *ABL* plasmid could not be amplified in all cases. For *ABL* amplification, three dilutions were prepared ranging from 10^5 to 10^3 copies/5 μl. For *BCR-ABL* M-BCR, four dilutions were sent to each laboratory ranging from 10^5 to 10 copies/5 μl. Each vial contained 100 μl of plasmid dilution; vials were dispatched at ambient temperature and stored at −20°C on receipt. Some laboratories used different calibrators to construct the *BCR-ABL* and *ABL* standard curves instead of the supplied EAC plasmid DNAs. Six laboratories used different plasmids (coded numbers 8, 9, 11, 12, 15 and 19) and one (coded number 5) used cell lines.

Comparison of lyophilized and fresh K562 cells for ABL, GUS and BCR-ABL expression

Trizol was used for extraction of RNAs from both fresh and lyophilized K562 cells. The RNA concentration was determined by measurement of the optical density at 260 nm and 1 μg of RNA was retrotranscribed following the EAC protocol.[17] The expression of control genes (*ABL* and *GUS*) and fusion transcript *BCR-ABL* was measured by RQ-PCR using the ABI7700 machine (ABI, Foster City, CA, USA) as previously described.[17,18] Standard curves for quantification were created using plasmid dilutions ranging from 10^5 to 10 copies and allowed the determination of copy number (CN) for each transcript.[17] Triplicate runs for *BCR-ABL* and duplicate runs for *ABL* were performed in each assay and results expressed as the mean CN value obtained in the respective number of replicates.

Accelerated degradation studies

In order to evaluate the stability of the lyophilized reagent, vials were incubated at +4, +20, +37 and +45°C (Table 2) at the NIBSC (UK), sent at ambient temperature and assayed in parallel with vials stored at −20°C at different time points using a RQ-PCR assay for *BCR-ABL, ABL* and *GUS* transcripts in one laboratory (Marseille, France) on the ABI7700 machine according to the EAC protocol, as described above.

International evaluation – study design

Participants were sent four vials of the lyophilized K562 cells (batch 01/604) along with the plasmid dilutions described above. Participants were requested to perform four independent assays, using a fresh vial on each day. Assays for the fusion gene, *BCR-ABL*, and the control gene, *ABL*, were to be run concurrently. Standard curves for the quantification of *BCR-ABL* and *ABL* CN were to be constructed from the plasmid samples, which were to be tested in duplicate. The majority of laboratories followed the common suggested design, although some laboratories included additional triplicate runs for *ABL*. Raw data (C_t values) for all assays and standard curves were to be returned. PCR efficiency was calculated according to the formula $E = 10^{(-1/\text{slope})} - 1$. The samples were to be tested undiluted and at 10^{-1} and 10^{-2} dilutions in water, with some laboratories reporting data from a 10^{-5} dilution. Throughout this report, participating laboratories are referred to by a code number allocated at random by the NIBSC statistical team, unrelated to the authorship listing order.

RQ-PCR machines used

Fourteen laboratories used the ABI 7700 (Applied Biosystems, Foster city, CA, USA)
Four the Light Cycler (Roche Diagnostics, Mannheim, Germany)
One the ABI 7900HT (Applied Biosystems)
One the ABI 7000 (Applied Biosystems)
One theABI 5700 (Applied Biosystems)
One laboratory used both ABI 7900HT (Applied Biosystems) and Light Cycler (Roche).

International evaluation – assay methods

The RNA extraction protocols included use of Trizol, silica-based methods (RNeasyMini kit, Qiagen, Hilden, Germany, MagNA Pure LC mRNA from Roche Diagnostics) and CsCl gradient ultracentrifugation. RNA was retro-transcribed following EAC (10 laboratories) or other protocols (11 laboratories) and the method used was not recorded by one laboratory. Sixteen laboratories used the EAC standardized RQ-PCR protocol and primer and probes for the two genes[17,18] which were centrally ordered (Applied Biosystems) and distributed to the participants upon request. Others used either published