Table 2 Impact of pretransplant factors on the incidence of acute GVHD by univariate analyses

	Incidence of acute GVHD	*P-value*
Sex		
Male	45%	0.55
Female	38%	
Age		
<40 years	44%	0.82
⩾40 years	42%	
Risk		
Standard	37%	0.15
High	51%	
Donor		
Related	36%	0.014
Unrelated	54%	
HLA		
Match	37%	0.0026
Mismatch	63%	
Stem cell		
BM	42%	0.33
PBSC	55%	
Regimen		
Non-TBI	30%	0.041
TBI	48%	
MTX		
<35 mg/m^2	60%	0.0025
⩾35 mg/m^2	36%	
CsA		
TD	27%	0.00022
CIF	56%	

BM = bone marrow, PBSC = peripheral blood stem cell, TBI = total body irradiation, MTX = methotrexate, CsA = cyclosporine A, TD = twice-daily infusion, CIF = continuous infusion.

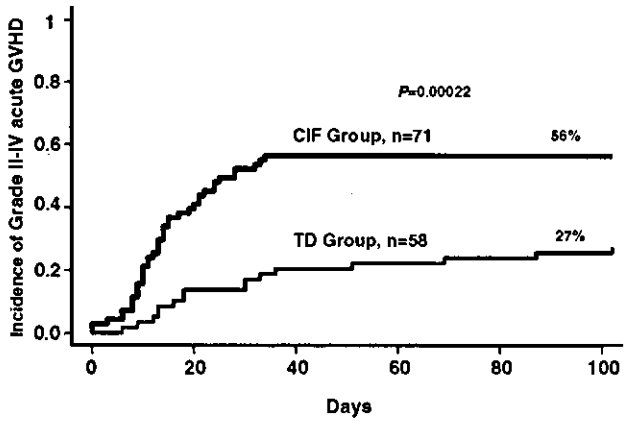

Figure 1 Cumulative incidence of grade II–IV acute GVHD grouped by the mode of CsA administration (TD = twice-daily infusion, CIF = contin-continuous infusion).

group was consistently significantly lower than that in the TD group during the first 4 weeks after transplantation (Figure 2).

Table 3 Impact of pretransplant factors on the incidence of acute GVHD by multivariate analysis: (a) independent significant risk factors identified by multivariate analysis using backward stepwise selection; (b) impact of HLA mismatch and the mode of cyclosporine A administration adjusted for the total methotrexate dose and the donor type (CsA = cyclosporine A, MTX = methotrexate, TD = twice-daily infusion, CIF = continuous infusion)

		Relative risk (95% CI)	*P-value*
	(A)		
HLA	Mismatch *vs* match	2.01 (1.15–3.53)	0.014
CsA	CIF *vs* TD	2.59 (1.46–4.60)	0.0011
	(B)		
HLA	Mismatch *vs* match	1.89 (1.04–3.45)	0.038
CsA	CIF *vs* TD	1.98 (0.98–4.00)	0.056
MTX	⩾35 mg/m^2 *vs* <35 mg/m^2	1.51 (0.80–2.87)	0.20
Donor	Unrelated *vs* related	1.36 (0.78–2.38)	0.28

Table 4 Difference in the incidence of renal dysfunction by the mode of cyclosporine A administration (TD = twice-daily infusion, CIF = continuous infusion)

		(−)	*(+)*	*P-value*
Incidence of serum creatinine > 1.5 × baseline value				
CsA	TD	20	38 (66%)	<0.001
	CIF	52	19 (27%)	
Incidence of serum creatinine > 2.0 × baseline value				
CsA	TD	34	24 (40%)	0.0002
	CIF	62	9 (13%)	

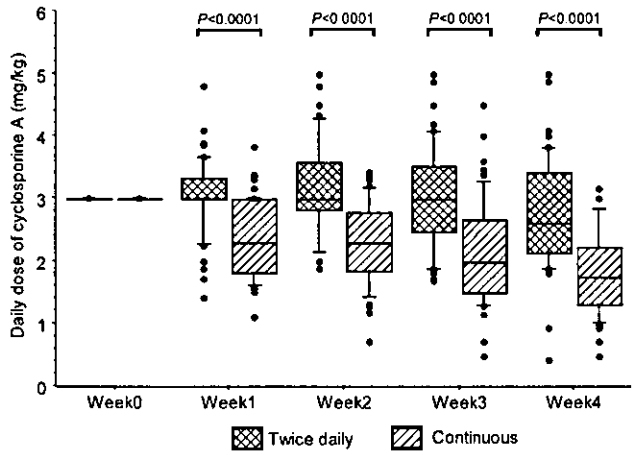

Figure 2 Actual daily dose of CsA grouped by the mode of administration. The box-and-whisker plot shows 10, 25, 50, 75, and 90 percentile values. Outliers are indicated by dots.

Transplant outcome

The CIF of CsA was shown to significantly decrease the incidence of relapse, after adjusting for disease status before transplantation (relative risk 0.41, 95% CI 0.18–0.95, $P = 0.038$). This resulted in significantly better disease-free

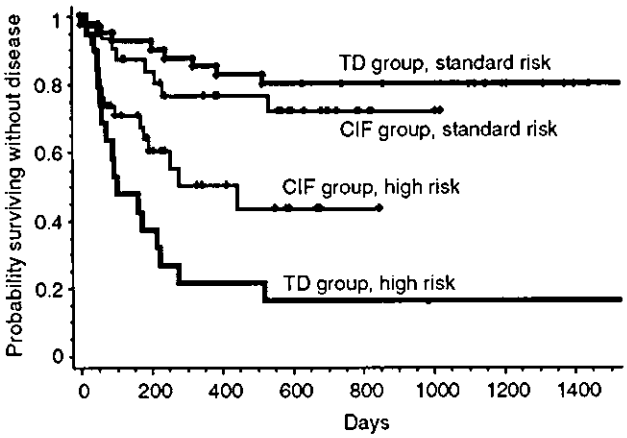

Figure 3 Disease-free survival grouped according to the mode of CsA administration, stratified by the disease status (TD = twice-daily infusion, CIF = continuous infusion).

survival in the CIF group than in the TD group among high-risk patients (43 *vs* 16% at 2 years, $P=0.039$, Figure 3), whereas there was no significant difference in disease-free survival between the two groups among standard-risk patients (72 *vs* 80% at 2 years, $P=0.45$).

Discussion

To summarize the findings of this study, the CIF of CsA with a target level of 250–400 ng/ml significantly increased the incidence of grade II–IV acute GVHD, but significantly decreased the incidences of renal dysfunction and relapse, which resulted in better disease-free survival in high-risk patients. However, disease-free survival was not improved in standard-risk patients, probably because the incidence of relapse was originally low in these patients. Therefore, this mode of CsA administration may not be appropriate for standard-risk patients.

There are at least two possible explanations for why the incidence of acute GVHD was higher in the CIF group. First, the total dose (or the area under the curve) of CsA may be important. Second, it may be important to achieve a peak CsA concentration. It was impossible to draw a definite conclusion from this study. However, considering that the actual daily dose of CsA was gradually decreased in the CIF group after transplantation, a target level of 250–400 ng/ml might be too low to prevent GVHD adequately, although this target level has been used in recent large randomized controlled trials.[6,7] Miller *et al*[8] adjusted the dose of CsA as a CIF to maintain the blood CsA level between 450 and 520 ng/ml. In this setting, the mean actual dose of CsA was maintained between 2.87 and 3.15 mg/kg during the first 4 weeks after transplantation. Therefore, this higher target level may be more appropriate when comparing the mode of CsA administration, although it needs to be confirmed by measuring the area under the curve.

The major shortcoming of this study was that this was not a randomized controlled trial and there were some uncontrolled variables that might have caused bias. However, the impact of the mode of CsA administration remained significant after adjusting for these uncontrolled variables, as shown in Table 3B. We are planning a randomized controlled trial to confirm these results.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare.

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Stem cell leukemia protein directs hematopoietic stem cell fate

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Stem cell leukemia (SCL) protein has been shown to be an essential transcription factor during hematopoietic development in the embryo. In adult hematopoiesis, however, the role for SCL has remained largely unknown, whereas it is expressed in bone marrow hematopoietic stem cells (HSCs). In this study, we performed HSC transplantation and an in vitro HSC differentiation assay using retrovirally transduced HSCs with wild-type (WT) and dominant-negative (DN) SCL. The transplantation experiments showed that SCL does not affect the long-term repopulating capacity of HSCs but that WT SCL and DN SCL increase the short-term contribution of the transduced HSCs in myeloid and lymphoid lineages, respectively. An in vitro single-cell assay using a fetal thymus organ culture system further demonstrated that WT SCL facilitates HSCs to differentiate into the myeloid lineage but that DN SCL facilitates HSCs to differentiate into the lymphoid lineage. We conclude that the up-regulation or down-regulation of SCL directs HSCs toward myeloid or lymphoid lineage, respectively, although SCL does not affect their long-term repopulating capacity. (Blood. 2004;103: 3336-3341)

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Introduction

Hematopoietic stem cells (HSCs) that can give rise to all lineages of blood cells are enriched in surface marker–defined populations, such as those that are lineage marker (Lin)–negative, c-Kit–positive, Sca-1–positive, and CD34–low/negative ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^{low/-}$ $[34^-KSL]$[1]) in adult mouse bone marrow. Recently, common myeloid progenitors (CMPs)[2] and common lymphoid progenitors (CLPs)[3] were identified by surface marker profiles as clonogenic cells that can generate all lineages of myeloid and lymphoid cells, respectively, without any potential to differentiate into one another. Therefore, the first step in blood production from an HSC must be initiated by commitment to a CMP[2] and a CLP.[3] This process, like other differentiation processes, is governed by an expression profile of transcription factors. Although a number of transcription factors are known to be involved in commitment and differentiation processes during hematopoiesis,[4] none have been identified that play roles in the progression from an HSC to a CLP and a CMP. It was reported that CLPs and CMPs differ in the expression pattern of transcription factors: stem cell leukemia (SCL), a basic helix-loop-helix (bHLH) motif–containing transcription factor,[5] and GATA2, a zinc finger motif–containing transcription factor, are expressed in HSCs and CMPs but not in CLPs.[2]

SCL appears to play roles in hematopoietic cell differentiation into myeloid, erythroid, megakaryocyte, and mast cell lineages because it is preferentially expressed during early cell stages in these lineages.[6-12] Studies in knockout mice have revealed that SCL is indispensable in the early stages of primitive embryonic hematopoiesis, when it functions in the specification of ventral mesoderm to blood cells and in the formation or maintenance of immature blood progenitors.[13,14] Studies using embryonic stem cells in vitro have demonstrated that SCL is necessary for the development of bipotent progenitors of blood and endothelial cells in vertebrates.[15,16] Furthermore, ectopic expression of SCL promotes the proliferation and inhibits the apoptosis of 32D, a late myeloid progenitor cell line; these effects are dependent on the bHLH domain.[17] Essential roles for SCL in megakaryopoiesis and erythropoiesis in the adult mouse bone marrow were recently demonstrated by conditional targeting of the *SCL* gene.[18,19]

To clarify the roles of SCL in the commitment process of an HSC to a CLP and a CMP, we performed HSC transplantation and an in vitro HSC differentiation assay using retrovirally transduced HSCs with wild-type (WT) and bHLH domain–deleted—that is, dominant-negative (DN)—SCL.[9,20] Our data show that the overexpression of WT SCL in HSCs strikingly promotes myelopoiesis. In sharp contrast, the overexpression of DN SCL (down-regulation of SCL) in HSCs results in a marked bias toward lymphopoiesis.

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†Hisamaru Hirai died suddenly on August 23, 2003. His students, fellows, and colleagues will greatly miss his energetic and nurturing leadership in the field of hematology. We dedicate this paper in his memory.

An Inside *Blood* analysis of this article appears in the front of this issue.

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Materials and methods

Mice

C57BL/6 (B6-Ly5.2) mice were purchased from SLC (Tokyo, Japan). Mice congenic for Ly5 locus (B6-Ly5.1) were bred and maintained at the University of Tsukuba Animal Research Center (Tsukuba, Japan).

Purification of HSCs and flow cytometric analysis

We purified 34$^-$KSL cells as HSCs (Figure 1A) from murine bone marrow cells and analyzed the surface phenotypes of cells from peripheral blood, bone marrow, spleen, and thymus as described.[21] All antibodies were purchased from PharMingen (San Diego, CA), as follows: biotinylated and unmodified sets of rat immunoglobulin G2b (IgG2b) antilineage markers Gr-1, B220, CD4, CD8, Mac1, and Ter119; fluorescence-labeled antibodies phycoerythrin (PE)–Gr-1, PE-Mac1, PE-B220, allophycocyanin (APC)–B220, APC-Thy1.2, PE-Ly5.1, PE-Sca-1, PE-CD71, APC-c-Kit, and fluorescein isothiocyanate (FITC)–antimurine CD34; biotinylated antimurine α subunit of the receptor for interleukin-7 (IL-7Rα); APC-streptavidin and peridin chlorophyll protein (PerCP)–Cy5.5-streptavidin. All cytokines except for recombinant mouse IL-7 were from Kirin Brewery Research Laboratory (Takasaki, Japan). IL-7 was purchased from PEPROTECH EC (London, United Kingdom).

Plasmid construction

The cDNA fragment for *SCL* was obtained from a library made from an embryonic day-14 (E14) mouse embryo (a gift from Kirin Brewery Research Laboratory) by using a polymerase chain reaction (PCR) method based on the published sequence. *DN SCL* devoid of the DNA-binding domain (amino acid residues 180-233) was constructed with 2 PCR fragments. One of the PCR fragments was generated with primers 1 and 2 (ACAGAATTCTCTAAATATGCCCCAGGATGACGGAG and CACTACTTTGGTGTGAGGACCATCAG); the other fragment was generated with primers 3 and 4 (AAGTACATCAATTTCCTGGCCAAGTTACT and AAACTCGAGTCACCGGGGGCCAGCCCCATCA). A Flag sequence 5′-GACTACAAAGACGATGACGATAAATGA-3′) was fused in-frame to the 3′ end of the cDNAs. The obtained *SCL* and *DN SCL* fragments were cloned into pMY IRES-GFP,[22] which allows the expression of green fluorescence protein (GFP) from an internal ribosomal entry site (IRES) (Figure 1B).

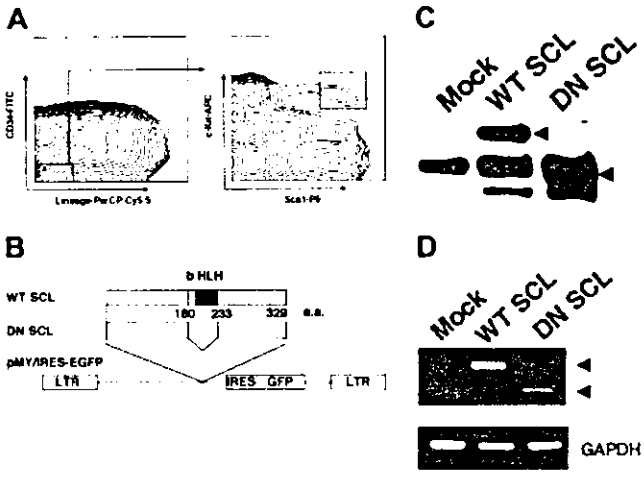

Figure 1. Construction of retroviral vector and target HSC for gene transduction. (A) Flow cytometric analysis of murine hematopoietic stem cells in adult bone marrow. Staining profile of lineage marker versus CD34 for lineage-depleted cells (left) and Sca-1 versus c-Kit in lineage$^-$/CD34^{-low}–gated populations (right) in bone marrow is shown. (B) Schematic representation of the retroviral vector, pMY/WT SCL and pMY/DN SCL, encoding WT SCL and DN SCL linked by IRES to a cDNA encoding enhanced GFP (EGFP). (C) Expression of WT SCL and DN SCL proteins in retrovirally transduced 32D cells. Arrowheads indicate approximately 37 kDa (lane WT SCL) and 29 kDa (lane DN SCL). The other bands commonly seen in all the lanes, including the Mock control lane, represent nonspecific reaction of the antibody. (D) Expression of SCL mRNA in retrovirally transduced HSCs. Arrowheads indicate 308–base pair (bp) (lane WT SCL) and 170-bp (lane DN SCL) transcripts, the latter of which resulted from the deletion of a 138-bp sequence corresponding to the bHLH domain.

Retroviral gene transfer

We produced WT and DN SCL retroviruses and the Mock GFP retrovirus from a packaging cell line, ψMP34. The 34$^-$KSL cells[1] were infected with these retroviruses under stimulation with mouse stem cell factor (SCF) and thrombopoietin (TPO), as previously described.[21]

Western blot analyses and reverse transcription–polymerase chain reaction

32D cells, which were infected with retrovirus and were selected by the expression of GFP, were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) with 10 ng/mL mouse interleukin-e (IL-3). They were harvested in TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA [ethylenediaminetetraacetic acid], 1% NP-40) with protease inhibitor cocktail (Sigma, St Louis, MO) and were sonicated by Handy Sonic HR-20P (Tomy Seiko, Tokyo, Japan). Cell lysates were immunoprecipitated with anti-FLAG M2-Agarose Affinity Gel (Sigma), and Flag fusion proteins were eluted by competition with Flag peptide (Sigma) according to the manufacturer's instructions. The proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using anti-Flag M2 monoclonal antibody (Sigma) and were detected by antimouse IgG antibody conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark).

Reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed as described previously.[21] Primer pairs were: GAPDH, 5′-GCATTGTGGAAGGGCTCATG-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG-3′; SCL, 5′-CTAGGCAGTGGGTTCTTTGG-3′ and 5′-TCCTCCTCCTGGTCATTGAG-3′.

Colony, noncompetitive and competitive reconstitution, and MLP assays

Colony and transplantation assays were performed as previously described.[21] To perform a competitive long-term reconstitution (CLTR) assay and to evaluate whether SCL affects the long-term repopulating capacity of 34$^-$KSL cells, competitor cells were prepared by culturing 34$^-$KSL cells without retroviral transduction in the same conditions used for transducing 34$^-$KSL cells. Competitor cells and GFP-sorted transduced cells (test cells) (1000 each) were then transplanted into irradiated recipient mice and were analyzed for the contribution of test cells against competitor cells (ie, ratio of GFP$^+$ cells among donor [Ly5.1] cells) in the recipients' blood at various times. For noncompetitive assays, we transplanted 2000 transduced cells and 10 000 lineage-depleted fresh bone marrow cells into lethally irradiated recipient mice. A multilineage progenitor (MLP) assay was performed according to the original method previously described.[23] In brief, to prepare hematopoietic cell–depleted fetus thymic lobes, thymi obtained from B6 fetuses 15 days after coitus were cultured on polycarbonate filters (pore size, 8 μm) (Nuclepore, Pleasanton, CA) floating on culture medium containing dGuo (1.35 mM) for 6 days. A single HSC placed in each well of a 96-well V-bottom plate was cultured together with a washed deoxyguanosine (dGuo)–treated lobe in the medium supplemented with recombinant murine IL-7 (rmIL-7) (100 U/mL). Plates were placed into a plastic bag (Ohmi Oder Air Service, Hikone, Japan) in which the air had been replaced by a gas mixture (70% O_2, 25% N_2, 5% CO_2) and were incubated for 12 days.

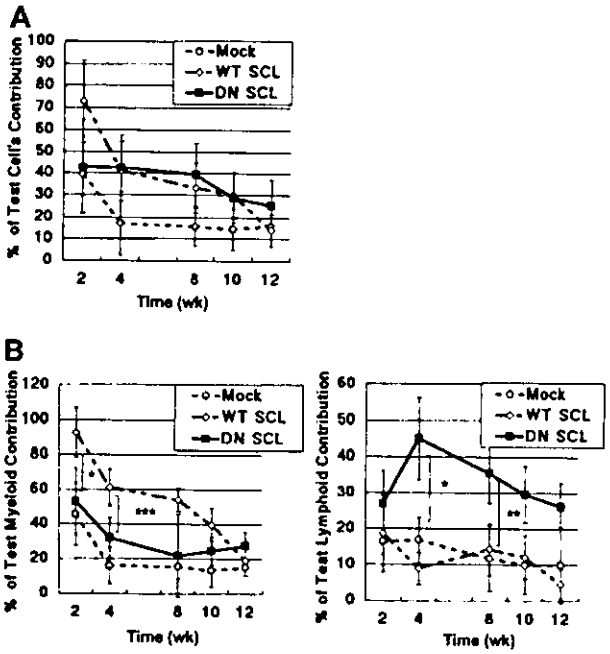

Figure 2. Effects of retrovirally mediated WT SCL and DN SCL on stem cell activities. (A) Analysis of total peripheral blood after competitive reconstitution assay. (B) Lineage-specific contribution in peripheral blood from recipient mice. (left) Percentage of GFP$^+$ cells in the donor-derived Mac1$^+$/Gr1$^+$ cells. (right) Percentage of GFP$^+$ cells in the donor-derived Thy1.2$^+$/B220$^+$ cells (percentage of GFP$^+$/CD45.1$^+$/Mac1$^+$/Gr1$^+$ or CD45.1$^+$/Thy1.2$^+$/B220$^+$ cells of recipient mice). Numbers of recipient mice in each group were between 6 and 9 in panels A and B. Plots are shown as the mean ± SD. $^*P < .1$; $^{**}P < .01$; and $^{***}P < .0001$.

Results

Effects of retrovirally transduced WT SCL and DN SCL on the properties of HSCs

First, we confirmed the expression of Flag-tagged WT SCL and DN SCL proteins in retrovirally transduced 32D cells (Figure 1C). The predicted molecular weight from an amino acid sequence of WT SCL is 34276.6 Da, and from an amino acid sequence of DN SCL it is 28764.46 Da. We next examined the expression of mRNA in retrovirally transduced HSCs (Figure 1D). In WT SCL–transduced HSCs, the level of SCL mRNA expression was higher than that of Mock GFP–transduced HSCs. mRNA for DN SCL was detected only in DN SCL-transduced HSCs.

In a CLTR assay, the contribution of WT SCL– and DN SCL–transduced HSCs was slightly greater than that of Mock GFP–transduced HSCs when the chimerism was accessed shortly after transplantation (Figure 2A). However, the ratios of GFP$^+$ cells gradually decreased, and, at 12 weeks after transplantation, they were as low as approximately 20%, irrespective of introduced genes (Figure 2A). We previously compared in detail the number of the GFP$^+$ cells and congenic marker (Ly5.1$^+$) cells at different time points after transplantation. Silencing of GFP expression did occur, but its ratio was less than 20% by 3 months after transplantation (data not shown). Thus, the decrease in GFP$^+$ cells in the current study is attributed mainly to the decrease in donor-derived cells rather than to the silencing of GFP expression, indicating that the long-term repopulating capacity of retrovirally transduced (ie, GFP$^+$) HSCs was less than that of nontransduced HSCs, regardless of whether WT SCL, DN SCL, or Mock virus was introduced.

We found, however, that the contribution of WT SCL–transduced cells was greater than that of the DN SCL– or the Mock GFP–transduced cells in the myeloid lineage (Figure 2B, left) and, conversely, that the contribution of DN SCL–transduced cells was greater than that of the WT SCL– or the Mock GFP–transduced cells in the lymphoid lineage (Figure 2B, right) until 12 weeks after transplantation if the chimerism was separately characterized in myeloid (Mac1$^+$ or Gr1$^+$) and lymphoid (Thy1.2$^+$ or B220$^+$) lineages.

In a methylcellulose colony-forming assay using the retrovirally transduced GFP$^+$ cells, we found that neither WT SCL nor DN SCL affected the number of immature progenitor-derived colonies compared with the Mock GFP vector (Figure 3A). WT SCL–transduced cells, however, gave rise to progenitors that formed 4 times as many mature myeloid and erythroid colonies than those of Mock GFP–transduced cells (Figure 3B-C).

Up-regulation or down-regulation of SCL in HSCs influences the distribution of progeny in recipient mice

CLTR assay results suggested that SCL had no effect on the maintenance of multipotent HSCs but that it affected the extent of contribution to mature cells from myeloid or lymphoid lineage progenitors. To focus on the lineage commitment of HSCs, we next performed a noncompetitive reconstitution assay. In the blood from recipients of transplanted Mock GFP-transduced HSCs, the ratio of Mac1$^+$ or Gr1$^+$ (hereafter Mac1$^+$/Gr1$^+$) cells in the GFP$^+$ cells was essentially the same as that in total blood cells 6 weeks after transplantation (Figure 4A, upper left panel). In GFP$^+$ cells from recipients of WT SCL–transduced HSCs, however, the Mac1$^+$/Gr1$^+$ cell ratio was markedly higher than that in the total blood cells (Figure 4A, upper middle panel). This corresponded to the decreased ratio of the sum of B220$^+$ cells and Thy1.2$^+$ cells (Figure 4A, lower middle panel). In contrast, the ratio of Mac1$^+$/Gr1$^+$ cells in the GFP$^+$ cells was markedly lower than that in the total blood cells from the recipients of transplanted DN SCL–transduced HSCs (Figure 4A, upper right panel), which corresponded to the increase in the sum of B220$^+$ cells and Thy1.2$^+$ cells (Figure 4A, lower right panel). These results were consistent in all recipients (Figure 4B).

Next, we examined the myeloid and lymphoid cell populations in each hematopoietic organ. In the GFP$^+$ bone marrow cells 6 weeks after transplantation, more than 75% Mac1$^+$/Gr1$^+$

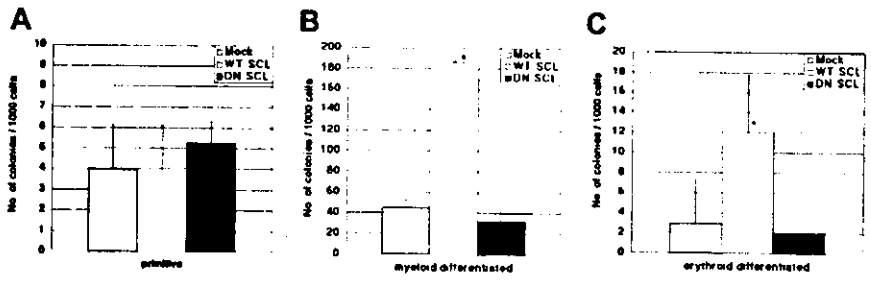

Figure 3. In vitro colony-forming potential of retrovirally gene-transduced cells. Numbers of mixed (A), myeloid (B), and erythroid (C) colonies are shown. Data show the mean ± SD of triplicates. Similar data were obtained in 2 independent experiments. $^*P < .1$.

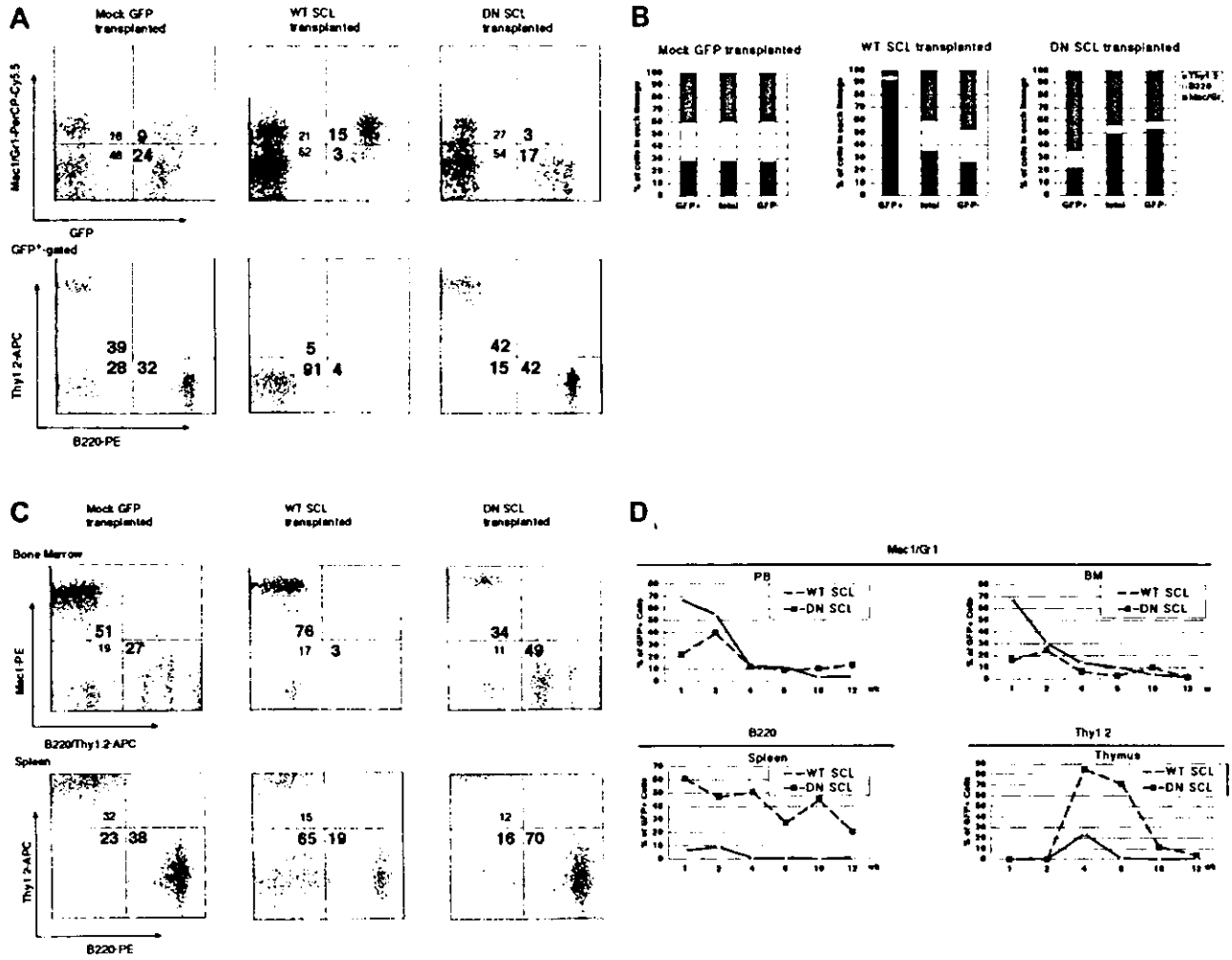

Figure 4. Contribution of WT SCL– and DN SCL–transduced donor-derived cells in myeloid and lymphoid lineages 6 weeks after transplantation. (A) Results of representative FACS analysis of peripheral blood. Profiles for GFP and Mac1 or Gr1 in peripheral blood cells (upper panels) and B220 and Thy1.2 in GFP$^+$-gated peripheral blood subpopulations (lower panels) are shown. (B) Mean ratios of T, B, and myeloid lineage cells in GFP$^+$-gated, nongated, or GFP$^-$-gated peripheral blood subpopulations in recipient mice receiving Mock GFP–transduced cells (SD = 0.02-0.04), WT SCL–transduced cells (SD = 0.19-0.60), and DN SCL–transduced-cells (SD = 0.21-0.22). (C) Results of representative FACS analysis of bone marrow and spleen. (D) Chimerism of GFP$^+$ cells in myeloid cells from peripheral blood and bone marrow (upper panels), in B220$^+$ cells from spleen (lower left), and in Thy1.2$^+$ cells from thymus (lower right). (A-D) Three, 7, and 5 recipient mice were analyzed during Mock GFP–transduced, WT SCL–transduced, and DN SCL–transduced cell transplantation, respectively. Numbers in each quadrant (A,C) represent the percentage of cells in the respective quadrants.

cells—less than 3% B220$^+$ or Thy1.2$^+$ (B220$^+$/Thy1.2$^+$) cells and less than 35% Mac1$^+$/Gr1$^+$ cells—more than 45% B220$^+$/Thy1.2$^+$ cells were observed in the recipients of transplanted WT SCL– and DN SCL–transduced HSCs, respectively, whereas 51% Mac1$^+$/Gr1$^+$ and 27% B220$^+$/Thy1.2$^+$ cells were in the GFP$^+$ cells from Mock GFP–transduced HSCs (Figure 4C, upper panel). In most of the recipients of DN SCL–transduced HSCs, the ratio of B220$^+$ cells was increased in the GFP$^+$ population compared with that in the total population of spleen cells (Figure 4C, lower panel). Changes in the ratios of Mac1$^+$/Gr1$^+$ cells in the blood and bone marrow, B220$^+$ cells in the spleen, and Thy1.2$^+$ cells in the thymus, collectively, showed that WT SCL and DN SCL facilitated the contributions from donor cells to myeloid and lymphoid lineages, respectively (Figure 4D). It was suggested that persistent up-regulation or down-regulation of SCL in HSCs influences their commitment to myeloid or lymphoid progenitors. Furthermore, we found marked decreases and increases in the number of cells expressing IL-7Rα in the GFP$^+$ bone marrow cells from recipients receiving WT SCL– and DN SCL–transduced HSCs (Figure 5A-B). This observation supports the possibility that SCL influences the cell fate of an HSC at the bifurcation of myeloid and lymphoid lineages because IL-7Rα is expressed in immature progenitor cells and because IL-7Rα is one of the key markers discriminating CLPs from CMPs.

In vitro single-cell progenitor assay demonstrates that WT SCL and DN SCL direct HSCs to opposite lineages

To obtain further evidence that SCL affects the commitment process of HSCs rather than the expansion of myeloid progenitors, we examined the developmental capacity of individual hematopoietic progenitors to generate T, B, and myeloid (M) cells using an MLP assay.[23] Cell growth sufficient to perform fluorescence-activated cell sorter (FACS) analysis was achieved in approximately 50% of the wells (data not shown), in each of which a single sorted cell was cultured together with a dGuo-treated fetal thymic lobe. A single cell was designated as p-M, p-T, p-B, p-MT, p-MB, p-TB, or p-MTB if the cell population in the well expressed only Mac1/Gr1, Thy1.2, B220, Mac1/Gr1 and Thy1.2, Mac1/Gr1 and

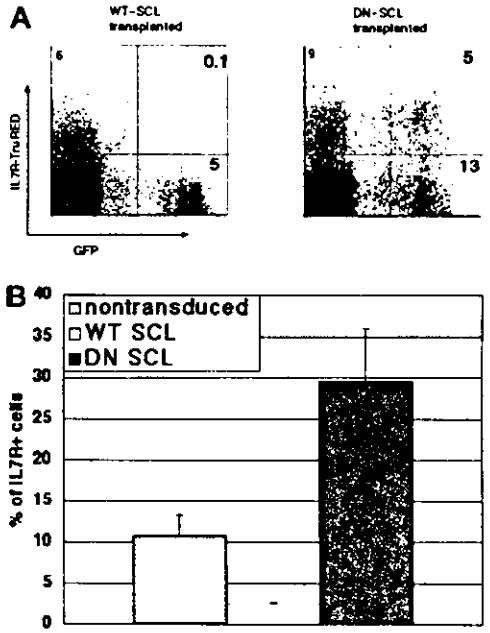

Figure 5. IL-7Rα–expressing cells in the donor-derived cells from bone marrow of recipients of transplanted WT SCL– and DN SCL–transduced HSCs. (A) Results of representative FACS analysis 10 weeks after transplantation. Numbers in quadrants represent the ratio of cells in the respective quadrants. (B) Mean of IL-7α–expressing cells in GFP+-gated or nongated subpopulations in lineage-depleted bone marrow cells from recipients. Data show the mean ± SD of 2 to 4 mice from 2 independent experiments.

B220, Thy1.2 and B220, or Mac1/Gr1, Thy1.2, and B220, respectively. WT SCL–transduced HSCs generated a higher number of p-M and a lower number of p-T than Mock GFP–transduced HSCs (Figure 6A-B). Indeed, p-T was not detected in the cells derived from WT SCL–transduced HSCs in repeated experiments. Conversely, DN SCL–transduced HSCs generated a higher number of p-T and a lower number of p-M than Mock GFP–transduced HSCs (Figure 6A-B). Generation of p-MT and p-MB was not affected by the introduction of WT SCL or DN SCL into HSCs. If only the expansion of myeloid or lymphoid progenitors takes place and the myeloid or lymphoid commitment is not affected by the up-regulation or down-regulation of SCL, the ratio of the number of p-M–containing wells to that of p-T–containing wells would not be altered, and only the cell number of each well would be affected. Results of the MLP assay combined with those of the reconstitution assay described here strongly suggest that the up-regulation of SCL actively promotes an HSC to the myeloid lineage and that the down-regulation of SCL results in the facilitated lymphoid commitment of HSCs.

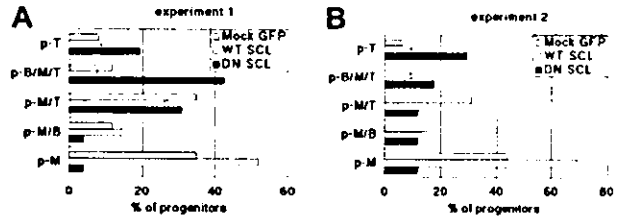

Figure 6. Frequency of different types of progenitors in WT SCL– and DN SCL–transduced HSCs. Progenitor types were determined by analyzing the cells generated from a single cell. Results of 2 independent experiments (A-B) are shown. Asterisks indicate the wells in which the indicated type of progenitor was undetectable. At least 3000 hematopoietic cells per well were analyzed using FACS.

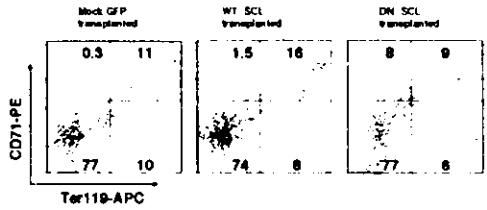

Figure 7. Perturbed erythropoiesis in bone marrow of recipients of transplanted DN SCL–transduced HSCs. FACS analysis of single-cell suspensions of GFP+-gated bone marrow cells immunostained with Ter119 and CD71 antibodies is shown. Numbers in quadrants represent the ratio of cells in the respective quadrants.

Erythroblast maturation of transduced HSCs in recipient bone marrow

SCL expression is closely associated with erythroid maturation. We analyzed bone marrow cells from mice that underwent transplantation for erythroid differentiation markers. We found an increase in the frequency of $Ter119^{low/-}$ $CD71^+$ cells representing an immature erythroid population[24] in GFP^+ bone marrow cells from recipients receiving DN SCL–transduced HSCs (Figure 7). In GFP^+ cells derived from WT SCL–transduced HSCs, however, the frequency of the $Ter119^+CD71^+$ cells, more mature erythroid cells, was slightly increased (Figure 7), suggesting that SCL also affects the maturation of erythroid cells, as expected from the previous report.[18,19]

The hypothesis of lineage commitment of an HSC through SCL signaling is consistent with the expression pattern of SCL, which is positive in HSCs, CMPs, and their progeny but is negative in CLPs.[2] The results demonstrated in this report suggest that SCL is the most obvious candidate molecule determining the cell fate of HSCs toward CMPs or CLPs.

Discussion

Mice without the *SCL* gene in adult HSCs were recently established by a conditional gene-targeting method.[18,19] In these studies, *SCL* was shown to be dispensable for HSC properties, such as long-term repopulating activity and multipotency, but not for proper erythroid and megakaryocyte generation. In the current study, we observed similar abnormalities induced by DN SCL during erythroid differentiation (Figure 7), but megakaryocytic differentiation was not changed by the transduction of WT SCL or DN SCL, as judged by the expression of CD61 (data not shown). Our major observation that the down-regulation of SCL results in predominant lymphoid commitment is also supported by results of a conditional knockout (cKO) mouse study.[19] Cre-mediated excision of *SCL* resulted in a significant increase in the number of T cells in the peripheral blood of HSC recipients. The number of B cells did not appear to be influenced in the cKO mice, and this result could represent a difference from our result. However, the number of mice analyzed in the cKO study[18,19] does not appear to be sufficiently large. Therefore, we speculate that the decrease in the number of B cells in the cKO mice might be observed if the number of mice examined were large enough, given that we observed large mouse-to-mouse variation in T- and B-cell ratios in the GFP^+ population. If so, data described in the cKO studies are very similar to ours using DN SCL–transduced HSCs, including the findings that continued expression of *SCL* is not essential for the maintenance of HSCs.

Our study, however, is the first to shed light on the fact that SCL influences the polarity of an HSC toward myeloid or lymphoid

commitment because WT SCL and DN SCL guide HSCs to opposing commitment pathways. Previously, the exogenous expression of SCL was reported to support proliferation and to inhibit apoptosis of 32D myeloid cells[17] and $CD34^+$ bone marrow cells[11,12] or to stimulate erythromegakaryocytic lineage progenitor cells and to inhibit monomyelocytic lineage cell differentiation. These data and ours apparently show a discrepancy. We used the most highly purified HSCs from bone marrow, which might be a plausible cause of the discrepancies, while others used less purified mouse cells or human cells. It is likely that we observed early differentiation events from HSCs rather than mature lineage differentiation events from progenitors.

Thus, we propose that SCL controls the generation of CMPs and CLPs from HSCs without affecting the self-renewal activity of HSCs. It is of interest whether other transcription factors such as GATA-2, NF-E2, and C/EBPα, which are expressed in CMP but not in CLP, and Aiolos and GATA-3, which are expressed in CLP but not in CMP, play a similar role.

The authors of the reports describing *SCL* cKO mouse characterization did not conclude that lymphoid commitment was facilitated at the expense of myeloid commitment.[18,19] This could suggest that DN SCL might be stronger than *SCL* cKO in influencing HSCs. If so, it is possible that DN SCL interferes with bHLH motif–containing transcription factors other than SCL and that such transcription factors substitute for the function of SCL in *SCL* cKO mice. Direct comparison of mice receiving DN SCL–transduced HSCs with *SCL* cKO mouse–derived HSCs could help to address this issue.

Although CMPs and CLPs as the direct progeny of HSCs has been described, no molecular backgrounds controlling the transition from HSC to progenitor has been characterized. Changes in the expression profiles of transcription factors, including bHLH motif–containing ones, must represent the most important determinant. Our data indicate that a change in SCL activity is the first candidate of such a determinant.

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Incidence of Invasive Aspergillosis after Allogeneic Hematopoietic Stem Cell Transplantation with a Reduced-Intensity Regimen Compared with Transplantation with a Conventional Regimen

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ABSTRACT

To evaluate the clinical characteristics of invasive aspergillosis (IA) after reduced-intensity stem cell transplantation (RIST) compared with those after conventional stem cell transplantation (CST), we examined the medical records of 486 CST and 178 RIST recipients. The overall incidence of IA after allogeneic transplantation was 35 (5.3%) of 664, which gave a 3-year cumulative incidence of 5.6%. The estimated 3-year incidence of IA in CST and RIST was 4.5% and 8.2% ($P = .045$), respectively, but the mortality rates were similar (76% and 86%). The median onset of IA after RIST (day 127) occurred significantly later than that after CST (day 97). A multivariate analysis revealed that IA was associated with age older than 50 years (relative risk, 2.12; 95% confidence interval, 1.08-4.17; $P = .03$) and the presence of acute and/or chronic GVHD (relative risk, 6.2; 95% confidence interval, 2.4-16.4; $P = .0002$). IA remains an important complication after allogeneic transplantation, regardless of the type of conditioning regimen.
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KEY WORDS

Invasive fungal infection • Invasive aspergillosis • Reduced-intensity stem cell transplantation • Graft-versus-host disease • Allogeneic stem cell transplantation

INTRODUCTION

Over the past few decades, many changes in transplantation strategies and supportive measures have been developed, and these may influence the epidemiology of fungal infection. Since prophylactic use of fluconazole for the prevention of candida infection has become a common practice in allogeneic hematopoietic stem cell transplantation (HSCT), candida and molds that are resistant to azoles have become the chief pathogens in HSCT [1]. Invasive fungal infection (IFI) has become a frequent complication of HSCT, and *Aspergillus* species are the most common causative organisms [2]. The reported incidence of invasive aspergillosis (IA) after HSCT has been 6.3% to 15.1% [3,4]. Reported risk factors for IA include neutropenia, transplantation without laminar air flow equipment, transplantation from a matched unrelated donor, presence of graft-versus-host disease (GVHD), use of corticosteroids, older age, and underlying diseases [3,4]. Routine screening with high-resolution chest computed tomography scanning [5] and the development of non–culture-based diagnostic methods [6,7] have improved our ability to diagnose IA. Furthermore, new investigational agents with impressive activity against *Aspergillus* species have also been

†*Hisamaru Hirai died in August 2003.*

shown to be useful in early clinical trials [8,9]. Despite these advances in diagnostic and treatment modalities for IA [5-7], its prognosis still remains poor, with high mortality rates ranging from 50% to >90% [3,4,10].

Reduced-intensity HSCT (RIST) is a new transplantation strategy that was developed to decrease regimen-related toxicity while preserving an adequate antitumor effect [11]. Different pioneering conditioning regimens for RIST have been investigated, including a combination of purine analogs [12] and total body irradiation with potent immunosuppressants [13]. It is assumed that elderly patients, who are not eligible for conventional stem cell transplantation (CST) with a myeloablative regimen, may be able to benefit from RIST. Favorable results have been reported in patients with various hematologic malignancies, including chronic myelocytic leukemia [14] and malignant lymphoma [15]. Among solid tumors, metastatic renal cell carcinoma has been shown to respond well to RIST [16], and on the basis of these early clinical results, studies are currently under way to evaluate the efficacy of RIST in a wide variety of hematologic and nonhematologic malignancies.

However, it remains unknown whether RIST carries the same risk of IA, because it is associated with a shorter neutropenic period and less regimen-related toxicity. Several studies have suggested that IA still occurs frequently after RIST [17,18], whereas others have reported no increase in IFIs despite an increase in the rate of cytomegalovirus (CMV) infection [19]. All of these studies on IA after RIST were small, with 12 to 163 patients, and there is still only limited information on this complication. In this study, we investigated the clinical characteristics of IA after RIST, with regard to its incidence, characteristics, and risk factors, in comparison to those after CST.

PATIENTS AND METHODS

Patients

The medical records of all of the patients who underwent allogeneic HSCT at the National Cancer Center Hospital, Toranomon Hospital, Tokyo Metropolitan Komagome Hospital, and the University of Tokyo Hospital between January 1999 and November 2002 were reviewed. Among the 664 patients, 486 underwent CST and 178 underwent RIST (Table 1). The patients in the RIST group were significantly older, and their malignancies were in more advanced stages than those in the CST group ($P < .01$ and $P < .01$, respectively). The 3-year non-IA mortality rates after CST and RIST were 41.2% and 50.2%, respectively ($P = .24$).

The factors that we considered to be associated with low risk for transplantation were as follows: acute myeloblastic leukemia or acute lymphoblastic leukemia in first or second complete remission, chronic myelogenous leukemia in chronic phase, and myelodysplastic syndrome refractory anemia. All other patients were defined as having high-risk diseases. All of the patients with solid tumors were incurable with conventional treatments.

Stem Cell Sources

In the CST group, 423 and 63 patients received transplants from an HLA-identical and 1 antigen-mismatched donor, respectively (Table 1). In the CST group, 96 patients received granulocyte colony-stimulating factor–mobilized peripheral blood stem cells, whereas 390 received bone marrow. In the RIST group, 153 patients had an HLA-identical donor and 25 had a mismatched donor, and the stem cell source was peripheral blood (n = 155) or bone marrow (n = 23).

Preparative Regimens

Conventional preparative regimens consisted of cyclophosphamide (CY) and total body irradiation (n = 295) or of busulfan and CY (n = 191), as shown in Table 1. Reduced-intensity preparative regimens included fludarabine/CY-based (n = 21), fludarabine/busulfan-based (n = 119), fludarabine/melphalan-based (n = 13), and cladribine/busulfan-based regimens (n = 25). Antithymocyte globulin was added to the preparative regimen in 7 CST and 74 RIST patients. Total body irradiation (4 to 8 Gy) was further added in 14 RIST patients.

Prophylaxis and Treatment of GVHD

Cyclosporine (CSP) and short-term methotrexate were primarily used for GVHD prophylaxis in the CST group (Table 1), whereas most of the RIST patients received CSP 3 mg/kg alone. The diagnosis of GVHD was clinically made in concert with biopsy of the skin or gastrointestinal tract. Acute and chronic GVHD were graded according to the consensus criteria [20,21]. Grade II to IV acute GVHD was treated with methylprednisolone 2 mg/kg/d in addition to CSP.

Management of Infections

Most of the patients were cared for in reverse isolation in a laminar airflow-equipped room, and they received prophylaxis with trimethoprim/sulfamethoxazole or pentamidine inhaler and ciprofloxacin against *Pneumocystis carinii* and bacterial infection, respectively. Herpesvirus prophylaxis with acyclovir was also given as previously described [22]. CMV pp65 antigenemia was routinely monitored once a week. When antigenemia was detected, preemptive therapy with ganciclovir was initiated as previously reported [22].

Absorbable antifungal agents, usually fluconazole,

Table 1. *Characteristics of Patients*

Variable	CST (n = 486)	RIST (n = 178)	P Value
Age, y, median (range)	36 (1-58)	53 (4-70)	<.01
Sex (male/female)	309/177	110/68	.67
Underlying diseases			
Acute myeloid leukemia	154	44	<.01
Acute lymphoblastic leukemia	111	5	
Chronic myelogenous leukemia	129	12	
Myelodysplastic syndrome	43	27	
Malignant lymphoma	31	36	
Solid tumors*	0	34	
Others	18	20	
Risk for transplantation (low/high)†	290/196	55/123	<.01
Stem cells (peripheral blood/bone marrow)	96/390‡	155/23	<.01
HLA (matched/mismatched)	423/63	153/25	.72
Donor (related/unrelated)	224/262	154/24	<.01
Conditioning regimen			
TBI/CY-based	295		
BU/CY-based	191		
2-CdA/BU-based		25	
Flu/BU-based		119	
Flu/CY		21	
Flu/Mel		13	
GVHD prophylaxis			
CSP	9	118	
CSP MTX	426	57	
FK506	3	0	
FK506 sMTX	46	0	
Others	2	3	

CST indicates conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; TBI, total body irradiation; CY, cyclophosphamide; BU, busulfan; 2-CdA, cladribine; Flu, fludarabine; Mel, melpharan; CSP, cyclosporine; sMTX, short-term methotrexate.

*All the patients with solid tumors had documented progressive lesions despite prior therapy.

†We divided the risk of transplantation into 2 groups. The low-risk group was as follows: acute myeloid or lymphoid leukemia in first and second remission, chronic myelogenous leukemia in chronic phase, and myelodysplastic syndrome refractory anemia. The other patients were defined as having high-risk diseases.

‡Four patients were infused with both peripheral and bone marrow. These patients were assigned to the bone marrow group.

were used for antifungal prophylaxis at a dose ranging from 200 to 400 mg/d. Neutropenic fever was treated according to the recommendation of Pizzo [23]. Briefly, we empirically started both a β-lactam antibiotic and an aminoglycoside at the time of the first febrile episode. Intravenous administration of amphotericin B at 0.5 mg/kg/d was added when the fever persisted for more than 5 to 7 days. If the diagnosis of aspergillus infection was confirmed, the dosage of amphotericin B was increased to 1.0 mg/kg/d. We used blood tests, enzyme-linked immunosorbent assay for galactomannan antigen, (1-3)-beta-D-glucan assay, and chest computed tomography for the early diagnosis of IA, as previously reported [5].

Diagnostic Criteria for IA

IA was categorized as proven, probable, or possible IA, according to the established European Organization for Research and Treatment of Cancer (EORTC)/National Institutes of Health (NIH)/Mycology Study Group (MSG) criteria [24]. Briefly, we diagnosed patients as having proven IA when there was histologic evidence of tissue invasion by small uniform, dichotomously branching hyphae, septate at regular intervals, and/or culture findings positive for *Aspergillus* species from autopsy specimens, mostly the lung, and a compatible clinical presentation. Probable IA was defined as radiographic findings compatible with IA on computed tomography with clinical symptoms and positive findings in enzyme-linked immunosorbent assay for galactomannan antigen. Aspergillus antigen positivity was defined as an optical density index of galactomannan >1.5 in 2 consecutive blood samples by an enzyme-linked immunosorbent assay (Platelia *Aspergillus* EIA; Bio-Rad Laboratories, Hercules, CA). Possible IA was not included in this study.

End Points and Statistical Analysis

The primary end point of this study was the incidence of IA after allogeneic HSCT, and the probability of IA after RIST was compared with that after CST. Another objective was to investigate the char-

Table 2. *Clinical Characteristics of Patients with Invasive Aspergillosis*

Variable	CST (n = 21)	RIST (n = 14)	P value
Age, y, median (range)	38 (22-57)	54 (37-70)	<.01*
Antifungal prophylaxis, fluconazole/others	21/0	14/0	
Pulmonary complication before HSCT, yes/no	2/19	1/13	1.00
Risk of transplantation†, low/high	7/14	2/12	.26
Neutropenia at onset of IA, yes/no	3/18	3/11	.66
Graft-versus-host disease (GVHD), acute/chronic/none	11/7/3	5/7/2	
Use of corticosteroid‡, yes/no	17/4	13/1	.63
CMV infection (antigenemia positive), yes/no	4/17	5/9	.43
Onset day of invasive aspergillosis, median (range)	97 (11-885)	127 (35-364)	<.01
Treatment			
deoxycholate amphotericin B	14	10	.67
liposomal *amphotericin* B	2	1	
itraconazole	5	2	
no treatment	0	1	
Response to antifungal therapy, yes/no	6/15	5/9	.72
Mortality after IA diagnosis, within 30 days	7	8	.19

CST indicates conventional stem cell transplantation, RIST indicates reduced-intensity stem cell transplantation, CMV indicates cytomegalovirus.

*Statistically significant.

†We divided the risk of transplantation into 2 groups. The low-risk group was as follows: acute myeloid or lymphoid leukemia in first and second remission, and myelodysplastic syndrome. The other patients were defined as having high-risk diseases.

‡Use of corticosteroid during administration of antithymocyte globulin was excluded from this analysis.

acteristics and risk factors of IA. The cumulative incidence curves of IA were produced by using Gray's method [25], considering death without IA as a competing risk. The median follow-up period after transplantation was 518 days (range, 2-1874 days), and surviving patients were censored on the last day of follow-up.

Risk factors associated with IA were identified in univariate and multivariate Cox regression models. The variables analyzed included age, sex, primary disease and its status at transplantation, stem cell source, donor type, and type of transplantation (CST versus RIST). To evaluate the influence of the development of grade II to IV acute or chronic GVHD, proportional hazard modeling was used, with the onset of GVHD treated as a time-dependent covariate. Variables with a P value of <.10 were subjected to a multivariate analysis with backward stepwise proportional hazard modeling. P values <.05 were considered significant.

RESULTS

Incidence of IA

Thirty-five (5.6%) patients were diagnosed as having IA, which gave a 3-year cumulative incidence of 5.9%. Five of the 35 cases had proven IA, and the remaining 30 had probable IA according to the EORTC/NIH/MSG criteria [24]. The diagnosis of IA was established after death in 4 of the 5 patients with proven IA, and 1 of these cases was diagnosed with a transbronchial lung biopsy. Three of the remaining 4 proven cases had been diagnosed as probable IA while the patients were alive. The day of diagnosis of IA was defined as the day when the first diagnostic test was performed. Thirty patients were diagnosed as having probable IA on the basis of clinical, radiologic, and microbiological findings. All the patients showed some subjective or objective symptoms. The circulating galactomannan antigen assay tested positive in 26 patients. *Aspergillus* species were cultured from sputum in 3 patients, and bronchoalveolar lavage fluid was positive for galactomannan antigen in a patient. All of the 30 patients underwent chest computed tomography scan, and all of them showed some abnormal findings including halo and multiple nodules.

Clinical Characteristics of IA

The clinical characteristics of patients with IA are shown in Table 2. All cases developed after engraftment, whereas 3 patients each in the RIST and CST groups were still neutropenic at the presentation of IA. The median time to the onset after RIST (127 days) was later than that after CST (97 days; $P < .01$). All of the patients had been receiving fluconazole prophylaxis when the diagnosis of IA was established. Of the 35 patients with IA, 30 (86%) had GVHD (acute in 16 and chronic in 14). Thirty patients had been receiving corticosteroid treatment at the time of IA diagnosis, all for the treatment of GVHD. CMV antigenemia was positive in 9 of the 35 patients, and 3 of these progressed to CMV disease. Five patients with IA showed no signs of GVHD and were not receiving corticosteroid treatment when the diagnosis

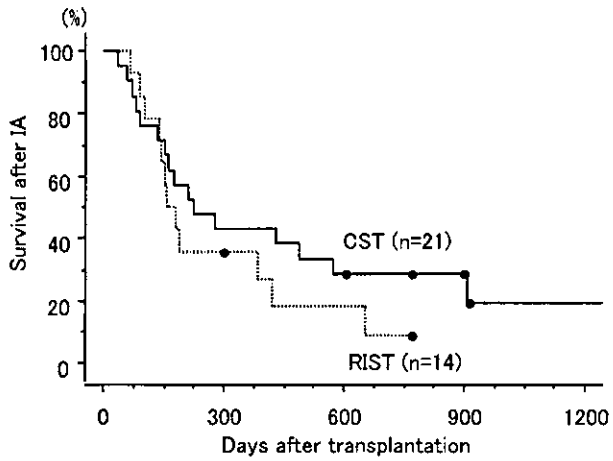

Figure 1. Overall survival after the diagnosis of IA. The survival rate at 1 year after the diagnosis of IA was similarly poor in both the RIST and CST groups (27% and 18%, respectively; $P = .24$).

of IA was established. The clinical characteristics of patients with IA after RIST (n = 14) were compared with those with IA after CST (n = 21), as summarized in Table 2. Except for age and onset of IA, no significant differences were observed between groups.

Treatment and Outcomes

Initial antifungal treatment consisted of deoxycholate amphotericin B (n = 24), liposomal amphotericin B (n = 3), and itraconazole (n = 7). One patient did not receive any antiaspergillus treatment because the diagnosis of IA was established after death. Sixteen (76%) of the 21 CST recipients and 12 (86%) of the 14 RIST recipients who developed IA died, and IA was the direct cause of death in 5 patients in each group. The 1-year survival rates after the diagnosis of IA were similarly poor in both groups (24% versus 19%; $P = .19$; Figure 1).

Risk Factors for IA

The actuarial frequency for the development of IA in the RIST group was 7.9% (14/178), which gave a 3-year cumulative incidence of 8.2%. In the CST group, 21 (4.3%) of 487 patients developed IA, to give a 3-year cumulative incidence of 4.5%. The probability of developing IA was significantly higher in the RIST group than in the CST group ($P = .045$; Figure 2). Old age ($P = .0068$), disease risk for transplantation ($P = .0043$), RIST ($P = .045$), and the development of GVHD ($P = .00014$) were significant risk factors for IA by univariate analysis (Table 3). Among these factors, only age >50 years and the development of GVHD were confirmed to be independently significant by proportional hazard models (Table 3). The difference in the types of preparative regimens was not significant after adjusting for age, donor sources, and the development of GVHD (RIST: relative risk, 1.52; 95% confidence interval, 0.64-3.6; $P = .34$).

DISCUSSION

This study was conducted to investigate the incidence and clinical features of IA after RIST compared with those after CST. IA is a significant complication in patients receiving CST, but little information is currently available on IA after RIST. Although the retrospective nature of this study is a limitation, this is one of the largest studies on IA after RIST and provides valuable information on this complication. We found that the incidence of IA was 4.3% and 7.9% in CST and RIST recipients, respectively. IA tended to be more common in older patients after RIST than after CST, suggesting that IA is still a significant complication in RIST, as well as in CST, in this setting. The incidence of IA in our study is lower than those in previous reports after CST [4] or RIST [18]. Although the reason for this difference is unclear, it might be associated with the lower incidence of GVHD and the consequent decrease in the use of steroids in our country [26]: both of these are known risk factors for IA [4].

The diagnostic criteria used in epidemiologic studies on IA are debatable. IA is difficult to diagnose while patients are alive, and it is frequently confirmed by autopsy. We strictly applied the diagnostic criteria recommended by EORTC/NIH/MSG [24]. However, in retrospective epidemiologic studies, the number of "IA" cases depends on whether both possible and probable IA cases are included. Because we included only probable and proven cases in this study and excluded possible cases, the rate of IA might have been underestimated. Nevertheless, we believe that the primary end point was achieved, because we com-

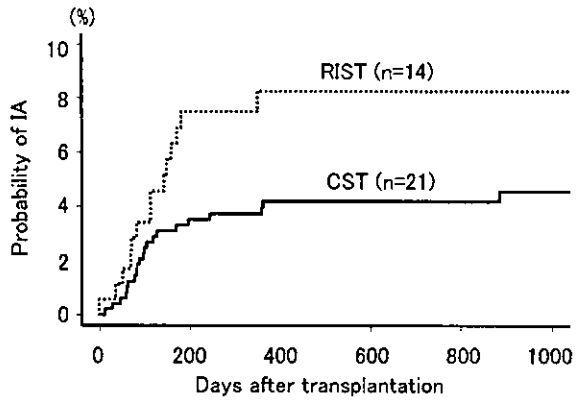

Figure 2. The incidences of invasive aspergillosis after RIST and CST. The cumulative incidences of IA at 3 years after RIST and CST were 8.2% and 4.5%, respectively, with a statistically significant difference ($P = .045$).

Table 3. *Risk Factors for Invasive Aspergillosis*

Factor	Variable	n	Incidences	P value
Univariate analysis				
Pretransplant factors				
Sex	Male	419	5.9%	.48
	Female	245	4.9%	
Age	<50	471	4.1%	.0068*
	≥50	193	9.2%	
Risk for transplantation	High-risk	486	7.2%	.0043*
	Low-risk	178	3.9%	
Donor	Matched related	343	5.6%	.39
	Mismatched related	39	11.7%	
	Matched unrelated	233	4.7%	
	Mismatched unrelated	49	4.1%	
Graft source	Bone marrow	413	4.1%	.091
	Peripheral Blood	251	7.8%	
Preparative regimen	Conventional	486	4.5%	.045*
	Reduced-intensity	178	8.2%	
Use of ATG	With	81	5.4%	.91
	Without	583	5.6%	
Posttransplant factor				
GVHD (acute and/or chronic)	Presence vs Absence		6.55 (2.49-17.2)	.00014*

Factor	Relative Risk	95% Confidence Interval	P value
Multivariate analysis			
Age older than 50	2.12	1.08-4.17	.03*
GVHD (acute and/or chronic)	6.2	2.4-16.4	.0002*

ATG indicates Antithymocyte globin; GVHD indicates graft-versus-host disease.
*Statistically significant.

pared the 2 groups of patients by using the same criteria.

The onset of IA is bimodal, peaking 16 and 96 days after transplantation [3]. Recent studies, including ours, have shown that the development of IA has shifted to a late onset [4,18]. Several factors have contributed to the shift of IA development. First, most recipients undergo transplantation in a laminar air flow–equipped room with or without high-efficiency particulate air filters, which have been demonstrated to be protective against aspergillus infection [3]. Second, early-onset IA tends to occur among patients with incubating or occult aspergillus infection, and these high-risk patients usually receive some antiaspergillus agents before or immediately after transplantation. Third, the development of GVHD, which frequently necessitates corticosteroid therapy, is delayed in RIST compared with CST [27]. Both GVHD and the use of corticosteroids are independent risk factors for IA after HSCT [4]. Finally, the development of CMV infection, which is associated with IA [28], is also delayed in RIST [29]. We should therefore recognize that IA has been well characterized as a late-onset complication in RIST, and any preventive strategy for IA should be extended to cover the possibility of late aspergillosis. Prolonged prophylaxis with antiaspergillus agents such as voriconazole and itraconazole might be beneficial for reducing its incidence.

We found that IA is associated with a poor prognosis in RIST as well as in CST. In this study, only 2 of the 14 RIST recipients and 5 of the 21 CST recipients who developed IA survived. Although most patients with IA had been intensively treated with intravenous amphotericin B, the response rate to treatment was only 36% and 29% in RIST and CST recipients, respectively. Despite the recent availability of new antifungal agents such as voriconazole, itraconazole, and lipid-complex amphotericin B preparations, treatment of IA in recipients of HSCT is still a difficult task without the recovery of host immunity.

It is important to determine the risk factors of IA to identify high-risk patients and to enable the introduction of prophylaxis for more intense infection. The primary risk factor for IA in our study was the development of GVHD and the closely associated use of corticosteroid, because they further delay immune recovery to open the way for the development of IA. Because the incidence of GVHD after RIST is similar to that after CST in this study, it is quite reasonable that the clinical significance of IA is comparable to that after CST. Although use of antithymocyte globulin might be associated with an increase in the risk of IFI, this was not observed in our study. We speculate that the decrease in the incidence of GVHD with the introduction of antithymocyte globulin (10% in this study) contributed to the decrease in the use of steroid and, hence, decreased the risk of IA. Because the dose

of antithymocyte globulin we used was lower than those used in other institutes [12,30], the mild suppression of immune recovery might be another reason for the lower incidence of IA.

We believe that this study will provide some useful information on IA; however, there are several limitations. We compared the incidence of IA between the 2 treatment groups. They had different backgrounds, and many different preparative regimens were used for different underlying diseases, thus making it difficult to draw definite conclusion on IA from this study. The reliability of patient diagnosis is another concern. Although we made an IA diagnosis by using the EORTC/NIH/MSG criteria [24], only 1 of 7 IA patients had histopathologic evidence of IA. The EORTC/NIH/MSG criteria are the best currently available consensus on IFI in immunocompromised patients. However, it is well known that it is difficult to make an accurate diagnosis of IA in recipients of allogeneic HSCT, and most cases are diagnosed by postmortem examination. Additionally, the retrospective nature of this study provides a potential bias. Development of IA is associated with GVHD and corticosteroid use. Further study is required to investigate the association between IA development and other variables, including the severity of GVHD and the dose and duration of corticosteroid use. A prospective evaluation is warranted to further clarify the clinical features of IA after RIST.

In conclusion, we showed that IA is a major complication of RIST that affects older patients and is associated with significant mortality. Special attention should be paid to the effect of steroid treatment on complicated GVHD, especially in older patients. Currently, high-efficiency particulate air filters and correctly sealed rooms are accepted as proper prophylaxis for IA. However, these measures are not realistic in RIST, in which the onset of IA averaged beyond 100 days, when patients are generally followed up in the outpatient clinic [31]. The effectiveness of chemoprophylaxis with newly developed drugs against aspergillus [32,33] and of changing the home environment to eliminate the routes of infection for aspergillosis should be seriously investigated.

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Prospective Comparison of the Diagnostic Potential of Real-Time PCR, Double-Sandwich Enzyme-Linked Immunosorbent Assay for Galactomannan, and a (1→3)-β-D-Glucan Test in Weekly Screening for Invasive Aspergillosis in Patients with Hematological Disorders

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The establishment of an optimal noninvasive method for diagnosing invasive aspergillosis (IA) is needed to improve the management of this life-threatening infection in patients with hematological disorders, and a number of noninvasive tests for IA that target different fungal components, including galactomannan, (1→3)-β-D-glucan (BDG), and *Aspergillus* DNA, have been developed. In this study, we prospectively evaluated the diagnostic potential of three noninvasive tests for IA that were used in a weekly screening strategy: the double-sandwich enzyme-linked immunosorbent assay (ELISA) for galactomannan (Platelia Aspergillus), a real-time PCR assay for *Aspergillus* DNA (GeniQ-Asper), and an assay for BDG (β-glucan Wako). We analyzed 149 consecutive treatment episodes in 96 patients with hematological disorders who were at high risk for IA and diagnosed 9 proven IA cases, 2 probable IA cases, and 13 possible invasive fugal infections. In a receiver-operating characteristic (ROC) analysis, the area under the ROC curve was greatest for ELISA, using two consecutive positive results (0.97; $P = 0.036$ for ELISA versus PCR, $P = 0.055$ for ELISA versus BDG). Based on the ROC curve, the cutoff for the ELISA could be reduced to an optical density index (O.D.I.) of 0.6. With the use of this cutoff for ELISA and cutoffs for PCR and BDG that give a comparable level of specificity, the sensitivity/specificity/positive predictive value/negative predictive value of the ELISA and the PCR and BDG tests were 1.00/0.93/0.55/1.00, 0.55/0.93/0.40/0.96, and 0.55/0.93/0.40/0.96, respectively. In conclusion, among these weekly screening tests for IA, the double-sandwich ELISA test was the most sensitive at predicting the diagnosis of IA in high-risk patients with hematological disorders, using a reduced cutoff of 0.6 O.D.I.

Invasive aspergillosis (IA) is one of the most serious complications in patients with hematological malignancies. It has an extremely high mortality rate (11) and affects not only terminally ill patients with refractory leukemia or lymphoma but also patients who could otherwise be expected to experience a potential cure of the underlying leukemia or lymphoma. Among several factors that contribute to the high mortality rate, difficulties in establishing a reliable diagnosis early enough for successful intervention have been repeatedly discussed (10). A definitive diagnosis usually requires invasive tissue sampling, which is often hampered by the critical condition of the patients, while a delay in initiating antifungal therapy, or, conversely, a hasty use of empiric or prophylactic amphotericin B before making a definitive diagnosis may result in treatment failure for full-blown infection or excess toxicity, respectively.

To overcome this problem and to improve the treatment outcome, advances have been made over the past decade in the fields of both diagnostics and therapeutics, including improvements in diagnostic imaging (7, 8, 18) and histopathology (1), and the development of broad-spectrum antifungal agents with low toxicities (4, 24, 29, 33). In the field of diagnostics, much attention has recently been given to the development of several types of noninvasive laboratory tests for IA. These tests are designed to sensitively detect circulating *Aspergillus* components and include a double-sandwich enzyme-linked immunosorbent assay (ELISA) for galactomannan (GM) antigen (Platellia Aspergillus) (30), tests for (1→3)-β-D-glucan (BDG) (β-glucan Wako or FungiTec G test) (23, 25), and a number of PCR-based assay systems for *Aspergillus* DNA (5, 6, 12, 34).

The ELISA for GM uses a rat monoclonal antibody directed against the 1→5-β-galactofuranoside side chains of the GM molecule as both the capture and detection antibodies for ELISA and can detect as little as 1.0 ng of circulating GM per ml (30). The excellent sensitivity and specificity of this assay have been repeatedly demonstrated and validated in tests of patients with hematological disorders (22, 27, 32). BDG is a ubiquitous component of diverse fungal species and a possible target for the diagnostic detection of IA. Two assay systems are currently available for the sensitive detection of circulating

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BDG, and both are based on the *Limulus* reaction, in which a trace amount of BDG can trigger a horseshoe crab coagulation cascade through factor G (23, 25). The BDG test is a useful method for screening for invasive fungal infection (IFI) and is widely used in Japan. The other test that has long been under intensive investigation for the sensitive detection of IA is PCR amplification of *Aspergillus* DNA, mainly of the 18S ribosomal gene (5, 6, 12, 34). Moreover, recently introduced real-time PCR designs have made it possible to quantitatively evaluate a fungal load with high sensitivity (9, 17, 21).

With regard to an antifungal strategy, it would be interesting to determine which of these tests is the best for diagnosing IA in patients with hematological disorders. Although high sensitivity and specificity are reported for PCR-based assays, the question whether PCR-based assays are superior to GM ELISA is still controversial (3, 5, 19, 34). Previously, we developed a sensitive real-time PCR system for detecting *Aspergillus* 18S ribosomal DNA, with which as few as 40 copies of aspergillus DNA per ml of plasma could be stably detected. We reported that the sensitivity of our real-time PCR for IA in 33 IA patients was higher than those of the double-sandwich ELISA for GM and the BDG test, with only a slightly lower specificity than that of GM ELISA (17). However, this previous study may have been biased by its partially retrospective design, limited sampling points in each case or infectious episode, and use of an inappropriately high cutoff value for ELISA. In the present purely prospective analysis, we consecutively enrolled 96 patients with hematological disorders who were at high risk for IA, monitored the levels of *Aspergillus* DNA, GM, and BDG in plasma, as well as the development of IA, at weekly intervals, and evaluated their diagnostic potentials by using receiver-operating characteristic (ROC) analyses.

MATERIALS AND METHODS

Study population and design. From March 2001 through April 2002, a consecutive series of adult patients with hematological disorders who had been admitted to our hospital and were thought to be at high risk for IA were enrolled in the study, and their levels of *Aspergillus* DNA in plasma and GM in serum, and BDG in plasma were monitored weekly. Patients were considered to be at high risk for IA if (i) they underwent chemotherapy and were expected to be neutropenic (less than 500 neutrophils per μl) for at least 10 days, (ii) they had refractory disease or were neutropenic and presented for more than 96 h with persistent fever that was refractory to appropriate broad-spectrum antibacterial treatments, (iii) they had presented with acute graft-versus-host disease (GVHD) of grade 2 or greater or had extensive chronic GVHD, or (iv) they had received corticosteroids for more than 3 weeks within the previous 60 days. Plasma *Aspergillus* DNA levels, serum GM levels, and plasma BDG levels were to be measured once weekly whenever the patients were thought to be at high risk. Each period during which measurement was performed was defined as one treatment episode. Omission of sampling was permitted unless two consecutive samples were lacking. Treatment episodes with only one or two samples for each test were excluded from the analysis.

The level of *Aspergillus* DNA in plasma was measured using real-time PCR, as described previously (17). The ELISA for GM (Platelia Aspergillus; Sanofi Diagnostics Pasteur, Marnes-La-Cosuette, France) and the β-glucan Wako test (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) were performed as specified by the manufacturers. Each sample was tested twice for GM and BDG, and the average of the two measurements was taken.

Antifungal prophylaxis consisted of daily administration of 200 mg of fluconazole or itraconazole capsules with or without 15 mg of aerosolized amphotericin B or 10 mg of intravenous amphotericin B for patients with a suspected history of IA. Neutropenic fever was treated with broad-spectrum antibiotics in accordance with the published guidelines (16). Blood samples were used for bacterial, mycobacterial, and fungal cultures prior to the initiation of antibiotics. When IFI was suspected, treatment with 1 mg intravenous amphotericin B per kg was initiated. During the febrile period, patients were intensively surveyed for possible sites of infection and causative microorganisms. Diagnostic procedures included routine cultures of urine and stools, repeated cultures of blood and sputum, weekly chest X rays, high-resolution computed tomography (CT) scan of the chest, and, when possible, bronchoscopic examinations and open biopsies.

Case definitions. For each treatment episode, a diagnosis was made following the published case definition criteria for invasive fungal infections from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC-IFICG and NIAID-MSG) (2), with the necessary modification that the plasma GM level was not included in the microbiological criteria.

Statistical analysis. As described by Maertens et al. (22), we made a set of different estimates (A/B, C, and D) for the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each test, where different definitions of disease status for an episode were used to calculate these statistical indexes, since there is an intrinsic uncertainty regarding the true disease status of IA so that the calculation of these values could be significantly affected by the definition of the disease status. Estimate A/B defines "proven IA" and "probable IA" as truly positive and only "no IA" as truly negative, whereas estimates C and D incorporate "possible IFI" into the truly positive and truly negative groups, respectively. In all of the estimates, "no-IA" episodes were considered truly negative. Since our objective was to validate and compare the potentials of different diagnostic tests in a setting where these tests are performed weekly to monitor the development of IA, the positivity or negativity of a test was defined for each episode, where an episode was considered positive if at least one sample (method I), or any two consecutive samples (method II) became positive. There is also a practical reason for this approach. The onset and resolution of an IA episode are not always clear and, indeed, are rather poorly defined in many cases. Even in proven cases, there might be several febrile episodes and the onset might be insidious. In this setting, the sample-based calculation of sensitivity and specificity might be severely biased. In addition, we determined a proper cutoff value for each test through a ROC analysis, in which sensitivity and specificity were calculated as a function of the cutoff value, (1 − specificity) was plotted against the sensitivity, and the areas under the ROC curves (AUCs) were calculated. The significance of the difference in the AUCs of any two diagnostic measures was statistically tested as described above, and *P* values were calculated by the paired method under the null hypothesis that the two ROC curves represent random samples from similar underlying data for sensitivities and specificities (13). Therefore, the *P* values can be used only to compare two ROC curves at a time. The calculated *P* values reflect the one-tailed significance of difference between two ROC curves.

RESULTS

Study episodes. There were 149 treatment episodes in 96 consecutive patients, including 9 proven IA, 2 probable IA, 13 possible IFI, and 125 no-IA episodes. Of these, 56 episodes (38%) were associated with stem cell transplantation. The patient characteristics and sample distributions are summarized in Table 1. Nineteen treatment episodes had no host factors. Overall, 1,251 samples were analyzed by the real-time PCR assay, 1,233 were analyzed by double-sandwich ELISA for GM, and 1,243 were analyzed by the BDG test. On average, approximately eight samples were examined for each treatment episode. The characteristics of the 24 episodes of proven IA, probable IA, and possible IFI are shown in Table 2. There were 24 fatal episodes, of which 8 were proven IA, 1 was probable IA, 4 were possible IFI, and 11 were no IA. Autopsies were performed in 14 episodes (58%), including 6 proven IA and 8 no-IA cases. In the remaining 10 fatal episodes, autopsy was not permitted by the patients' families. The 3 proven IA episodes were diagnosed based on histopathology of a pharyngeal biopsy specimen, a surgical specimen of the brain, and a skin biopsy specimen, respectively. Although postmortem examinations disclosed superinfections of disseminated *Trichosporon* infection and atypical mycobacteriosis in episode 1 and

TABLE 1. Patient characteristics

Characteristic	Patients with:				Total[b]
	Proven IA	Probable IA	Possible IFI	No IA	
No. of episodes	9	2	13	125	149 (96)
No. of deaths	8	1	4	11	24
No. of autopsies	6	0	0	8	14
Age (yr)					
Mean	46	47	43	45	45
Median	42	47	40	47	46
Range	19–69	40–53	18–68	17–74	17–74
Sex (no. male/no. female)	6/3	2/0	12/1	82/43	102/47 (67/29)
No. with disease[a]					
AML	3	1	5	48	57 (29)
ALL	1	0	4	26	31 (19)
CML	0	1	2	8	11 (9)
MDS	3	0	2	11	16 (14)
NHL	2	0	0	28	30 (21)
AA	0	0	0	2	2 (2)
Other	0	0	0	2	2 (2)
No. with allografts	4	2	6	44	56
Duration of episode (days)					
Mean	126	92	78	50	57
Median	135	92	57	37	43
Range	36–234	50–134	35–172	11–181	11–234
No. with host factor:					
Neutropenia	7	1	8	86	102
Fever	6	1	7	37	51
GVHD	2	2	5	17	26
Steroid	2	1	4	28	35
None	1	0	0	18	19
Duration of neutropenia (days)					
Mean	63	10	42	16	21
Median	37	10	18	14	15
Range	0–205	0–20	0–162	0–120	0–205
No. of samples tested					
PCR	154	25	146	926	1,251
Mean (per episode)	17.1	12.5	11.2	7.4	8.4
Median (per episode)	17	13	9	6	6
Range (per episode)	7–32	6–19	4–24	3–26	3–32
GM	155	24	140	914	1,233
Mean (per episode)	17.2	12.0	10.8	7.3	8.3
Median (per episode)	18	12	9	5	6
Range (per episode)	7–30	5–19	5–24	2–26	2–30
BDG	158	24	147	914	1,243
Mean (per episode)	17.6	12.0	11.3	7.3	8.3
Median (per episode)	19	12	9	6	6
Range (per episode)	7–31	5–19	6–24	3–23	3–31

[a] AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; CLL, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; AA, aplastic anemia.
[b] Values in parentheses are numbers of patients. Other values refer to numbers of episodes.

episode 9, respectively, no invasive candidiasis was documented during the study period.

Among the 125 no-IA episodes, 11 deaths occurred, and the diagnosis of no IA was confirmed by autopsy in 8. The other three fatal episodes were not confirmed by autopsy and included two respiratory failures following chemotherapy and one case of severe stomatitis following a second bone marrow transplantation. One respiratory failure was due to bacterial pneumonia, in which *Pseudomonas aeruginosa* was cultured from the sputum and the blood. In the other episode, respiratory failure developed in association with rapid tumor growth. Although no pathogen was identified despite repeated cultures, we could not completely exclude a possible infectious origin of this episode. The episode of severe stomatitis became suddenly fatal after the patient aspirated the clot and was asphyxiated.

ROC analysis. Figure 1 shows ROC curves for each test, using different definitions of the disease status. First, we examined the behaviors of the ROC curves for different diagnostic tests by using an "ideal" estimate (estimate A/B), in which episodes were expected to be most accurately defined. ELISA has a larger AUC in both method I (ELISA, 0.93; PCR, 0.81; BDG, 0.85) and method II (ELISA, 0.97; PCR, 0.76; BDG, 0.79). To increase the sensitivity for GM, we could more easily decrease its cutoff value with a small decrease in specificity. In contrast, a higher sensitivity could be obtained for the PCR and BDG tests by decreasing their cutoff values, but this would be at a significant cost in terms of specificity. When we shifted the diagnostic algorithm from method I (one positive sample) to method II (two consecutive positive samples), the AUC for the GM test was further increased while those for the PCR and BDG tests decreased, indicating that the GM test has higher

TABLE 2. Diagnosis of IA and its documentation

Episode no.	Patient characteristics[a]: Age (yr)	Sex	IA	Primary disease	Status of primary disease	Outcome	Host factors	Clinical evidence	Culture and its source	Histological evidence	Maximum value (method I/method II) PCR (copies/ml)	GM (O.D.L)	BDG (ng/ml)
1	41	F	P	AML M1	Post-allo, RD	Dead	NF	Erosion of sinus walls	*A. flavus* and *A. fumigatus* from pharyngeal mucosa	Biopsy	2,000/200	3.8/3.6	19.7/4.7
2	32	M	P	MDS (RAEB-t)	Post-allo, CR	Dead	GS	Dyspnea, pleural effusion		Autopsy	32/0	1.3/1.0	60.5/36.5
3	58	M	P	AML M1	RD	Dead	NF	Halo sign		Autopsy	90/42.5	7.7/6.4	25/1.5
4	38	F	P	AML M2	Post-allo, CR	Alive	NS	Cavity within area of consolidation	*A. fumigatus* from broncheal lavage fluid	Biopsy	33.5/0	1.9/1.7	2.8/0
5	51	M	P	Macroglobulinemia	Stable disease	Dead	None	Extensive skull base destruction	*A. fumigatus* from epidural abscess	Biopsy	0/0	1.2/0.8	37.4/7.1
6	19	M	P	MDS RA	RD	Dead	NF	Multiple nodular lesions in the lung field, pleural effusion		Autopsy	3,500/1,000	2.5/1.5	155.5/59.2
7	42	M	P	MDS/AML	Post-allo, RD	Dead	NFG	Dyspnea, pleural effusion		Autopsy	24/9	2.4/0.6	0/0
8	63	F	P	ATL acute type	RD	Dead	NF	Dyspnea, pleural effusion		Autopsy	50/12.5	1.9/0.7	2.4/0
9	69	M	P	ALL PreB	RD	Dead	NF	No specific clinical evidence		Autopsy	100,000/5,000	4.2/1.1	171.7/12.6
10	53	M	PP	AML M2	Post-allo, CR	Dead	FG	Dyspnea, pleural effusion	*A. spergillus* spp. from broncho-alveolar lavage fluid	NA[b]	5/0	5.3/0.7	4.5/2.2
11	40	M	PP	CML CP1	Post-allo, CR	Alive	NGS	Halo sign	*A. fumigatus* from sputum	NA	11.5/7.5	2.3/2.0	0/0
12	68	M	PPP	MDS/AML	RD	Dead	NF	Multiple nodular lesions in the lung field, intraparenchymal brain mass lesion, seizure, hemiparesis		NA	155/100	2.2/1.5	18.3/16.6
13	24	M	PPP	AML M4E	CR, HDAraC	Alive	NF	Nodular skin lesion without any other explanation, multiple nodular lesions in the lung field		NA	20.5/0	4.5/0.3	0/0
14	61	M	PPP	AML M4E	CR, HDAraC	Alive	N	Halo sign		NA	1,000/9	0.2/0.1	3.5/2.9
15	30	M	PPP	ALL precursor B	Post-allo, CR	Alive	NFGS	Nonspecific abnormal shadow in lung field, pleural effusion		NA	60/60	0.6/0.4	0/0
16	61	M	PPP	AML M2	RD	Dead	NF	Multiple nodular lesions in the lung field, halo sign, cavity within area of consolidation		NA	84.5/0	1.1/0.7	2/0
17	68	M	PPP	CML BC	RD	Dead	NS	Dyspnea, pleural effusion		NA	165/0	0.3/0.2	0/0
18	25	M	PPP	ALL precursor B	RD	Alive	NG	Cavity within area of consolidation		NA	400/0	0.7/0.6	3.2/0
19	32	M	PPP	ALL PreB	Post-allo, CR	Dead	FGS	Dyspnea, pleural effusion		NA	27/1	0.7/0.5	3.7/2.4
20	18	F	PPP	AML M2	CR, HDAraC	Alive	N	Halo sign		NA	0/0	0.6/0.1	0/0
21	55	M	PPP	MDS RA	Stable disease	Alive	F	Cough, dyspnea, pleural effusion		NA	19/4	0.8/0.3	0/0
22	28	M	PPP	CML CP1	Post-allo, CR	Alive	G	Cough, dyspnea, pleural effusion		NA	0/0	0.4/0.3	0/0
23	40	M	PPP	CML CP1	Post-allo, CR	Alive	GS	Cough, dyspnea, new infiltrate not fulfilling the major radiological criteria without an alternative diagnosis		NA	6/0	0.5/0.4	0/0
24	54	M	PPP	ALL precursor B	Post-allo, CR	Alive	F	Dyspnea, new infiltrate not fulfilling the major radiological criteria without an alternative diagnosis		NA	10.5/0	0.5/0.3	0/0

[a] F, female; M, male; P, proven; PP, probable; PPP, possible; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB-t, RA with excess of blasts in transformation; ALL, acute lymphoblastic leukemia/lymphoma; CML, chronic myelogenous leukemia; CP, chronic phase; BC, blastic crisis; allo, allogencic hematopoietic stem cell transplantation; CR, complete remission; RD, refractory disease; HDAraC, high-dose cytrabine; N, neutropenia; F, persistent fever; G, GVHD, S, prolonged use of corticosteroid.

[b] NA, not available.