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Mobilization of Human Lymphoid Progenitors after Treatment with Granulocyte Colony-Stimulating Factor[1]

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Hemopoietic stem and progenitor cells ordinarily residing within bone marrow are released into the circulation following G-CSF administration. Such mobilization has a great clinical impact on hemopoietic stem cell transplantation. Underlying mechanisms are incompletely understood, but may involve G-CSF-induced modulation of chemokines, adhesion molecules, and proteolytic enzymes. We studied G-CSF-induced mobilization of $CD34^+CD10^+CD19^-Lin^-$ and $CD34^+CD10^+CD19^+Lin^-$ cells (early B and pro-B cells, respectively). These mobilized lymphoid populations could differentiate only into B/NK cells or B cells equivalent to their marrow counterparts. Mobilized lymphoid progenitors expressed lymphoid- but not myeloid-related genes including the G-CSF receptor gene, and displayed the same pattern of Ig rearrangement status as their bone marrow counterparts. Decreased expression of VLA-4 and CXCR-4 on mobilized lymphoid progenitors as well as multipotent and myeloid progenitors indicated lineage-independent involvement of these molecules in G-CSF-induced mobilization. The results suggest that by acting through multiple *trans*-acting signals, G-CSF can mobilize not only myeloid-committed populations but a variety of resident marrow cell populations including lymphoid progenitors. ***The Journal of Immunology,*** **2005, 175: 2647–2654.**

Hemopoietic stem and progenitor cells (HPC),[5] which usually reside within bone marrow (BM), can be released into circulation after treatment with cytokines, cytotoxic agents, or both (1). Among a number of agents, G-CSF is the cytokine most commonly used clinically to mobilize HPC in a variety of transplantation settings because of its potency and lack of serious toxicity. Especially in allogeneic stem cell transplantation, G-CSF-mobilized peripheral blood stem and progenitor cells (PBPC) now are replacing marrow-derived HPC as a stem cell source.

G-CSF acts by binding to its receptor (G-CSFR), a member of the class I cytokine receptor family expressed on various hemopoietic cells such as stem cells, multipotent progenitors, myeloid-committed progenitors, neutrophils, and monocytes (2–4). Liu et al. (5) showed a significant effect of G-CSF signals on HPC mobilization by demonstrating that mice deficient in G-CSFR failed to mobilize HPC in response to G-CSF. Conversely, they also reported that chimeric mice with both wild-type and deleted G-CSFR can mobilize equal numbers of HPC with or without the receptor in response to G-CSF (6). Thus, G-CSFR expression on HPC may not be crucial for their mobilization by G-CSF. These data indicate that G-CSF can induce HPC mobilization by pathways not limited to transmittal of G-CSF signals directly onto the target cells. Recent insights using experimental animal models are provided suggesting that HPC mobilization by G-CSF could be mediated by indirect effects involving generation of multiple *trans*-acting signals in the marrow microenvironment (7–11). Proteolytic enzymes such as neutrophil elastase, cathepsin G, and matrix metalloproteinase (MMP)-9 released from the activated neutrophils and monocytes can degrade and/or inactivate the adhesion molecules such as VCAM-1/VLA-4, chemokines such as stromal-derived factor (SDF)-1/CXCR-4, and soluble Kit ligand, resulting in the disruption of contact between HPC and the BM microenvironment. HPC then would be released to migrate into peripheral blood (PB). However, details of the mechanisms of HPC mobilization by G-CSF are not yet fully understood, especially in humans.

Marrow is the primary lymphohematopoietic organ where B lymphoid lineage development occurs. Under ordinary steady-state conditions, immature lymphoid progenitors in various stages of differentiation as well as multipotent and myeloid progenitors are confined to BM microenvironments in which they undergo further differentiation; then the mature cells leave the BM and circulate in the blood. In this context, considering the broad spectrum of target cells affected by G-CSF and involvement of changes affecting adhesion molecules in HPC mobilization, G-CSF might be expected to mobilize not only G-CSFR-possessing cells but a variety of cell populations including lymphoid cells and nonhematopoietic cells residing within the BM. Accordingly, we evaluated populations of G-CSF-mobilized blood cells in detail using multicolor flow cytometry to better understand the mechanism of G-CSF-induced mobilization in humans. We identified small but significant populations possessing immature lymphoid phenotypes such as

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[5] Abbreviations used in this paper: HPC, hematopoietic stem and progenitor cells; BM, bone marrow; PBPC, peripheral blood stem and progenitor cells; G-CSFR, G-CSF receptor; MMP, matrix metalloproteinase; SDF, stromal-derived factor; PB, peripheral blood; MNC, mononuclear cells; MPP, multipotent progenitor cells; SCF, stem cell factor; FL, flt3/flk2-ligand; LTC-IC, long-term culture initiating-cell; CLP, common lymphoid progenitors; MFI, mean fluorescence intensity.

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$CD34^+CD10^+CD19^-$ and $CD34^+CD10^+CD19^+$ cells among G-CSF-mobilized cells in blood; these have been defined as early B and pro-B cells in the BM, respectively. The mobilized $CD34^+CD10^+CD19^-$ and $CD34^+CD10^+CD19^+$ cells are capable of differentiation into B/NK cells or B cells that are equivalent to their BM counterparts. Furthermore, these mobilized lymphoid progenitors express lymphoid-related genes but not myeloid-affiliated genes including G-CSFR. In addition, Ig gene rearrangements were detected in these mobilized progenitors. Expression of adhesion molecules such as VLA-4 and CXCR-4 on the mobilized lymphoid progenitors as well as multipotent and myeloid progenitors was down-regulated compared with their steady-state BM and even G-CSF-treated BM counterparts. Thus, G-CSF can mobilize not only myeloid progenitors but also early B and pro-B progenitor cells by modulation of adhesion molecules in a lineage-independent manner. These findings also support the hypothesis that G-CSF can mediate HPC mobilization indirectly in humans as well as mice.

Materials and Methods

Patients

G-CSF-mobilized PB samples were collected from 82 healthy allogeneic PBPC donors who received G-CSF (Filgrastim; Kirin) s.c. at 400 $\mu g/m^2$ per day for 5 days. G-CSF-treated BM samples also were collected from three of these volunteer donors on day 5 of G-CSF administration. Steady-state BM and PB samples were collected from 14 and 34 healthy adults, respectively. Informed consent was obtained from all subjects.

Cell preparation and staining

PB and BM mononuclear cells (MNC) were prepared by gradient centrifugation. For analysis of myeloid progenitor cells, cell samples were stained with a Cy5-PE-conjugated lineage (Lin) mixture (anti-CD3, -CD4, -CD8, -CD11b, -CD16, -CD20, -CD56, and glycophorin A; Caltag Laboratories), FITC-conjugated anti-CD13 (BD Pharmingen), PE-conjugated anti-CD33 (BD Pharmingen), allophycocyanin-conjugated anti-CD34 (BD Pharmingen), and biotin-conjugated anti-CD38 (Caltag Laboratories) Abs. B lymphoid progenitors were stained with the same Cy5-PE-conjugated lineage mixture followed by FITC-conjugated anti-CD10 (Ancell), PE-conjugated anti-CD19 (BD Pharmingen), and anti-CD38 and -CD34 as described above. For analysis of T-lymphoid progenitors, Lin^- cells were stained with FITC-conjugated anti-CD7 (BD Pharmingen), PE-conjugated anti-CD2 (BD Pharmingen), and anti-CD38 and -CD34 as described above. Streptavidin-conjugated Cy7-allophycocyanin (Caltag Laboratories) were used for visualization of biotinylated Abs. Nonviable cells were excluded by propidium iodide staining. Expression of adhesion molecules was detected on progenitors staining by PE-conjugated anti-CXCR-4, VLA-4, and c-Kit (BD Pharmingen), together with anti-CD10 or -CD13 and anti-CD38 and -CD34 as described above.

For sorting cells, $CD34^+$ cells were enriched from MNC using immunomagnetic beads according to the manufacturer's instructions ($CD34^+$ selection kit; Miltenyi Biotec) followed by staining specific for progenitors of each lineage as described above. $CD34^+CD38^{-/low}CD13^-CD10^-CD19^-Lin^-$ multipotent progenitor cells (MPP), $CD34^+CD38^+CD13^+CD10^-CD19^-Lin^-$ myeloid progenitor cells, $CD34^+CD38^+CD10^+CD19^-CD13^-Lin^-$ early B cells, and $CD34^+CD38^+CD10^+CD19^+CD13^-Lin^-$ pro-B cells were sorted by a highly modified triple laser (488-nm argon laser, 633-nm helium-neon laser, and UV laser) FACS (FACSVantage SE; BD Pharmingen). Five-color sorting using both positive and negative gates in multiple channels usually gives rise to cells with >98% purity, avoiding cosorted cells stained in a nonspecific manner. The sorted cells were subjected to an additional round of sorting using the same gate to eliminate contaminating cells and doublets (12).

In vivo and in vitro assays to determine differentiation potential

Clonogenic progenitor assay was performed using a methylcellulose culture system as reported previously (12, 13). Cells also were cultured on the irradiated Sys-1 or MS-5 stromal cell layers (14, 15). Human cytokines such as stem cell factor (SCF) (20 ng/ml), IL-3 (30 ng/ml), IL-2 (50 ng/ml), GM-CSF (20 ng/ml), erythropoietin (2 U/ml), thrombopoietin (20 ng/ml; Kirin), IL-7 (20 ng/ml), flt3/flk2-ligand (FL; 10 ng/ml), and IL-11 (10 ng/ml; R&D Systems) were added at the start of the culture.

Long-term culture initiating-cell (LTC-IC) assays were performed in human long-term culture medium (Myelocult H5100; StemCell Technologies) supplemented with hydrocortisone on irradiated M2-10B4 stromal layers as described previously (13). At 6 wk of culture on the stromal layers, cells were transferred to the methylcellulose medium, and colonies were counted 14 days later.

For limiting dilution analysis, variable numbers of double-sorted early B cells were deposited on the MS-5 stromal cell layers in the presence of IL-7, IL-11, SCF, FL, and IL-2, using an automatic cell deposition unit system (BD Pharmingen). All cultures were incubated at 37°C in a humidified atmosphere including 7% CO_2.

For reconstitution assays, FACS-sorted cells were injected into irradiated (350 rad) NOD/SCID/β_2-microglobulin knockout (NOD/SCID/$\beta 2^{-/-}$) mice as described previously (16). At 6–8 wk after transplantation, BM and spleen were collected for analysis by flow cytometery. Cells were stained with mAbs to human leukocyte differentiation Ags, including CD45, CD19, CD10, CD15, CD56, and CD3.

Gene expression profile by RT-PCR

To examine gene expression profile of each population, total RNA was purified from 1000 double-sorted cells and was amplified by RT-PCR (12). The primer sequences were reported previously (13). PCR products were electrophoresed on an ethidium bromide-stained 2.0% agarose gel. PCR amplification was repeated at least twice for at least two separately prepared samples.

PCR analysis of IgH gene rearrangement

To analyze IgH gene rearrangements status of each population, DNA was extracted from double-sorted 5000 cells, and PCR amplification of VDJ_H and DJ_H rearrangement was performed as described previously (17, 18). This PCR analysis can detect incomplete DJ_H rearrangements of IgH gene with a mixtures of upstream D_H primers and a consensus J_H primer, resulting in a ladder of different sized products ranging from 70 to 100 bp depending on the length of the DJ_H rearrangements. The GAPDH gene primers were used as control for DNA integrity.

Statistical analyses

Levels of significance were measured using paired t test. $p < 0.05$ was considered significant.

Results

Phenotypic analysis

Using a five-color cell sorter, we analyzed the distribution of each cell population including multipotent, myeloid, and lymphoid progenitors. Under unstimulated conditions, few $CD34^+Lin^-$ cells circulated in the periphery (0.023 ± 0.014% of MNC; $n = 34$), whereas the number of circulating $CD34^+Lin^-$ cells increased up to 0.59 ± 0.35% of MNC after G-CSF administration (Table I). $CD34^+Lin^-$ fractions are subdivided into two fractions according to expression of CD38: $CD34^+CD38^{-/low}Lin^-$ fractions contain hemopoietic stem cells with multipotent, self-renewing capacity, whereas $CD34^+CD38^+Lin^-$ cells include lineage-committed progenitors that have lost self-renewing capacity (Fig. 1*A*) (13).

In BM, G-CSF administration increased the number of MNC up to ~2.4-fold, with dominant expansion of myeloid-committed progenitors and mature granulocytes/macrophages; this reflected a relatively reduced percentage of the primitive $CD34^+CD38^{-/low}Lin^-$ population (Table I). Although $CD34^+CD38^{-/low}Lin^-$ cells were mobilized into the periphery by G-CSF, their percentage was significantly lower than in steady-state BM (Table I). Following G-CSF, most $CD34^+$ cells were $CD13^+CD38^+$ myeloid-committed progenitors; myeloid progenitors constituted 92.30 ± 3.76% and 96.70 ± 6.62% of $CD34^+CD38^+Lin^-$ cells in BM and PB, respectively (Table I and Fig. 1*B*). These data showed that myeloid-committed progenitors expanded within BM and were the main population mobilized by G-CSF administration.

Lymphoid progenitors mobilized by G-CSF

Under ordinary conditions, after commitment to the B lymphoid differentiation pathway, $CD34^+CD38^{-/low}Lin^-$ MPP cells become $CD34^+CD38^+CD10^+CD19^-CD20^-$ early B cells or common lymphoid progenitors (CLP) (14, 19) and differentiate within

Table I. *Subsets of $CD34^+CD38^+Lin^-$ fraction among cells from steady-state BM, G-CSF-treated marrow, and G-CSF-mobilized PB*[a]

	Steady-State PB (n = 34)	Steady-State BM (n = 14)	G-CSF-Treated BM (n = 3)	G-CSF-Mobilized PB (n = 82)
% $CD34^+Lin^-$ cells/MNC	0.023 ± 0.014	1.49 ± 0.62	1.03 ± 0.12	0.59 ± 0.35[b]
% $CD34^+CD38^-Lin^-$/MNC	ND	0.15 ± 0.072	0.078 ± 0.015	0.060 ± 0.036[b]
% $CD13^+$/$CD34^+CD38^+Lin^-$	80.51 ± 7.59	89.21 ± 6.91	92.3 ± 3.76	96.70 ± 6.62[b]
% $CD10^+CD19^+$/$CD34^+CD38^+Lin^-$	ND	8.93 ± 5.37	5.52 ± 3.04	1.49 ± 1.30[b]
% $CD10^+CD19^-$/$CD34^+CD38^+Lin^-$	ND	0.82 ± 0.56	0.44 ± 0.11	0.14 ± 0.09[b]

[a] Data are expressed as mean ± SD. ND, not detected.
[b] Significantly different from steady-state BM ($p < 0.05$).

the BM through a $CD34^+CD38^+CD10^+CD19^+CD20^-$ pro-B phenotype into a $CD34^{-/low}CD38^+CD10^-CD19^+CD20^+$ pre-B phenotype (Fig. 1*A*) (20). Then mature B cells are released from the BM into the circulation. In our analysis, early B and pro-B cells were undetectable in the PB of 34 healthy volunteers (data not shown). In the steady-state BM, early B cells and pro-B cells comprised 0.82 ± 0.56% and 8.93 ± 5.37% of $CD34^+CD38^+Lin^-$ cells, respectively (n = 14; Table I). Following G-CSF administration, despite the relatively reduced percentage of lymphoid progenitors reflecting expansion of myeloid lineage cells, absolute numbers of early B and pro-B cells in BM were not significantly different from those in steady-state BM (Table I).

Surprisingly, the G-CSF-mobilized PB contained small but significant populations possessing the same lymphoid phenotypes as BM early B and pro-B cells in the $CD34^+CD38^+Lin^-$ fractions: $CD10^+CD19^-$ and $CD10^+CD19^+$ cells were detectable in 60 and 80 of 82 cases, respectively. A representative FACS analysis is shown in Fig. 1*B*. These $CD10^+CD19^-$ and $CD10^+CD19^+$ cells constituted 0.14 ± 0.09% (0–0.68%) and 1.49 ± 1.30% (0–8.58%) of $CD34^+CD38^+Lin^-$ cells, and ~0.001% and 0.01% of G-CSF-mobilized PB MNC, respectively (Table I). The percentage of circulating lymphoid progenitors was 10 and 6 times less than that in steady-state BM and in G-CSF-treated BM, respectively. T-lineage progenitor coexpressing CD34 and CD7 or CD2 was undetectable in the G-CSF-mobilized PB (data not shown). These lymphoid progenitors were doubly sorted and subjected to analyses of differentiation capacity and gene expression profiles as follows.

Change in expression of adhesion molecules during G-CSF mobilization

We next evaluated expression of c-Kit and adhesion molecules such as VLA-4 and CXCR-4 on different progenitors during G-CSF administration. Fig. 2 shows the mean fluorescence intensity (MFI) for these molecules among MPP, myeloid progenitors, and lymphoid progenitors.

Under physiological conditions, c-Kit was expressed at a low level on the BM $CD34^+CD38^{-/low}Lin^-$ primitive MPP. Its expression was up-regulated in myeloid progenitors but shut down in $CD10^+$ lymphoid progenitors (Fig. 2*A*); these results are consistent with those reported previously (14, 21). CXCR-4, a receptor for SDF-1 that is critical for homing of HPC as well as B lymphopoiesis (22), was highly expressed on lymphoid progenitors, whereas MPP and myeloid progenitors showed low CXCR-4 expression (Fig. 2*B*). VLA-4 was expressed on all three types of progenitors; frequency of expression did not differ significantly among them (Fig. 2*C*).

Following G-CSF administration, MFI for c-Kit expression on MPP and myeloid progenitors was decreased in PB compared with that in steady-state BM (Fig. 2*A*). In contrast, lymphoid progenitors showed low to absent c-Kit expression, and MFI between the tissues was not different. As shown in Fig. 2, *B* and *C*, MFI for

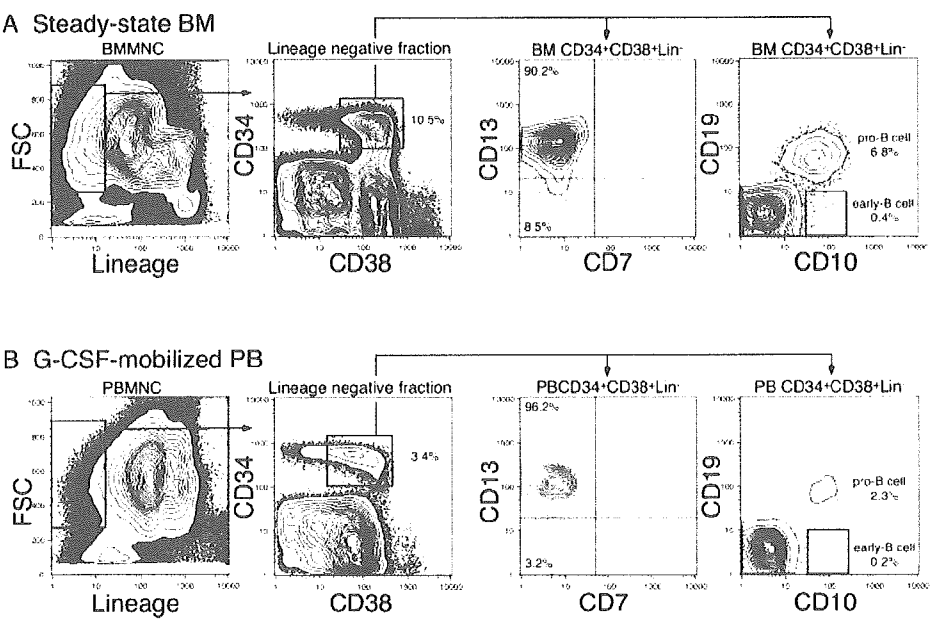

FIGURE 1. Five-color flow cytometric analyses of BM (*A*) and G-CSF-mobilized PB cells (*B*). Cells first were gated by lack of expression of lineage-related Ags. Most $CD34^+CD38^+$ BM cells are $CD13^+$ myeloid-committed cells. Small lymphoid-committed populations such as $CD10^+CD19^-$ early B and $CD10^+CD19^+$ pro-B cells exist in the $CD34^+CD38^+$ fraction but are absent from the peripheral circulation under steady-state condition (*A*). Following G-CSF administration, minor populations possessing the same phenotype of BM early B and pro-B cells were mobilized into the PB (*B*).

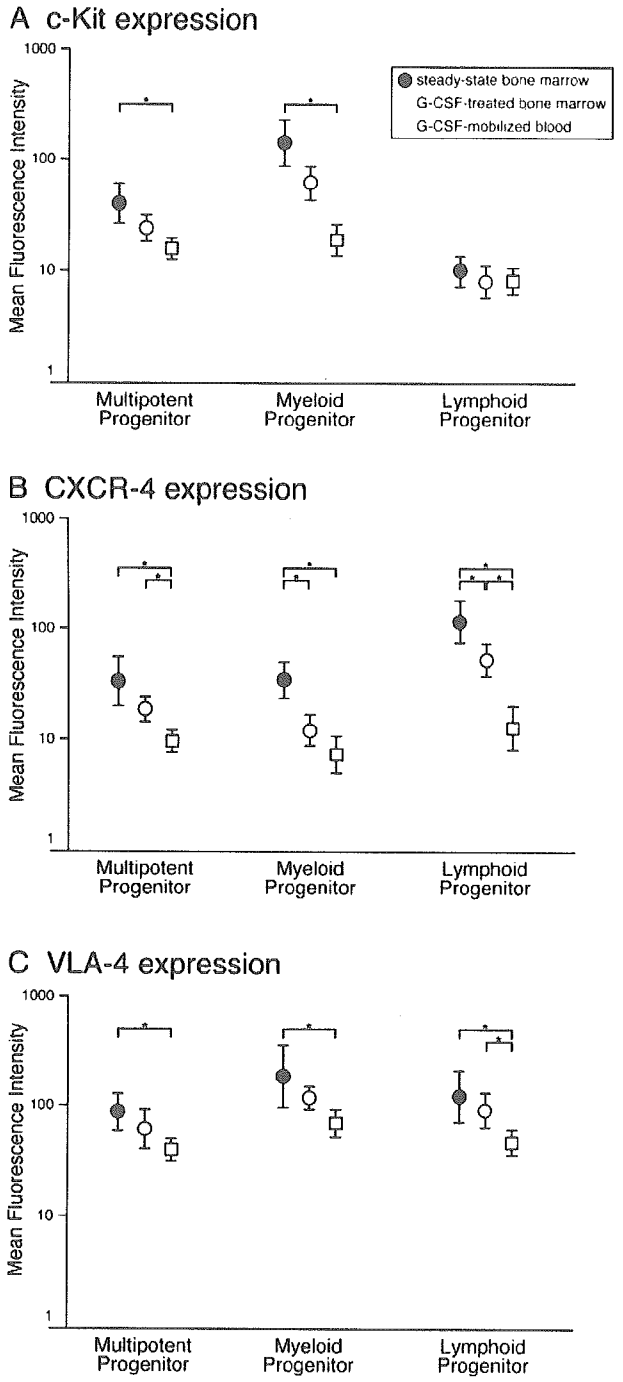

FIGURE 2. Comparative expression of c-Kit (*A*), VLA-4 (*B*), and CXCR-4 (*C*) on $CD34^+CD38^{-/low}Lin^-$ multipotent progenitors, $CD34^+CD38^+CD13^+$ myeloid progenitors, and $CD34^+CD38^+CD10^+$ lymphoid progenitors in the steady-state BM (●), G-CSF-treated BM (○), and G-CSF-mobilized PB (□). Circles and boxes indicate median MFI for these molecules among progenitors, and bars show SD. *, $p < 0.05$, significantly different compared with each tissue.

CXCR-4 and VLA-4 expression for all three progenitor types in BM declined after G-CSF administration; further decrease was observed in the G-CSF-mobilized progenitors. These results suggest that adhesion molecule expression on HPC in BM decreased during G-CSF administration in a lineage-independent manner. As a result, a fraction of cells with lesser expression of these molecules may be mobilized into the circulation.

Mobilized lymphoid cells are restricted to B/NK cell lineage

To test the differentiation capacity of mobilized cells possessing immature lymphoid phenotypes, doubly sorted $CD34^+CD10^+CD19^-$ and $CD34^+CD10^+CD19^+$ cells were cultured on methylcellulose with IL-7, SCF, IL-11, IL-3, GM-CSF, erythropoietin, thrombopoietin, and FL. One hundred $CD34^+Lin^-$ or $CD34^+CD38^+Lin^-$ cells purified from BM and G-CSF-mobilized PB gave rise to a variety of colonies including all types of myeloid lineages (13). In contrast, 500 $CD34^+CD10^+CD19^-$ and $CD34^+CD10^+CD19^+$ cells sorted from the steady-state BM and G-CSF-mobilized PB did not form any colonies after 14 days of culture under this condition (data not shown).

After 4 wk of culture on MS-5 or Sys-1 stromal layers in the presence of IL-7, SCF, IL-11, IL-3, IL-2, GM-CSF, and FL, BM $CD34^+Lin^-$ cells differentiated into $CD15^+$ myeloid cells, $CD19^+$ B lymphoid cells, and $CD56^+$ NK cells (Fig. 3, *A* and *B*). In contrast, early B cells sorted from BM and G-CSF-mobilized PB gave rise to $CD10^+CD19^+$ pro-B cells and $CD10^-CD19^+$ pre-B cells as well as NK cells but not myeloid cells (Fig. 3, *C*, *D*, *G*, and *H*). Pro-B cells from BM and G-CSF-mobilized PB also formed $CD19^+$ B cell-containing colonies, but neither myeloid nor NK cells were detected in cultured cells (Fig. 3, *E*, *F*, *I*, and *J*). Production of T cells was not observed in any of these cultures (data not shown).

Six to 8 wk after transplantation of FACS-sorted progenitors into irradiated NOD/SCID/$\beta2^{-/-}$ mice, animals were sacrificed to assess reconstitution of human hematopoiesis. FACS analysis of spleen and BM cells showed the presence of human $CD45^+$ cells in all mice transplanted with 50,000 $CD34^+Lin^-$ cells, $CD34^+CD38^+CD10^-CD19^+Lin^-$ pro-B cells, and $CD34^+CD38^+CD10^+Lin^-$ cells (including early B and pro-B cells). Strikingly, mice transplanted with $CD34^+CD38^+CD10^+Lin^-$ cells exhibited massive splenomegaly (5- to 10-fold enlargement) in contrast to mice receiving $CD34^+Lin^-$ cells, indicating that lineage-committed B lymphoid progenitors might proliferate rapidly in the spleen. In mice receiving human $CD34^+Lin^-$ cells, most $CD45^+$ cells in the spleen and BM were positive for CD19 (68 ± 7% and 45 ± 14%, respectively), but $CD45^+CD15^+$ and $CD45^+CD56^+$ cells also were found in both spleen and BM; thus, human $CD34^+Lin^-$ cells could differentiate into myeloid cells, B cells, and NK cells in these mice (Fig. 4*A*). In contrast, mice transplanted with 50,000 pro-B cells sorted from BM or G-CSF-mobilized PB exhibited only B lymphoid reconstitution (Fig. 4*B*). However, human $CD45^+$ cells could not be detected in animals transplanted with up to 3000 $CD34^+CD38^+CD10^+CD19^-Lin^-$ early B cells: the number of injected early B cells might not be sufficient to engraft mice. Because early B cell population was too tiny to sort cells enough for engraftment in xenogeneic hosts, we tested the differentiation potential of early B cells in vivo by injecting $CD34^+CD38^+CD10^+Lin^-$ cells, containing both $CD19^-$ early B and $CD19^+$ pro-B cells. Mice transplanted with $CD34^+CD38^+CD10^+Lin^-$ cells sorted from BM or G-CSF-mobilized PB reconstituted both $CD19^+CD56^-CD15^-$ B cells and $CD19^-CD56^+CD15^-$ NK cells in the BM and spleen (Fig. 4*C*). These in vitro and in vivo data revealed that G-CSF-mobilized $CD34^+CD38^+CD10^+CD19^+$ and $CD34^+CD38^+CD10^-CD19^+$ cells can rapidly differentiate in a B/NK and B lineage-restricted manner, which represents the same functional properties as in their BM counterparts, early B and pro-B cells.

Lymphoid progenitors have no self-renewing capacity

To test limited self-renewal activity, BM and G-CSF-mobilized early B and pro-B cells were plated in limiting dilution in LTC-IC assays and transferred to methylcellulose after 6 wk of culture as described previously (13). The estimated frequency of LTC-IC

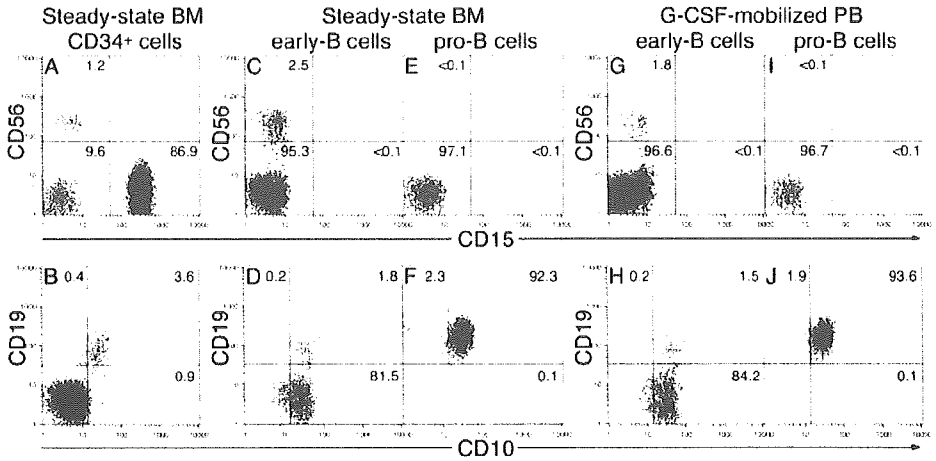

FIGURE 3. Differentiation potential of FACS-sorted progenitors in stroma-supported cultures. Cells cocultured on MS-5 and Sys-1 stromal cell layers with IL-7, SCF, IL-11, IL-3, IL-2, GM-CSF, and FL were harvested after 28 days, and FACS analysis was gated for viable $CD45^+$ cells. $CD34^+Lin^-$ BM cells differentiated into $CD15^+$ myeloid, $CD56^+$ NK cells (*A*), and $CD10^+CD19^-$ early B and $CD10^+CD19^+$ pro-B cells (*B*). Steady-state BM early B and pro-B cells gave rise to more differentiated lymphoid-restricted cells such as $CD10^+CD19^+$ pro-B and $CD10^-CD19^+$ pre-B cells and $CD56^+$ NK cells, but not myeloid cells (*C–F*). Similarly, $CD10^+CD19^-$ and $CD10^+CD19^+$ cells sorted from G-CSF-mobilized PB possessed B lymphoid-restricted differentiation potential in the stromal cell cultures (*G–J*).

was 1 in 20 for $CD34^+CD38^-Lin^-$ cells, but no LTC-IC activity was detected in early B and pro-B cells from either BM or G-CSF-mobilized PB (data not shown).

Differentiation potential of early B cells in limiting dilution analysis

We next evaluated B cell differentiation capacity of BM and G-CSF-mobilized early B cells at limiting dilution in culture on MS-5 stromal cell layers, which can support differentiation of B lymphoid progenitors from human $CD34^+$ cells (15). As shown in Fig. 3, early B cells were capable of differentiation into $CD10^+CD19^+$ pro-B cells and $CD10^-CD19^+$ pre-B cells after 4 wk of culture under this condition. In a limiting dilution assay, we estimated that one in eight BM early B cells and one in 10 G-CSF-mobilized early B cells could read out B cell differentiation in this culture condition (Fig. 5). Frequency of B cell development did not differ significantly between BM and G-CSF-mobilized early B cells.

Mobilized lymphoid progenitors show lineage-specific gene expression profiles

We tested expression of several genes in early B and pro-B cells sorted from the G-CSF-mobilized PB and BM by RT-PCR. Lymphoid lineage-specific genes such as IL-7R, TdT, Pax-5, and VpreB were expressed in early B and pro-B cells from both BM and G-CSF-mobilized PB (Fig. 6). Granulocyte lineage-related genes including G-CSFR, GM-CSF receptor, and myeloperoxidase were not detected in early B or pro-B cells from either BM or G-CSF-mobilized PB (Fig. 6).

IgH rearrangement in G-CSF-mobilized B lymphoid progenitors

We examined the IgH rearrangement status in the mobilized B lymphoid progenitors, because Ig genes rearrangement status reflect well the differential stages in the B cell development pathway. In general, along with B cell differentiation pathway, Ig genes rearrangements proceed from DJ_H at the early B cells or CLP stage through VDJ_H at the pro-B cells stage and to L chain gene at the pre-B cells stage (17, 20, 23, 24).

In our experiments, DJ_H and VDJ_H rearrangements were undetectable in the most immature $CD34^+CD38^-Lin^-$ cells sorted from both BM and the G-CSF-mobilized PBPC. In contrast, a ladder of DJ_H rearrangement bands ranging from 70 bp to 100 bp was observed in BM and G-CSF-mobilized $CD34^+CD10^+CD19^-$ early B cells (Fig. 7). VDJ_H and DJ_H rearrangements were detected in both BM and G-CSF-mobilized $CD34^+CD10^+CD19^+$ pro-B cells. The mobilized B lymphoid progenitors undergo IgH gene rearrangements in parallel with their BM counterparts.

Discussion

In this study, using multicolor flow cytometry, we demonstrated that administration of G-CSF to human subjects induced mobilization into PB of tiny but significant cell populations possessing the same immature lymphoid phenotypes as those of B/NK and B lymphoid-committed progenitors that are well defined in the BM. These populations are phenotypically identified as $CD34^+CD10^+CD19^-Lin^-$ early B cells (or CLP) and $CD34^+CD10^+CD19^+Lin^-$ pro-B cells and do not circulate in PB under steady-state condition (14, 20). Importantly, however, a phenotypically defined population of mobilized blood cells may not necessarily have the same functional properties as its BM counterpart. Mobilized $CD34^+CD10^+CD19^-Lin^-$ cells and $CD34^+CD10^+CD19^+Lin^-$ cells had prominent B/NK and B lymphoid differentiation potential in vivo and in vitro, respectively. Neither of these two populations exhibited self-renewal or LTC-IC capacity, indicating that the mobilized cells had characteristics of B/NK and B lineage-committed progenitors. In addition, in a limiting dilution assay, the mobilized cells had a differentiation potential for B/NK lymphoid lineage equivalent to that of BM early B cells. Furthermore, mobilized B lymphoid progenitors expressed only B lymphoid lineage-affiliated genes, with no expression of myeloid-lineage restricted genes including G-CSFR. IgH genes rearrangements were detected in early B cells and pro-B cells sorted from the G-CSF-mobilized PBPC, respectively. Collectively, these results demonstrate that a phenotypically defined lymphoid progenitor in the G-CSF-mobilized PB had the same functional properties as its BM counterpart, indicating that BM-resident B lymphoid progenitors could be released into the periphery by administration of G-CSF.

HPC express various molecules such as VLA-4/VCAM-1, SDF-1/CXCR-4, and Kit-ligand, and anchor to the BM through adhesive contact with their respective ligands in the BM microenvironment. Several studies have demonstrated that following G-CSF

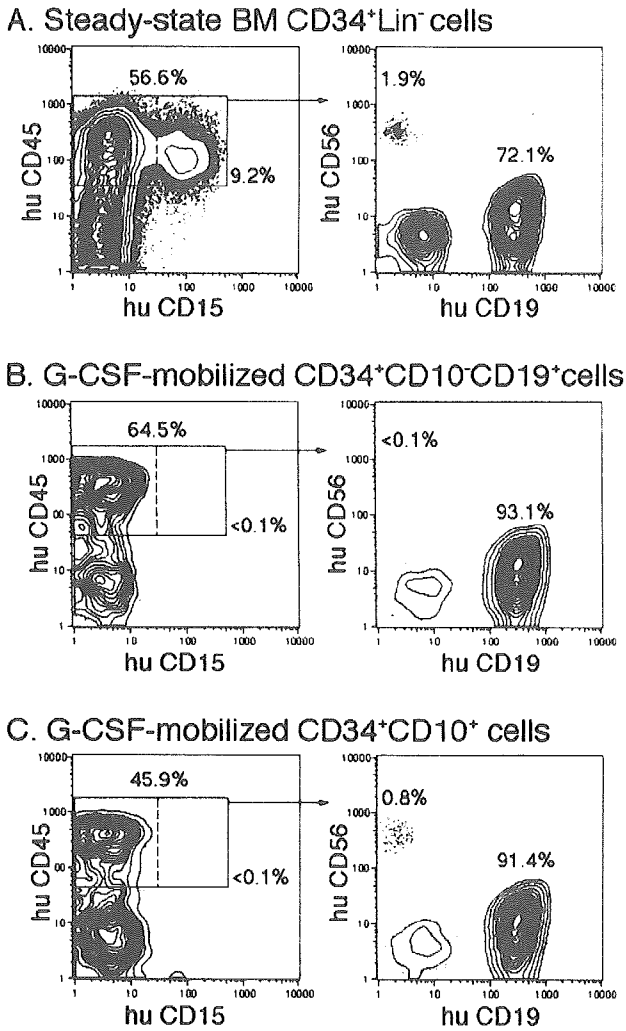

FIGURE 4. Engraftment and reconstitution potential of human different progenitors in NOD/SCID/$\beta 2^{-/-}$ mice. Six to 8 wk after i.v. injection of 50,000 $CD34^+Lin^-$ cells, $CD34^+CD38^+CD10^-CD19^+Lin^-$ pro-B cells, and $CD34^+CD38^+CD10^+Lin^-$ cells (including early B and pro-B cells) into irradiated mice, analysis was gated for viable human $CD45^+$ cells. Human $CD15^+$ myeloid, $CD56^+$ NK, and $CD19^+$ B cells were detected in the mice transplanted with BM $CD34^+Lin^-$ cells (*A*). In contrast, only B cells were generated in mice transplanted with G-CSF-mobilized $CD34^+CD38^+CD10^-CD19^+$ cells (*B*). Mice transplanted with $CD34^+CD38^+CD10^+Lin^-$ cells reconstituted both B and NK cells (*C*). Representative analyses of mice BM are shown.

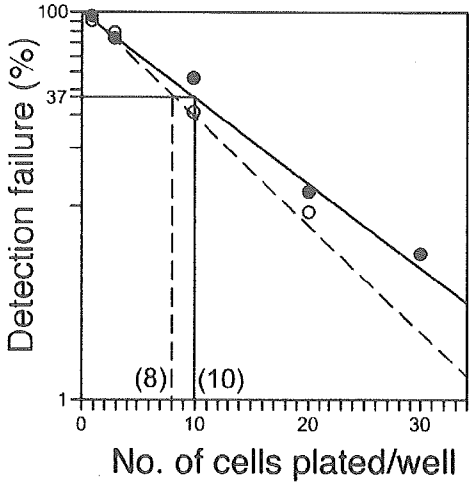

FIGURE 5. Limiting dilution analysis of early B cells from steady-state BM and G-CSF-mobilized PB. $CD34^+CD38^+CD10^+CD19^-$ cells plated by limiting dilution on MS-5 stromal layers were cultured in the presence of IL-7, IL-2, IL-11, SCF, and FL for 28 days. The differentiation potential of cells in individual wells was determined as $CD19^+$ mature B cells as shown in Fig. 3, *D* and *H*. We estimated that one in eight BM early B cells (○) and one in 10 G-CSF-mobilized $CD10^+CD19^-$ cells (●) could undergo B cell differentiation under this condition, indicating that G-CSF-mobilized $CD10^+CD19^-$ cells possess the same B cell differentiation potential as early B cells from BM. Numbers in parentheses represent limiting numbers.

administration, activated neutrophils and monocytes release proteolytic enzymes such as neutrophil elastase, cathepsin G, and MMP-9, which cleave and/or inactivate adhesion molecules expressed on the HPC (25–31). In fact, decreased expression of VLA-4 (32–35), CXCR-4 (28, 36), and c-Kit (30, 37, 38) on mobilized HPC has been reported during G-CSF administration in humans. Altered expression of adhesion molecules and consequent modification of their adhesion capacity might lead to release and migration of HPC into the circulation (7–11). Our study showed decreased expression of VLA-4 and CXCR-4 on circulating progenitors including MPP, myeloid, and lymphoid progenitors following G-CSF administration compared with that of BM, suggesting possible involvement of adhesion molecules in mobilization of at least three different types of immature progenitors. However, the extent to which each of these molecules contributed to mobilization of each hemopoietic lineage (11), or whether specific adhesion molecules are modulated in a lineage-dependent fashion, was not clear. Among the adhesion molecules that we examined, a dramatic decrease was observed in CXCR-4 expression on the lymphoid progenitors mobilized by G-CSF administration. SDF-1/CXCR-4 interactions also are involved in B lymphopoiesis, as substantiated by studies in CXCR-4-deficient mice that demonstrated reduced numbers of B lymphoid progenitors in the BM but abnormally high numbers of B lymphoid progenitors as well as the presence of mature B cells in blood and spleen (22, 39, 40). This suggests that CXCR-4 is required to retain B lymphoid progenitors within BM microenvironment for further maturation, as opposed to direct signaling to promote B cell development. These results agree with our findings that despite reduced expression in CXCR-4, the same differentiation capacity was preserved in mobilized lymphoid progenitors as their counterparts had in the steady-state BM. Thus, a decrease in CXCR-4 expression could be induced in a lineage-independent fashion following G-CSF, with resulting modulations contributing to migration of HPC without loss or alteration of differentiation capacity.

Importantly, hemopoietic growth factors can affect growth and/or properties of hemopoietic progenitors and cells. G-CSF has been characterized as a pivotal cytokine in proliferation, maturation, and survival in the myeloid lineage development pathway. Thus, G-CSF may affect potential or manifest characteristics of HPC during G-CSF mobilization, and HPC may have different abilities to develop and function. For example, mobilized $CD34^+$ cells have been reported to show decreased cell cycling compared with their BM counterparts (38, 41). In addition, numerous recent studies have demonstrated differentiation plasticity of committed progenitors, suggesting that hemopoietic progenitors retain a latent *trans* differentiation potential making them susceptible to diversion from their developmental fate (42–46). These observations suggest that circulating B lymphoid progenitors exposed to extremely high concentrations of G-CSF might show a different potential

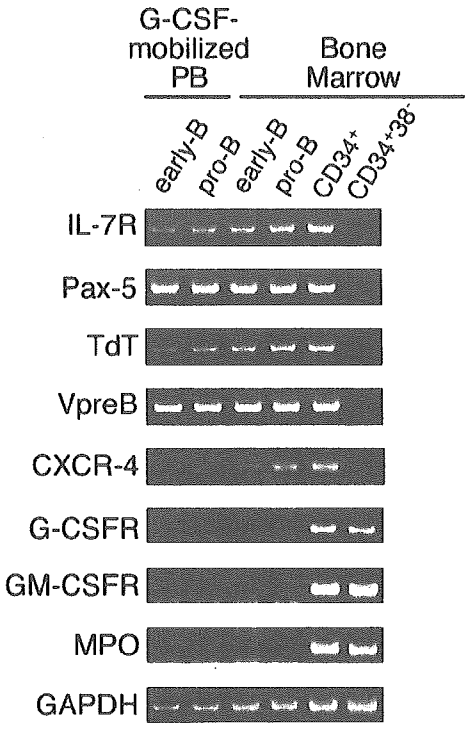

FIGURE 6. Differential expression of hematopoiesis-affiliated genes in BM and G-CSF-mobilized lymphoid progenitors as shown by RT-PCR analysis. BM $CD34^+$ cells were used as controls. In both BM and G-CSF-mobilized PB, lymphoid progenitors expressed lymphoid-affiliated genes but not myeloid-affiliated genes. MPO, myeloperoxidase; GM-CSFR, GM-CSF receptor.

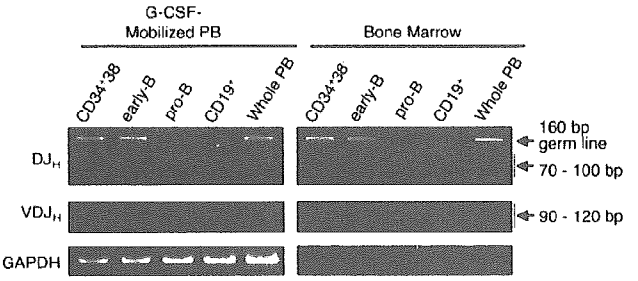

FIGURE 7. PCR analysis of DJ_H and VDJ_H genes rearrangement on DNA from BM and G-CSF-mobilized lymphoid progenitors. $CD34^-CD19^+$ cells were used as controls. None of the Ig genes rearrangements was observed in $CD34^+CD38^-$ cells. Partial DJ_H rearrangement initiated at the stage of $CD34^+CD10^+CD19^-$ early B cells followed by the rearrangement of VDJ_H genes at $CD34^+CD10^+CD19^+$ pro-B cells along with B cell development pathway. G-CSF-mobilized B lymphoid progenitor displayed the same pattern of Ig rearrangement status as its BM counterpart.

than their BM counterparts under physiological conditions, or might be *trans*-differentiated from other lineages. In our analysis, expression of B lineage-specific differentiation programs was preserved, and no myeloid genes were activated in G-CSF-mobilized lymphoid progenitors. By limiting dilution assay, we also demonstrated that the B cell differentiation potential of G-CSF-mobilized lymphoid progenitors was equivalent to that of their BM counterparts. Thus, G-CSF can mobilize B lymphoid progenitors without loss or alteration of the original characteristics of B lymphoid progenitors in BM. For that reason, B lymphoid progenitors, as opposed to all $CD34^+$ cells or myeloid cells, represent a good population for analysis of mechanisms of G-CSF-induced mobilization, because, lacking the receptor, lymphoid progenitors would be less affected by G-CSF signals during mobilization.

Recent insights have increased understanding of the important role of the BM microenvironment, or niche, in retention and development of HPC within the BM. Regulation of cell-fate determination and trafficking of the primitive HPC may be governed by complex interactions between HPC and the surrounding BM niche (47, 48). As discussed above, SDF-1/CXCR-4 signaling is crucial for retention of B lymphoid progenitors within the BM, which can support further B cell development within the BM microenvironment (22, 39, 40). However, whether G-CSF can change BM microenvironments themselves to promote or inhibit HPC mobilization remains largely unknown. Our findings indicated that G-CSF can mobilize cell populations that do not possess G-CSFR from the BM into the circulation. Accordingly, G-CSF-mobilized blood cells can include a variety of populations such as mesenchymal stem and progenitor cells, which can differentiate into nonhematopoietic cells such as vascular endothelial cells, cardiac muscle cells, and hepatocytes. Such mobilized blood cells conceivably might serve as a therapeutic agent in the treatment of various degenerative disorders as opposed to BM cells as a stem cell source (49). Up to now, G-CSF has been the HPC mobilizer of choice in clinical settings, based upon its potency and safety. However, poor mobilization has been reported in ~10–20% of healthy donors, representing a major problem (50, 51). To address these unresolved issues, further investigation of mechanisms of G-CSF-induced mobilization may lead to more effective and safer mobilization methods and agents, and clarify the usefulness of G-CSF-mobilized PB cells as an alternative source of a variety of cells for regenerative medicine.

In summary, our data provide further evidence for an indirect effect of G-CSF on human HPC mobilization by demonstrating mobilization of lymphoid progenitors. Lineage-independent modulation of adhesion molecules such as VLA-4 and CXCR-4 might be involved in G-CSF-induced mobilization. These findings suggest that G-CSF can mobilize not only HPC but also nonhematopoietic cells residing in the BM by indirect effects involving multiple *trans*-acting signals that affect cell interactions with the marrow microenvironment.

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Disclosures

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Hyperacute GVHD and emergence of peripheral CD3+CD56+ T cells and activated natural killer cells are useful markers for early diagnosis of post-transplant hemophagocytic syndrome

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Hemophagocytic syndrome (HPS) is a cytokine-related disorder characterized by sustained high-grade fever, pancytopenia, hepatomegaly, coagulopathy and hemophagocytosis in the marrow, spleen, or lymph nodes.[1] The development of HPS is related to underlying immune dysregulation and inappropriate activation of macrophages. In adults, viral, bacterial, and fungal infections and/or malignant lymphoma are associated with HPS.[1]

HPS is a rare complication following stem-cell transplantation (SCT), and occasional case reports only have been published.[2,3] Besides infection and hematologic malignancies, immune reactions particularly hypercytokinemia following SCT have been associated with HPS. Although some patients with early-onset HPS respond to corticosteroids, HPS is often fatal. At present, its clinical characteristics remain unknown. We report a patient who developed early-onset HPS after allogeneic SCT. A 30-year-old man with chemorefractory acute myeloid leukemia was referred to our hospital for allogeneic SCT in November 2003. After cytoreduction using cytarabine, he underwent peripheral blood SCT from his one-locus-mismatched sister in January 2004. The preparative regimen consisted of busulfan 4 mg/kg/day for 4 days and cyclophosphamide 60 mg/kg for 2 days. Graft-versus-host disease (GVHD) prophylaxis was tacrolimus 0.03 mg/kg/day. Methotrexate was omitted from GVHD prophylaxis to enhance a graft-versus-leukemia effect. The numbers of infused CD34+ cells and CD3+ cells were 2.6×10^6/kg and 3.2×10^8/kg, respectively.

His clinical course was uneventful until day 3, when he developed a high-grade fever. Lacy blanching erythema and diarrhea appeared on day 4. White blood cell (WBC) count was 100/mm^3 on the day. Hyperacute GVHD was suspected. Painful hepatomegaly, weight gain, and jaundice appeared on day 7, and hepatic veno-occlusive disease (VOD) was diagnosed. When renal dysfunction developed on day 7, we switched tacrolimus to methylprednisolone 0.5 mg/kg. On day 8, the WBC increased to 1600/mm^3 including 90% lymphocytes (Figure 1). Double X signals were shown by sex chromosome fluorescence *in situ* hybridization in 100% of the peripheral leukocytes. Fluorescence-activated cell sorter (FACS) showed that 44, 36 and 8% of the lymphocytes were CD3−CD56+, CD3+CD56−, and CD3+CD56+, respectively (Figure 1), and that 31% of cytoplasmic CD3+ cells expressed NKp46, a specific marker of activated natural killer cells (NK cells).[4] While the skin rash and diarrhea improved, his renal and respiratory functions deteriorated. Chest radiographs revealed bilateral pulmonary edema and pleural effusions. Serum creatinine increased to 5.9 mg/dl. We suspected worsening hyperacute GVHD and VOD, and increased the dose of methylprednisolone to 2.0 mg/kg. He achieved neutrophil engraftment on day 10, when the WBC was 5700/mm^3. The numbers of neutrophils, surface CD3+, and CD20+ cells were 4500, 780, and 70/mm^3, respectively. Despite intensive immunosuppression, he rapidly deteriorated and died of multiple-organ failure on day 11. Throughout his clinical course, the spleen was not palpable. Repeated blood cultures failed to detect any organisms.

Post-mortem examination showed normocellular marrow with an increase in activated macrophages. In the liver, fibrous obliteration of terminal hepatic venules, dilatation of centrilobular sinusoids, and necrosis of zone 3 hepatocytes were evident. These findings were consistent with VOD. No findings suggested hepatic involvement by acute GVHD. A massive infiltration of activated macrophages was present in the liver, while neither CD3+ nor CD56+ cells were observed. Serum cytokine levels were: interferon gamma 0.2 IU/ml (normal range: <0.1 IU/ml), interleukin (IL) −2 <0.8 U/ml (normal range: <0.8 U/ml), IL-6 467 pg/ml (normal range: <4.0 pg/ml), tumor necrosis factor alpha 7 pg/ml (normal range: <5.0 pg/ml), and macrophage colony-stimulating factor 10 000 pg/ml (mean levels in healthy volunteers, 670 pg/ml).

Hyperacute GVHD preceded the onset of HPS in this patient, which is an immunological syndrome overlapping with engraftment syndrome, capillary leak syndrome, and periengraftment clinical abnormalities.[5] The previously reported patients with HPS following allo-SCT, and our patient had high-grade fever, skin eruption, and diarrhea in common; these are typical manifestations of hyperacute GVHD,[2,3] although the possibility of toxicity from the preparative regimen cannot be excluded. The negative post-mortem findings for GVHD in our patient cannot exclude the diagnosis of hyperacute GVHD because it had resolved before death and because the pathological findings of hyperacute GVHD are not always identical with those of conventional GVHD.[5,6] High-dose corticosteroid is usually effective for hyperacute GVHD. However, this persisted despite corticosteroids in this patient and finally resulted in fatal HPS, although the time from the onset of hyperacute GVHD to steroid initiation might have influenced the outcome. Since this patient had a one-locus-mismatched related donor and GVHD prophylaxis with tacrolimus and no methotrexate,[6] he was at high risk of hyperacute GVHD. Severe hyperacute GVHD might have aggravated cytokine dysregulation, contributing to the development of HPS. This hypothesis is consistent with previous observations, suggesting that a severe alloimmune response resulted in HPS.[2]

It should be noted that the patient developed lymphocytosis with CD3+CD56+ T cells. The number of activated NK cells expressing NKp46 was also elevated in the peripheral blood. These findings are comparable with

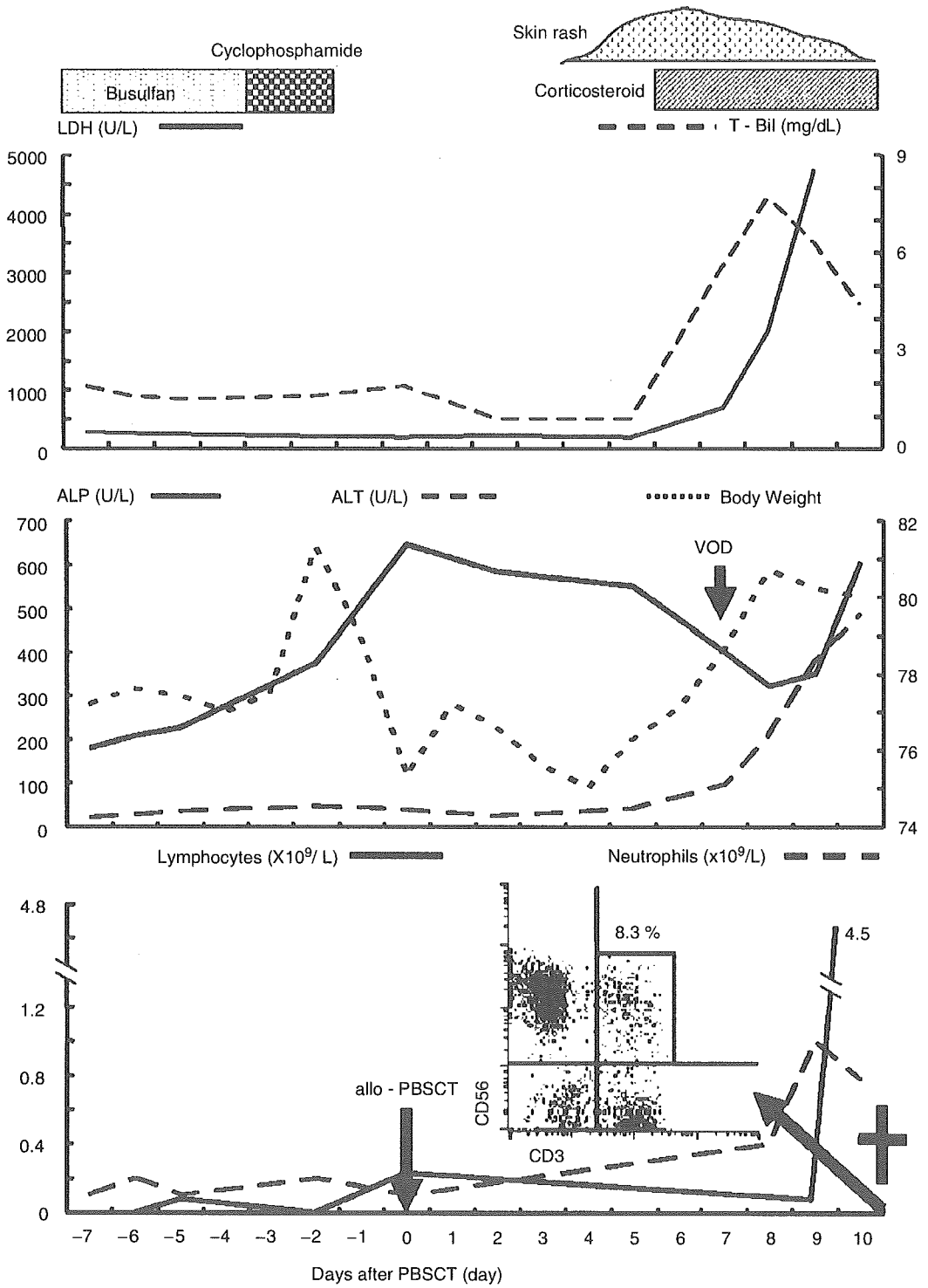

Figure 1 Clinical course of this patient. On day 8, the WBC increased to 1600/mm^3 including 90% lymphocytes. Double X signals were shown by sex chromosome fluorescence *in situ* hybridization in 100% of the peripheral leukocytes. FACS showed that 44, 36, and 8% of the lymphocytes were CD3−CD56+, CD3+CD56−, and CD3+CD56+.

our previous case report.[7] While CD3+CD56+ T cells have been described in both murine and human tissues,[8,9] their clinical significance has not been fully clarified. Some of these lymphocytes are classified into cytokine-induced killer (CIK) cells, which are a unique population of cytotoxic T-lymphocytes with higher proliferative and

cytolytic activities in comparison to the reported CD3−, CD56+ lymphokine-activated killer cells, which are essentially activated NK cells.[10] Since CIK cells can be generated *in vitro* using exogenous IL-2, IL-7, or IL-12, cytokine storm associated with severe hyperacute GVHD might have promoted the generation of CIK cells, causing HPS and severe organ damages in this patient. Alternatively, an increase in CD3+CD56+ lymphocytes and NK cells might have reflected activated immune reactions, and these cells might not have caused severe organ damages. At present, the exact mechanisms of these immune reactions remain unknown, and further investigation is required to clarify the clinical significance. Post-mortem examination, which failed to show infiltration of CD3+CD56+ T cells in the liver, supports the latter hypothesis. The clinical course of this patient suggests that the development of hyperacute GVHD, and proliferation of CD3+CD56+ T cells and activated NK cells with NKp46 in the peripheral blood are useful markers for early diagnosis of HPS.

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bjh research paper

Outcomes of patients with acute leukaemia who relapsed after reduced-intensity stem cell transplantation from HLA-identical or one antigen-mismatched related donors

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Summary

The characteristics of relapse following reduced-intensity stem-cell transplantation (RIST) remain to be clarified. We reviewed the medical records of 19 patients with acute leukaemia [acute myeloid leukaemia (AML), 16; acute lymphoblastic leukaemia (ALL), 3] who relapsed after RIST from related donors using purine-analogue-based regimens. Their median age was 55 years (range, 29–65 years). Median interval between RIST and relapse was 4·9 months (range, 1·8–24·9 months). Three chose not to receive interventions. The remaining 16 patients received withdrawal of immunosuppression ($n = 3$), chemotherapy ($n = 2$), donor lymphocyte infusion ($n = 10$) and second transplantation ($n = 7$), alone ($n = 9$) or in combination ($n = 7$). Four are alive with a median follow-up of 27·6 months (range, 16·0–28·9 months); three in remission and one in relapse. The 2-year overall survival after relapse was 28·9%. Causes of death in 15 patients included progressive disease ($n = 7$), graft-*versus*-host disease ($n = 5$) and infections ($n = 3$). Cumulative incidences of relapse-related and non-relapse-related deaths at 2 years after relapse were 37% and 32% respectively. Two prognostic factors were identified on univariate analysis: age [$P = 0·017$; hazard ratio (HR), 1·16; 95% confidence interval (CI), 1·03–1·32], and ALL as underlying disease ($P = 0·011$; HR, 10·4; 95% CI, 1·73–62·4). Some AML patients who relapse after RIST achieve durable remission with allogeneic immunotherapy-based interventions; however they carry a significant risk of non-relapse mortality.

Keywords: graft-*versus*-host disease, graft-*versus*-leukaemia effect, donor lymphocyte infusion, second allogeneic transplantation, non-myeloablative haematopoietic stem cell transplantation.

The relapse of underlying haematological malignancies after allogeneic haematopoietic stem-cell transplantation (allo-SCT) is a significant problem. Adults with acute leukaemia who relapsed after allo-SCT had a median survival of 3–4 months if no treatment was given (Mortimer *et al*, 1989). Approaches to treating patients in relapse after allo-SCT include rapid tapering of immunosuppressive agents, donor lymphocyte infusion (DLI), re-induction chemotherapy and second transplantation. Standard chemotherapy sometimes results in complete remission (CR), but long-term disease-free survival (DFS) is unlikely, because of regimen-related toxicity (RRT) and recurrence (Frassoni *et al*, 1988). Although a second allograft produces sustained molecular remission in a proportion of patients, transplant-related mortality (TRM) is high with 100-d mortality rates of 25–50% and a DFS of 10% (Mrsic *et al*, 1992; Radich *et al*, 1993). Poor prognostic factors after second allo-SCT include an interval between the procedures of <1 year, resistance to re-induction chemotherapy, older age and poor performance status (Michallet *et al*, 2000). Immunotherapy, such as cessation of immunosuppressive agents and DLI, is beneficial for patients with early relapse or those with chronic myeloid leukaemia (CML). DLI can result in a high CR rate of 60% in CML; however, it is less effective in acute leukaemia with an estimated rate of CR of only 15%

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(Collins *et al*, 1997). Graft-*versus*-leukaemia (GVL) effects seem to be weak, or rapid growth of leukaemic clones exceeds an effective immune response, which manifests 5–6 weeks after DLI (Kolb *et al*, 1995; Collins *et al*, 1997).

A new strategy for transplantation, reduced-intensity stem-cell transplantation (RIST) (Slavin *et al*, 1998; Giralt *et al*, 2001), has been developed to reduce RRT while preserving an adequate GVL effect. It appears to be promising for a variety of haematological malignancies, if disease activity is controlled prior to transplant (Michallet *et al*, 2001). In contrast, most physicians believe that RIST is insufficient in controlling advanced haematological malignancies, and small pilot studies showed that RIST was unsuccessful for advanced haematological malignancies (Giralt *et al*, 1997; Nagler *et al*, 2000). Relapse is a significant concern in RIST; however, little is known of the prognosis of patients who relapse after RIST, or of the value of interventions aimed at re-inducing remission (Bethge *et al*, 2003). We investigated the clinical characteristics of patients with acute leukaemia who relapsed following RIST.

Patients and Methods

Data collection

We retrospectively reviewed the medical records of 19 patients who had a relapse of acute leukaemia of 111 patients who achieved morphological CR following RIST from a human leucocyte antigen-identical or one antigen-mismatched related donor at the National Cancer Centre Hospital and Toranomon Hospital between September 1999 and March 2003. All patients had acute leukaemia that was incurable with conventional treatments, and were considered inappropriate for conventional allo-SCT because of age >50 years and/or organ dysfunction. Transplantation procedures, supportive care and chimaerism analysis were reported previously (Saito *et al*, 2002; Hamaki *et al*, 2004). Bone marrow examination was performed 1 and 3 months after transplantation, or when relapse was suspected. Minimal residual disease (MRD) in bone marrow was monitored by flow cytometry, cytogenetics and reverse transcription-polymerase chain reaction (RT-PCR), when MRD markers were available. The intervention selected for relapsed acute leukaemia after RIST was based on patient condition. All patients and donors gave their written informed consent in accordance with the requirements of our Institutional Review Board.

Definition

Diagnosis of acute leukaemia was based on the World Health Organization classification (Brunning *et al*, 2001a,b). Treatment responses were evaluated according to Cheson *et al* (2003). CR was defined as morphological complete remission: patients achieved the morphological leukaemia-free state and had an absolute neutrophil count $>1{\cdot}0 \times 10^9$/l. Recovery of platelets of $\geq 100 \times 10^9$/l was not required.

Graft-*versus*-host disease (GVHD) was diagnosed by clinical judgment as well as skin or digestive tract biopsies to support the clinical diagnosis. Acute and chronic GVHD were graded according to the consensus criteria (Sullivan *et al*, 1991; Przepiorka *et al*, 1995).

Endpoints and statistical analysis

The aims of this study were (i) to describe clinical characteristics of relapse following RIST, and (ii) to identify its prognostic factors. The probability of overall survival was calculated using the method of Kaplan and Meier. Overall survival was defined as the duration of survival between the first relapse after RIST and either death or last follow-up. Cumulative incidences of relapse-related and non-relapse-related mortality were calculated as reported previously (Gooley *et al*, 1999). An initial analysis comparing potential prognostic factors was carried out using the log-rank test. Acute GVHD was included as a time-dependent covariate. Multivariate analysis was not conducted because of the small number of patients. $P < 0{\cdot}05$ were considered significant.

Results

Patient characteristics

Nineteen patients relapsed after RIST. Their backgrounds are shown in Table 1. Clinical characteristics of relapse after first RIST are shown in Table 2. All 19 patients had achieved morphological remission after first RIST, while platelets counts had not normalized ($>100 \times 10^9$/l) in four patients (case 6, 12, 16 and 19). In all 19 patients, MRD analyses using cytogenetics and flow cytometry were negative at morphological remission after first RIST. MRD was monitored by RT-PCR in two patients (*AML1-MTG8* and *E2A/PBX1* in cases 6 and 18 respectively). In these patients, the chimaeric transcripts had been positive at morphological remission after first RIST.

Treatment of relapse

Treatment of relapse was heterogeneous and varied depending on the individual patients' condition (Table 2).

No treatment

Three patients (cases 4, 11 and 14) chose not to receive further intervention after relapse; one patient (case 11) is currently alive in non-remission without any intervention, and the remaining two (cases 4 and 14) died of underlying disease.

Intervention

The other 16 patients received the firstline treatments. At diagnosis of relapse, three patients (cases 5, 17 and 18) who were still receiving immunosuppression had the drugs discon-

Table I. Characteristics of patients (n = 19) who relapsed after RIST.

Age (years) [median (range)]	55 (29–65)
Sex (male/female)	15/4
Reasons for RIST	
Age >50 years/organ dysfunction	17/2¶
Numbers of cytotoxic chemotherapies prior to first RIST	5 (0–7)
Diagnosis at first RIST	No. of patients
Acute lymphoblastic leukaemia	
Second complete remission	3
Acute myeloid leukaemia	
Second complete remission	2
Induction failure	4
Relapse	8
Myelodysplastic syndrome§	
Refractory anaemia	1
Refractory anaemia with excess blasts	1
Conditioning regimen	
Fludarabine/busulphan*	15
Fludarabine/melphalan†	2
Cladribine/busulphan‡	2
Graft-*versus*-host disease prophylaxis	
Ciclosporin	19
Donor (matched sibling/one-antigen mismatched related)	13/6
History of GVHD prior to relapse (0-I/II-IV)	16/3
Interval between RIST and relapse (months)	4·9 (1·8–24·9)

*The preparative regimen comprised fludarabine 30 mg/m^2 for 6 d and busulphan 4 mg/kg for 2 d. Three patients received rabbit ATG (Thymoglobulin; Imtix-Sangstat, Lyons, France) 2·5 mg/kg for two consecutive days.

†The preparative regimen comprised fludarabine 30 mg/m^2 for 6 d and melphalan 80 mg/m^2 for 1 d.

‡The preparative regimen comprised cladribine 0·11 mg/kg for 6 d and busulphan 4 mg/kg for 2 d. Two received rabbit ATG (Thymoglobulin; Imtix-Sangstat, Lyons, France) 2·5 mg/kg for two consecutive days.

§These two patients are those described in ‡ above.

¶The complications included renal dysfunction and hepatic dysfunctions.

tinued. Two patients (cases 17 and 18) received secondary intervention (chemotherapy and DLI) following rapid tapering of cyclosporine. The chemotherapy regimen comprised cytarabine and idarubicin. The other patient (case 5) refused to receive secondary intervention, and died 9·0 months after relapse.

The remaining 13 patients received one or more of the following treatments, based on the physicians' discretion after consideration of their general status, aggressiveness of the underlying disease and presence of comorbidity.

Two patients (cases 1 and 2) received re-induction chemotherapy comprising cytarabine and idarubicin. Both patients underwent secondary interventions including second RIST from the same donor and DLI, and achieved durable remission.

Eight patients (cases 7–10, 13, 15, 16 and 19) received DLI from their original donors. The median number of DLI was one (range, 1–3). The median dose of lymphocytes transfused was 0·8 × 10^8/kg (range, 0·4–1·4 × 10^8/kg). Two patients (cases 9 and 10) achieved durable remission. Another two (cases 8 and 15) and one patient (case 19) died of acute GVHD and infection during myelosuppression respectively. The other three patients (cases 7, 13 and 16) did not achieve remission after DLI, and underwent second RIST as secondary intervention. The stem cell sources were granulocyte colony-stimulating factor-mobilized peripheral blood (case 7), marrow from a matched unrelated donor (case 13), and umbilical cord blood (case 16). One patient (case 13) achieved durable remission. Two patients (cases 7 and 16) died of septicaemia and progressive disease respectively.

Three patients (cases 3, 6 and 12) underwent second RIST as first intervention. All the three patients tolerated transplantation procedures. One (case 3) achieved durable remission, but died of chronic GVHD. The other two patients (cases 6 and 12) did not achieve remission; one (case 6) was alive in relapse 22·3 months after second RIST, and the other (case 12) died of disease progression.

Responses and survival

Six of the 19 patients (cases 1–3, 9, 10, 13) achieved complete morphological remission after first and/or second interventions. The association between GVHD and response was evaluable in 11 patients. All the four patients with acute GVHD (cases 1, 2, 9, 10) achieved CR, while five of the seven patients without GVHD showed progressive disease (cases 3, 5, 6, 12, 13, 16, 18).

In these six patients, duration of CR following the interventions was longer than that from the first RIST to relapse (Table 2). Four of the 19 patients were alive at a median follow-up of 27·6 months (range, 16·0–28·9 months); three in CR, and one in relapse. The 2-year overall survival rate after relapse was 28·9% (95% confidence interval; 7·3–50·5%) (Fig. 1).

Causes of deaths

Causes of death in 15 patients included progressive disease (n = 7), acute GVHD (n = 3), chronic GVHD (n = 2), and infections (n = 3; Table 2).

Cumulative incidences of relapse-related and non-relapse-related deaths at 2 years after post-transplant relapse were 37% and 32% respectively.

Prognostic factors

Results of univariate analysis on overall survival are shown in Table 3.

Discussion

The present study shows that some patients with relapsed acute myeloid leukaemia (AML) after RIST can achieve remission

Table II. Outcomes of relapse following RIST.

Case	Age (years)	Sex	Underlying disease	Disease status at 1st RIST	Grade II-IV acute GVHD after 1st RIST	Chimaerism analysis after 1st RIST (% of donor type)			Haematologic findings at relapse after 1st RIST			Interval between RIST and relapse (months)	Intervention		Response	Acute GVHD after intervention	Survival after relapse (months)	Outcomes/ cause of death
						Day 30	Day 60	Day 90	Blast in marrow (%)	Leucocytes in peripheral blood ($\times 10^9$/l)	Blast in peripheral blood (%)		First	Second				
1	55	F	AML	Relapse	Absent	100	Not performed	Not performed	44	2·4	0	10·9	Chemotherapy	SCT*	CR	Grade 4	15·3	Acute GVHD
2	29	M	AML	Relapse	Absent	100	100	100	7	3·8	4	15·7	Chemotherapy	DLI	CR	Grade 2	30·6	Leukaemia
3	58	M	AML	2nd remission	Absent	100	100	100	32	3·9	0	4·5	SCT*	None	CR	None	23·8	Chronic GVHD
4	53	M	AML	Induction failure	Absent	45	Not performed	95	56	3·2	4	17·4	None	None	PD	NA	11·3	Leukaemia
5	65	M	AML	2nd remission	Absent	100	56	Not perfomed	26	3·2	1	2·6	Tapering of ciclosporin	None	PD	None	9·0	Leukaemia
6	55	F	AML	Relapse	Grade 2	82	92	100	59	5·3	39	7·5	SCT*	None	PD	None	27·8+	Alive in relapse
7	57	M	AML	Relapse	Absent	100	100	85	63	4·8	5	12·4	DLI	SCT*	ND†	None	2·5	Septicaemia
8	51	M	AML	Relapse	Grade 2	100	88	100	15	2·2	0	4·9	DLI	None	ND†	Grade 4	1·1	Acute GVHD
9	52	M	AML	Induction failure	Absent	Not performed	Not performed	Not performed	48	1·4	5	4·0	DLI	None	CR	Grade 3	16·0+	Alive in remission
10	49	M	MDS	RA	Absent	100	100	100	9	3·1	0	24·9	DLI	None	CR	Grade 2	30·2	Chronic GVHD
11	64	F	MDS	RAEB-2	Absent	88	82	92	59	3·7	10	14·8	None	None	PD	NA	27·4+	Alive in relapse
12	56	M	AML	NR	Absent	65	0	Not performed	6	2·7	0	2·5	SCT*	None	PD	None	4·9	Leukaemia
13	53	F	AML	Relapse	Absent	Not performed	Not performed	not performed	30	14·1	0	6·0	DLI	UBMT‡	CR	None	28·9+	Alive in remission
14	58	M	ALL	2nd remission	Absent	100	100	Not performed	22	7·6	3	7·9	None	None	PD	NA	0·5	Leukaemia
15	55	M	ALL	2nd remission	Grade 2	100	Not performed	Not performed	54	27·6	7	2·8	DLI	None	ND†	Grade 4	1·8	Acute GVHD
16	54	M	AML	Induction failure	Absent	88	100	100	Dry tap	3·6	8	3·3	DLI	UCBT§	PD	None	5·6	Leukaemia
17	54	M	AML	Relapse	Absent	100	90	Not performed	8	8·9	1	2·9	Tapering of ciclosporin	SCT*	ND*†	None	3·0	Invasive aspergillosis

18	63	M	ALL	2nd remission	Absent	100	Not performed	Not performed	87	5	3·9	1·6	9	1	3·1	1·8	Tapering of ciclosporin DLI	None	PD	None	1·8	Leukaemia
19	60	M	AML	Relapse	Absent	100	66	Not performed										None	ND†	None	0·7	Pneumonia

RIST, reduced intensity stem cell transplantation; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukaemia; SCT, stem-cell transplantation; CR, complete remission; PD, progressive disease; ND, not determined; UCBT, umbilical cord blood transplantation; SCT, stem cell transplantation; UBMT, unrelated bone marrow transplantation; DLI, donor lymphocyte infusion.
*Donors and preparative regimens were same as the first transplantation.
†These patients died of infection or GVHD during neutropenia following DLI or chemotherapy. We were not able to determine the responses to interventions for post-transplant relapses.
‡The patient was transplanted from a matched unrelated donor following fludarabine 30 mg/m^2 for 6 d, busulphan 4 mg/kg for 2 d and 4 Gy total body irradiation.
§The patient underwent umbilical cord blood transplantation following fludarabine 25 mg/m^2 for 6 d, melphalan 80 mg/m^2 and 4 Gy total body irradiation.

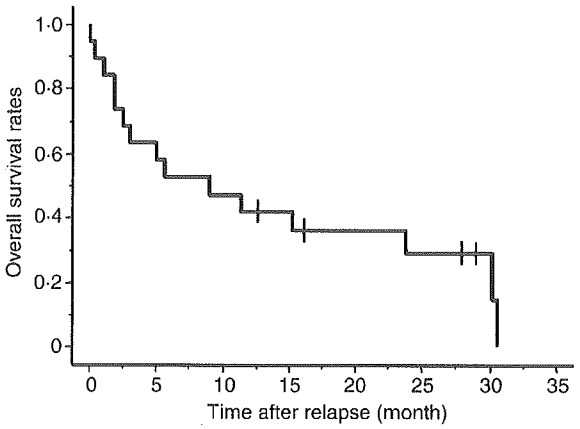

Fig 1. Probability of survival for 19 patients who relapsed after reduced-intensity stem-cell transplantation. The 2-year overall survival rate after relapse was 28·9% (95% confidence interval; 7·3–50·5%).

Table III. Univariate analyses on overall survival.

Factor	Relative risk (95% confidence interval)	*P*-value
Age	1·16 (1·03–1·32)	0·017*
Sex (female *versus* male)	0·17 (0·022–1·34)	0·093
Interval from diagnosis to transplant (months)	0·91 (0·81–1·02)	0·10
HLA disparity (Matched *versus* mismatched)	1·73 (0·37–8·06)	0·48
Use of ATG at first RIST (ATG *versus* non-ATG)	1·52 (0·50–4·68)	0·46
Underlying disease (Lymphoid *versus* myeloid)	10·4 (1·73–62·4)	0·011*
Disease status at first RIST (Non-remission *versus* remission)	0·63 (0·21–1·94)	0·42
Grade I-IV acute GVHD (absent/present)	1·39 (0·38–5·01)	0·72

*Statistically significant
ATG, antithymocyte globulin; RIST, reduced intensity stem cell transplantation; GVHD, graft-*versus*-host disease.

and even long-term survival. Seven patients (cases 1–3, 6, 9, 10 and 13) achieved remission after relapse following RIST. The remission duration after secondary interventions was longer than a year and longer than the duration between the first RIST and relapse. This observation supports that the interventions after relapse has improved the outcomes. In contrast to those who achieved long-term survival with currently available interventions, patients with acute lymphoblastic leukaemia (ALL) and older patients had poor outcomes. The three patients with ALL who underwent RIST in the second CR died 2·8–7·9 months after relapse. As ALL probably has low susceptibility to allogeneic immunity, long-term survival cannot be expected after relapse following RIST as well as

conventional myeloablative allo-SCT (Kolb *et al*, 1995; Slavin *et al*, 1995; Collins *et al*, 1997). In the present study, six of the eight patients who survived longer than 12 months after relapse following RIST were younger than 55 years old. The outcomes of older patients are poor, probably because of the high biological malignancy of leukaemia at advanced ages and because of the reduced tolerance to GVHD and chemotherapies. Further investigations are necessary to improve the treatment outcome in these patients.

The appropriate intervention for the relapsed leukaemia after RIST has not been established. The primary physicians decide the treatment according to the conditions of the primary malignancies and performance status of the patients. Of the seven patients who survived longer than 1 year after the secondary interventions (cases 1–3, 6, 9, 10 and 13), two received chemotherapies and four underwent a second RIST. As it is unlikely that the long-term remission was maintained solely by the effects of chemotherapies and conditioning regimens before RIST, allogeneic immunity must have contributed to suppression of AML progression. While two underwent a second RIST from a different donor, it should be noted that five patients achieved long-term remission after the second RIST or DLI from the same donor as in the first RIST. The outcomes contrast with the observation that some AML patients who relapse after conventional myeloablative allo-SCT can achieve remission by secondary interventions, such as DLI, but the remission is short. Although the reason is unclear, the delay in the manifestation of GVHD/GVL effects after RIST, compared with conventional myeloablative allo-SCT, may partly explain the difference. The median onset of GVHD was 2 months after RIST with our conditioning regimens, which was 1 month later than that after conventional allo-SCT (Nakai *et al*, 2003). As the tumour reduction by the conditioning regimens for RIST is limited and allogeneic immunity manifests late after RIST compared with conventional allo-SCT, the probability of early relapse may be high after RIST. When AML relapses after RIST, leukaemic cells have not been exposed enough to allogeneic immunity and may not be resistant to allogeneic immunity. While the duration from conventional allo-SCT to relapse is associated with the prognosis, that is not necessarily true of RIST (Mortimer *et al*, 1989; Levine *et al*, 2002). The GVL effects of DLI for AML manifest 1 month later. As a GVL effect plays a crucial role in reducing the risk of relapse after RIST for AML and myelodysplastic syndrome (Martino *et al*, 2002), DLI from the identical donor may be promising for slowly progressive AML and/or in cases where AML progression can be suppressed by chemotherapies or the conditioning regimen for RIST.

The present study showed that interventions for relapsed acute leukaemia following RIST carry a significant risk of TRM; five and three patients died of GVHD and infection respectively. Of particular note is that four of the seven patients who underwent second RIST died of TRM. These findings were in contrast to previous reports (Bethge *et al*, 2003; Feinstein *et al*, 2003). In the report by the Seattle group on the outcomes of relapsed haematological malignancies after non-myeloablative stem-cell transplantation (NST) using 2 Gy total body irradiation with or without fludarabine, 46 of 66 patients who underwent interventions after relapse died: 41 of progressive disease and five of TRM. The Seattle researchers also reported that the rate of TRM was 6% in patients who received NST as second allo-SCT (Feinstein *et al*, 2003). TRM in their studies (Bethge *et al*, 2003; Feinstein *et al*, 2003) was much lower than that in our study, although the comparison of these studies with different patient characteristics is not appropriate. TRM after interventions for patients with relapsed acute leukaemia after RIST is high, at least partly because the conditioning regimens for RIST are more intense than those for NST. Our study suggests that control of GVHD and management of infection are important to improve prognosis of those patients with acute leukaemia who relapse after RIST. Intensification of GVHD prophylaxis using potent immunosuppressive agents will contribute to improving GVHD-related outcomes (Kottaridis *et al*, 2000; Nakai *et al*, 2003); however, use of these agents might diminish a GVL effect, and could increase the rate of relapse and infections (Chakraverty *et al*, 2002). It should be noted that responses to interventions for relapse after allo-SCT are frequently associated with the development of GVHD (Luznik & Fuchs, 2002; Bethge *et al*, 2003). Further studies are warranted to establish a strategy which enhances a GVL effect without causing GVHD.

Although this study is hampered by its small size and heterogeneity of patients' background, the results are still informative. It demonstrated that some patients with relapsed AML after RIST can survive with allogeneic immunotherapy. These observations provide a rationale for continuing our clinical trials on this treatment for relapsed AML, which should be modified to focus on minimizing toxicities, preventing GVHD and enhancing a GVL effect. There were no significant differences in prognosis between patients who were given DLI alone and those who underwent second RIST. Considering the high TRM of second RIST, we should be careful in choosing RIST as intervention for relapsed acute leukaemia after RIST.

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