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

Rie Imamura,1,3,++ Toshihiro Miyamoto,2,3,‡ Goichi Yoshimoto,† Kenjiro Kamezaki,*,++ Fumihiko Ishikawa,‡ Hideho Henzan,*,‡ Koji Kato,† Ken Takase,*‡ Akihiko Numata,*‡ Koji Nagafuji,† Takashi Okamura,‡ Michio Sata,‡ Mine Harada,† and Shoichi Inaba4*  

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 hemoopoietic 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-CSF 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-CSF 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/CD106, 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 and 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...
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 μg/m2 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, -CD16, -CD20, -CD56, and glycophorin A; Caltag Laboratories), FITC-conjugated anti-CD13 (BD Pharmingen), PE-conjugated anti-CD33 (BD Pharmingen), allophtocyanin-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 (Anell), 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 -CD10 (BD Pharmingen), together with anti-CD34 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+CD10+CD19− Lin− multipotent progenitor cells (MPP), CD34+CD10+CD19− CD13+ Lin+ myeloid progenitor cells, CD34+CD10+CD19− CD13+ CD10+ Lin− early B cells, and CD34+CD10+CD19− CD13+ Lin+ pro-B cells were sorted by a highly modified triple laser (488-nm argon laser, 363-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; Caltag Laboratories), IL-12 (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 Technology) 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% CO2.

For reconstitution assays, FACS-sorted cells were injected into irradiated (350 rad) NOD/SCID/β2-microglobulin knockout (NOD/SCID/β2−/−) mice as described previously (16). At 6–8 wk after transplantation, BM and spleen were collected for analysis by flow cytometry. 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% 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 VDJH and DJJ rearrangements was performed as described previously (17, 18). This PCR analysis can detect incomplete DJH rearrangements of IgH gene with a mixtures of upstream DJH primers and a consensus JH primer, resulting in a ladder of different sized products ranging from 70 to 100 bp depending on the length of the DJH 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−/lowLin+ fractions contain hematopoietic stem cells with multipotent, self-renewing capacity, whereas CD34+CD38+Lin− cells include lineage-committed progenitors that have lost self-renewing capacity (Fig. 1A) (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−/lowLin− population (Table I). Although CD34+CD38−/lowLin− 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 92.50 ± 3.76% and 96.70 ± 6.62% of CD34−CD38−Lin− cells in BM and PB, respectively (Table I and Fig. 1B). 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−/lowLin+ MPP cells become CD34−CD38−CD10−CD19−CD20+ early B cells or common lymphoid progenitors (CLP) (14, 19) and differentiate within
the BM through a CD34+/CD138−CD10−CD19+/CD20+ pro-B phenotype into a CD34+/lowCD38−CD10−CD19+/CD20+ pre-B phenotype (Fig. 1A) (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+/CD19− 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+/CD19− 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. 1B. 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+/CD19− 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+/CD19− primitive MPP. Its expression was up-regulated in myeloid progenitors but shut down in CD10+/CD19− lymphoid progenitors (Fig. 2A); 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. 2B). VLA-4 was expressed on all three types of progenitors; frequency of expression did not differ significantly among them (Fig. 2C).

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. 2A). 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

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**Table I. Subsets of CD34+/CD38−Lin− fraction among cells from steady-state BM, G-CSF-treated marrow, and G-CSF-mobilized PB**

<table>
<thead>
<tr>
<th>Subset</th>
<th>Steady-State PB (n = 34)</th>
<th>Steady-State BM (n = 14)</th>
<th>G-CSF-Treated BM (n = 3)</th>
<th>G-CSF-Mobilized PB (n = 82)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD34+/Lin− cells/MNC</td>
<td>0.023 ± 0.014</td>
<td>1.49 ± 0.62</td>
<td>1.03 ± 0.12</td>
<td>0.59 ± 0.35b</td>
</tr>
<tr>
<td>% CD34+/CD38−Lin− MNC</td>
<td>ND</td>
<td>0.15 ± 0.072</td>
<td>0.078 ± 0.015</td>
<td>0.060 ± 0.036b</td>
</tr>
<tr>
<td>% CD13+/CD34+/CD38−Lin−</td>
<td>80.51 ± 7.59</td>
<td>89.21 ± 6.91</td>
<td>92.3 ± 3.76</td>
<td>96.70 ± 6.62b</td>
</tr>
<tr>
<td>% CD10+/CD19−/CD34+/CD38−Lin−</td>
<td>ND</td>
<td>8.93 ± 5.37</td>
<td>5.52 ± 3.04</td>
<td>1.49 ± 1.30b</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SD. ND, not detected.
*Significantly different from steady-state BM (p < 0.05).
CD34 may be mobilized into the circulation. As a result, a fraction of cells with lesser expression of these molecules served in the G-CSF-mobilized progenitors. These results suggest that CXCR-4 and VLA-4 expression for all three progenitor types in lymphoid progenitors in the steady-state BM (Fig. 4). Comparative expression of c-Kit (A), VLA-4 (B), and CXCR-4 (C) on CD34/CDC38⁻/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/β²⁻ /− 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. 4A). In contrast, mice transplanted with 50,000 pro-B cells sorted from BM or G-CSF-mobilized PB exhibited only B lymphoid reconstitution (Fig. 4B). 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. 4C). 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
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Pre-B cells stage (17, 20, 23, 24). Through DJHJ at the pro-B cells stage and to L chain gene at the
lymphoid progenitors, because Ig genes rearrangement status re-
lect well the differential stages in the B cell development pathway.

We examined the IgH rearrangement status in the mobilized B
lymphoid progenitors. In general, along with B cell differentiation pathway, Ig genes
rearrangements proceed from DJH at the early B cells or CLP stage
and G-CSF-mobilized PB (Fig. 6). Granulocyte lineage-related
genes including G-CSFR, GM-CSF receptor, and myeloperoxidase
were expressed in early B and pro-B cells from both BM and the G-CSF-mobilized PB (Fig. 6).

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 re-

lect well the differential stages in the B cell development pathway.

In general, along with B cell differentiation pathway, Ig genes
rearrangements proceed from DJH at the early B cells or CLP stage
through VDJH at the pro-B cells stage and to L chain gene at the
pre-B cells stage (17, 20, 23, 24).

In our experiments, DJH and VDJH rearrangements were unde-
tectable in the most immature CD34+CD38-Lin- cells sorted from both BM and the G-CSF-mobilized PBPC. In contrast, a
ladder of DJH 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). VDJH and DJH 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
cell 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 did not differentiate 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-
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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 in-
cluding 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 pheno-
typically defined lymphoid progenitor in the G-CSF-mobilized PB
had the same functional properties as its BM counterpart, indicat-
ing 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 adhe-
sive contact with their respective ligands in the BM microenviron-
ment. Several studies have demonstrated that following G-CSF
CD34 cells were generated in mice transplanted with 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.

FIGURE 4. Engraftment and reconstitution potential of human different progenitors in NOD/SCID/β2⁻/⁻ mice. Six to 8 wk after i.v. injection of 50,000 CD34⁺Lin⁻ cells, CD34⁺CD38⁻CD10⁻CD19⁻ 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
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<sup>+</sup> 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
The authors have no financial conflict of interest.

References

![FIGURE 6.](http://www.jimmunol.org/) Differential expression of hematopoiesis-affiliated genes in BM and G-CSF-mobilized lymphoid progenitors as shown by RT-PCR analysis. BM CD34<sup>+</sup> 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.](http://www.jimmunol.org/) PCR analysis of DJ<sub>μ</sub> and VDJ<sub>μ</sub> genes rearrangement on DNA from BM and G-CSF-mobilized lymphoid progenitors. CD34<sup>+</sup> CD19<sup>+</sup> cells were used as controls. None of the Ig genes rearrangements was observed in CD34<sup>+</sup>CD38<sup>+</sup> cells. Partial DJ<sub>μ</sub> rearrangement initiated at the stage of CD34<sup>+</sup>CD10<sup>+</sup>CD19<sup>+</sup> early B cells followed by the rearrangement of VDJ<sub>μ</sub> genes at CD34<sup>+</sup>CD10<sup>+</sup>CD19<sup>+</sup> 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.
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