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Comparison of CD34$^+$ Subsets and Clonogenicity in Human Bone Marrow, Granulocyte Colony-Stimulating Factor-Mobilized Peripheral Blood, and Cord Blood

To compare the clonogenicity and distribution of CD34$^+$ subsets in bone marrow (BM), granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood (PB) and cord blood (CB), we analyzed in vitro colony formation and CD34$^+$ cells co-expressing differentiation molecules (CD38, HLA-DR), myeloid associated molecules (CD13, CD33), a T-cell associated molecule (CD3), and a B-cell associated molecule (CD19) from mononuclear cells (MNCs) in the three compartments. The proportions of CD34$^+$CD38$^-$ cells (BM: 4.4±2.8%, PB: 5.3±2.1%, CB: 5.9±3.9%) and CD34$^+$HLA-DR$^-$ cells (BM: 4.7±3.4%, PB: 5.5±2.3%, CB: 6.1±3.7%) did not differ significantly among the compartments. In contrast, a significantly higher proportion of CD34$^+$ cells of PB and CB co-expressed CD13 (75.0±11.4%, 77.7±17.3%) and CD33 (67.1±5.7%, 56.8±10.3%) compared with those of BM (43.0±6.3%, 27.6±5.1%) and a significantly higher number of granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) were detected in MNCs derived from PB and CB compared with those from BM (p<0.01). The proportion of CD34$^+$CD19$^+$ cells was higher in BM (34.9±11.9%) than those in PB (5.6±3.0%) and CB (4.7±2.1%) (p<0.05). The proportion of CD34$^+$CD3$^+$ was comparable in all three compartments. In conclusion, our findings show that MNCs of mobilized PB and CB display similar phenotypic profiles of CD34$^+$ subsets and clonogenicity, different from those of BM.

Key Words: *Antigens, CD34; Stem cells; Granulocyte-macrophage colony-stimulating factor; Erythroid progenitor cells*

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INTRODUCTION

CD34$^+$ hematopoietic cells can reconstitute hematopoiesis in lethally irradiated animals or humans treated with myeloablative therapy (1, 2). CD34$^+$ cells are found in normal human bone marrow (BM), mobilized peripheral blood (PB), and cord blood (CB). CD34$^+$ cells contain lineage-committed and multilineage progenitor cells, as well as pluripotent stem cells (3-5). These CD34$^+$ cells are heterogeneous and have been fractionated, using several monoclonal antibodies against cell surface molecules by multicolor flow cytometric analysis (6, 7). Comparing transplantation with BM, mobilized PB, and CB, the time for engraftment varies. The minimal number of CD34$^+$ cells or clonogenic cells needed to assure complete and stable recovery is still unclear and may differ between grafts from different compartments (8-10).

Several studies have analyzed the CD34$^+$ cells presence in the different cell sources used for transplantation (7, 11, 12). These data indicate that, due to characteristic differences between the progenitor cells, the flow cytometric quantification using a simple one-color CD34 analysis may not provide sufficient information. CD34$^+$ cells are highly heterogeneous and various roles of CD34$^+$ subsets in hematopoietic recovery is not well defined. Analysis of CD34$^+$ subset is important for progenitor cell characterization. In this study, we compared the distribution of CD34$^+$ subsets in BM, mobilized PB and CB. Comparative analyses were performed with respect to co-expression of differentiation molecules (CD38, HLA-DR), myeloid associated molecules (CD13, CD33), a T-cell associated molecule (CD3), and a B-cell associated molecule (CD19). In addition, to analyse the number of clonogenic cells present in the three compartments, the number of granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) generated from mononuclear cells (MNC) were compared.

MATERIALS AND METHODS

Cell sources

BM samples (n=15) were collected from healthy adult BM donors or solid tumor patients without any evidence of tumor cell invasion to bone marrow on bone marrow biopsy.

PB samples (n=15) were collected from seven healthy adults. They received recombinant human granulocyte colony-stimulating factor (rhG-CSF), administered subcutaneously at 500 μg (8.1-10 μg/kg/day) for five to six days. Hematologic indices and the number of $CD34^+$ cells in PB were recorded at baseline and daily for a total of seven days. Cells were collected on day four to six of rhG-CSF administration, corresponding to the peak of $CD34^+$ cells in PB.

CB samples (n=10) were obtained from eight full-term infants delivered via normal vaginal delivery and two via caesarean section.

All sample donors gave informed consent, and the separate protocols were approved by The Institutional Review Board at Chonnam National University Hospital, Korea.

Cell preparation and staining

MNCs were prepared by gradient centrifugation (Lymphoprep, 1.077 g/mL, Nycomed, Oslo, Norway) for flow cytometry. MNCs were stained with fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated anti-CD34 (anti-HPCA-2) and one of the following: FITC-conjugated anti-CD3 (Leu5b), anti-CD19 (Leu12) and PE-conjugated anti-CD13 (LeuM7), anti-CD33 (LeuM9), anti-CD38 (Leu17), and anti-HLA-DR. For isotype controls, we used FITC-conjugated mouse IgG1 and PE-conjugated mouse IgG2a, which were purchased from Becton Dickinson (San Jose, CA, U.S.A.). Monoclonal antibody incubations were performed in the dark for 30 min followed by lysis of the erythrocytes with isomolar amonium chloride buffer (1 mL) for 10 min. All incubations were performed at 4℃, and after each incubation, the cells were washed with phosphate-buffered saline (PBS).

Two-color flow cytometric analysis

Flow cytometric analysis was performed with a FACScan (Becton Dickinson) with an argon-ion laser tuned at 488 nm. Analysis of the data was performed using Consort 30 research software (Becton Dickinson). The frequency of $CD34^+$ cells was determined by subtracting the percentage of cells stained with the control reagents from the percentage stained positively after labeling with anti-CD34-FITC. To determine the phenotype of $CD34^+$ cells, a minimum of 3,000 $CD34^+$ cells was analyzed. A marker was set at the first log decade and the percentage of $CD34^+$ cells that co-expressed a specific antigen was assessed after correction for the percentage of cells reactive within isotype control. $CD34^+$% was expressed as a percentage of total MNCs as calculated by the formula ($CD34^+$ events/total MNC processed)×100 and $CD34^+$ subset % was expressed as a percentage of total $CD34^+$ cells as calculated by the formula ($CD34^+$ subset events/total $CD34^+$ cells processed)×100.

Clonogenic assays

Clonogenic assays for CFU-GM and BFU-E were performed, using a modification of the culture method originally described by Iscove et al. (13). The 1×10^5 MNCs from BM, mobilized PB and CB were plated in triplicate dishes in 0.9% methylcellulose in IMDM (MethoCult, Stem Cell Technologies, Vancouver, Canada) supplemented with 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 10% agar leukocyte conditioned medium (LCM) and 3 units/mL erythropoietin. Plates were incubated for 14 days in a humidified atmosphere (37℃, 5% CO_2 in air). Both CFU-GM and BFU-E were counted under an inverted microsope.

Statistics

The data between the groups were compared using one-way ANOVA followed by Duncan's multiple range tests to discriminate inter-group differences. Values were expressed as mean±standard error. Differences were considered significant for p values <0.05.

RESULTS

Flow cytometric analysis of $CD34^+$ cells

Quantitation of $CD34^+$ cells

The highest percentage of $CD34^+$ cells was found in BM, which differed significantly from the values determined in the other sources (p=0.001): BM 2.9±0.51% (mean±SE), mobilized PB 1.38±0.3%, and CB 0.82±0.2% of MNCs.

Subsets of $CD34^+$ cells

There were no significant differences among the three compartments, according to proportions of $CD34^+CD38^-$ and $CD34^+HLA\text{-}DR^-$ (Table 1 and Fig. 1D-E). The pro-

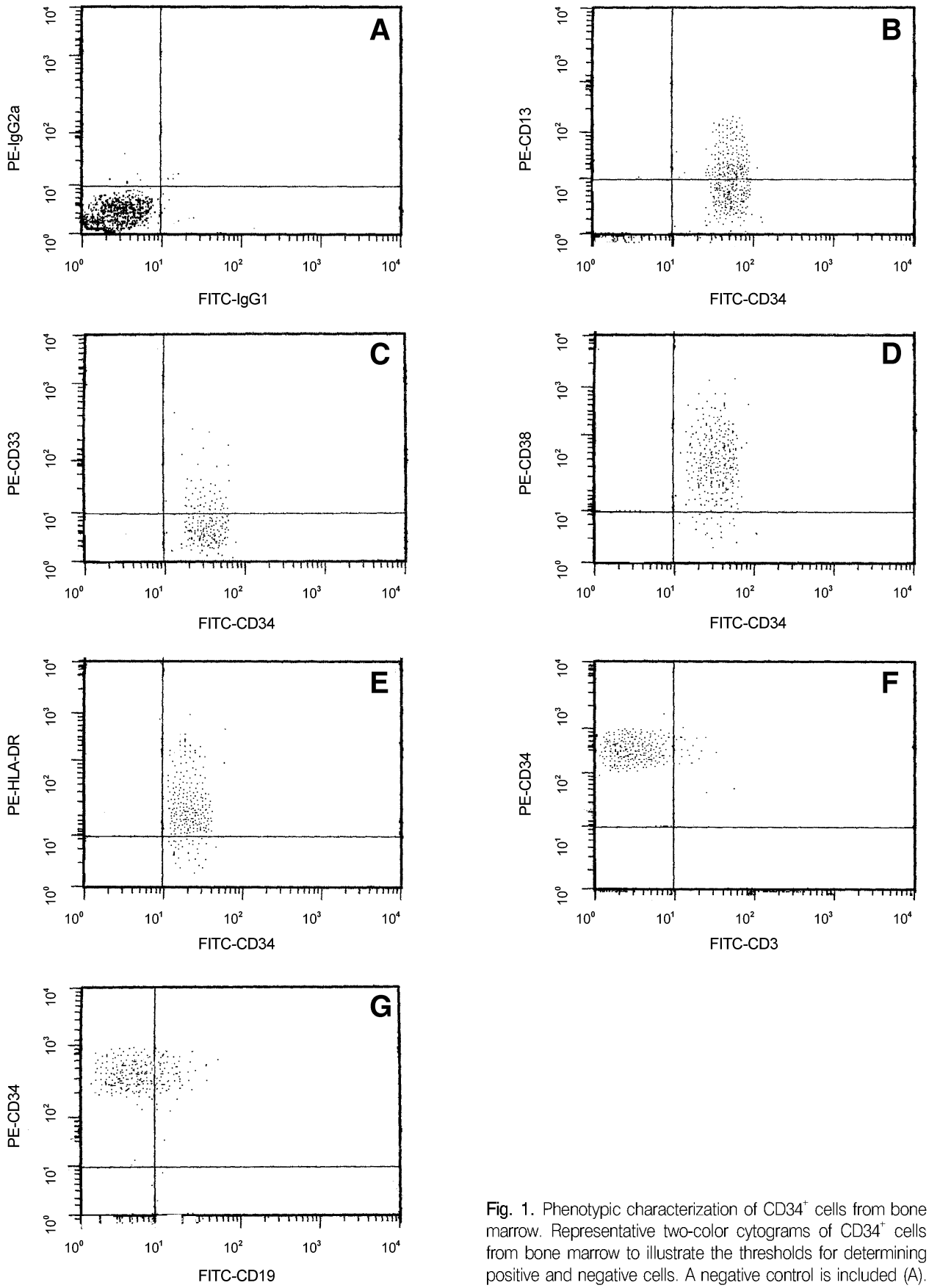

Fig. 1. Phenotypic characterization of $CD34^+$ cells from bone marrow. Representative two-color cytograms of $CD34^+$ cells from bone marrow to illustrate the thresholds for determining positive and negative cells. A negative control is included (A).

Table 1. $CD34^+$ subsets of BM, mobilized PB and CB

Subsets	% of $CD34^+$ cells			
	BM (n=15)	Mobilized PB (n=15)	CB (n=10)	*p* value
$CD34^+CD38^-$	4.4±2.8	5.3±2.1	5.9±3.9	NS
$CD34^+HLA\text{-}DR^-$	4.7±3.4	5.5±2.3	6.1±3.7	NS
$CD34^+CD13^+$	43.0±6.3*	75.0±11.4	77.7±17.3	0.003
$CD34^+CD33^+$	27.6±5.1*	67.1±5.7	56.8±10.3	0.001
$CD34^+CD3^+$	7.1±4.0	5.6±3.8	6.4±4.1	NS
$CD34^+CD19^+$	34.9±11.9*	5.6±3.0	4.7±2.1	0.001

Results are mean±SE
BM, bone marrow; PB, peripheral blood; CB, cord blood; NS, not significant
*$p<0.05$ vs. PB, CB by ANOVA

Table 2. Colony formation of MNCs from BM, mobilized PB and CB

Colony formation	BM (n=15)	Mobilized PB (n=15)	CB (n=10)
CFU-GM/10^5 cells	107.5±25.5*	172.3±33.9	179.1±36.6
BFU-E/10^5 cells	49.5±23.5*	71.6±13.3	65.9±10.8

Results are mean±SE
MNCs, mononuclear cells; BM, bone marrow; PB, peripheral blood; CB, cord blood; CFU-GM, granulocyte-macrophage colony-forming unit; BFU-E, erythroid burst-forming unit
*$p<0.05$ vs. PB, CB by ANOVA

portions of myeloid lineage precursors such as $CD34^+$ $CD13^+$, $CD34^+CD33^+$ cells were significantly higher in mobilized PB and CB than in BM (Table 1 and Fig. 1B-C).

The CD3 cell surface molecule, associated with the T-lymphocyte lineage, was expressed on a comparable and low proportion of $CD34^+$ cells from all three compartments (Table 1 and Fig. 1F). The relative number of $CD34^+$ cells expressing CD19, associated with the B-lymphocyte lineage, was significantly higher in BM compared with both mobilized PB and CB (Table 1 and Fig. 1G).

Colony formation

MNCs prepared from the three compartments were analyzed for in vitro colony formation (CFU-GM and BFU-E). A significantly higher number of CFU-GM and BFU-E were detected in MNCs derived from mobilized PB and CB compared with BM (Table 2).

DISCUSSION

Originally, the number of nucleated cells infused per kg of the recipient (14) or the number of myeloid clonogenic cells (CFU-GM) as estimated in semisolid culture assays were measures used to quantify transplantations (10, 15, 16). The production of monoclonal antibodies against epitopes of the CD34 antigen allowed a more rapid detection of $CD34^+$ cells by flow cytometric analysis (3). The CD34 antigen is a 115 kDa surface glycoprotein expressed in early lymphohematopoietic stem and progenitor cells in BM, mobilized PB and CB (17). Although $CD34^+$ cells are necessary for engraftment, these progenitor cells are highly heterogenous and various roles of $CD34^+$ subsets in hematopoietic recovery is not well defined. Analysis of $CD34^+$ subset is important for progenitor cell characterization. In this study we analyzed the distributions of $CD34^+$ subsets in BM, mobilized PB and CB.

$CD34^+$ cells that lack activation antigens (CD38, HLA-DR) are considered to be more primitive hematopoietic cells, which are precursors to clonogenic cells and contribute to late phases of hematopoietic reconstitution (6, 18-20). Mounting evidence indicates that long-term reconstituting hematopoietic stem cells are found among $CD34^+CD38^-$ and $CD34^+HLA\text{-}DR^-$ cells in BM (18, 19), PB (6), and CB (20). Proportions of these primitive subsets among $CD34^+$ cells were low and comparable in all three compartments. Data by Steen et al. (21) are in line with our data of $CD34^+CD38^-$ subsets, but they contrast our data on $CD34^+HLA\text{-}DR^-$ subsets. They showed that the proportion of $CD34^+HLA\text{-}DR^-$ cells was comparable in CB and mobilized PB, but higher in CB than in BM. Kinniburgh and Russell have reported no difference in the number of $CD34^+CD38^-$ cells between CB and BM (22), but Payne et al. reported that the proportion of

CB $CD34^+CD38^-$ cells was significantly higher than has been reported for BM (23). The $CD34^+CD38^-$ immunophenotype is consistent in defining a highly primitive subset of $CD34^+$ cells in all sources of hematopoietic stem cell (HSC) studied to date, i.e., fetal liver, fetal BM, adult BM, mobilized PB, and CB (6, 24-28). In contrast, HLA-DR expression on highly primitive progenitors vary among various hematopoietic sources. Traycoff et al. showed that, contrary to BM, CB $CD34^+$HLA-DR^+ cells contain the majority of primitive hematopoietic progenitors (29). The study by Payne et al. is in line with this observation, suggesting that primitive hematopoietic progenitors in CB are confined to the $CD34^+$HLA-DR^+ cell fraction (23). According to the fact that the proportion of $CD34^+$HLA-DR^+ among $CD34^+$ cells is definitely higher than that of $CD34^+$HLA-DR^- (23), the primitive hematopoietic progenitor cell subfraction of CB $CD34^+$ cells is probably larger than that of BM $CD34^+$ cells.

The proportion of $CD34^+$ cells, expressing the T-lymphocyte associated molecule CD3, was comparable in all three compartments.

The most striking difference observed regarding the proportions of $CD34^+$ subsets in the three compartments was the very high percentage of $CD34^+$ cells coexpressing CD13 and/or CD33 molecules and the correspondingly low percentage of $CD34^+$ cells coexpressing CD19 molecule in mobilized PB and CB as compared with BM. This is in line with previous observations (21, 30). It has been shown that the vast majority of CFU-GM are confined to the $CD34^+CD33^+$ cell population (31). In our study, the high presence of $CD34^+CD33^+$ myeloid progenitor cells, together with high numbers of CFU-GM and BFU-E in mobilized PB and CB compared with BM, parallel this finding. The content of CFU-GM and $CD34^+CD33^+$ cells are correlated to rapid hematologic reconstitution after myeloablative therapy (10, 32). Hence, we predict that hematologic reconstitution will be more rapid using mobilized PB and CB than BM after myeloablative conditioning.

In conclusion, our findings show that MNCs of mobilized PB and CB display similar phenotypic profiles of $CD34^+$ subsets and clonogenecity in contrast to BM. The relative proportions of myeloid progenitors which are important in early engraftment are higher at the expense of B-lymphocyte progenitors in mobilized PB and CB. These features should make mobilized PB and CB excellent sources for the transplantation of hematopoietic progenitor cells, because rapid engraftment can be expected, providing that adequate numbers of $CD34^+$ cells are transplanted. Further precise study using multicolor flow cytometry and cell sorting will be required to fully identify and compare the $CD34^+$ subsets in various stem cell sources.

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