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Multipotent and Committed CD34$^+$ Cells in Bone Marrow Transplantation

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In order to study the role of CD34$^+$ cells in hematological recovery following bone marrow transplantation (BMT), bone marrow cells stained with HPCA-1 (CD34) and MY-9 (CD33) monoclonal antibodies were analyzed by using a fluorescence-activated cell sorter on or about days 14 and 28, as well as at later times, following BMT in 6 recipients. Single cell cultures of CD34$^+$ cells were also performed to evaluate their *in vitro* hematopoietic function. CD34$^+$ cells were detectable in bone marrow cells on day 14. More than 80% of CD34$^+$ cells co-expressed the CD33 antigen, and macrophage (Mac) colony-forming cells predominated among total colony-forming cells of CD34$^+$ cells. In normal bone marrow cells, CD34$^+$,CD33$^+$ cells amounted to about 40% of CD34$^+$ cells, and the incidences of erythroid bursts, granulocyte/macrophage (GM) colonies, and Mac colonies were similar to each other. After more than 10 weeks, CD34$^+$,CD33$^-$ cells gradually recovered, as erythroid burst colony-forming cells increased following GM colony-forming cells. This phenomenon was well-correlated with the time course of peripheral blood cell recovery. CD34$^+$,CD33$^+$ cells as committed progenitors and CD34$^+$,CD33$^-$ cells as multipotent stem cells have distinctive biological behaviors in BMT.

Key words: Bone marrow transplantation — CD34$^+$ cells — Hematopoietic stem cells

CD34 antigen (MY-10) monoclonal antibody against the KG-1a cell line produced by Civin *et al.*[1] has been widely applied to the study of hematopoietic stem cells. The CD34 antigen is expressed on approximately 1% of normal human bone marrow cells. Some CD34$^+$ cells showed the ability to form colonies *in vitro* upon stimulation of hematopoietic factors[2] and were able to reconstitute hematopoiesis in lethally irradiated baboons.[3] Siena *et al.*[4] suggested that expanded peripheral CD34$^+$ cells could be relevant in the field of stem cell transplantation. Andrews *et al.*[5] reported that the precursors of colony-forming cells in long-term culture were CD34$^+$,CD33$^-$ cells. We previously reported[6] that CD34$^+$,CD33$^-$ cells respond to interleukin-3 (IL-3) but not to granulocyte colony-stimulating factor (G-CSF), while CD34$^+$, CD33$^+$ cells respond to both IL-3 and G-CSF. From these findings, multipotent stem cells seem to be included among CD34$^+$,CD33$^-$ cells.

For the purpose of clarifying the role of CD34$^+$ cells in the hematological recovery of recipients of bone marrow transplants (BMT), we serially analyzed bone marrow CD34$^+$ cells following the infusion of bone marrow cells, using a fluorescence-activated cell sorter (FACS). Co-expression of the CD33 antigen on CD34$^+$ cells was also examined. Furthermore, we examined the colony-forming ability of single CD34$^+$ cells sorted by a FACS clone-sorting system as previously described.[6] Significant differences in bone marrow CD34$^+$ cells were found between normal adults and recipients of BMT.

MATERIALS AND METHODS

Patients Six patients undergoing BMT were studied after informed consent had been obtained. Table I shows the characteristics of the patients: 4 patients with acute leukemia in complete remission, 1 patient with Hodgkin's disease in partial remission, and 1 patient with chronic myelogenous leukemia in the accelerated phase. Sibling donors were HLA-identical in 5 cases, but major ABO-mismatched in cases 1 and 5. In case 4, autologous marrow was transplanted. The conditioning regimens consisted of cyclophosphamide (CY) plus total body irradiation (TBI) in cases 1 and 2, CY plus busulfan in cases 3 and 5, and TBI plus cytarabine (Ara-C) in case 6. In case 4, Ara-C, nimustine hydrochloride, etoposide, and thiotepa were combined for conditioning. To deplete red blood cells, a Ficoll gradient was used in cases 1, 4 and 5. For the prophylaxis of graft-versus-host disease, cyclosporin A was given except for case 4. Granulocyte colony-stimulating factor (G-CSF, supplied by Kirin

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Brewery Co., Ltd., Tokyo) was administered in all cases. The doses and durations of G-CSF injected were as follows: at 7 μg/kg/day for 21 days (days 1–21) in case 1; at 8 μg/kg/day for 5 days (days 21–25) in case 2; at 4 μg/kg/day for 7 days (days 7–13) in case 3; at 5 μg/kg/day for 23 days (days 25–47) in case 4; at 5 μg/day for 12 days (days 16–27) in case 5; and at 10 μg/kg/day for 8 days (days 15–22) with a tapering follow-up of 4 days in case 6. Granulocyte-macrophage colony-stimulating factor (GM-CSF, supplied by Schering-Plough Co., Osaka) was administered at a dose of 500 μg/body/day from days 2 to 24 in case 4. Complete blood cell counts and bone marrow aspirations were serially performed after the BMT.

Cells Bone marrow samples were obtained from donors at the time of bone marrow harvest as well as from patients at various phases following the BMT. Bone marrow cells were also obtained from normal healthy volunteers who had given informed consent. Bone marrow cells were diluted with phosphate-buffered saline (PBS), layered over Ficoll-Metrizoate (Lymphoprep, Nyegaard, Oslo) and centrifuged. Interface cells were collected and washed with staining medium: 3% fetal calf serum (FCS, Flow Laboratories, North Ryde, NSW) and 0.1% sodium azide in PBS. Mononuclear cells were pelletted for staining with monoclonal antibodies.

Staining with monoclonal antibodies Cells were incubated with HPCA-1 (mouse IgG1 CD34 monoclonal antibody, kindly provided by Dr. C. I. Civin, Division of Pediatric Oncology, The Johns Hopkins Oncology Center, Baltimore, MD) and avidin phycoerythrin (PE)-conjugated MY-9 (mouse IgG2a CD33 monoclonal antibody, Coulter Immunology, Hialeah, FL). After washing with staining medium, cells were incubated with fluorescein isothiocyanate (FITC) conjugated rat anti-mouse IgG1 (Zymed Laboratories, Inc., South San Francisco, CA). Cells were then suspended in staining medium containing 7-aminoactinomycin D. For negative controls, unstained cells or cells stained only with anti-IgG1-FITC were included.

Analysis by FACS Stained cells and negative controls were subjected to analysis by using a FACStar^{plus} (Becton Dickinson Immunocytometry Systems, Inc., Mountain View, CA). Multiparameter data were collected and analyzed by using FACS-DESK (ver. 1.8) run on a Digital Micro VAX-II configured as described.[7] Fluorescence intensity of individual cells was measured as relative fluorescence units. Data from more than 50,000 cells were collected and analyzed. The gate was set using the light scattering properties of $CD34^+$ cells as described.[6]

Methylcellulose culture of mononuclear cells and single $CD34^+$ cells Methylcellulose cultures were performed as previously described.[8] Briefly, 1 ml of culture medium contained 1.2% methylcellulose (Aldrich Chemical Co., Milwaukee, WI), 30% human platelet-poor plasma (PPP), 1% deionized bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO), 5×10^{-5} *M* 2-mercaptoethanol (2-ME, Sigma Chemical Co.), 5% phytohemagglutinin-stimulated conditioned medium (PHA-LCM),[9] and 2 U of recombinant human erythropoietin (Epo, provided by Snow Brand Co., Ishibashi, Tochigi) in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Long Island, NY). Mononuclear cells (5×10^4/1 ml of culture medium) were plated into 35-mm non-tissue culture dishes (Falcon, Oxnard, CA). One $CD34^+$ cell was deposited in each well of a 96-well microtiter plate (A/S Nunc, Kampstrup, Denmark), containing 0.1 ml of culture medium, using the FACStar^{plus} and an automatic cell deposition unit (Becton Dickinson Immunocytometry Systems). Colonies derived from granulocyte/macrophage colony-forming units (CFU-GM), pure macrophage colony-forming units (CFU-Mac), erythroid burst-forming units (BFU-E), megakaryocyte colony-forming units (CFU-Meg) and mixed colony-forming units (CFU-Mix) were scored on day 14 of culture at 37°C in a humidified atmosphere of 5% CO_2 and 5% O_2 using an inverted microscope. The colonies were defined as aggregates consisting of 40 or more cells except for megakaryocyte colonies, which consisted of 3 or more cells. Colonies were lifted from semi-solid medium, and stained with May-Grünwald-Giemsa to confirm cell lineages.

RESULTS

Hematological recovery All recipients showed pancytopenia when the first bone marrow aspiration was performed (day 11 after BMT in case 3 and day 14 for the other cases). Bone marrow smears demonstrated severe hypoplastic marrow in all cases. The days to hematological recovery are shown in Table I. Granulocyte, reticulocyte, and platelet counts recovered within 24–38 days after the BMT. In case 4, the patient remained dependent on platelet transfusions for more than 10 weeks after the BMT, but the recovery of magakaryocytes was observed in the bone marrow on day 77. In no case did we observe graft failure.

$CD34^+$ cells of bone marrow mononuclear cells $CD34^+$ cells ($CD34$ bright cells) accounted for 0.58 ± 0.23% (mean$\pm$SD) of bone marrow cells, and $CD34^+$,$CD33^-$ cells were 60.0 ± 14.0% of the total $CD34^+$ cells in 12 normal adults examined. In all recipients of BMT, $CD34^+$ cells could be detected on day 14 after the BMT. The percentage of these cells among the bone marrow cells varied widely and below the normal range in half of the 6 cases (Fig. 1A). These values decreased around day

Table I. Characteristics of Patients

Case	Age (yr)	Sex	Diagnosis (status)	BMT	Number of cells ($\times 10^7$/kg)	Days to recovery: Granulocytes (>500/μl)	Days to recovery: Retic. (>2$\times 10^4$/μl)	Days to recovery: Platelets (>5$\times 10^4$/μl)
1	31	M	AML (CR)	allo	3.8[a]	16	22	24
2	21	M	ALL (CR)	allo	21.5	22	23	28
3	22	M	AMLL (CR)	allo	23.8	12	16	18
4	34	F	HD (PR)	auto	4.0[a]	25	38	NR
5	36	M	CML (AP)	allo	8.2[a]	14	26	27
6	9	M	AML (CR)	allo	20.5	19	25	38

Number of cells, numbers of nucleated cells per kg of recipient body weight infused. *a*) Mononuclear cells separated from nucleated cells by Ficoll-Metrizoate. Retic., absolute numbers of reticulocytes; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; AMLL, acute mixed lineage leukemia; CML, chronic myelogenous leukemia; CR, complete remission; PR, partial remission; AP, accelerated phase; allo, allogeneic; auto, autologous. NR, the number of platelets did not reach 5$\times 10^4$/μl during the observation period.

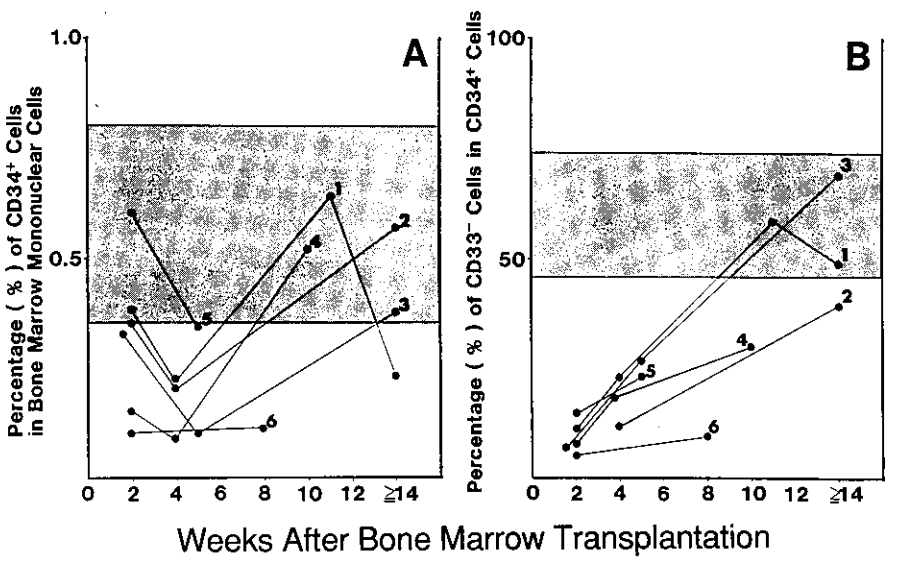

Fig. 1. Serial follow-up of CD34$^+$ cells in the bone marrow cells (A) and CD33$^-$ cells in the total CD34$^+$ cells (B). Shaded areas show the normal ranges obtained from 12 normal adults: CD34$^+$ cells among bone marrow cells amounted to 0.58$\pm$0.23% (mean$\pm$SD). CD33$^-$ cells among total CD34$^+$ cells amounted to 60.0$\pm$14.0%. Numbers near lines indicate case numbers. The percentages of CD34$^+$,CD33$^-$ cells in the total CD34$^+$ cells on day 14 after BMT in recipients were significantly lower than that in normal adults (Student's *t* test).

28 in all cases, and then increased toward the normal range. In all cases, on day 14, CD34$^+$,CD33$^-$ cells were less than 20% of the total CD34$^+$ cells with a gradual increase of CD34$^+$,CD33$^-$ cells requiring more than 8 weeks. The analysis of more than 10^4 CD34$^+$ cells collected showed that the proportion of CD34$^+$,CD33$^-$ cells to total CD34$^+$ cells was similar to that of the data on 5$\times 10^4$ bone marrow cells analyzed. In normal adults, CD34$^+$,CD33$^-$ cells amounted to 60.0$\pm$14.0% (mean$\pm$SD, n=12) of total CD34$^+$ cells (Fig. 1B and Fig. 2).

Colony formation of single CD34$^+$ cells Table II shows the results of colony assay of single CD34$^+$ cells isolated from bone marrow cells obtained from 12 normal adults and the recipients in cases 1–3. Colony-forming cells amounted to 31.9$\pm$10.0% (mean$\pm$SD) of CD34$^+$ cells in normal adults. BFU-E, CFU-GM and CFU-Mac each

amounted to approximately 10% of $CD34^+$ cells, as described previously.[6] In $CD34^+$, $CD33^+$ cells separated from total $CD34^+$ cells, CFU-Mac and CFU-GM predominated significantly compared with those in $CD34^+$, $CD33^-$ cells. In contrast, BFU-E significantly predominated in $CD34^+$,$CD33^-$ cells with a P-value of < 0.01. The colony formation of $CD34^+$ cells in donors was within the normal range (data not shown). The total number of colony-forming cells (CFC) among $CD34^+$ was similar to that of normal adults on day 14 in cases 1 and 2, but smaller on day 11 in case 3. The incidence of total CFC was persistently below the normal range. The proportions of BFU-E, CFU-GM and CFU-Mac in $CD34^+$ cells on days 11 or 14 in these cases were different from those of normal adults. In this early post-BMT, CFU-Mac predominated (15–30%) among $CD34^+$ cells. This proportion gradually normalized in each case. In particular, an increase in CFU-Mac was observed followed by an increase of BFU-E with a decrease in CFU-Mac.

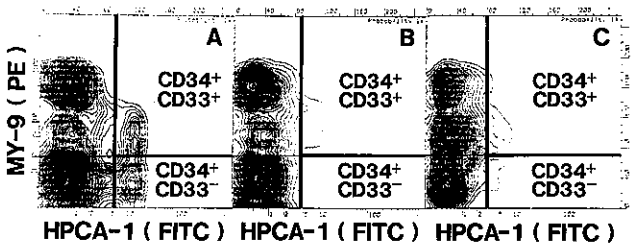

Fig. 2. Representative FACS analyses of bone marrow cells in a donor and a recipient. Bone marrow cells were stained with HPCA-1 (CD34) and MY-9 (CD33). The FACS map of donor bone marrow cells in case 1 (A) represents the analyses of normal bone marrow cells. (B) and (C) show bone marrow cells of the same recipient analyzed on days 14 and 77, respectively. $CD34^+$,$CD33^-$ cells are seen in (A) and (C), but not in (B). A probability level of 1% was used in each analysis.

Colony formation of unfractionated bone marrow mononuclear cells In case 1, 48 ± 18 BFU-E-derived colonies, 32 ± 6 GM colonies, 66 ± 13 Mac colonies, 2 ± 1 Meg colonies, and 5 ± 2 mixed colonies were formed by 5×10^4 mononuclear cells (MNCs) of the donor's bone marrow. No colonies were formed from 5×10^4 MNCs by day 14 and only 2 ± 1 GM colonies and 14 ± 3 Mac colonies were formed by day 28 in the recipient's bone marrow. The incidence of such colonies eventually reached normal

Table II. Colony Formation of Single $CD34^+$ Cells Isolated from Bone Marrow Cells in Normal Adults and Recipients of BMT

Source of cells	Incidence (%) of colony-forming cells in $CD34^+$ cells					
	BFU-E	CFU-GM	CFU-Mac	CFU-Meg	CFU-Mix	Total CFC
Normal adults	10.0 ± 5.7	10.2 ± 3.3	10.7 ± 5.7	0.0	1.0 ± 1.0	31.9 ± 10.0
$CD34^+$,$CD33^+$	3.0 ± 1.2	17.8 ± 5.6	27.3 ± 1.0	0.0	0.0	48.0 ± 4.3
$CD34^+$,$CD33^-$	16.5 ± 2.5	2.5 ± 1.3	3.5 ± 2.1	0.5 ± 1.0	0.3 ± 0.5	23.3 ± 4.6
Recipients						
Case 1						
Day 14	1.7	4.2	30.8	0.0	0.0	36.7
Day 28	2.5	8.3	11.7	0.0	1.7	24.2
Day 77	6.7	10.8	11.7	0.0	1.7	30.9
Case 2						
Day 14	0.0	6.7	16.7	0.0	0.0	23.4
Day 28	3.3	11.7	15.0	0.0	0.0	30.0
Day 190	7.5	9.2	10.8	0.8	0.8	29.1
Case 3						
Day 11	0.0	1.6	15.0	0.0	0.0	16.6
Day 35	5.8	10.0	10.8	0.0	0.0	26.6
Day 109	8.3	8.3	10.8	0.0	0.0	27.4

In normal adults, incidences of colony-forming units were expressed as mean $\pm$ SD ($n=12$ in total $CD34^+$ cells, $n=4$ in $CD34^+$,$CD33^+$ cells and $CD34^+$,$CD33^-$ cells). Incidences were calculated based on the culture of 120 single $CD34^+$ cells. BFU-E, erythroid burst-forming units; CFU-GM, granulocyte/macrophage colony-forming units; CFU-Mac, pure macrophage colony-forming units; CFU-Meg, megakaryocyte colony-forming units; CFU-Mix, mixed colony-forming units; CFC, colony-forming cells.

levels by day 77 following the BMT. Similar findings were observed in the colony formation of bone marrow MNCs for cases 2 and 3.

DISCUSSION

We studied the recovery of hematopoiesis in patients receiving allogeneic or autologous bone marrow transplants. CD34$^+$,CD33$^+$ cells were initially detected in the bone marrow around day 14 after BMT. These cells predominantly formed Mac colonies *in vitro*. At this point, the bone marrow showed a severe hypocellularity. Around days 28–35, the percentage of CD34$^+$ cells in bone marrow mononuclear cells decreased from the initial level, presumably due to dilution by a large number of mature cells other than CD34$^+$ cells and the percentage of CD34$^+$,CD33$^-$ cells in total CD34$^+$ cells became larger than that on day 14, but did not yet reach the normal range. The incidences of CFU-Mac and CFU-GM were normalized, but that of BFU-E still remained low. Ten or more weeks after BMT, total CD34$^+$ cells gradually increased to the normal range with an accompanying increase in CD34$^+$,CD33$^-$ cells. It was suggested that the prior appearance of CD34$^+$,CD33$^+$ cells, the ability to form Mac and GM colonies, and the initial recovery of monocytes and granulocytes were well correlated with each other. It seems reasonable that CFU-Mac first appeared in the bone marrow in the course of hematological recovery. This is compatible with the facts that CFU-Mac generates monocytes/macrophages capable of secreting cytokines,[10] that macrophage-derived stromal cells construct hematopoietic microenvironments,[11] and that macrophages defend against infection. In BMT, CD34$^+$,CD33$^+$ cells might play a role different from that of CD34$^+$,CD33$^-$. It was reported that CD34$^+$,CD33$^-$ cells are more primitive than CD34$^+$,CD33$^+$ cells.[5] In mice, Jones *et al.*[12] reported the existence of two phases of engraftment produced by committed progenitors and pluripotent stem cells. The two-phase engraftment of subsets of CD34$^+$ cells might correspond to that observed in mice.

Colony-stimulating factors such as G-CSF, GM-CSF, and macrophage colony-stimulating factor were proliferative stimuli for committed progenitor cells and their progeny.[13] G-CSF affects CD34$^+$ cells, especially CD34$^+$,CD33$^+$ cells, *in vitro*.[6] The *in vivo* effect of this factor on CD34$^+$ cells is not well known. G-CSF was administered early after BMT (days 1–14) in case 3; during a later period (days 15–27) in cases 2, 5, and 6; and throughout both periods in case 1. The percentages of CD34$^+$ cells in bone marrow cells and CD34$^+$,CD33$^-$ cells to total CD34$^+$ cells seemed unrelated to G-CSF injection. It was assumed that G-CSF merely supported the differentiation of CD34$^+$ cells, thus not affecting precursors of CD34$^+$ cells or self-renewing cells if these cells existed in CD34$^+$ cells, because it did not accelerate the recovery of CD34$^+$ cells.

Our preliminary study has shown that CD34$^+$,CD33$^-$ cells differentiated into CD34$^+$,CD33$^+$ cells on the human stromal layer, and this appears to represent one differentiation pathway from multipotential stem cell to committed myeloid progenitors. Taken together, it is suggested that CD34$^+$,CD33$^+$ cells function as committed progenitors in the early phases following BMT, and CD34$^+$,CD33$^-$ cells function as multipotent stem cells in late phases.

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