Bone Marrow Stromal Cells Induce Myeloid and Lymphoid Development of the Sorted Hematopoietic Stem Cells In Vitro

By Ryuhei Okuyama, Masahito Koguma, Nobuaki Yanai, and Masuo Obinata

Regulation of development of hematopoietic stem cells was examined by culturing Lin− c-Kit− Sca1+ stem cells sorted from bone marrow (BM) cells by fluorescence-activated cell sorting on a layer of TBR95, a BM stromal cell line established from simian virus 40 T-antigen gene transgenic mice. The sorted stem cells did not show self-renewal, but two waves (at 7 and 13 days) of a cobblestone formation were induced by the stromal cell layer. The cobblestones were formed by finite cell division (eight divisions on average) of sorted Lin− c-Kit− Sca1+ stem cells, and divided cells were still immature. The c-Kithigh stem cell population was induced to form the first wave of cobblestone formation committed to myeloid lineage, whereas c-Kitlow population was induced to form the second wave of this formation committed to lymphoid lineage. Both cobblestone formations require c-Kit function, but very late activation antigen-4–vascular cell adhesion molecule-1 interaction plays different parts in the two lineages.

MATERIALS AND METHODS

Antibodies and cytokines. Anti-B220 (RA3-6B2, kindly provided by Dr. H. Yagiya, Juntendo University School of Medicine, Japan), anti–c-Kit antibody (ACK2, kindly provided by Dr. S.-I. Nishikawa, Kyoto University, Kyoto, Japan), Mac1 (M1/70; Caltag Laboratories, South San Francisco, CA), and three lineage markers [Gr1(RB6-8C5), L3T4 (GK1.5), Ly-2 (53-6.72); Pharmingen, San Diego, CA] were purchased or provided as indicated. Antibody PS/2, which recognizes mouse c4 subunit of very late activation antigen-4 (VLA-4), and antibody M2K2, which recognizes mouse vascular cellular adhesion molecule-1 (VCAM-1) were gifts of Dr. H. Yagiya.26,27 Murine recombinant stem cell factor (SCF), murine recombinant interleukin-3 (IL-3), and human recombinant IL-6 were generously supplied by KIRIN-Amgen CO, Ltd (Tokyo, Japan).

Maintenance of BM stromal cells. Mouse BM stromal cell lines (TBR series) were established from ts simian virus 40 (SV40) T-antigen transgenic mice as described previously.17 TBR cell lines were maintained in RITC 80-7 (Kyoruto Pharmaceutical CO, Ltd, Tokyo, Japan) medium as described previously.18 The cultures were incubated at 33°C, which is a permissive temperature for SV40 ts T-antigen.

Enrichment of hematopoietic stem cells. A highly enriched stem cell fraction was obtained from BMs of C57BL/6 mice by the method of Okada et al19 with a slight modification. BM cells were incubated with a cocktail of MoAbs, specific to lineage markers (B220, Mac1, Gr1, L3T4, Ly-2, and TER119). Lin− cells were eliminated by immunomagnetic beads (Dynabeads M-450, coated with anti-mouse IgG, Dynal AS, Oslo, Norway). Quality of Lin− cells was monitored by fluorescence-activated cell sorting (FACS) analysis with several lineage markers. Contamination of either Gr1+ or Mac1+ cells was less than 1% and B220+, Mac1+, TER119+, CD4+, CD8+ cells were not detected. Lin− cells were successively incubated with fluorescein isothiocyanate–conjugated Sca1 and biotinated ACK2, phycoerythrin-streptavidine (Pharmingen), and propidium iodide. c-Kit−
Scal+ cells were analyzed and sorted on a FACStar (Becton Dickinson, San Jose, CA) with an automated cell deposition unit.

Coculture of the sorted stem cells on the stromal cells. For cocultivation of hematopoietic stem cells or BM cells on stromal cell lines, confluent cell layers of TBR cell lines were maintained at 37°C by replacing the maintenance medium with α-minimum essential medium (α-MEM; Flow Laboratories, Irvine, CA) supplemented with 10% fetal bovine serum (FBS) and 50 mmol/L 2-mercaptoethanol (2-ME). The medium was changed at 3-day intervals. After seeding of Lin− c-Kit+ Scal+ cells, dishes were scanned with an inverted-phase microscope for foci containing more than 10 hematopoietic cells attached to or beneath the stromal cells (cobblestone area).1

In vitro methylcellulose assay. Methylcellulose assay of the hematopoietic cells was performed according to the method of Iscove et al with a slight modification. Hematopoietic cells adhered to the stromal cells were recovered by dispase with an inverted-phase microscope for foci containing more than 50 cells were counted in triplicate as colony-forming units in culture (CFU-C), and colonies greater than 2 mm in diameter were regarded as high proliferative potential colony-forming cells (HPP-CFC).20

Analysis of cobblestone formation. The number of cells attached to or beneath the stromal cells in the cobblestone area was counted by daily photographing and the maximal colony size was determined. For histochemical analysis of the cells released into the medium from the cobblestones, cytospin preparations were made and stained with May-Grunwald-Giemsa. At least 300 cells from the cobblestones were counted.

Effect of MoAbs on the cobblestone formation. To examine the effect of c-Kit, VLA4, and VCAM-1 on the cobblestone formation, these antibodies were added at 3-day intervals to the coculture of the Lin− c-Kit+ Scal+ cells on the stromal cell layers, and the cobblestones were counted.

RESULTS

Selection of BM stromal cells to support hematopoietic stem cells. The establishment and characterization of these stromal cell lines from temperature sensitive (ts) SV40 T-antigen transgenic mice have been reported previously.17 To select BM stromal cell lines that support self-renewal or proliferation and differentiation of stem cells, 34 BM stromal cell lines were examined by coculturing BM nonadherent cells on each stromal cell layer. Each stromal cell line was observed for the presence of Lin− Scal+ c-Kit+ cells among more than 300 cells examined as described in Materials and Methods. The quality of the Lin− cells was confirmed by the absence of lineage markers in the FACS analysis as described in Materials and Methods and the sorted cells were of medium size and histologically immature hematopoietic cell type. In addition, no B220+ cells were observed among more than 300 sorted stem cells analyzed by immunostaining (data not shown). The colony-forming ability of the sorted stem cells in the semi-solid culture showed 35 to 45 colonies (CFU-Cs)/100 sorted cells, which was in agreement with the previous report.23 In HPP-CFC assay, three to five colonies were generated from 100 sorted cells. We used these preparations as

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Fig 1. Maintenance of cobblestone formation in coculture of BM cells with BM stromal cell (TBR) lines established from ts T-antigen transgenic mice. Nonadhesive BM cells (1 × 10^6 cells/flask) were cocultured with the stromal cell layer in 25-cm² flasks, and presence of the cobblestones was observed by phase-contrast microscopy. Values are the average number of colonies (CFU-CS)/flask ± SEM. A control stromal cell line, the preadipocyte cell line, PA6, was included. The results of 23 among 34 clones examined are shown in the figure.
sorted hematopoietic stem cells for the coculture with TBR59 stromal cells. When the HPP-CFC was measured during coculture, the value dropped gradually with the coculture (Table 1). Thus, it seemed that self-renewal of the sorted stem cells did not occur. However, we were able to identify the small colonies (CFU-Cs) containing more than 50 cells. The CFU-C increased for 1 week, then fell, and without stromal cells there was no colony formation. Thus, the stromal cells induced a transient increase in the CFU-C-forming cells from the sorted stem cells within 7 days of coculture. We then examined directly the fate of the sorted stem cells that were on the TBR59 stromal cells.

**Cobblestone formation of the sorted stem cells on the stromal cells.** During the coculture, we observed cobblestone formation of the hematopoietic cells. The formation was observed in three stromal cells among 34 stromal cell lines (data not shown). The round cells in the hematopoietic cobblestones were phase dense, indicating they lay underneath or attached to TBR59 cells. The hematopoietic cells within the cobblestone area had indented nuclei and no distinct features of mature hematopoietic cells, but were morphologically different from the initial sorted cells. The cobblestone formation was dependent on mostly the stem cell population because only c-Kit⁺ Sca1⁺ cell populations formed the cobblestones; c-Kit⁺ Sca1⁻ and c-Kit⁻ Sca1⁺ cell populations of the Lin⁻ hematopoietic cells rarely formed cobblestones containing a large number of cells (Fig 3A). The cobblestone formation continued for more than 30 days of the coculture. The maximum cobblestone size was measured by counting the number of phase-dense cells in each cobblestone as shown in Fig 4. In most cobblestones, the cell division cycles were 11 at most, with an average of 8. Thus, a cobblestone containing a maximum of 1,000 cells was generated by the rapid expansion of the sorted cells.

**Two waves of cobblestone formation on the stromal cells.** Generation of the cobblestones seemed to have two waves: one major peak on day 7 and a second peak on day 13 (Fig 3A). Cells were released from the cobblestones into the overlaying medium during coculture on TBR59 stromal cells. In the medium, the percentage of mature granulocytes was 80% on day 9 (2 days after the first peak) and slightly decreased to 70% on day 15 (2 days after the second peak), whereas the percentage of lymphocytes slightly increased from day 9 (20%) to day 15 (30%) (Fig 5, A and B). After 30 days of culture, the number of the cobblestones decreased to ≈10, but they were maintained for over 30 days. The two waves of cobblestone formation implied either the presence of two cell populations or two-step hematopoietic cell development responding to the stromal cells. We sorted the Lin⁻ c-Kit' Sca1⁻ stem cells into Lin⁻ c-Kitʰ Sca1ʰ (0.16% of Lin⁻ cells) and Lin⁻ c-Kitʰ Sca1ʰ (0.06% of Lin⁻ cells) populations and examined their colony formation on TBR59 stromal cell layers. The result indicated that the c-Kitʰ population formed the first wave of cobblestones and the c-Kitʰ population formed the second wave (Fig 3B), confirming that the two stem cell populations respond to stimulation by the stromal cells. The Lin⁻ c-Kitʰ Sca1ʰ population released mostly granulocytes, whereas the Lin⁻ c-Kitʰ Sca1ʰ population released mostly lymphocytes (Fig 5, A and B). Immunostaining showed that the released cells from c-Kitʰ were Gr1⁺-granulocytes (Fig 5C), whereas those from the c-Kitʰ population were B220⁺-lymphocytes (Fig 5D). The Lin⁻ c-Kitʰ Sca1ʰ population produced megakaryocytes, macrophages, and monocytes, most of which were attached to the stromal cells; few of them were released into the overlaying medium (data not shown).

**Molecules involved in the interaction between the stromal cells and stem cells that are required for cobblestone formation.** To know molecules involved in the interaction between the stromal cells and stem cells, MoAbs were added to the coculture. Addition of the anti-c-Kit antibody almost completely inhibited the two waves of cobblestone formation. The inhibition was observed in both the number (Fig
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Fig 3. Cobblestone formation of the sorted stem cell fractions. (A) Kinetics of cobblestone formation of the sorted stem cell fractions. Five hundred cells in each fraction of Lin- population were seeded on TBR59 cell layer. Cocultures were maintained at 37°C with α-MEM supplemented with 10% FBS and 2-ME. Foci containing more than 10 phase-dense cells were counted as cobblestones by inverted-phase microscopy. Lin- c-Kit+ Sca1+ fraction (□) generated cobblestones in two waves (days 7 and 13). Lin- c-Kit+ Sca1- (○) and Lin+ c-Kit- Sca1+ (△) fractions showed low cobblestone formation and Lin+ c-Kit- Sca1- (□) formed no cobblestones. The values were taken from three independent experiments. (B) Cobblestone-forming activities of c-Kithigh and c-Kitlow populations of Lin- Sca1+ cells. Five hundred cells of each of Lin- c-Kit+ Sca1+, Lin- c-Kitlow Sca1+ and Lin- c-Kitlow Sca1- populations were cocultured on TBR59 cell layer and cobblestones were counted. Lin- c-Kit+ Sca1+ (□) showed a single peak on day 7 and Lin- c-Kitlow Sca1+ (○) showed a single peak on day 11. Values are the average of four dishes from two independent experiments. (C) The effect of anti-c-Kit antibody on cobblestone formation. Five hundred Lin- c-Kit+ Sca1+ cells were cocultured on TBR59 cell layers. c-Kit antibody was added at 0 (□), 1 (○), 10 (△) or 50 (△) µg/mL. At each medium change (3-day intervals), the antibody was added to the culture and the cobblestones were counted. Values are the mean of two dishes.

Fig 4. Number of phase-dense cells in a cobblestone. Pictures taken daily allowed determination of the maximum number of phase-dense cells in each cobblestone; distribution of the cells in a single cobblestone was shown in 21 cobblestones.

3C) and size (data not shown) of cobblestones. Then the involvement of the adhesion molecules on the cobblestone formation was examined. The inhibitory activities of anti-VLA-4 antibody were saturated at the concentration of 1 µg/mL, but anti-VLA-4 antibody inhibited partially (40%) in the first wave of cobblestone formation even at a high dose and inhibited completely (80% to 90%) in the second wave (Fig 6A). The inhibitory effect of this antibody suggested the presence of ligand molecules for this integrin in the
A 9 Days  Percentage

Granulocyte

Monocyte / Macrophage

Lymphocyte

B 15 Days  Percentage

Granulocyte

Monocyte / Macrophage

Lymphocyte

DISCUSSION

By definition, stem cells have extensive capacity for self-renewal that extends throughout the life of the organism and, thus, is essentially unlimited. In contrast, the number of rounds of divisions that a transient amplifying cell undergoes is finite; transient amplifying keratinocytes undergo terminal differentiation within 15 cell generations in culture\(^\text{11}\): the O-2A progenitor cells of rat optic nerve undergo a finite number of divisions in the presence of platelet-derived growth factor-produced by type 1 astrocytes before differentiating into oligodendrocytes.\(^\text{32}\) The mechanism that determines the number of divisions in these situations is unknown, but one possibility is that the cells are able to count cell divisions and after an appropriate number undergo terminal differentiation.\(^\text{32,33}\)

In the present work, we showed that an established stromal cell line, TBR59, can induce a finite number of divisions committed to myelopoiesis and lymphopoiesis of the BM hematopoietic stem cells sorted as Lin\(^-\) c-Kit\(^+\) Sca1\(^+\) cells.\(^\text{25}\) This finite division was observed by a cobblestone formation in the absence of exogenously added soluble factors and was promoted by particular stromal cells established from BM because we found that only three stromal cells among 34 established stromal cell lines that could induce cobblestone formation of the sorted stem cells. We established many BM stromal cell lines from ts T-antigen transgenic mouse that showed characteristics of endothelial cells, preadipocytes, and fibroblasts,\(^\text{17}\) but in our analysis, the stem cell supporting ability was only observed in three preadipocyte cell lines (data not shown). In TBR59 cells, adipocyte conversion was induced at 39°C or in the presence of dexamethasone (data not shown). Even at 37°C, TBR59 cells differentiated slightly to adipocytes, and a slight temperature shift from 37°C affected the stem cell supporting ability (R. Okuyama, Fig 5. Characterization of the differentiated blood cells from c-Kit\(^{high}\) and c-Kit\(^{low}\) populations of Lin\(^-\) Sca1\(^+\) cells. Sorted cells were cocultured on the TBR59 cell layers. The hematopoietic cells released to the overlying medium from the coculture of Lin\(^-\) c-Kit\(^+\) Sca1\(^+\) (III), Lin\(^-\) c-Kit\(^{low}\) Sca1\(^+\) (II), and Lin\(^-\) c-Kit\(^{low}\) Sca1\(^+\) (I) were recovered by centrifugation onto glass slides at day 9 (A) and day 15 (B) and stained with May-Grünwald-Giemsa. Characterization of the differentiated blood cell types was performed by microscopic observation of more than 100 cells in every fraction. In addition, released cells were immunologically stained with anti-Gr1 antibody (C) or B220 antibody (D).
unpublished observation, June 1994), suggesting that the differentiated phenotype of the stromal cells is important in regulation of the stem cells.

We observed two waves of cobblestone formation from Lin<sup>-</sup> c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells; the first wave was derived from the c-Kit<sup>high</sup> population, whereas the second was from the c-Kit<sup>low</sup> population. The mature cells released from c-Kit<sup>high</sup> population were mostly myeloid lineages, whereas those from c-Kit<sup>low</sup> population were mostly lymphocytes, indicating that the c-Kit<sup>high</sup> stem cell population may be committed to myeloid lineages on the stromal cells, whereas the c-Kit<sup>low</sup> population may be committed to lymphopoiesis. Because B220<sup>+</sup> lymphoid progenitor cells were not detected in the sorted stem cells, lymphopoiesis may not be caused by expansion of the contaminated lymphocyte progenitors in the absence of hydrocortisone. In addition, supplementation of IL-3 to the coculture-stimulated cobblestone formation in the first wave with an increase in the released cells of myeloid lineage, but was not stimulatory for the second wave (R. Okuyama, unpublished observation, August 1994). Because a previous report suggested that the c-Kit<sup>low</sup> population contains more immature stem cells, our result could not exclude the possibility that c-Kit<sup>low</sup> population starts developing at a later time point and has the potential to generate both lymphoid and myeloid cells, but it is more likely that the two populations have different potentials for the lineage commitment. This is the first demonstration of clear separation of the stem cell populations into lymphopoiesis and myelopoiesis. We recently found that another stromal cell line also predominantly supported lymphopoiesis of the sorted stem cells (Koguma et al, manuscript in preparation). These coculture systems will be useful for analysis of commitment of the hematopoietic stem cells in vitro.

Whereas myelopoietic and lymphopoietic development of the stem cells could be induced by the stromal cells, we were unable to induce self-renewal of the stem cells by the stromal cells, because their HPP-CFC formation dropped rapidly during coculture. This is consistent with previous reports that the sorted stem cells cannot self-renew, even in the presence of stromal cells, although Fraser et al reported that the stem cells can self-renew in vitro on the BM stromal cells. There may be several possible reasons for the inability of sorted stem cells to self-renew: (1) the stromal cells that can support self-renewal of the sorted stem cells have not yet been obtained, and thus, more BM stromal cells must be screened of this ability; (2) self-renewal may require a mixture of stromal cells with different abilities (although this does not seem likely from the previous report on Dexter’s culture, the possibility is testable by a mixed culture of a variety of established stromal cells); (3) in addition to the stromal cells, a factor produced by other blood cells may be required for stem cell self-renewal.

We examined molecules involved in the interaction between the stromal and stem cells. c-Kit is thought to be essential for stem cell proliferation and we showed that c-Kit is essential for cobblestone formation; thus, c-Kit and its ligand, SCF, whose expression in TBR59 cells was confirmed by reverse-transcriptase polymerase chain reaction (R. Okuyama, unpublished observation, January 1995), are essential for proliferation of the sorted stem cells on the stromal cells. The importance of adhesive interactions in hematopoiesis has been well studied in lymphoid organs; integrins VLA-4, VLA-5, and VLA-6 are responsible for proliferation and activation of lymphocytes. The involvement of VLA-4 and V-CAM-I in the association of hematopoietic stem cells and stromal cells as well as in the stroma-dependent erythropoiesis has recently been reported. We confirmed the expression of VCAM-1 in TBR59 cells by FACS analysis (data not shown). Antibody against VLA-
4 inhibited cobblestone formation in the first and second waves, but interestingly, in the first wave (myelopoiesis), inhibition of VLA-4 was partial, whereas that of VLA-4 was complete in the second wave (lymphopoiesis). The inhibition of antibody against VCAM-1, a ligand for VLA-4, gave essentially the same result, suggesting that VLA-4–VCAM-1 interaction participates more strongly in lymphopoiesis than in myelopoiesis. It is likely that in myelopoiesis the hematopoietic cells must adhere to the stromal cells through VLA-4–VCAM-1 interaction, but their continuous interaction is not required for further proliferation and differentiation of the hematopoietic cells. However, their continuous interaction may be required for lymphopoiesis. Because expression of SCF and VCAM-1 may not be restricted to the stromal cells that induced the cobblestone formation, they alone may not be sufficient to support the sorted stem cells, although their expression is essential. The search for the molecules whose expression is restricted to TBR59 cells may be important for understanding the molecules involved in the stromal–stem cell interaction.

In conclusion, we established a new in vitro system for myelopoietic and lymphopoietic development of sorted stem cells using cloned BM stromal cells. This system will be valuable for analyzing the mechanism of lineage commitment of multipotent hematopoietic stem cells in vitro at the cellular and molecular levels.

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