Enhanced Hematopoiesis In Vivo and In Vitro by Splenic Stromal Cells Derived From the Mouse With Recombinant Granulocyte Colony-Stimulating Factor

By Naoshi Fukushima, Hiromichi Nishina, Yasuo Koishihara, and Hiroyuki Ohkawa

Splenic stromal cells (CF-1 cells) were established from a mouse administered recombinant human granulocyte colony-stimulating factor (rG-CSF) to clarify the mechanism of splenic extramedullary hematopoiesis induced by the factor. The cells were negative for alkaline phosphatase, factor VIII-related antigen, mac I, and phagocytosis. They were positive for acid phosphatase, collagen type I, collagen type III, and fibronectin. CF-1 cells were not converted to adipocytes in a confluent culture with 10⁻² mol/L hydrocortisone. [³⁵S]rG-CSF bound to CF-1 cells specifically in the growth phase but not in the resting phase. The CF-1 cells had greater colony-stimulating activities than the normal splenic stromal cells. When CF-1 cells were added to bone marrow cells in the spleen colony-forming cells (CFU-S) assay, the number of colonies in the spleen increased between 1.4 and 1.8 times the control without these stromal cells. On the other hand, the normal splenic stromal cells had no effect on increasing the number of CFU-S colonies. Therefore, these data suggest that a factor-dependent hematopoietic microenvironment is generated in the spleen by rG-CSF, and the stromal cells that have the hematopoietic potency become dominant in splenic extramedullary hematopoiesis induced by rG-CSF.

A LARGE NUMBER of studies have suggested that hematopoiesis is supported by hematopoietic microenvironment of the bone marrow (BM).1-3 Dexter et al4 and other investigators5-6 have shown that BM stromal cells support the growth of hematopoietic stem cells in in vitro long-term BM cultures. Several stromal cell lines have been established from BM to investigate the mechanism of hematopoiesis. These data show that BM stromal cells secrete hematopoietic cytokines that stimulate the proliferation and differentiation of hematopoietic stem cells and progenitor cells,7 12 and regulate hematopoiesis by cell-to-cell interactions13-17 or by extracellular matrix.16,18-21 In the case of splenic stromal cells, some data have been reported which show that these cells in the newborn mouse selectively support erythroid colony formation.22 However, splenic stromal cells in the adult mouse have not been well studied for hematopoietic effects in support of granulocyte-macrophage (GM) progenitor and hematopoietic stem cells. The spleen microenvironment in the adult mouse could have potency supportive of hematopoietic stem cells because an assay of spleen colony-forming cells (CFU-S) has been used to detect hematopoietic stem cells. Furthermore, in increased hematopoiesis in mice administered granulocyte colony-stimulating factor (G-CSF) exogenously, hematopoietic stem cells and all of the progenitor cells have been seen to increase greatly in the spleen, but not in the BM.23,24 Therefore, it should be pointed out that there exist dormant splenic stromal cells which can support hematopoietic stem cells and that these stromal cells become dominant by stimulation of this hematopoietic factor.

In this report, we describe the hematopoietic potential of splenic stromal cells investigated to clarify the mechanism of extramedullary hematopoiesis by recombinant human G-CSF (rG-CSF). We hypothesize that a factor-dependent hematopoietic microenvironment can be generated to maintain hematopoiesis in the spleen by stimulation with rG-CSF. For this study, we established splenic stromal cells derived from a mouse administered rG-CSF and show that these cells support the proliferation and differentiation of GM progenitor cells and maintain hematopoietic stem cells.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice were purchased from Shizuoka Animal Farm (Shizuoka, Japan) and kept under specific pathogen-free conditions. An 8-week-old mouse was administered rG-CSF 100 µg/kg for 5 days daily subcutaneously to isolate the spleen stromal cells. Mice that were 7 to 9 weeks old were used as the control splenic stromal cell and BM cell cultures and as the recipient mice for the CFU-S assay.

rG-CSF

rG-CSF was purified to homogeneity from media conditioned by Chinese hamster ovary cells transfected with human G-CSF cDNA. rG-CSF was dissolved in phosphate-buffered saline (PBS) to a final concentration 10 µg/mL.

Establishment of Stromal Cells

A splenic stromal cell line was established from primary culture of the spleen cells of a C57BL/6J mouse administered rG-CSF 100 µg/kg for 5 days. After the administration of rG-CSF, this spleen was removed under germ-free conditions, and cultured in a 25-cm² plastic flask (Corning Co, Corning, NY) for 6 weeks. This culture was maintained in Iscove's modified Dulbecco's medium (IMDM) (Boehringer-Mannheim Co, Mannheim, Germany) with 10% heat-inactivated fetal bovine serum (FBS) (Sanko Jyunyaku, Tokyo, Japan), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5.0% CO₂ atmosphere, and the medium was exchanged for fresh growth medium twice a week. In the confluent culture, the adherent cell populations (stromal) were harvested from the flask by using 0.05% trypsin plus 0.02% EDTA (Sigma Chemical Co, St Louis, MO) in Ca₂⁺-, Mg-free PBS, and were transferred into new flasks. These passages were repeated approximately once or twice a week. In the early passages (1st through 10th passages), the split ratio of the cells was 1/4 to 1/8, and subsequently the ratio was...
1/16 to 1/32. After approximately the 10th passage, the stromal cells became homogeneous and fibroblastoid. At the 20th passage, the stromal cells were harvested as described above and forwarded to cell cloning by using a limiting dilution technique. Cell cloning was repeated twice. After this, a CF-1 cell line was established. These cells were maintained in 5 mL of IMDM supplemented with 10% heat-inactivated FBS in a 25-cm² flask (Corning). They were harvested and subcultured once every 5 days at the split ratio of 1/32. Control splenic stromal cell lines were established from the spleens of the normal C57BL/6J mice using the same method as for the CF-1 cells. Two cell lines (H and K cells) were established from each normal animal and used as the control of the CF-1 cells.

Examination of the Characteristics of the CF-1 Cells

CF-1 cells were examined for alkaline phosphatase, acid phosphatase, ?-glucuronidase, ?-naphthyl acetate esterase, and oil red O using standard cytotoxic techniques. CF-1 cells were also characterized by immunoenzymatic histochemistry using the following monoclonal and polyclonal antibodies: mac I (SeroTec, Oxford, England); factor VIII-related antigen (Dakopatts, Glostrup, Denmark); and collagen type I, collagen type III, and fibronectin (Chemicon International, Inc, Temecula, CA). Phagocytosis was tested by latex bead uptake (particle diameter: 1.09 mm, Sigma). The ability of CF-1 cells to convert to adipocytes was tested by exposure to 10⁻⁶ mol/L hydrocortisone phosphate (Sigma) in a 25-cm² flask for 4 weeks after the confluent culture.

Binding of ³⁵S]rG-CSF to CF-1 Cells

A binding assay was performed to investigate the specific binding of G-CSF to CF-1 cells. CF-1 cells were removed from dishes with a rubber policeman at the subconfluent and over-confluent stage, and suspended with PBS. [³⁵S]rG-CSF was supplemented into this cell suspension and incubated on ice for 5 hours. To assay the nonspecific binding of r-G-CSF, [³⁵S]rG-CSF and cold r-G-CSF (3,000 times as much as [³⁵S]rG-CSF) were supplemented and incubated by the same procedure as described above. After centrifugation (320g for 5 minutes), the supernatants were removed and the cells were suspended with the extraction buffer (0.5% NP-40 buffer). Cell suspensions were homogenized and centrifuged (8,050g for 5 minutes). The supernatants were then examined by molecular-sieve high-performance liquid chromatography (HPLC). A TSK-gel G3000SW column (7.5 ? 300.0 mm; Tosoh, Tokyo, Japan) was used to examine the samples with 10 mmol/L phosphate buffer (pH 7.2) at a flow rate of 1 mL/min on the HPLC radio system (Tosoh radio LC system; Tosoh).

CFU in Culture (CFU-C) Assay

To investigate the production of colony-stimulating activity (CSA) from CF-1 cells, CFU-C assays with feeder layers were undertaken in 24-well plastic dishes (Corning). In the subconfluent culture of CF-1, H, and K cells, the cells, as the feeder layers, were overlaid with 0.5 mL of 0.3% Matrigel (DIFCO, Detroit, MI) in complete growth medium (IMDM with 20% heat-inactivated FBS, 20 µg/mL transferrin [Boehringer Mannheim], and 20 µg/mL soybean-leipids [Boehringer Mannheim]) containing 5.0 x 10⁴ or 2.5 x 10⁴ fresh mononuclear BM cells. The number of colonies was counted after 6 days of culture at 37°C.

Assay for Interleukin-3 (IL-3) Activity

IL-3 activity was evaluated by using an IL-3-dependent cell line, FDC-P2 cells. FDC-P2 cells were kindly provided by Dr K. Kumagai (Tohoku University, Sendai, Japan). These cells were maintained in IMDM supplemented with 10% heat-inactivated FBS and WEHI-3 conditioned medium. The cells were washed intensively in IMDM and FDC-P2 cells were cultured at a concentration of 2.8 x 10⁴ cells/0.1 mL (IMDM supplemented with 10% heat-inactivated FBS)/96 microwell (Corning), and the conditioned media of CF-1, H, and K cells, at 10x concentration, were supplemented in this culture by 10%. The conditioned media were prepared from supernatants of stromal cells without FBS for 3 days after the confluent culture. After incubation for 4 days, the growth of the cells was evaluated by the MTT method.

Assay for GM-CSF Activity

GM-CSF activity was evaluated by using an IL-3-dependent and GM-CSF-dependent cell line, DA-1 cells. DA-1 cells were kindly provided by Dr M. Kawakita (Kumamoto University, Kumamoto, Japan). These cells were maintained in IMDM supplemented with 10% heat-inactivated FBS and IL-3. The cells were washed intensively in IMDM, and DA-1 cells were cultured at a concentration of 7.0 x 10³ cells/0.1 mL (IMDM supplemented with 10% heat-inactivated FBS)/96 microwell and the conditioned media of CF-1, H, and K cells, at 10x concentration, were supplemented in this culture by 10%. The conditioned media were prepared from supernatants of stromal cells supplemented with IL-1α (1.0 U/mL) (Boehringer Mannheim) without FBS for 3 days after the confluent culture. After incubation for 4 days, the growth of the cells was evaluated by the MTT method. Furthermore, to determine whether the growth of DA-1 cells caused by GM-CSF, growth inhibition tests were undertaken using rabbit antiserum GM-CSF polyclonal antibody, which was able to neutralize GM-CSF activity completely.

Assay for IL-6 Activity

IL-6 activity was evaluated by using an IL-6-dependent mouse hybridoma clone, MH60.BSF2.28 MH60.BSF2 cells were kindly provided by Drs T. Hirano and T. Kishimoto (Osaka University, Osaka, Japan). These cells were maintained in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FBS and IL-6. The cells were washed intensively in RPMI-1640, and MH60.BSF2 cells were cultured at a concentration of 1.0 x 10⁶ cells/0.2 mL (RPMI-1640 supplemented with 10% heat-inactivated FBS) and the conditioned media of CF-1, H, and K cells. The conditioned media were prepared from supernatants of stromal cells supplemented with IL-1α (1.0 U/mL) for 3 days were also examined. After incubation for 2 days, 1 µCi H-thymidine was supplemented during the last 4 hours of the culture. The cells were then harvested and the radioactivity was counted with a scintillation counter (Betaplate, LKB-1205; Pharmacia, Turku, Finland).

Observation of Coculture Between CF-1 Cells and BM Cells

To investigate whether the hematopoietic stem and progenitor cells proliferate on CF-1 cells or not, BM cells were seeded onto a confluent culture of CF-1 cells at a concentration of 1.0 x 10⁶/25-cm² flask. A confluent culture of CF-1 cells was used for the cocultivation experiment. This culture was maintained for 3 weeks and hematopoiesis was observed by phase-contrast microscopy.

Assay for the Maintenance of Hematopoietic Stem Cells

To examine the hematopoietic stem cells maintained by CF-1 cells, CFU-S assays were performed by the technique of Till and McCulloch.29 Ten mice per group were irradiated with 900 cGy (MBR-1520R; Hitachi, Tokyo, Japan) and injected intravenously.
(IV) with BM cells (1.0 × 10⁵/head, 5.0 × 10⁴/head, or 2.5 × 10⁴/head) and CF-1 cells (1.0 × 10⁵/head). Colonies in the spleen were counted on the 12th day as CFU-S. H and K cells were examined as controls in the same manner.

**Lodgment of CF-1 Cells in the Spleen**

To investigate whether CF-1 cells maintain hematopoietic stem cells in the spleen directly or not, we examined the lodgment of CF-1 cells in the spleen by using the cells labeled with ⁵¹Cr or ³H-thymidine.

**Experiment using ⁵¹Cr.** CF-1 cells were harvested from flask and suspended with PBS. ⁵¹Cr was supplemented into this cell suspension (10 μCi/mL at the final concentration) and incubated at 37°C in a 5.0% CO₂ atmosphere for 4 hours. After the incubation, the cells were washed by PBS four times, suspended with PBS, and injected IV into the mice. After 2, 4, 24, and 48 hours, six mice per group were killed to remove spleen, bilateral femurs (BM), lung, liver, kidneys, brain, heart, thymus, and testes. The radioactivities of these organs were counted with γ-counter (ARC-300; Aloka Co Ltd, Tokyo, Japan). By the radioactivities of the dilution of CF-1 cells, the cell numbers were estimated in every organ.

**Experiment using ³H-thymidine.** CF-1 cells were cultured supplemented with ³H-thymidine (1 μCi/mL at the final concentration) for 2 days. After that, CF-1 cells were washed by PBS three times, harvested, and injected IV (1 × 10⁵/head) into the irradiated mice (900 cGy). Shortly after the injection, the mice were killed to remove spleen, lung, liver, and kidney (BM was not examined). These were fixed with 20% phosphate-buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The presence of CF-1 cells was examined by using the autoradiography technique.

**RESULTS**

**Establishment of Splenic Stromal Cell Lines**

A CF-1 cell line was established from primary spleen culture of a mouse administered rG-CSF. A phase-contrast micrograph shows the appearance of CF-1 cells (Fig 1). CF-1 cells have a fibroblastoid appearance at low concentrations, but they become epithelioid in confluent culture. On the other hand, H and K cells (control splenic stromal cells) have the fibroblastoid appearance even in the confluent culture.
Table 1. Characteristics of CF-1 Cells

<table>
<thead>
<tr>
<th>Staining (characteristics)</th>
<th>CF-1 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>+</td>
</tr>
<tr>
<td>α-Naphthyl acetate esterase</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>–</td>
</tr>
<tr>
<td>Oil red O</td>
<td>±</td>
</tr>
<tr>
<td>Factor VIII-related antigen</td>
<td>–</td>
</tr>
<tr>
<td>Mac I</td>
<td>–</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>–</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>+</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>+</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>–</td>
</tr>
<tr>
<td>Adipose conversion (10^-6 mol/L hydrocortisone)</td>
<td>–</td>
</tr>
</tbody>
</table>

Characteristics of CF-1 Cells

The histochemical and immunohistochemical profiles of CF-1 cells are summarized in Table 1. CF-1 cells were negative for alkaline phosphatase, factor VIII-related antigen, Mac I, and phagocytosis, whereas they were positive for acid phosphatase, β-glucuronidase, α-naphthyl acetate esterase, collagen type I, collagen type III, and fibronectin. CF-1 cells were not converted to adipocytes during 4 weeks in a confluent culture with 10^-6 mol/L hydrocortisone, although CF-1 cells had only traces of lipids. From these data, CF-1 cells do not have the characteristics of preadipocytes, macrophages, and endothelial cells.

Binding of[^35S][rG-CSF to CF-1 Cells

When CF-1 cell suspension with[^35S][rG-CSF was applied to HPLC, the peak was eluted at the 10-minute position, which was in a larger molecular weight position than that of[^35S][rG-CSF (18-minute position) (Fig 3A). The 10-minute peak is the peak of the complex of G-CSF receptor and[^35S][rG-CSF. Because this peak decreased, when cold rG-CSF was supplemented 3,000 times as much as[^35S][rG-CSF (Fig 3B); furthermore, the same phenomenon occurred in BM cells (data not shown). In the over-confluent culture of CF-1 cells, the peak was hardly eluted at the 10-minute position (Fig 3C). From these data, CF-1 cells express the G-CSF receptor in the growth phase, but not in the resting phase.

CSA of CF-1 Cells

In the CFU-C assay containing no stimulators, CF-1 cells stimulated the formation of greater numbers of colonies (GM, granulocyte, and macrophage colonies) than H and K cells (Table 2).

FDC-P2 cells, an IL-3-dependent cell line, were proliferated very slightly by supplement with a conditioned medium of CF-1 cells, but not at all by those of H and K cells. CF-1 cells produced IL-3 at 0.7 U/mL for 3 days in IMDM without FBS (Fig 4).

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**Fig 3.** The specific binding of[^35S][rG-CSF to CF-1 cells. At the subconfluent culture, the peak was eluted at the 10-minute position, which was in a larger molecular weight position than that of[^35S][rG-CSF (18-minute position) (A). This peak decreased by supplement with cold rG-CSF 3,000 times as much as[^35S][rG-CSF (B). At the over-confluent culture of CF-1 cells, the peak was hardly eluted at the 10-minute position (C).
Table 2. Potential Effect of CF-1 Cells on Colony Formation of Committed Hematopoietic Progenitor Cells In Vitro

<table>
<thead>
<tr>
<th>Feeder Layer</th>
<th>BM Cells (1 x 10⁴/well)</th>
<th>No. of Colonies (/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GM</td>
</tr>
<tr>
<td>None</td>
<td>5.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>H cells</td>
<td>5.0</td>
<td>30.8 ± 5.6</td>
</tr>
<tr>
<td>K cells</td>
<td>5.0</td>
<td>14.4 ± 4.3</td>
</tr>
<tr>
<td>CF-1 cells</td>
<td>5.0</td>
<td>106.0 ± 11.9*</td>
</tr>
<tr>
<td>None</td>
<td>2.5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>H cells</td>
<td>2.5</td>
<td>12.2 ± 2.6</td>
</tr>
<tr>
<td>K cells</td>
<td>2.5</td>
<td>9.8 ± 4.1</td>
</tr>
<tr>
<td>CF-1 cells</td>
<td>2.5</td>
<td>54.6 ± 4.4*</td>
</tr>
</tbody>
</table>

Abbreviations: No. of Colonies, mean ± SD of CFU-C colonies/well; GM, number of granulocyte-macrophage colonies; G, number of granulocyte colonies; M, number of macrophage colonies.

*pSignificantly different from H and K cells (P < .01).

In the proliferation assay using DA-1 cells, a GM-CSF- and IL-3-dependent cell line, conditioned media supplemented with IL-1α were used. DA-1 cells were greatly proliferated by supplement with the conditioned medium of CF-1 cells, which had been previously stimulated for 3 days by IL-1α (1.0 U/mL), although not by those of H and K cells with IL-1α (1.0 U/mL). CF-1 cells produced GM-CSF at 25.0 U/mL for 3 days in IMDM supplemented with IL-1α (1.0 U/mL) without FBS. However, the production of GM-CSF by H and K cells was not detected in this experiment. Furthermore, the proliferation of DA-1 cells by a conditioned medium of CF-1 cells with IL-1α was almost completely inhibited by the rabbit antimouse GM-CSF polyclonal antibody (Fig 5).

IL-6 Activity

In the IL-6 assay, MH60.BSF2 cells, an IL-6-dependent cell line, were proliferated by conditioned media of CF-1, H, and K cells. When the conditioned media of CF-1, H, and K cells previously stimulated by IL-1α (1.0 U/mL) were supplemented, the proliferations of MH60.BSF2 cells were strikingly increased to 3.6, 20.9, and 4.7 times those without stimulation with IL-1α (Fig 6). Furthermore, these IL-6 activities were completely inhibited by the rat antimouse IL-6 monoclonal antibody (data not shown).

Coculture of CF-1 Cells and BM Cells

The formation of cobblestones was observed for 3 weeks. As shown in Fig 7, the cobblestones were found to adhere to CF-1 cells. This shows that CF-1 cells have the potential to make hematopoietic cells adhere and proliferate in vitro.

Maintenance of Hematopoietic Stem Cells by CF-1 Cells

When BM cells and CF-1 cells were transplanted into irradiated mice, the number of spleen colonies significantly increased (between 1.4 and 1.8 times) compared with that of the mice without CF-1 cells at every group of BM cells. Furthermore, on the 12th day, the survival ratios of the mice transplanted with BM and CF-1 cells were higher than those with only BM cells. In fact, the number of spleen colonies in the mice transplanted with BM cells and H or K
cells decreased slightly, compared with mice transplanted with only BM cells (Table 3).

**Lodgment of CF-1 Cells in the Spleen**

The lodgment of CF-1 cells was recognized in the spleen, femurs (BM), lung, liver, and kidneys, but not in the brain, testes, thymus, and heart. The cell numbers in the spleen and bilateral femurs (BM) increased significantly with time (Fig 8). On the other hand, the cell numbers in the lung decreased greatly with time, and those of liver and kidneys did not change, although their cell numbers were numerous (data not shown). These data showed that CF-1 cells lodged specifically in the spleen and BM.

In the autoradiography study, CF-1 cells labeled with \(^3\)H-thymidine were observed in the spleen (Fig 9). Besides the spleen, the cells also were present in the lung, liver, and kidney of the mice (data not shown).

**DISCUSSION**

The hematopoietic microenvironment in the spleen was investigated through stromal cells for extramedullary hematopoiesis in mice administered rG-CSF. We established the splenic stromal cells, ie, CF-1 cells, derived from the mouse administered rG-CSF, and show clearly that CF-1 cells have a potential effect on hematopoiesis and have the G-CSF receptor. These cells supported the proliferation and differentiation of GM progenitor cells in vitro and maintained hematopoietic stem cells in vivo. On the other hand, normal splenic stromal cells (H and K cells) did not show this hematopoietic effect. These data suggest that modification of the hematopoietic microenvironment occurred, and that stromal cells supporting GM progenitor and hematopoietic stem cells become dominant in the spleen by stimulation with rG-CSF.

Hematopoietic tissue consists of hematopoietic cells and stromal cells. Recently, it has been shown that some

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**Table 3. Maintenance of Hematopoietic Stem Cells by CF-1 Cells in BM Transplantation**

<table>
<thead>
<tr>
<th>No. of BM Cells* (x10^5/h)</th>
<th>Stromal Cells† (1.0 x 10^5/h)</th>
<th>No. of CFU-S</th>
<th>No. of Surviving Animals on Day 12 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>K cells</td>
<td>7.0 ± 2.5</td>
<td>90</td>
</tr>
<tr>
<td>10.0</td>
<td>H cells</td>
<td>7.0 ± 4.9</td>
<td>90</td>
</tr>
<tr>
<td>10.0</td>
<td>CF-1 cells</td>
<td>13.3 ± 3.0†</td>
<td>100</td>
</tr>
<tr>
<td>5.0</td>
<td>CF-1 cells</td>
<td>3.6 ± 1.5‡</td>
<td>80</td>
</tr>
<tr>
<td>2.5</td>
<td>CF-1 cells</td>
<td>6.1 ± 2.5‡</td>
<td>100</td>
</tr>
<tr>
<td>2.5</td>
<td>CF-1 cells</td>
<td>3.6 ± 1.6</td>
<td>70</td>
</tr>
<tr>
<td>0.0</td>
<td>CF-1 cells</td>
<td>0.0 ± 0.0</td>
<td>50</td>
</tr>
</tbody>
</table>

The number of CFU-S are the mean ± SD of spleen colonies of surviving animals.

*Donor BM cells were transplanted IV at 1.0 x 10^5, 5.0 x 10^5, 2.5 x 10^5/head.
†Stromal cells were transplanted IV at 1.0 x 10^5/head, and the nontreated group was not.
‡Significantly different from control (P < .05).
The hypothesis that a factor-dependent hematopoietic microenvironment is generated in the spleen by stimulation with rG-CSF. It follows that stromal cells (CF-1 cells) should be able to be proliferated by rG-CSF. CF-1 cells could only be established when administered with rG-CSF, and could not be established from the normal mouse spleen. Furthermore, by analysis of the G-CSF receptor, it has become clear that CF-1 cells express the G-CSF receptor in the growth phase but not in the resting phase.

The origin of CF-1 cells would appear to have to be from splenic stromal cells. According to examinations of cytochemical and immunoenzymatic histochemistry, CF-1 cells are negative for alkaline phosphatase activity and do not have the characteristics of preadipocytes, macrophage, and endothelial cells, whereas a lot of mouse BM stromal cell lines reported to date are preadipocytes, and almost all are positive for alkaline phosphatase activity. In addition, CF-1 cells may be too large to migrate freely through blood vessels from the BM, based on our observation that some mice died of infarction soon after the transplantation of the cells. Considering the origin and the hematopoietic potential of CF-1 cells, CF-1 cells may be a critical part of the spleen hematopoietic microenvironment required for CFU-S colony formation in the mouse spleen.

To speak of the hematopoietic potential of CF-1 cells, two mechanisms seem possible. One is caused by the cytokines that CF-1 cells produce. CF-1 cells produce GM-CSF from the results of the CFU-C assay and the DA-1 cells’ proliferation assay. Furthermore, CF-1 cells produce IL-3 and IL-6, and it is thought that these factors play an important role in maintaining hematopoietic stem cells. It has been reported that IL-6 has synergistic activity with IL-3 and stimulates hematopoietic stem cells to shorten the period of G0. We have examined the expression of G-CSF mRNA in CF-1 cells by Northern blot analysis, but the cells did not express G-CSF mRNA at all (data not shown). The other mechanism might be caused by the adhesion molecules that play a role in the homing of hematopoietic stem cells. It has been shown that hematopoietic stem cells adhere to stromal cells by the homing receptor. In the coculture of CF-1 and BM cells, cobblestones were observed. This shows that CF-1 cells have the potential to make the hematopoietic stem cells adhere and proliferate in vitro. Furthermore, we have shown that CF-1 cells lodge in the spleen and BM specifically by the IV injection of the cells and we think that the cells support the hematopoietic stem cells in the spleen directly. In the present study, these two mechanisms are related to maintenance of the hematopoietic stem cells by CF-1 cells, and study on the role of the adhesion molecules is ongoing.

In conclusion, our data show that CF-1 cells, established from the mouse spleen with rG-CSF, have hematopoietic potential in support of proliferation and differentiation of
GM progenitor cells and in maintenance of hematopoietic stem cells. These data suggest that rG-CSF generates a factor-dependent hematopoietic microenvironment that provides a suitable milieu for hematopoietic cells.

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ACKNOWLEDGMENT

We thank Dr K. Kumagai for providing FDC-P2 cells, and Dr M. Kawakita for providing DA-1 cells. We also thank Drs T. Hirano and T. Kishimoto for providing MH60.BSF2 cells.
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Enhanced hematopoiesis in vivo and in vitro by splenic stromal cells derived from the mouse with recombinant granulocyte colony-stimulating factor

N Fukushima, H Nishina, Y Koishihara and H Ohkawa