Murine Spleen Stromal Cell Line SPY3-2 Maintains Long-Term Hematopoiesis In Vitro

By Junjiro Tsuchiyama, Masaharu Mori, and Shigeru Okada

The hematopoietic microenvironment (HIM) of mouse spleen predominantly induces the differentiation of hematopoietic progenitors into erythroid lineage in vivo. However, the mechanisms of this phenomenon have not been fully explored because of the lack of an adequate in vitro system mimicking the spleen hematopoiesis. To reconstruct the HIM of mouse spleen in vitro, we established spleen stromal cell lines from a three-dimensional (3D) spleen primary culture in collagen gel matrix. Of these, SPY3-2 cells were negative for preadipocytic and endothelial markers, had a fibroblastic morphology, and were not converted to adipocytes in the presence of 1 μmol/L hydrocortisone. They supported the maintenance and multilinear differentiation of hematopoietic progenitor cells for more than 8 weeks in vitro. The differentiated hematopoietic cells in the coculture medium were predominantly monocytes rather than granulocytes.

Furthermore, erythropoiesis was predominantly induced in the presence of 2 U/mL erythropoietin and continued for more than 12 weeks. The number of burst-forming units–erythroid (BFU-E) was increased 10 times after 3 weeks of coculture, which was followed by pronounced production of erythroid cells in the coculture after week 4. SPY3-2 expressed high levels of c-kit ligand and low levels of granulocyte macrophage colony-stimulating factor and interleukin-3, and these molecules were all involved in this long-term erythropoiesis. Thus, the clonal SPY3-2 cell line will provide a novel HIM in vitro analogous to that of mouse spleen in vivo. These results suggest that 3D collagen gel culture may facilitate the establishment of functioning stromal cell lines of hematopoietic organ.

© 1995 by The American Society of Hematology.
After further washing with PBS, they were incubated with ABC for pretreated with normal serum and incubated with the first antibody. Avidin-biotin-peroxidase complex (ABC) method. Briefly, these coverslips were body (Advance, Tokyo, Japan), employing the avidin-biotin-peroxidase, acid phosphatase, and a-naphthyl butyrate esterase, or were drochloride and 0.02% hydrogen peroxide in PBS. Cover slips 12-mm in diameter in a 24-well plate (Falcon 3047; Becton Dickinson, Lincoln Park, NJ) with 1 mL of IMDM containing 20% FBS. The cells reached confluence after 60 minutes. After further washing with PBS, they were stained for hematopoietic cells from the cocultures were injected into mice lethally irradiated with 8.5 Gy, and macroscopic colonies on the surfaces of spleens were counted after 12 days. The numbers of CFU-C in cocultures were assayed by using a soft agar culture system, as reported previously. Hematopoietic cells procured from cocultures were inoculated into 35-mm Petri dishes with 1 mL of αMEM containing 20% FBS, 100 μL/mL of 2-mercaptoethanol, 30% FBS; (Stemcell Technologies Inc, Vancouver, Canada), 2 U/mL of rhEPO, 20 ng/mL of mouse recombinant c-kit ligand, 100 U/mL of mouse recombinant interleukin-3 (IL-3; Genzyme Corp, Boston, MA). Erythroid colonies and bursts were then counted at day 2 and day 7, respectively. Assay of burst-promoting activity (BPA) and colony-stimulating activity (CSA) in the conditioned media of stromal cell line stimulated with IL-1α. Confluent monolayers of stromal cell line were stimulated with IL-1α for 24 hours and the conditioned medium was collected and filtered through Millipore filters and used for the following experiments.

The BPA and CSA of IL-1CM were examined using a methylcellulose culture system. For BPA assay, the hematopoietic cells were procured at week 16 from erythroid-inducing coculture, which contained very large number of burst-forming units—erythroid (BFU-E), washed three times with IMDM, and cultured in the presence of 50% IL-1CM and 2 U/mL of rhEPO using methylcellulose medium.
as mentioned above. For GM-CSA assay, 5 x 10^4 BM nonadherent cells fractionated as described above were cultured in the presence of 20% IL-1CM using methylcellulose medium as mentioned above. After 7 days, the numbers of erythroid burst colonies and GM colonies were counted. To investigate the role of cytokines in colony formation, we added 1 µg/mL of control rat IgG, monoclonal neutralizing anti-IL-3 antibody (AIL-3; Genzyme), or anti-GM-CSF antibody (AGM; Boehringer-Mannheim, Mannheim, Germany) to this methylcellulose medium.

Detection of cytokine antigen in stromal cell lines or in conditioned media by Western blotting analysis. Stromal cell lines were seeded onto a 75-cm² tissue-culture flask (Falcon 3024) and cultured with IMDM containing 20% FBS. After confluent monolayers had formed, cells were scraped with a rubber policeman, rinsed three times with PBS, and collected by centrifugation at 1,500 rpm; 5 to 7 x 10^6 cells/ flask were collected. For SDS-PAGE, the pellet of each stromal cell line was mixed with 1 mL of nonreducing 1X gel sample buffer and analyzed according to the method of Laemmli using multi-gel 15-25 (Daichikayaku, Tokyo, Japan). After electrophoresis, proteins were transferred onto nitrocellulose membranes, as reported previously and stained with monoclonal neutralizing antiserum to c-kit ligand (AKL; Genzyme), AIL-3, or AGM (described above), using the ABC method. IL-1CM of SPY3-2 noted above was also examined with Western blotting.

Detection of IL-3 and GM-CSF proteins by enzyme-linked immunosorbent assay (ELISA). For the assay of IL-3 or GM-CSF in IL-1CM or coculture media, we purchased ELISA kits (Endogen, Boston, MA); the sensitivity for IL-3 was less than 3 pg/mL; the sensitivity for GM-CSF was less than 5 pg/mL (described in the data sheets).

Detection of cytokine mRNA in SPY3-2 by reverse-transcriptase polymerase chain reaction (RT-PCR) method. Total RNA was extracted from 10^7 cells of SPY3-2 stimulated with rhIL-1α for 12 hours by the guanidinium thiocyanate-/CsCl method. RNA samples were transcribed in a total volume of 20 µL in buffer containing 50 mmol/L TRIS-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl₂, 10 mmol/L dithiothreitol, 10 mmol/L deoxynucleotide mixture, 100 pmol/L random hexamer oligonucleotides, and 200 U Moloney murine leukemia virus reverse transcriptase (Clontech, Palo Alto, CA). The cDNA was amplified by PCR using specific oligonucleotide primers for rat c-kit ligand, 5'-ATGAGAAAGACA CAAACTT-3' (sense), 5'-AATATITGAAAACTTGTCCA-3' (antisense), mouse IL-3, 5'-ACCAGCATCACCACCATGCT-3' (sense), 5'-AGATGATAGGCAGGCAACAGT-3' (antisense), mouse GM-CSF, 5'-CATCAGTGTCACCACCGCTT-3' (sense), 5'-TGT CTATGAATCCGGCATG-3' (antisense), and β-actin, 5'-ATG GATGATGATATCGCTGC-3' (sense), 5'-CATGATCTGGGT CATCTTTT-3' (antisense) as reported previously. The PCR cycle was repeated 35 times for β-actin and c-kit ligand, and 45 times for IL-3 and GM-CSF; a fraction of each sample was electrophoresed in 3% agarose, stained with ethidium bromide, and visualized under ultraviolet illumination. The length of RT-PCR products were β-actin, 340 bp; c-kit ligand, 272 bp; IL-3, 310 bp; GM-CSF, 299 bp.

Inhibition of hematopoiesis by neutralizing anticytokine antibodies. To investigate the role of cytokines in the long-term hematopoiesis supported by SPY3-2, we added AKL, AIL-3, or AGM to the coculture medium. AKL was added at a concentration of 0.2 or 2 µg/mL; AIL-3 and AGM were each added at a concentration of 1 µg/mL and preincubated with the stromal cells for 30 minutes before inoculation of BM nonadherent cells. Twice weekly, half of the medium was exchanged for fresh medium containing antibody. Four weeks after the beginning of coculture, the numbers of hematopoietic cells in the coculture media were counted. The numbers of day 8 CFU-S for the standard coculture or erythroid progenitors for erythroid-inducing coculture were assayed at week 3 as described above.

Detection of c-kit ligand antigen in mouse spleen. Spleens were embedded in OCT compound (Miles Inc, Elkhart, IN) immediately after excision at room temperature and frozen in hexane at -80°C. Sections of 6-mm thickness were cut in a cryostat, flattened against the glass slides, air dried, and fixed in acetone at room temperature. These sections were used for immunohistochemical staining with AKL, using the ABC method as noted above.

RESULTS

Morphologic features of stromal cell lines. Stromal cell lines, SPY3-2 and SPY8-1, were obtained from spleen primary cultures in collagen gel. SPY3-2 had a fibroblastoid morphology and SPY8-1 had an endothelialoid morphology as shown by phase-contrast microscopy (Fig 1, A and B). Macrophages in the primary stroma had disappeared during the process of passage.

The immunochemical and cytochemical characteristics of these stromal cell lines are shown in Table 1. Both cell lines were negative for endothelial cell markers. SPY3-2 was negative for alkaline phosphatase activity and did not differentiate into adipocytes in the presence of 1 mmol/L HC, ie, SPY3-2 did not have the characteristics of endothelial cells or BM preadipocytes. The characteristics of SPY8-1 were partly those of endothelial cells.

Capacity of spleen stromal cell lines to support long-term hematopoiesis in vitro. To investigate the capacity of spleen stromal cell lines to support long-term hematopoiesis in vitro, we cocultured nonadherent BM cells with stromal cell lines using Dexter-type medium.

A small number of discrete hematopoietic foci in the adherent layer appeared 3 days after the beginning of coculture with SPY3-2. Their size and number gradually increased with the coculture period, reaching a plateau at 3 weeks (data not shown). Some of these hematopoietic foci were maintained for more than 8 weeks. Some of the hematopoietic cells constituting these foci became nonadherent and were released into the coculture medium. The number of nonadherent hematopoietic cells in the coculture medium was maintained at around 1 x 10^6 cells per dish (Fig 2A). These cells consisted of approximately 70% monocytes, 20% granulocytes, 10% blasts, and these proportions remained fairly constant throughout the experimental period (data not shown). Megakaryocytes were also consistently produced throughout the culture period; the number gradually increased from 3 x 10^3/dish at week 1 to 3 x 10^3/dish at week 5, and then decreased to 2 x 10^3/dish at week 6 (data not shown). Although the number gradually decreased, reaching 20% of the inoculated number at week 8, the number of day 12 CFU-S in the coculture was maintained for more than 6 weeks. The number of CFU-C in the coculture was maintained at a level comparable with that of inoculated number throughout the first 6 weeks (Fig 2B).

When we used SPY8-1 or primary spleen stroma as the adherent layer for coculture, hematopoietic foci appeared in the adherent layer, but were maintained for only 3 weeks. Small numbers of CFU-C were detected in the coculture at week 1, but not at week 4 (Fig 2B). The number of nonadherent hematopoietic cells in the coculture was less than that
of SPY3-2 (Fig 2A) and the cells consisted of more than 90% monocytes (data not shown).

These results showed that spleen stromal cell line SPY3-2 supported the multilineal differentiation and survival of hematopoietic progenitor cells in vitro for more than 8 weeks.

Erythropoiesis-inducing activity of SPY3-2. When 2 U/mL of rhEPO was added to the coculture medium, newly formed, nonresidual clusters of erythroblasts adhering to SPY3-2 were observed by the phase-contrast microscopy after 10 days of coculture and were released into the coculture medium after 2 weeks (Fig 3A). The differentiation of erythroid cells was accelerated after week 4, which was preceded by a 10-fold increase in BFU-E at week 3 (Fig 4A). This erythropoiesis continued for more than 12 weeks, but the maturation of many erythroid cells was arrested at the stage of proerythroblast after week 12 (data not shown). The percentage of erythroid cells in the coculture medium increased from 24% before culture to 80% at week 5 (Fig 4B). At all differentiation stages, the clusters of erythroid cells consisted of erythroblasts surrounding some central macrophages (Fig 3B), some of which were enucleated and maturated into reticulocytes and red blood cells. Most of these erythroid cells, other than proerythroblasts, bore band 3 protein on their plasma membranes (Fig 3B).

Table 1. Comparison of Cytochemical Characteristics in Stromal Cell Lines

<table>
<thead>
<tr>
<th>Characteristics of Cell Line</th>
<th>3-2</th>
<th>8-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>α-Naphthyl butyrate esterase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Laminin</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Adipocytic conversion</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

SPY8-1 or primary spleen stroma, on the other hand, did not support erythroid differentiation or the survival of BFU-E even in the presence of Epo (data not shown). These results showed that SPY3-2, rather than SPY8-1 or primary stroma, supported the differentiation and proliferation of erythroid progenitors in the presence of erythropoietin.

Immunocytochemical and cytochemical analysis of adherent hematopoietic cells in coculture with SPY3-2. Hematopoietic cells in coculture with SPY3-2 had monocytic (α-naphthyl butyrate), granulocytic (naphthol-ASD chloroacetate), and megakaryocytic (von Willebrand factor) markers; an erythroid (band 3 protein) marker was also shown only in the presence of rhEPO. However, lymphoid markers (Thy-1 and B220) were not detected (data not shown). Cytochemical analysis confirmed that the hematopoietic foci adhering to SPY3-2 contained hematopoietic cells of three lineages.

CSA and BPA in IL-1CM. When we used the BM cells for the detection of BPA in preliminary experiments, IL-1CM plus 2 U/mL of rhEPO did not support the erythroid burst formation at a constant frequency. To detect the low level of BPA in the IL-1CM of stromal cell lines, we procured hematopoietic cells from erythroid-inducing coculture at week 16, more than 5% of which were BFU-E (Table 2), and cultured in methylcellulose medium containing 50% of IL-1CM and 2 U/mL of rhEPO. To detect the GM-CSA in the IL-1CM of stromal cell lines, we cultured BM cells in methylcellulose medium containing 20% IL-1CM. To investigate the role of cytokines in colony formation, we added monoclonal neutralizing antimouse cytokine antibodies or control rat IgG to the methylcellulose culture medium. In the presence of 1 ng/mL of IL-3 or GM-CSF, 0.2 μg/mL of AIL-3 or 10 μg/mL of AGM inhibited 100% or 60% to 95% of FDCP-2 cell proliferation, respectively (as described in the supplied data sheet). In the preliminary experiments, we confirmed that 1 μg/mL of AIL-3 or 5 μg/mL of AGM inhibited the CSA of 200 U of IL-3 or GM-CSF and did not cross-react with each other. These results were consistent with previous studies using these antibodies.25,26
As shown in Table 2, SPY3-2 IL-1CM supported the GM colony and erythroid burst formation at a level comparable with WEHI3 CM. GM colony formation and erythroid burst formation were inhibited by AIL-3 or AGM at a comparable level. SPY8-1 IL-1CM also supported erythroid burst formation and GM colony formation at a level comparable with SPY3-2 IL-1CM.

These results indicated that SPY3-2 and SPY8-1 produced IL-3 and GM-CSF, both of which played a role as BPA or GM-CSA, but not enough for the long-term hematopoiesis and erythropoiesis in the coculture system, because SPY8-1 did not support the long-term hematopoiesis and erythropoiesis.

Expression of c-kit ligand molecule by stromal cell lines. On Western blotting analysis, AKL detected a band of 53 kD and some minor bands between 50 and 70 kD in the cell lysate of SPY3-2, whereas no bands were detected in the cell lysate of SPY8-1 (Fig 5). The molecular weight of the c-kit ligand was not changed when reducing buffer was used (data not shown). These results were consistent with those of a previous study and showed that SPY3-2 expressed c-kit ligand of various molecular weights, which were not modified by disulfide bonds.27 c-kit ligand was produced in the absence of IL-1α stimulation. In the IL-1CM of SPY3-2, c-kit ligand was not detected by Western blotting analysis. IL-3 or GM-CSF were not detected in the IL-1CM or cell lysate of SPY3-2 by Western blotting.

These results indicated that SPY3-2 constitutively produced c-kit ligand, predominantly in the cell-bound form.

IL-3 and GM-CSF protein level in IL-1CM or coculture media. To detect small amounts of IL-3 or GM-CSF, we performed ELISA on IL-1CM and coculture media. As shown in Table 3, SPY3-2 and SPY8-1 produced small amounts of IL-3 and GM-CSF.
31  12 TSUCHIYAMA, MORI,  AND  OKADA

A Erythroid cells -“E- BFU-E

10^7

CN-E

0

1011

100

0

1

2

3

4

5

6

Weeks

Fig 4. (A) The number of erythroid cells, CFU-E, and BFU-E or (B) differential morphology of hematopoietic cells in coculture with SPY3-2 in the presence of 2 U/mL of human recombinant erythropoietin. E, erythroid cells; G, granulocytes; Mo, monocytes; Meg, megakaryocytes; Bla, blasts.

Table 2. Support of Erythroid and Granulocyte-Monocyte Progenitor Growth by IL-1α-Stimulated SPY3-2 CM

<table>
<thead>
<tr>
<th>CSA</th>
<th>BFU-E^*</th>
<th>CFU-GM^t</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-2 IL-1 CM + Control</td>
<td>258 ± 19</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>+ AIL-3</td>
<td>176 ± 24†</td>
<td>30 ± 34†</td>
</tr>
<tr>
<td>+ AGM</td>
<td>174 ± 26†</td>
<td>33 ± 21†</td>
</tr>
<tr>
<td>8-1 IL-1 CM</td>
<td>232 ± 28</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>WEH13 CM</td>
<td>293 ± 31</td>
<td>39 ± 7</td>
</tr>
</tbody>
</table>

Control, 1 μg rat IgG; AIL3, 1 μg neutralizing antimouse IL-3 antibody; AGM, 1 μg neutralizing antimouse GM-CSF antibody.

* 5 x 10^5 hematopoietic cells procured from erythroid-inducing coculture at week 16 were stimulated with 50% IL-1CM or WEH13 CM. Mean ± SD (n = 4).

† 5 x 10^4 nonadherent BM cells were stimulated with 20% IL-1CM or WEH13 CM. Mean ± SD (n = 4).

Means < .01 compared with control antibody.

amount of IL-3 and GM-CSF in response to IL-1α stimulation. IL-3 and GM-CSF production was also detected in the erythroid-inducing coculture with SPY3-2 in the first 6 weeks. The IL-3 level in the erythroid-inducing coculture media at week 3 was comparable with that of IL-1CM, but the GM-CSF level was less than 10% of IL-1CM. SPY8-1 produced level of IL-3 and GM-CSF comparable with that of SPY3-2. Such low levels of IL-3 or GM-CSF do not support the colony formation of BM cells by itself. Thus, SPY3-2 must produce other humoral factors augmenting the activity of IL-3 or GM-CSF.

Expression of cytokine mRNA by SPY3-2. By RT-PCR, mRNA expression of c-kit ligand, IL-3, and GM-CSF were detected in SPY3-2 (Fig 6). The level of c-kit ligand mRNA expression was comparable with that of β-actin. In contrast, it was necessary to increase the cycle of PCR to 45 times for the detection of IL-3 or 48 times for GM-CSF mRNA. This result indicated that SPY3-2 stimulated with IL-1α expressed IL-3 and GM-CSF mRNA at levels of roughly less than 0.1% of c-kit ligand mRNA.

Inhibition of SPY3-2–supported hematopoiesis by anticytokine antibodies. To investigate the role of c-kit ligand, IL-3, and GM-CSF in the in vitro hematopoiesis supported by SPY3-2, we used a neutralizing monoclonal antimouse c-kit ligand antibody, antimouse IL-3 antibody and antimouse GM-CSF antibody. In the presence of 5 ng/mL of c-kit ligand, 40 μg/mL of AKL inhibited 50% of Mo-7E cell proliferation (as described in supplied data sheet). The inhibitory activities of AIL-3 and AGM were described above. In preliminary experiments, we confirmed that hematopoiesis and the number of CFU-C in LTBMC decreased to 25% of control culture when 2 μg/mL of AKL was added to the culture medium, whereas the growth of stromal cells was

MW 3-2 8-1

46 kD

30 kD

Fig 5. Western blotting analysis of cell lysate in stromal cell lines, using anti-c-kit ligand antibody. Fifteen micrograms of total protein was applied to each lane of gel.
Table 3. IL-3 and GM-CSF Level in the IL-1-Stimulated Conditioned Medium of Stromal Cell Lines or Coculture Media

<table>
<thead>
<tr>
<th>Conditioned Medium</th>
<th>IL-3 (pg/mL)</th>
<th>GM-CSF (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-2 IL-1 CM</td>
<td>2.1</td>
<td>18.9</td>
</tr>
<tr>
<td>8-1 IL-1 CM</td>
<td>3.0</td>
<td>17.5</td>
</tr>
<tr>
<td>Coculture medium at week 1</td>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Coculture medium at week 3</td>
<td>2.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Coculture medium at week 6</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Coculture medium at week 16</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WEHI3CM</td>
<td>689.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not detected.

Table 4. Effects of Neutralizing Anticytokine Antibodies on the Erythropoiesis Supported by SPY3-2

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Hematopoietic Cells (×10⁶, week 3)</th>
<th>Day 8 CFU-S⁺ (week 4)</th>
<th>Erythroid Cells (×10⁶, week 4)</th>
<th>BFU-E⁺ (week 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17</td>
<td>88 ± 11</td>
<td>74.5</td>
<td>900 ± 12</td>
</tr>
<tr>
<td>0.2 µg/mL AKL</td>
<td>9</td>
<td>53 ± 2t</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2 µg/mL AKL</td>
<td>3</td>
<td>26 ± 4t</td>
<td>1.4</td>
<td>180 ± 52t</td>
</tr>
<tr>
<td>1 µg/mL AIL-3</td>
<td>4</td>
<td>63 ± 6</td>
<td>32.1</td>
<td>410 ± 42t</td>
</tr>
<tr>
<td>1 µg/mL AGM</td>
<td>6</td>
<td>59 ± 10</td>
<td>29.4</td>
<td>315 ± 12t</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* Mean ± SD (n = 4).
† P < .05 compared with no addition of antibodies.
‡ P < .001 compared with no addition of antibodies.

not impaired (data not shown). On the basis of these results, CSA or BPA inhibition by neutralizing anticytokine antibodies, and the IL-3 and GM-CSF assay by ELISA noted above, we added anticytokine antibodies at various concentrations to cocultures with SPY3-2; the cocultures were continued, maintaining the concentration of antibody equal to that at the beginning of coculture.

As shown in Table 4, AKL dose-dependently reduced the numbers of nonadherent hematopoietic cells, hematopoietic foci in the adherent layer (data not shown), and day 8 CFU-S in the standard coculture, without any toxic effects on SPY3-2. In the presence of 2 µg/mL of AKL, the percentages of granulocytes, megakaryocytes, and blasts constituting nonadherent hematopoietic cells markedly decreased, with a reciprocal increase in monocytes (data not shown), and the proportion of nonadherent hematopoietic cells was comparable with that in the coculture with SPYS-1, which did not produce c-kit ligand (described above). In contrast, AIL-3 or AGM slightly inhibited the maintenance of day 8 CFU-S and the differentiation of granulocytes and megakaryocytes.

Erythropoiesis was suppressed by AKL, AGM, or AIL-3 throughout the culture period. The suppression of erythropoiesis by AGM and AIL-3 was prominent after week 4 (data not shown). As shown in Table 3, AKL markedly suppressed the proliferation of BFU-E and differentiation of erythroid cells in the coculture, whereas the inhibition by AIL-3 or AGM was limited.

These results showed that c-kit ligand molecule played a crucial role in the long-term hematopoiesis and erythropoiesis supported by SPY3-2; IL-3 and GM-CSF played definite roles, especially in the erythropoiesis after week 4, despite their low concentration in the coculture media (as described above).

**DISCUSSION**

The HIM in mouse spleen is considered to be constructed by various types of stromal cells such as fibroblasts, reticulum cells, endothelial cells, macrophages, and lymphocytes other than preadipocytes and adipocytes. Of these cells, central macrophages²⁸⁻³⁰ and endothelial cells³¹,³² have been shown to play an important role in erythropoiesis. However, stromal cells that support the long-term survival and differentiation of HSCs have not been shown, and the histologic identity of such stromal cells has not been defined, although much in vivo data has suggested their presence.² In this study, using Dexter’s culture medium, we showed that a clonal spleen stromal cell line, SPY3-2, supported the long-term survival and multilineal differentiation of hematopoietic progenitor cells, predominantly to erythroid cells in the presence of rhEPO; SPY3-2 was morphologically and cytochemically distinct from macrophages, endothelial cells, and preadipocytic stromal cells of BM. Therefore, SPY3-2 fulfills the requirements of stromal cells that support constitutive hematopoiesis in mouse spleen. This cell line appears to be a counter-part of preadipocytic cell lines from BM that support constitutive hematopoiesis.

The function of c-kit and its ligand in constitutive hematopoiesis in BM has been the subject of recent investigations. Both in vivo and in vitro data have shown that c-kit and its ligand play a crucial role in the survival and self-renewal of hematopoietic progenitor cells in BM.¹⁰,³³,³⁴ It has also been suggested in an in vivo experiment with the steel mouse² and in recent experiments with anti-c-kit antibodies³³,³⁴ that hematopoiesis in mouse spleen is largely dependent on c-kit and its ligand. In contrast, in an in vitro study in which
Fig 7. Immunohistochemical staining of c-kit ligand in mouse spleen. c-kit ligand was distributed in the hematopoietic foci beneath the capsule (A) or around the trabecula (B) in red pulp, and these foci were surrounded by hematopoietic cells. Arrow, capsule; T, trabecula. Original magnification × 100.

Hematopoiesis in spleen culture was compared in steel and athymic mice, it was suggested that hematopoiesis in mouse spleen was dependent on T lymphocytes rather than steel factor (c-kit ligand). This discrepancy between the in vivo and in vitro findings has not been adequately explained. However, we believe that the findings presented here support the former hypothesis, and that they suggest that spleen stroma expressing the c-kit ligand also plays a crucial role in supporting the survival and differentiation of hematopoietic progenitor cells in mouse spleen.

It was shown that CFU-S transplanted into lethally irradiated mice lodged and proliferated in subcapsular or peritrabecular region of red pulp. This site was comparable with the distribution of c-kit ligand in mouse spleen as noted above. Thus, SPY3-2 seemed to be derived from stromal cells in the hematopoietic foci beneath the capsule or around the trabecula.

The capacity of SPY3-2 to support the maintenance and differentiation of multipotential and myeloid progenitors in vitro was lower than that of a preadipocytic cell line reported by Kodama et al but comparable with that of a BM stromal cell line reported by Rios et al or to that of LTBMC. However, the capacity to support the proliferation of BFU-E and differentiation to erythroid cells in vitro was much higher than that of BM stromal cell line and long-term BM culture and was comparable with that of spleen in vivo. This result indicates that SPY3-2 must produce cytokines with BPA. In addition to the high-level expression of c-kit ligand, SPY3-2 produced low levels of IL-3 and GM-CSF. All of these molecules have been reported to have BPA and have played important roles in the erythropoiesis under our coculture conditions. The level of IL-3 or GM-CSF in the coculture media was too low to induce the in vitro colony formation by itself, but such low levels of IL-3 or GM-CSF have been reported to have a biologic activity and to be involved in the constitutive hematopoiesis in LTBMC.

It was shown that CFU-S transplanted into lethally irradiated mice lodged and proliferated in subcapsular or peritrabecular region of red pulp. This site was comparable with the distribution of c-kit ligand in mouse spleen as noted above. Thus, SPY3-2 seemed to be derived from stromal cells in the hematopoietic foci beneath the capsule or around the trabecula.

The capacity of SPY3-2 to support the maintenance and differentiation of multipotential and myeloid progenitors in vitro was lower than that of a preadipocytic cell line reported by Kodama et al but comparable with that of a BM stromal cell line reported by Rios et al or to that of LTBMC. However, the capacity to support the proliferation of BFU-E and differentiation to erythroid cells in vitro was much higher than that of BM stromal cell line and long-term BM culture and was comparable with that of spleen in vivo. This result indicates that SPY3-2 must produce cytokines with BPA. In addition to the high-level expression of c-kit ligand, SPY3-2 produced low levels of IL-3 and GM-CSF. All of these molecules have been reported to have BPA and have played important roles in the erythropoiesis under our coculture conditions. The level of IL-3 or GM-CSF in the coculture media was too low to induce the in vitro colony formation by itself, but such low levels of IL-3 or GM-CSF have been reported to have a biologic activity and to be involved in the constitutive hematopoiesis in LTBMC.

Two mechanisms have been reported to explain the activity of such low levels of cytokine in the long-term culture; first, the presence of other cytokines augmenting the effect of IL-3 or GM-CSF in the coculture such as c-kit ligand (mentioned above) and, probably, IL-6 (neutralizing antimouse IL-6 antibody markedly suppressed the erythropoiesis in the coculture [data not shown]) second, the adhesion of secretory cytokines to extracellular matrix resulting in the increased local concentration in stromal cell layer. In the coculture with SPY3-2, the first mechanism was involved as mentioned above. The second mechanism is excellent for explaining the role of IL-3 and GM-CSF in the long-term culture system, but was not confirmed in the coculture with SPY3-2. Thus, expression of these three cytokines may define the pattern of hematopoiesis and erythropoiesis supported by SPY3-2. Recently, a primate BM stromal cell line was established, which has the fibroblastoid morphology and supports the long-term proliferation of human erythroid progenitors. A novel cytokine, IL-11, was cloned from this primate stromal cell line and was reported to stimulate the multiple phases of erythropoiesis.

SPY3-2 had similar characteristics to this primate stromal cell line. Thus, SPY3-2 may produce IL-11. This possibility must be examined in subsequent studies.

Dexter et al reported that erythropoiesis was induced in LTBMC by addition of EPO and normal mouse serum to the medium, normal mouse serum being required for the differentiation of erythroid cells. In contrast, SPY3-2 induced erythropoiesis in the absence of normal mouse serum, indicating that this cell line may produce those humoral factors essential for erythropoiesis that are contained in normal mouse serum.

SPY3-2 constitutively supported megakaryopoiesis, and the capacity to support the proliferation of megakaryocyte was much higher than those of LTBMC or LTBMC in collagen gel. Stromal cell lines derived from BM have not been reported to support long-term differentiation of megakaryocyte in vitro. Thus, it seems likely that SPY3-2 reconstructs the HIM preferential to megakaryopoiesis in vitro.

It has been reported that hematopoiesis-supporting stromal cells might be slow-growing, and some investigators have successfully established stromal cell lines from BM on the
basis of this hypothesis. SPY3-2 is also a slow-growing cell line (data not shown). Considering this feature of hematopoiesis-supporting cells, taken together with the observations of LTBMNC in collagen gel, SPLEEN STROMAL CELL LINE MAINTAINS LTBMC was thought to provide suitable conditions for the growth of functioning stromal cells that were slow growing and highly differentiated. Primary culture in collagen gel may be suitable for the culture of the stromal cells of human BM and other lympho-hematopoietic organs.

ACKNOWLEDGMENT

We thank for Dr Y. Sadahira for his helpful suggestions and for monoclonal antime-thyrocyte band 3 antibody. We also thank Y. Watanabe for her skillful assistance in the laboratory, and the department of radiology which institutes for irradiation of mice.

REFERENCES

33. Ogawa M, Matsuzaki Y, Nishikawa S, Hayashi S, Kunisada T, Sudo T, Kina T, Nakauchi H, Nishikawa SI: Expression and
Murine spleen stromal cell line SPY3-2 maintains long-term hematopoiesis in vitro

J Tsuchiyama, M Mori and S Okada