Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver

Hiroshi Kawamoto, Koichiro Ohmura and Yoshimoto Katsura

Department of Immunology, Chest Disease Research Institute, Kyoto University, Kyoto 606-01, Japan

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Abstract

We established an experimental system in vitro to examine the developmental capacity of individual hematopoietic progenitors to generate T, B and myeloid (M) cells. By using this system we analyzed the process of lineage commitment of hematopoietic progenitors in murine fetal liver (FL). It is known that small numbers of B and M cells, in addition to T cells, are generated in a co-culture of hematopoietic progenitors and a deoxyguanosine-treated fetal thymus (FT) lobe. We tried to enhance the growth of B and M cells by the addition of IL-7, IL-3 and stem cell factor into the co-culture. This cytokine-supplemented FT organ culture was used to examine the developmental capacity of individual hematopoietic progenitors in FL. Single cells of lineage marker (Lin)– c-kitF Sca-1F (Sca-1+) and Lin–c-kitF Sca-1– (Sca-1–) populations from the FL harvested at day 12 of gestation were cultured for 10 days, and the phenotypes of cells generated in each lobe were analyzed with a flow cytometer. All progenitors in the Sca-1+ population were shown to be committed to generate only T, B or M cells. On the other hand, multipotent progenitors, which are capable of generating T, B and M cells, as well as unipotent progenitors committed to the T, B or M lineage were found in the Sca-1+ population. Bipotent progenitors generating M and T cells and those generating M and B cells were also found in the Sca-1+ population, which probably represent progenitors in the process of commitment. However, no bipotent progenitors generating T and B cells were detected.

Introduction

All hematopoietic cells are derived from hematopoietic stem or progenitor cells originating either in yolk sac or intra-embryonic tissues (1–7). Recent studies indicate that multipotent hematopoietic progenitors emerge at ~9 days post-coitum (d.p.c.) in the para-aortic splanchnopleura (7) and that stem cells with long-term reconstitution ability appear at 10 d.p.c. in aorta–gonad–mesonephros region of fetuses (8). The stem cells then accumulate and expand in the liver and bone marrow (4,9–12) in the fetus and adult respectively. It is well accepted that all hematopoietic cells are produced from a stem cell through lineage-committed progenitors (1,13,14). However, the investigation of the process of lineage commitment has mostly been restricted to myelo-erythroid lineages. This is because a variety of in vitro assay systems are available only in these lineages (15–17). Our knowledge of the mechanism(s) in the earlier stages of hematopoiesis including commitment to T, B and myeloid (M) lineages has been mainly acquired from gene marking and gene knockout experiments. Gene marking studies have provided circumstantial evidence for the existence of progenitors committed to T lineage (p-T), M lineage (p-M) and possibly also B lineage (p-B) (18–20), and gene knockout experiments indicate that lineage commitment is controlled by expression of transcription factors (21, 22). However, such committed progenitors are not easily identifiable nor has the process of commitment been corroborated by other experimental systems such as in vitro cell cultures. Identification and isolation of such progenitors of different developmental capabilities will greatly contribute to clarifying the mechanisms of lineage commitment and differentiation.

Differentiation of T, B and M lineage cells can be induced in human thymus fragment culture (23), but this culture system has not been tried for clonal assay of progenitor cells. Attempts to induce different lineage cells in a single-cell
culture system using a cytokine cocktail or a combination of stromal cells and cytokines have also been carried out (24–26). These systems, however, are difficult to apply for examining multilineage differentiation, because no cytokine nor stromal cell line has been discovered that effectively supports T cell development. Recently, Cumano and her colleagues succeeded in inducing both B and M cells by culturing single progenitors on a monolayer of a stromal cell line supplemented with cytokines, and analyzed theforking point for B and M lineages (7,25,27,28). Their findings suggest that commitment to the M lineage begins at the Sca-1+ stage, whereas commitment to the B lineage occurs after losing the Sca-1 antigen (27). They further showed that the progenitors capable of developing into both B and M cells on a stromal cell layer were also able to generate T cells by transferring a portion of generated cells into fetal thymus (FT) organ cultures (28), thus representing the pluripotent stem cells. In this experimental system, however, the T cell producing capacity of progenitors can only be examined when a clone shows extensive growth on a stromal cell line. Since p-T usually do not grow on a stromal cell monolayer, they would not be detected using this method.

To investigate the earlier stages of hematopoiesis including commitment to T, B and M lineages, it is a prerequisite to establish a culture system which supports the development of all T, B and M cells. Moreover, the system is required to be capable of assaying the hematopoiesis from single stem/progenitor cells. In the present study, we established such a culture system by modifying the FT organ culture. The deoxyguanosine (dGuo)-treated FT lobe can support the differentiation of not only T but also B and M cells (23 and this paper), although it preferentially supports the proliferation of T cells. By adding IL-7, IL-3 and stem cell factor (SCF) to the organ culture, we were able to construct an environment supporting the development of T, B and M cells. With this culture system, it was possible to detect multipotent progenitors (p-Multi) as well as committed progenitors for T, B and M lineages in the fetal liver (FL) from 12 d.p.c. fetuses.

**Methods**

**Mice**

C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan) and B6Ly5.1 mice were maintained in our animal facility. The FT lobes from 15 d.p.c. B6 fetuses were used in organ culture experiments as the source of FT lobes. FL and FT were obtained from B6Ly5.1 mice at 12 d.p.c., and were used as the source of progenitors.

**Antibodies**

The following antibodies were used: anti-Ly5.1 (A20-1.7; donated by Dr Y. Saga, Banyu Seiyaku, Tokyo, Japan), anti-Ly5.2 (ALI-4A2; donated by Dr I. L. Weissman, Stanford University, San Francisco, CA), anti-B220 (RA6B2; obtained from ATCC, Rockville, MD), FITC-anti-Mac-1 (M1/70; Caltag, San Francisco, CA), FITC-anti-Gr-1 (RA3-8CS, PharMingen, San Diego, CA), phycoerythrin (PE)-anti-B220 and FITC-anti-B220 (RA-8B2, Caltag), FITC-anti-IgM (Organon Tecnica, West Chester, PA), allophycocyanin (APC)-anti-Thy-1.2 and FITC-anti-Thy1.2 (5a-8; Caltag), PE-anti-CD4 (GK1.5; Caltag), FITC-anti-CD25 (PC61; PharMingen), FITC-anti-CD45 (30F11.1; PharMingen), PE-anti-Sca-1 (E13-161.7; PharMingen), anti-FcγII/III (FcR) (2.4G2) (29), anti-c-kit (ACK-2; donated by Dr S.-I. Nishikawa, Kyoto University) (30) and anti-erythroid lineage cells (TER119; established by T. Kina in our laboratory). Anti-Ly5.1, anti-FcR and TER119 were labeled with FITC as described (31). Anti-c-kit, anti-B220 and anti-Ly5.2 were labeled with Cyanine 5 (Cy5) (Cy5 labeling kit; Biological Detection Systems, Pittsburgh, PA) whose fluorescence characteristics are similar to those of APC.

**Cell lines**

Stromal cell lines derived from adult bone marrow (PA6) (32) and fetal thymus (TSt-4) (33) were used. PA6 and TSt-4 are capable of supporting the generation of M cells and B and M cells respectively. Monolayers of TSt-4 and PA6 were prepared in a six-well plate (Costar). The culture medium was RPMI 1640 supplemented with 5% FCS, 2-mercaptoethanol (5×10^−5 M), streptomycin (100 µg/ml) and penicillin (100 U/ml).

**Growth factors**

Recombinant murine IL-7 and murine granulocyte colony stimulating factor (G-CSF) were kindly donated by Dr Sudo (Basic Research Lab., Toray, Kanagawa, Japan). Commer- cially available recombinant murine SCF (Genzyme, Cambridge, MA), murine IL-3 (Genzyme), murine granulocyte macrophage colony stimulating factor (GM-CSF; Gibco/BRL, Gaithersburg, MD) and human macrophage colony stimulating factor (M-CSF) (Cellular Products, Buffalo, NY) were also used.

**Cytokine-supplemented FT organ culture: culture conditions for multilineage progenitor (MLP) assay**

The high oxygen submersion (HOS) organ culture has been described in detail (34). In the present study, the HOS organ culture was modified in order such that not only T, but also B and M cells grow. RPMI 1640 medium supplemented with 10% FCS, sodium pyruvate (1 mM), sodium bicarbonate (2 mg/ml), non-essential amino acid solution (1 mM), 2-mercaptoethanol (5×10^−5 M), streptomycin (100 µg/ml) and penicillin (100 U/ml) was used as complete medium. FT lobes obtained from B6 fetuses (15 d.p.c.) were cultured with dGuo (1.35 mM) for 6 days to deplete all hematopoietic cells in the lobes. Single dGuo-treated lobes were submersed in 0.2 ml of complete medium supplemented with or without cytokines in a well of a 96-well V-bottom plate (Costar, Cambridge, MA). Sorted FL or FT cells from B6Ly5.1 fetuses were suspended in complete medium. A single cell or 30 cells were inoculated into each well. For inoculation of a single cell into wells, a cell was lifted using a fine capillary tube under direct microscopic visualization and seeded into a well. Plates were centrifuged at 150 g for 5 min at room temperature, placed into a plastic bag (Ohmi Oder Air Service, Hikone, Japan), and the air was exchanged with a gas mixture of 5% CO₂, 70% O₂ and 25% N₂. The plastic bag was then incubated in a 37°C incubator. Half of the medium was replaced with fresh medium on the fifth day.
Cell sorting and analysis

Surface staining of cells, and flow cytometric analysis and sorting of stained cells were performed as previously described (35). FL cells were stained with FITC–anti-CD45, PE–anti-Sca-1 and Cy5–anti-c-kit. Sca-1− and Sca-1+ cells in the c-kit−/CD45− population were sorted using a FACS Vantage (Becton Dickinson). Since the FL cells expressing c-kit at high levels (c-kit+) do not express lineage markers (Lin) (see Fig. 1), Lin staining was not performed for cell sorting. Nona-viable cells were excluded by forward and side scatters. FcR high positive (FcRhi) c-kit+ CD45+ FL cells and c-kit−/CD25+ FT cells were similarly sorted. Sorted cells were reanalyzed to check their purity and were always found to be >98% pure.

Cells grown both in and outside the FT lobe were harvested from each well 10 days after the culture, and single-cell suspensions were made. One-third of each cell sample was stained with FITC–anti-Ly5.1 and Cy5–anti-Ly5.2, and analyzed by flow cytometry. The samples containing Ly5.1+ cells were selected for further analysis. The remaining two-thirds of cells from the selected samples were divided into two lots. One lot was stained with FITC–Ly5.1, PE–anti-B220 and APC–anti-Thy1.2, and the other stained with FITC–anti-Ly5.1, PE–anti-Mac-1, PE–anti-Gr-1 and Cy5–anti-B220. Surface phenotypes were analyzed with a FACS Vantage. Computer-assisted data analysis was done on a Power Macintosh 8500/180 (Apple Computer, Cupertino, CA) with CELLQuest software (version 1.2.2). Cells showing Ly5.1+ Thy-1− B220− and Ly5.1+ Thy-1− B220+ phenotypes were tentatively regarded as donor derived T and B cells respectively. These populations were then checked for forward/side scatter and expression of Mac-1/Gr-1. Those falling within the lymphocyte area and expressing neither Mac-1 nor Gr-1 were considered to be either T or B cells. Ly5.1+ Thy-1− B220− Mac-1−/Gr-1− cells were judged as donor-derived myeloid cells. This population contained cells with low level staining for B220 or Thy-1, but such low level staining was regarded as non-specific, since these Mac-1−/Gr-1− cells were found to fall in the area of myeloid cells in forward/side scatter analysis.

In one experiment, more mature phenotypes of T and B cells were analyzed after re-culturing cells derived from a p-Multi with a dGuo-treated lobe or a stromal cell line TS-4.

Results

Multipotent progenitor activity of c-kit+Sca-1+ CD45+ (Sca-1+) FL cells

It is well known that progenitors capable of generating T, B and M cells are enriched in the Sca-1+ population in both the FL and bone marrow (10,36). We confirmed this observation by using standard methods. Sca-1+ cells were sorted from FL cells of 12 d.p.c. fetuses (Fig. 1), and were cultured on the monolayers of PA6 and TSt-4 at 30 cells/well. Co-culture with a dGuo-treated FT lobe was also performed. Cells grown in these cultures were harvested, and assayed for their expression of T, B and M cell markers.

Generation of M cells on PA6 monolayers indicates that progenitors capable of generating M cells are included in the 30 Sca-1+ FL cells (Table 1). The finding that both B and M cells are generated in cultures on TSt-4 monolayers may indicate either that p-M and p-M are present or that a common progenitor for M and B lineage is present. A small number of Thy-1− B220+ cells (1.5%) are usually disregarded. T cell generation in FT lobes provides evidence that progenitors generating T cells are present, and again small numbers of B and M cells recovered from the lobes are disregarded. The results from these three independent experiments indicate that the 30 Sca-1+ cells contain progenitors for T, B and M lineages, and suggest that p-Multi are present in this population. However, these experiments do not provide any definitive evidence elucidating the presence of p-Multi nor lineage-committed progenitors, because the experiments performed here are not designed to discriminate the p-Multi from a mixture of unipotent progenitors giving rise to different lineage cells.

An environment supporting the development of T, B and M cells

For establishing a clonal assay system to examine the developmental potential of individual progenitor cells, it is necessary to construct an environment that supports the development of all T, B and M lineage cells. We frequently observed the generation of small numbers of B and M cells in co-cultures of progenitors with dGuo-treated lobes (e.g. see Table 1). These results may reflect the capacity of FT lobes to support the differentiation of all T, B and M cells,
although the lobes promote the extensive growth of only T cells. It was supposed that an environment supporting the development of T, B and M cells can be constructed by the addition of cytokines promoting the growth of B and M cells to the FT organ culture.

Sca-1+ FL cells from B6Ly5.1 mice were added to each well of a V-bottom plate (30 cells/well) in which a dGuo-treated FT lobe (B6) had been placed. Various cytokines were added to the culture medium. Cells were harvested 10 days later from each well, stained with various mAb and analyzed by flow cytometry. Results are shown in Table 2 and Fig. 2.

In the absence of exogenous cytokines, a large majority of the cells were T cells, and the mean proportions of B and M cells were 3.0 and 2.0% respectively. By the addition of IL-7, the proportion of B cells prominently increased without affecting either the total number of recovered cells or the proportion of M cells. Generation of M cells was preferentially enhanced by the addition of GM-CSF. G-CSF and M-CSF were only slightly effective in promoting the growth of B or M cells. By contrast, IL-3 (3 ng/ml) strongly enhanced the generation of both B and M cells, although a higher amount of IL-3 (10 ng/ml) resulted in higher skewing towards the M lineage.

Although the addition of a low dose (1–3 ng/ml) of IL-3 to the FT organ culture appeared to be sufficient for establishing an environment which supported the generation of T, B and M cells, we further investigated the effect of various combinations of cytokines. Results of these experiments are shown in Table 3 and Fig. 2 (bottom lane). Treatment with SCF alone as well as combinations of cytokines more or less promoted the growth of B and M cells. We adopted the combination of IL-7 (200 U/ml), IL-3 (3 ng/ml) and SCF (10 ng/ml) as the cytokine cocktail in the clonal assay (bottom lane of Table 3). We choose this protocol because in pilot clonal assay experiments, the seeding efficiency was highest with this particular combination of cytokines (data not shown).

In analyzing the surface phenotypes of cells generated in the co-cultures, a large gating area was set in forward/side scatter, in order to include all viable T, B and M cells (Fig. 2A). Examples of flow cytometric profiles are shown in Fig. 2(B). Regardless of the group, all Thy-1+ , B220+ and Mac-1/Gr-1+ cells were Ly5.1+ , indicating that they are derived from progenitor cells added to the culture. M cells generated in these cultures are morphologically mature (not shown). On the other hand, large proportions of Thy-1+ and B220+ cells seen at day 10 of the cytokine-supplemented organ cultures did not express markers of mature cells. Nevertheless, we regarded the Thy-1+ and B220+ cells as being T and B cells respectively as these cells gave rise to mature cells when continuing the culture in appropriate conditions (see Fig. 4).

Cytokine-supplemented FT organ cultures do not alter the differentiation potential of committed progenitors

In order for MLP assay cultures to be useful for analyzing the differentiation capacity of a progenitor, it should be ensured that these culture conditions do not induce fluctuation of commitment. c-kit+CD25+ cells in FT have been shown to be committed to the T lineage (37,38) and we have found that p-M exist in FL as Lin-c-kit+FcRhi cells (H. Kawamoto et al., manuscript in preparation). These cells and, as a control, Sca-1+ FL cells, were cultured together with a dGuo-treated lobe in the presence of a cytokine cocktail (IL-7, IL-3 and SCF), and the cells grown in these cultures were assayed for their expression of T, B and M markers. Table 4 shows that these progenitors produce only the progeny of the committed lineage, indicating that the culture condition used here did not induce transdifferentiation.

Clonal analysis of the progenitor cells in Sca-1+ and Sca-1- populations of FL

Single Sca-1+ and Sca-1− FL cells were put into each well of a V-bottom plate in which a dGuo-treated FT lobe (B6) had been placed, and cultured under HOS conditions. Culture medium was supplemented with a cytokine cocktail (IL-7, IL-3 and SCF). On day 5, half of the medium was changed with fresh medium containing IL-7 and IL-3, and cells were harvested from each lobe on day 10 for flow cytometric analysis.

Figure 3 shows representative surface profiles of six different types of clones observed in this experiment. The clone in the extreme left lane (p-Multi) contains T, B or M cells, indicating that the progenitor seeded in this well is of the multipotent type. Wells containing exclusively T, B or M cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Dose</th>
<th>Recovered cells ($\times 10^3$)</th>
<th>Percentage of cells expressing</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>80 ± 29</td>
<td>89 ± 7.4</td>
</tr>
<tr>
<td>IL-7</td>
<td>20</td>
<td>73 ± 15</td>
<td>68 ± 25</td>
</tr>
<tr>
<td>IL-7</td>
<td>200</td>
<td>85 ± 31</td>
<td>46 ± 23</td>
</tr>
<tr>
<td>IL-3</td>
<td>1</td>
<td>82 ± 35</td>
<td>68 ± 25</td>
</tr>
<tr>
<td>IL-3</td>
<td>3</td>
<td>84 ± 25</td>
<td>66 ± 20</td>
</tr>
<tr>
<td>IL-3</td>
<td>10</td>
<td>76 ± 30</td>
<td>24 ± 15</td>
</tr>
<tr>
<td>G-CSF</td>
<td>10</td>
<td>75 ± 11</td>
<td>9 ± 8</td>
</tr>
<tr>
<td>M-CSF</td>
<td>10</td>
<td>70 ± 26</td>
<td>24 ± 15</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10</td>
<td>28 ± 8</td>
<td>24 ± 15</td>
</tr>
</tbody>
</table>

*Thirty Sca-1+ FL cells (B6Ly5.1) were cultured in each well in the presence of a dGuo-treated lobe and a cytokine for 10 days.

*bMean cell number ± SD of three wells.
were considered to be seeded by p-T, p-B or p-M respectively. Lineage commitment seems very strict. For example, in the case of p-T, B220⁺ or Mac-1⁺/Gr-1⁺ cells were not at all generated. In addition to these multipotent and unipotent progenitors, the presence of apparently bipotent p-MT and p-MB progenitors was elucidated. On the other hand, we failed to detect any p-TB type bipotent progenitor. Recovered cell numbers per well were very high for p-Multi, p-MT, p-MB and p-T (10⁵–10⁶/well). However, the cell recoveries in other cases were smaller: 10³–10⁴ for p-M and 200–10⁴ for p-B. These recovered cell numbers may not necessary reflect the growth potential of each progenitor type but may be dependent upon the congeniality between progenitors and the environment artificially constructed in the present study.

A more detailed characterization of cells derived from a single p-Multi was made and the results are shown in Fig. 4. Thy-1⁺ cells and B220⁺ cells recovered on day 10 of culture fall in the area of lymphoid cells in forward/side scatter analysis, whereas Mac-1⁺/Gr-1⁺ cells represent larger and morphologically more complex cells (Fig. 4A). Lymphoid cells observed in the lobe at day 10 of culture are immature, in that a large majority of these cells do not express CD3 or IgM on their surface (not shown). These cells, however, gave rise to mature T and B cells by culturing with a fresh dGuo-treated lobe and on a TSt-4 monolayer respectively. Representative FACS profiles of these re-cultured cells are shown in Fig. 4(B and C).

Fig. 4A shows the number of different types of progenitors detected among 124 Sca-1⁺ and 160 Sca-1⁻ FL cells assayed in the preceding section. Detection of p-Multi and unipotent p-T, p-B and p-M as well as bipotent p-MT and p-MB in the Sca-1⁺ population strongly suggests that lineage commitment progresses at the Sca-1⁺ stages. Concordant with previous findings obtained by culturing progenitors on a stromal cell monolayer (27), commitment to the M lineage is preferred to the B lineage. The data shown in Fig. 5(A) could further indicate that the T lineage commitment is preferred to B lineage commitment. In the Sca-1⁻ population, all progenitors detected were unipotent, suggesting that the process of T, B and M lineage commitment has finished during the Sca-1⁺ stage. These results are concordant with previous findings that progenitors in the Sca-1⁺ population are more primitive than those in the Sca-1⁻ population (10,27,39). Regardless of Sca-1 expression, a majority of unipotent progenitors are p-M. This may reflect the active erythropoiesis and myelopoiesis in FL. Although the frequency of p-B is the lowest among committed progenitors, the absolute number of p-B in FL does not seem very small, since ~1000 per 12 d.p.c. FL determined here is comparable or slightly higher than that determined by another group (27). p-T have not been enumerated. About 3000 p-T per 12 d.p.c. FL estimated in the present work seems high enough to produce T cells in the FT, since it has been suggested that a single progenitor is able to repopulate the FT to produce a whole repertoire of T cells (40).

**Discussion**

We have established a culture system in which the development of T, B and M cells is supported, and with this culture system we investigated the developmental capacity of individual 124 Sca-1⁺ and 160 Sca-1⁻ FL cells. The presence of not only p-Multi but also unipotent p-T, p-B and p-M was observed. Apparently bipotent p-MT and p-MB, but not p-TB were also detected. The present success in identifying different types of progenitors will contribute to further investigations of the mechanism of lineage commitment and differentiation of hematopoietic cells at cellular and molecular levels.
Table 3. Combinations of cytokines effectively supporting the growth of T, B and M cells in FT organ cultures

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Recovered cells (×10^3)b</th>
<th>Percentage of cells expressing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy-1</td>
<td>B220</td>
</tr>
<tr>
<td>IL-7 (U/ml)</td>
<td>IL-3 (ng/ml)</td>
<td>SCF (ng/ml)</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>10</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>50</td>
</tr>
<tr>
<td>200</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>200</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>3</td>
<td>10c</td>
</tr>
</tbody>
</table>

*aThirty Lin−c-kit−Sca-1− FL cells (B6Ly5.1) were cultured in each well with a dGuo-treated lobe and cytokine(s) for 10 days.
bMean cell number ± SD of three wells.
cSCF was added at the beginning of culture and omitted from cytokine cocktail added at the medium change on the fifth day of culture.

Table 4. MLP assay cultures do not induce transdifferentiation of committed progenitors

<table>
<thead>
<tr>
<th>Progenitorsa</th>
<th>Seeded cells</th>
<th>Recovered cells (×10^3)b</th>
<th>Percentage of cells expressing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thy-1</td>
<td>B220</td>
<td>Mac-1/Gr-1</td>
</tr>
<tr>
<td>FL c-kit^+FcRhi</td>
<td>50</td>
<td>13 ± 5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>FT c-kit+CD25</td>
<td>50</td>
<td>63 ± 15</td>
<td>94 ± 2.1</td>
</tr>
<tr>
<td>FL Sca-1^−</td>
<td>30</td>
<td>147 ± 19</td>
<td>35 ± 26</td>
</tr>
</tbody>
</table>

*aCells enriched for M lineage-committed progenitors (FL c-kit^+FcRhi) and those enriched for T lineage-committed progenitors (FT c-kit+CD25^+) were cultured with a dGuo-treated lobe in the presence of cytokine cocktail (IL-7, IL-3 and SCF). FL Sca-1^− cells were used as the control which contain various types of progenitors.
bMean cell number ± SD of four wells.

Studies on the process of lineage commitment of hematopoietic stem cells tend to be restricted to myelo-erythroid lineages (16,17), since clonal assay systems have only been available for these lineages. Recently, the monolayer of a fibroblast stromal cell line was found to provide an environment supporting the development of M and B cells (27,28), and with this culture system it was suggested that p-M and p-B were generated at the Sca-1^+ and Sca-1^− stages respectively. However, to investigate the process of differentiation including commitment to T, B and M lineages, it is a prerequisite to establish an assay system where the development of all these cells is supported. Because T cell development is highly dependent upon the thymic architecture (41), it was thought that only the modification of FT organ cultures may provide the MLP assay for T, B and M lineages.

We have repeatedly found that M as well as B cells are generated, albeit in a small number and sometimes only transiently, in co-cultures of a dGuo-treated FT lobe and progenitors from FL or adult bone marrow. These observations suggest that the thymic environment is at least partially able to support the development of M and B cells. A similar finding has also been obtained in human thymus fragment cultures (23). In the present study, we showed that the growth of B and M cells was greatly enhanced by the addition of cytokine cocktail into FT organ cultures. Nearly 30% of Sca-1^+ FL cells were found to have progenitor activity in the MLP assay. A slightly higher level of seeding (~40%) is obtained when single Sca-1^+ FL cells were cultured on monolayers of the stromal cell line TSt-4 (data not shown). The difference may reflect the fact that some of the progenitors failed to differentiate in the MLP assay. However, considering the culture conditions of the MLP assay that only one cell was added to a V-bottomed well, the seeding efficiency in the MLP assay seems high enough.

The MLP assay enabled us to easily detect and enumerate
Clonal assay of stem/progenitor cells

Fig. 4. Further characterization of cells generated from a p-Multi in the MLP assay culture. The cells analyzed in this figure are the same as those shown in the extreme left lane of Fig. 3. (A) Thy-1\(^{+}\), B220\(^{+}\) and Mac-1\(^{+}/Gr-1^{+}\) cells enclosed with ellipses in upper panels are displayed for forward/side scatters in lower panels. It is indicated that both Thy-1\(^{+}\) and B220\(^{+}\) cells are lymphoid, whereas most of Mac-1\(^{+}/Gr-1^{+}\) cells represent large cells. (B and C) Since Thy-1\(^{+}\) cells and B220\(^{+}\) cells recovered on day 10 in the MLP assay culture are immature, the recovered cells were re-cultured together with dGuo-treated lobes or on monolayers of TSt-4. CD4- and/or CD8-expressing cells are generated by culturing for 7 days with a dGuo-treated lobe (B). Surface IgM-expressing cells are generated by culturing for 7 days with TSt-4 cells (C).

p-Multi. The p-Multi were detected only in the Sca-1\(^{+}\) population, and this type of progenitor retains characteristics of the hematopoietic stem cells delivering T, B and M cells. The phenotype of the p-Multi in FL is Lin\(^{-}\)c-kit\(^{+}\)Sca-1\(^{+}\), and is similar to that of the stem cells in FL (10) and bone marrow (9,42), which are capable of reconstituting the hematopoietic cells of a lethally irradiated recipient mouse with a few cells. Although Morrison and colleagues (10) did not determine the number of such stem cells in a FL of a 12 d.p.c. fetus, the number can be estimated as ~1200 from the data shown in their paper. We found that ~2000 p-Multi exist in the FL of a 12 d.p.c. fetus. Thus, the number of p-Multi is comparable to that of stem cells. These findings in addition to their common characteristics may support the idea that p-Multi represent hematopoietic stem cells. Various in vitro systems have been developed in order to assay progenitor/stem cells, which include cultures for long-term culture initiating cells (43,44), cobblestone-area-forming cells (45) and colony-forming unit-Dexter (46). These methods may be effective in detecting the hematopoietic stem cells, since the capacity of stem cells to deliver hematopoietic cells for a long period is expressed in these cultures. The MLP assay is unique in that it is effective in not only detecting the multipotent activity of stem cells but is also capable of discriminatively detecting different type of progenitors. If the p-Multi are proved to exactly represent the stem cells, the MLP assay will also be useful as the most time-saving method for detecting the stem cells.

The numbers of p-M and p-B detected in the present study are comparable to those previously detected by cultures on stromal cell monolayers (27). p-M are the most abundant in FL of this embryonal age. This seems reasonable, because myelopoiesis has already begun in 10 d.p.c. FL (47). The presence of a relatively small number of p-B may also be concordant with the finding that only a very small number of immature B cells are present in 12 d.p.c. FL (48,49).

The presence of p-T in FL was determined by the MLP assay. Prethymic commitment of stem cells to T lineage has long been suggested (18–20), but p-T have to date not been identified in FL or adult bone marrow. The only extrathymic cell population reported so far to contain p-T is the Thy-1\(^{-}\)c-kit low positive (c-kit\(^{lo}\)) cells in the blood of 15.5 d.p.c. fetuses (50). The p-T found in the present study are phenotypically distinct from those in 15.5 d.p.c. fetal blood. Our p-T are c-kit\(^{+}\) (Fig. 1) and should be Thy-1\(^{-}\) because Thy-1\(^{-}\) cells

Fig. 5. Lineage commitment occurs at a c-kit\(^{+}\)CD45\(^{+}\)Sca-1\(^{+}\) stage. Single Sca-1\(^{+}\) and Sca-1\(^{-}\) cells were served for MLP assay. The culture period was 10 days. The numbers of different types of progenitors detected by assaying 124 Sca-1\(^{+}\) and 160 Sca-1\(^{-}\) cells are scored. In calculating the total numbers of progenitors in FL, the number of cells in FL from a 12 d.p.c. fetus was regarded as 2.5\(\times\)10\(^6\), and the proportions of both Sca-1\(^{+}\) and Sca-1\(^{-}\) cells were regarded as 2.0% (see Fig. 1). The data are from four independent experiments.
were undetectable in FL of 12 d.p.c. fetuses (data not shown). It is thought that the most immature cells in FT represent the immediate progeny of prethymic p-T. As previously shown, the FT cells from 12 d.p.c. fetuses which do not express the transcription factors TOF-1 or GATA-3 are also Thy-1·c-kit+ (35,51). Thus, it is likely that the p-T found in FL of 12 d.p.c. fetuses represent the progenitors immigrating into FT at an early stage, which are distinct from Thy-1·c-kit+ cells found in the blood of 15.5 d.p.c.

Of interest is the presence of bipotent p-MT and p-MB progenitors. The presence of these bipotent progenitors at easily detectable levels suggests that the commitment to T or B lineage may occur along with the commitment to M lineage. On the other hand, we failed to detect any p-TB type progenitors, which have been speculated or presumed to be present (22,52). So far, it is unclear whether or not p-TB type progenitors exist. It is probable that the number of p-TB is much smaller than other types of progenitors, or that the period of p-TB stage is very short. It should be pointed out, however, that our failure to detect any p-TB is consistent with that of Lemischka et al. (20) made in gene marking experiments in that a common gene integration site was found between T and M lineage or B and M lineages, but not between T and B lineages. Further investigation is necessary to clarify whether the development of T and B cells progresses through a common route.

The total number of lineage-committed progenitors is much higher than that of p-Multi. This may reflect the capacity of p-Multi for repeated production of committed progenitors with retaining self-renewal capacity. Among committed progenitors, p-M are the most abundant, followed by p-T, while p-B are the most scarce. It is still unclear whether cell interactions or growth factors play any specific roles in commitment of stem cells to T, B or M lineages. If the commitment to T, B and M lineages occurs at the same frequency through a stochastic process, such a large difference in numbers among p-T, p-B and p-M may be attributable to a function of the FL environment in maintaining these progenitors. Lineage commitment of hematopoietic progenitors is thought to be controlled by transcription factors (21,53). Details of the role of these molecules have not yet been clarified and it is probable that additional specific molecules will be discovered. Isolation of different types of progenitors is one of the most important steps for investigation of the molecular mechanisms of commitment and differentiation of hematopoietic cells. The present success in the identification of multipotent and lineage committed, as well as bipotent progenitors, has provided a basic tool for isolation of these progenitors.

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Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>Cy5</td>
<td>cyanine 5</td>
</tr>
<tr>
<td>dGuo</td>
<td>deoxyguanosine</td>
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<tr>
<td>d.p.c.</td>
<td>days postcoitum</td>
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<td>FL</td>
<td>fetal liver</td>
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<td>FT</td>
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<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>HOIS</td>
<td>high oxygen submersion</td>
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<td>M</td>
<td>myeloid</td>
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<tr>
<td>M-CSF</td>
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<td>phycoerythrin</td>
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<td>stem cell factor</td>
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References
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