Support of Early B-Cell Differentiation in Mouse Fetal Liver by Stromal Cells and Interleukin-7

By Yuji Gunji, Tetsuo Sudo, Junko Suda, Yuji Yamaguchi, Hiromitsu Nakauchi, Shin-Ichi Nishikawa, Nobuyuki Yanai, Masuo Obinata, Masayoshi Yanagisawa, Yasuasa Miura, and Toshio Suda

We compared the development of B-cell progenitors with that of myeloid progenitors in fetal liver cells at various gestational ages. Day 12 to 14 fetal liver cells did not form pre-B-cell colonies. Pre-B-cell colonies were developed from day 15 fetal liver cells. The incidence of colonies increased with increases in gestational age and reached a maximum on days 13 to 21 of gestation. After coculturing day 13 fetal liver cells with IL-7-producing stromal cell line ST-2, they could respond to IL-7 and proliferate. Analysis of the phenotypes showed that day 13 fetal liver cells were B220−, IgM−, while culturing day 13 fetal liver cells with ST-2 and untreated day 18 fetal liver cells contained the population of B220− cells. Even in the presence of IL-7−defective stromal cell line FLS-3, IL-7−responsive cells could be induced from day 13 fetal liver cells. IL-7 acted on B220− cells and induced pre-B-cell colonies that contained IgM+ cells in the methylcellulose culture. IL-7 mRNA was expressed in days 13 and 18 fetal liver cells but not in pre-B cells or adult liver cells. From these findings, it is suggested that stromal cells or stromal-derived factors but not IL-7 were required for the differentiation from B220− cells to B220+ cells. In the second stage, B220+ IgM+ cells proliferated and some of them differentiated to IgM+ cells in the presence of IL-7 alone. The two-step model can apply to in vivo early B lymphopoiesis.

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MATERIALS AND METHODS

Mice. BALB/c mice were purchased from SLC Co (Shizuoka, Japan). Female BALB/c mice were mated with males on day 0. The pregnant mice were killed on days 12 to 21 by cervical dislocation. Livers with pigmentation were removed and single cell suspensions were prepared.

Hematopoietic growth factors. The cDNA clones encoding the IL-7 gene were obtained from CDNA of a stromal cell line (ST-2) that had been stimulated with lipopolysaccharide (LPS) and 12-0-tetradecanoylphorbol 13-acetate (TPA). We recloned the IL-7 coding region into the expression vector pCDL-SRas and the resulting clone, designated pSRas mlIL-7, was transfected into COS-1 cells. The pooled culture supernate was further chromatographically purified using diethyl aminoethyl (DEE)-cellulose, SP-sephadex, and Blue A gels up to 5 × 105 U/mL and in most of the proliferation assay, the partially purified recombinant (r) IL-7 was used. Murine rIL-3 was kindly provided by Dr K. Ariz (DNAx, Palo Alto, CA), specific activity being 105 U/ml. Purified human recombinant erythropoietin (Ep) was generously provided by Dr M. Ueda (Snow Brand Milk Product Co, Tochigi-ken, Japan). It had a specific activity of 81,600 U/mg protein. Standard concentrations of hematopoietic factors used in our cultures were as follows: 20 U/mL murine rIL-7, 100 U/mL murine rIL-3, and 2 U/mL human rEp.

Phenotype of untreated and cultured fetal liver cells. After lysis of the red blood cells by an ammonium chloride-potassium buffer, single cell suspensions of fetal liver cells were prepared in phosphate-buffered saline (PBS) supplemented with 3% fetal calf serum (FCS; Flow Laboratories, North Ryde, Australia) and 0.1% sodium azide. The cells were stained with fluorescein isothiocyanate (FITC)- or biotin-conjugated monoclonal antibody (MoAb) to B-220, human Thy-1, or mouse Thy-1, and 331.12 (antimouse IgM).

 Colony assays. Methylcellulose culture was performed by using
a modification of a technique described previously. Briefly, bone marrow cells and spleen cells were incubated in 1 mL of culture medium containing alpha medium (GIBCO Laboratories, Grand Island, NY), 1.2% methylcellulose (1,500 centipoises; Aldrich Chemical Co, Milwaukee, WI), 30% FCS, 1% deionized bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), 5 × 10⁻⁵ mol/L 2-mercaptoethanol (2-ME; Sigma Chemical Co) and hematopoietic factors. On day 7 of culture, aggregates consisting of 40 or more cells were scored as a colony. After scoring the number of colonies, the colonies were lifted from the methylcellulose cultures using an Eppendorf pipet under microscopic visualization, and suspended in 0.1 mL of alpha medium. The cell suspensions were spun in a centrifuge from Shandon Southern Instruments Inc (Sewickley, PA) and stained with May-Grünwald-Giemsa. A myeloid colony was defined as a colony consisting of neutrophils, macrophages, eosinophils, mast cells, and/or megakaryocytes. A lymphoid colony was defined as a colony consisting of lymphocytes. Cell suspensions of pooled colonies were analyzed for surface phenotypes by FACS.

Coculture for fetal liver cells and stromal cells. ST-2 is a stromal cell line established from long-term cultures of BC8 mouse bone marrow cells by the method of Whitlock and Witte. ST-2 cells were grown to confluence in a T25 flask (Corning Glass Work, Corning, NY). Day 13 fetal liver cells were cultured on this stromal layer with 10 mL of RPMI containing 5% FCS and 5 × 10⁻⁵ mol/L 2-ME. Five to 13 days after coculture, fetal liver cells were harvested and plated in a methylcellulose medium containing 20 U/mL IL-7. To clarify whether cell contact is essential for B-cell development, fetal liver cells and ST-2 cells were separated with a 0.45-μm filter membrane (Intercell, Kurabo, Osaka, Japan) in the presence or absence of IL-7.

Instead of the bone marrow-derived stromal cell line (ST-2) that was shown to produce IL-7, we performed the coculture of fetal liver cells on another stromal cell line that was established from fetal liver cells (FLS-3). Moreover, we attempted to culture fetal liver cells on the Matrigel- (Collaborative Research Inc, Bedford, MA) or collagen-coated 24-well plate (Toyobo Engineering Co, Osaka, Japan).

Expression of IL-7 mRNA in primary fetal liver cells and stromal cells. Total cellular RNA was extracted from fetal liver cells and stromal cells (ST-2, FLS-3) by the LiCl/Urea method, and poly(A)⁺ was purified by oligo-dT-cellulose column chromatography. ST-2 was incubated with 1 μg/mL LPS, Escherichia coli 055:B5 (Difco Laboratories, Detroit, MI) and 1 ng/mL TPA (Sigma Chemical Co) or with 2 × 10⁶ of DW34 cells for 3 days. FLS-3 was stimulated with 10 U/mL human IL-1α (Dainippon Pharmaceutical Co, Osaka, Japan) for 3 days. Two or four micrograms of each poly(A)⁺ RNA sample was subjected to electrophoresis on 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond-N; Amersham, Tokyo, Japan), and hybridized with the IL-7 probe. The Pru II restriction fragment of CDM mIL-7 was used as a probe.

In vitro amplification of mRNA by reverse transcription-polymerase chain reaction (RT-PCR). We used two oligonucleotide primers (A, 5’CCATGTTCCATGTTTCTTTAAG3’; B, 5’CGTATTATCTGCGCTC3’) that flanked the region to be amplified. When mouse IL-7 mRNA was present in the poly(A)⁺ RNA of each sample, a 471-bp fragment was amplified. ssDNA was
Table 1. Colony Formation of Fractionated Day 18 Fetal Liver Cells

<table>
<thead>
<tr>
<th>IL-7</th>
<th>IL-3 + Ep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>B220⁺</td>
<td>112 ± 2</td>
</tr>
<tr>
<td>B220⁻</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

Number of pre-B-cell and myeloid colonies formed by 1 × 10⁶ cells per dish in the presence of IL-7 and IL-3 plus Ep, respectively.

Table 2. Colony Formation by Coculturing Day 13 Fetal Liver Cells With Stromal Cells

<table>
<thead>
<tr>
<th>Days of Culture With ST-2</th>
<th>0</th>
<th>5</th>
<th>9</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-7</td>
<td>0</td>
<td>12 ± 3*</td>
<td>36 ± 1*</td>
<td>132 ± 13*</td>
</tr>
<tr>
<td>IL-3 + Ep</td>
<td>18 ± 2†</td>
<td>58 ± 15†</td>
<td>1 ± 1†</td>
<td>0</td>
</tr>
</tbody>
</table>

Day 12 fetal liver cells were cocultured with stromal cells (ST-2) for 5 to 13 days. On each day, cells attached to ST-2 cells were harvested and cultured in methylcellulose medium with IL-7 or IL-3 plus Ep.

RESULTS

We examined the pre-B-cell colony formation of fetal liver cells at various gestational ages by the method of methylcellulose culture in the presence of IL-7. As shown in Fig 1A, days 12 to 14 fetal liver cells did not form pre-B-cell colonies. However, pre-B-cell colonies developed from day 15 fetal liver cells. The incidence of colonies increased with increases of gestational age and reached a maximum on days 18 to 19. Thereafter, the number of colonies decreased. Pre-B-cell colony formation was not tested after birth, because single cell suspensions could not be obtained without the proteinase treatment. We concluded that IL-7-responsive B-cell progenitor cells developed on day 15 of gestation. In contrast, the incidence of myeloid colonies formed by fetal liver cells in the presence of IL-3 and Ep did not change significantly during days 13 to 21 of gestation, except that day 12 fetal liver cells formed smaller numbers of colonies (Fig 1B).

We compared the phenotypes of fetal liver cells between day 13 and day 18. As shown in Fig 2A and B, B220⁺, IgM⁻ cells were detected in day 18 fetal liver cells but not in day 13 fetal liver cells. To confirm that pre-B-cell colonies were formed by B220⁺ cells, day 18 fetal liver cells were fractionated into B220⁺ and IgM⁻ cells. As shown in Table 1, pre-B-cell colonies were formed by B220⁺ cells in the presence of IL-7. In contrast, myeloid colonies were formed by B220⁻ cells in the presence of IL-3 and Ep.

Pre-B-cell colonies were not observed in the presence of IL-7, even when 1 × 10⁶ cells obtained from day 13 fetal livers were plated in a dish. To clarify whether B-cell progenitors were present in day 13 fetal liver cells or not, we transferred 1 × 10⁶ fetal liver cells onto an ST-2 cell layer and cultured them. Figure 3 shows that small cells developed from day 13 fetal liver cells on stromal cells. To determine when day 13 fetal liver cells can respond to IL-7, we harvested hematopoietic cells grown on ST-2 cells sequentially and replated them into the methylcellulose medium with IL-7.
To clarify whether the cell contact is essential for the growth of early B-cell progenitors, we attempted to separate day 13 fetal liver cells from ST-2 cells with a millipore membrane. B cells did not develop even when IL-7 was added to this culture system (Table 3). This result indicated that contact with stromal cells is essential for early B-cell development.

In our previous works, ST-2 was shown to produce IL-7 by the stimulation of IL-1α or the contact of hematopoietic cells. Therefore, to clarify whether early B-cell progenitors can be grown only in the presence of stromal cells, the coculture of fetal liver cells with one of fetal liver stroma cell lines (FLS-3) was performed. As shown in Fig 4A, IL-1α-stimulated FLS-3 did not produce IL-7 mRNA at the level of the Northern blot analysis. However, a band of IL-7 was detected in FLS-3 by RT-PCR. The amount was quantified by densitometry of the band in the autoradiography after Southern blot analysis of PCR amplified mL-7 cDNA. Expression of IL-7 mRNA in FLS-3 was less than 5% of that in ST-2 (Fig 5). In the presence of FLS-3 alone, lymphocyte did not develop from day 13 fetal liver cells (Table 3). However, by adding IL-7 to this culture system on days 0, 14, and 21, small lymphocyte developed 1 week later. Moreover, after various days of preincubation of day 13 fetal liver cells with FLS-3, we transferred these cells and cultured them in the methylcellulose medium containing IL-7. As shown in Table 4, only 3 to 5 days of culture of day 13 fetal liver cells with FLS-3, IL-7-responsive cells were

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**Table 3. Development of B Lymphocytes on Stromal Cell Lines**

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-2</td>
<td>+</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>ST-2 separated by millipore membrane</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ST-2 separated by membrane + IL-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FLS-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FLS-3 + IL-7 on day 0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLS-3 + IL-7 on day 14</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FLS-3 + IL-7 on day 21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Collagen + IL-7 on day 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Matrigel + IL-7 on day 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ST-2 separated by millipore membrane: separate fetal liver cells from ST-2 cells with a millipore membrane. FLS-3 (collagen or matrigel) + IL-7. IL-7 was added on day 0, 14, and 21 in the coculture system of fetal liver cells with FLS-3 (collagen or matrigel).

Abbreviations: +, growth of small lymphocytes from fetal liver cells; -, no growth; /, not observed.

culture system containing IL-7 or IL-3 plus Ep. On day 5, IL-7-responsive cells were already detected in addition to IL-3-responsive myeloid cells (Table 2). Thereafter, the incidence of IL-7-induced pre-B-cell colonies increased, while IL-3-induced myeloid colonies decreased.

After the coculture of day 13 fetal liver cells with ST-2 cells, 66.66% of these cells expressed B220 antigen (Fig 2C). IL-7-induced colonies formed by day 18 fetal liver cells were lifted and analyzed for surface phenotypes, and it was found that these colonies contained 27% IgM+ cells (Fig 2D).
detected by methylcellulose culture. When day 13 fetal liver cells were cultured on collagen layer or Matrigel, lymphocytes did not develop even in the presence of IL-7.

Whether IL-7 was produced in fetal liver cells or not, expression of IL-7 mRNA was examined by the Northern blot hybridization technique. IL-7 mRNA was detected in days 13 and 18 fetal liver cells (Fig 4D). In contrast, IL-7 mRNA was detected neither in pre-B cells nor adult liver cells (data not shown).

**DISCUSSION**

Using a soft agar or methylcellulose culture systems, Lee et al and we showed that IL-7 acts on B220⁺ cells and supports pre-B-cell colony formation. It has been demonstrated that pro-B cells can differentiate in the presence of bone marrow stromal cells. We examined B-cell generation in murine fetal liver to determine whether this two step model can be applied to in vivo lymphopoiesis.

We showed that IL-7-responsive B-cell progenitor cells developed on day 15 fetal liver cells, while days 12 to 14 fetal liver cells did not form pre-B-cell colonies. Pre-B-cell colony forming ability in fetal liver cells correlated well with the data on phenotype analysis that B220⁺ cells were not detected until day 15 of gestation. Recently, Goodwin et al isolated cDNA clones encoding the IL-7 receptor and showed that IL-7 receptor mRNA was detected in murine fetal liver but not in adult liver tissue.

From the finding that day 13 fetal liver cells were able to differentiate into mature B cells in the presence of stromal cells, it is concluded that day 13 fetal liver cells contained pro-B cells that were not responsive to IL-7 but to stromal cells. Sudo et al previously showed that ST-2 cells expressed IL-7 mRNA by the stimulation of contact with pre-B cells. When day 13 fetal liver cells were separated from ST-2 cells with a millipore membrane, B cells did not develop, even when IL-7 was added. IL-7-responsive pre-B cells developed from the day 13 fetal liver cells after the coculture with FLS-3 that did not produce IL-7. These results clearly indicate that IL-7 is not required for the differentiation of pro-B cells to pre-B cells and that contact with stromal cells

**Table 4. Colony Formation of Cocultured Day 13 Fetal Liver Cells With Fetal Liver Stroma Cell Line (FLS-3)**

<table>
<thead>
<tr>
<th>Days of Culture With FLS-3</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>11</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-7</td>
<td>0 ± 0*</td>
<td>6 ± 1*</td>
<td>9 ± 1*</td>
<td>11 ± 1*</td>
<td>26 ± 1*</td>
</tr>
<tr>
<td>IL-3 + Ep</td>
<td>36 ± 4t</td>
<td>79 ± 10t</td>
<td>8 ± 0t</td>
<td>4 ± 1t</td>
<td>26 ± 0t</td>
</tr>
</tbody>
</table>

*Number of lymphoid colonies per 4 x 10⁶ cells.

†Number of myeloid colonies per 4 x 10⁶ cells.
was essential for early B-cell development. So far, we could not replace the role of stromal cells with collagen or matrigel. In our preliminary study, an MoAb against murine c-kit-encoded molecule suppressed the supporting capacity of IL-7-responsive cells by stromal cells. However, it remains to be clarified whether signal transduction of c-kit/stem cell factor is essential in the early differentiation of B cells or not.17

We showed that the expression of IL-7 mRNA was detected in the murine fetal liver but not in adult liver. It has been reported that significant expression of IL-7 messenger was found in the murine thymus and that IL-7 has potent costimulatory activity for T cells. In this context, IL-7 produced by fetal liver cells may affect B lymphopoiesis in vivo. However, from our experiments it is clear that IL-7 alone does not affect B220− cells. Stromal cells or stromal-derived factor(s) is required for the differentiation from B220+ cells to B220+ cells. We concluded that the two-step model can be applied to in vivo B lymphopoiesis.

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REFERENCES
Support of early B-cell differentiation in mouse fetal liver by stromal cells and interleukin-7

Y Gunji, T Sudo, J Suda, Y Yamaguchi, H Nakauchi, S Nishikawa, N Yanai, M Obinata, M Yanagisawa and Y Miura