Murine Embryonic Yolk Sac Cells Promote In Vitro Proliferation of Bone Marrow High Proliferative Potential Colony-Forming Cells

By Mervin C. Yoder, Barbara King, Kelly Hiatt, and David A. Williams

To examine the influence of the hematopoietic microenvironment on hematopoietic cell proliferation and differentiation during the yolk sac phase of hematopoiesis, we have recently established cell lines from embryonic yolk sac visceral endoderm (YSE) and mesoderm (YSM). In the present experiments, we compared in vitro growth of adult murine bone marrow high proliferative potential colony-forming cells (HPP-CFC) in coculture with YSE- and YSM-derived or adult bone marrow stromal cell lines. Whereas both yolk sac-derived and adult stromal cell lines supported the proliferation of HPP-CFC during coculture, YSE- and YSM-derived cells stimulated a significant increase in total HPP-CFC compared with adult bone marrow stromal cell lines. Conditioned media from both YSE- and YSM-derived cell lines also stimulated the growth of HPP-CFC in vitro, but only in combination with exogenous recombinant hematopoietic growth factors. Although multiple hematopoietic growth factor mRNAs were detected in the yolk sac-derived cells by polymerase chain reaction, only macrophage colony-stimulating factor (M-CSF) activity was detected in conditioned media using an enzyme-linked immunosorbent assay. A neutralizing polyclonal antibody against M-CSF did not diminish the YSE- or YSM-derived cell line conditioned media promotion of HPP-CFC colony formation. These results suggest that murine yolk sac-derived cell lines produce a novel soluble factor(s) that recruits primitive bone marrow hematopoietic cells to grow in vitro in response to a combination of hematopoietic growth factors.

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Submitted December 9, 1994; accepted April 3, 1995.

Supported by National Institutes of Health Grants No. P01 HL45168 (M.C.Y.) and R01 HL65282 (D.A.W.) and by Basic Research Grant No. J-1235 from the March of Dimes-Birth Defects Foundation (D.A.W.)

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YOLK SAC CELLS PROMOTE BM PROLIFERATION

In this report, we compared the growth of adult murine bone marrow HPP-CFC in vitro in coculture with cell lines derived from murine embryonic yolk sac and adult bone marrow stroma. We report that YSE- and YSM-derived cell lines support in vitro growth of adult murine bone marrow HPP-CFC to a greater extent than did adult bone marrow stromal cell lines and that conditioned media from the cultured yolk sac-derived cell lines contains some of the growth-promoting factor(s). These results indicate that nonhematopoietic cells of the hematopoietic microenvironment exert a potent influence on multipotent hematopoietic progenitor cell proliferative behavior.

MATERIALS AND METHODS

Murine stocks. C3H/HeJ mice were obtained at 8 to 10 weeks of age from Jackson Laboratory (Bar Harbor, ME). Animals were housed 3 to 5 per cage and received food and water ad libitum. This study was approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Cell lines. Four YSE and five YSM cell lines were maintained as previously described in high glucose Dulbecco’s modified Eagle’s medium (hgMEM) with 10% fetal bovine serum (FBS), 5% fetal calf serum (FCS), 1 mmol/L nonessential amino acids, 1 mmol/L L-glutamine, and 10^{-4} mol/L 2-β-mercaptoethanol (Sigma, St Louis, MO). Cells were passaged twice weekly at 70% to 80% confluence.

The adult murine bone marrow stromal cell lines U2, U3, and U5 have been previously described.13,14 In the current experiments, U2 through U5 were cultured in MEM 10% FCS and passed twice weekly when at 70% to 80% confluence. These cell lines were used as control stromal elements representing the adult bone marrow hematopoietic microenvironments.

Coculture experiments. Established YSE, YSM, and U cell lines grown to 80% confluence were treated with mitomycin C (Sigma) at a concentration of 10 μg/mL for 4 hours at 37°C, washed three times with phosphate-buffered saline (PBS), detached with trypsin (0.5%), and plated to confluence in 6-well tissue culture plates with each cell line plated in triplicate wells as previously described.15 To obtain a population of adult murine bone marrow cells enriched in HPP-CFC, C3H-HeJ male mice were injected via the tail vein with 150 mg/kg 5-fluourouracil (5-FU; Aldrich Chemicals, Milwaukee, WI). Two days later, marrow cells were harvested as described, washed in DME + 10% FCS, and counted. 5-FU–resistant bone marrow cells (1 × 10^5) were plated in coculture with the mitomycin C–treated cell monolayers in Iscove’s modified Dulbecco’s medium (IMDM) with 15% horse serum, 5% FCS, 400 mg/mL human iron-saturated transferrin (Boehringer Mannheim, Indianapolis, IN), 10^{-4} mol/L 2-β-mercaptoethanol, and 10^{-4} mol/L hydrocortisone (Upjohn, Kalamazoo, MI). One-half of the media and nonadherent cells were carefully removed, discarded, and replaced with fresh media after 7 days of culture. On day 14, adherent and nonadherent hematopoietic cells were counted using a Coulter counter (Coulter, Hialeah, FL), morphologic cell differentials were performed using Wright-Giemsa stain, and cells were aliquoted in triplicate wells for in vitro assay of HPP-CFC.

In some experiments, 1 × 10^3 5-FU–resistant bone marrow cells were added to each of three wells of a 6-well culture plate containing confluent monolayers of the YSE, YSM, and U cell lines. After 2 hours of incubation at 37°C, nonadherent cells were recovered, washed, pooled, counted, and plated in double-layer agar cultures for HPP-CFC analysis. Adherent hematopoietic cells were removed using cell dissociation buffer (GIBCO, Grand Island, NY), and the suspended hematopoietic cells from triplicate wells were pooled, washed, counted, and plated for HPP-CFC enumeration.

HPP-CFC assay. Double-layer agar cultures were prepared as previously described.16 Briefly, the recombinant hematopoietic growth factor human macrophage colony-stimulating factor-1 (M-CSF; 1,600 U; Genetics Institute, Boston, MA), IL-3 (200 U) and IL-1α (500 U; Genzyme, Cambridge, MA), and rat SCF (100 ng; Amgen, Thousand Oaks, CA) were added to 10 × 35 mm gridded tissue culture dishes followed by the addition of 0.5% agar (Bacto-agar; Difco, Detroit, MI). Nonadherent or adherent hematopoietic cells (50,000/plate) from the cocultures were suspended in 0.3% agar and applied as an overlay to the 0.5% agar/growth factor-containing dishes and triplicate cultures were plated and incubated in a 5% O_2, 10% CO_2, and 85% N_2 humidified environment. On day 14 of culture, the plates were stained with 1 mg/mL piodonitrotetrazolium violet (Sigma) and colonies greater than 0.5 cm were scored as HPP-CFC. The results from the 4 YSE, 5 YSM, and 3 U cell lines were pooled for each of four experiments. In all experiments, statistically significant differences were determined using the Student’s t-test with a level of significance set at P < .05.

RNA isolation and polymerase chain reaction (PCR) analysis. RNA was isolated from monolayers of YSE, YSM, and U cell lines using TRI REAGENT (Molecular Research Center, Cincinnati, OH) and the method of Chomczynski and Sacchi.21 One microgram of total RNA was reverse transcribed using 2.5 μmol/L random hexamers, 2.5 U Moloney’s murine leukemia virus (MMLV) reverse transcriptase, and resents from a commercial RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). PCR amplification of the cDNA was then performed using oligonucleotide primers (Table 1) for several hematopoietic growth factors. Standard PCR conditions were used with 1 minute of denaturation at 95°C, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. Analysis of PCR products was performed by comparing predicted PCR fragment lengths with actual PCR products after ethidium bromide staining of PCR products separated by electrophoresis in a 2% agarose gel. In addition, electrophoresed PCR products were transferred to n nylon membranes and hybridized with sequence-specific radiolabeled “nested” oligonucleotides to confirm PCR product identity. Briefly, the internal oligonucleotide primers were end-labeled with 32P using T4 polynucleotide kinase and a commercial kit (Boehringer Mannheim). The membranes were prehybridized in 50% formamide, 2.5 X SSC, 2 X Denhardt’s solution, 0.1% sodium dodecyl sulfate, 100 μg/mL yeast RNA, 150 μg/mL salmon sperm DNA, and 1% dextran sulfate at 68°C for 30 minutes and then hybridized with the labeled probe overnight at 42°C. In the morning, the membranes were washed with 6 X SSC, 0.1% sodium dodecyl sulfate (SDS) at 50°C for 1 hour and then exposed to Kodak film at ~70°C with intensifying screens overnight (Eastman Kodak, Rochester, NY).

Conditioned media experiments. Conditioned media from each of the YSE, YSM, and U stromal cell lines was prepared by culturing the cells to 80% confluence, adding the spent media, and the cells overnight. In the morning, the conditioned media was removed, centrifuged at 2,000g for 20 minutes, and passed through a 0.2-μm filter before freezing in aliquots at −80°C. For some experiments, conditioned media was concentrated 20-fold (Micro-Prodicon; Spectrum Industries, Houston, TX) before filtering and freezing.

Assay of the conditioned media from YSE, YSM, and U cell lines was performed using growth factor specific commercially available enzyme-linked immunosorbent assays (ELISAs) as previously described by Hunt et al.10 The sensitivity of the assays and results are indicated in Table 5. Assay for each growth factor was performed with undiluted conditioned media or with 10× concentrated media.

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Table 1. Oligonucleotide Primers for Hematopoietic Growth Factor mRNA

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Antisense and Sense Primer</th>
<th>Bases Spanned (size)</th>
<th>Probe</th>
<th>Bases Spanned Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF</td>
<td>5'-ATCTTCTCCAGCAGTCAGAGGTCT-3'</td>
<td>66-93 (381)</td>
<td>5'-TCGGTCTCTCATAGTCTGTTGGAAGC-3'</td>
<td>358-384 23</td>
</tr>
<tr>
<td></td>
<td>5'-GGTCTGTCCTCTCATAGCAGGAGTATT-3'</td>
<td>418-445 (381)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>5'-CCGAGATGTAGGCGACAGACTTAAGTG-3'</td>
<td>49-75 (300)</td>
<td>5'-TGTCTCAGGGATCCACTTTTCCTGGC-3'</td>
<td>278-304 24</td>
</tr>
<tr>
<td></td>
<td>5'-ACCCGATCCACCCACTGCTGCTTCTG-3'</td>
<td>333-360 (300)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5'-GGCTGTCATGCTACCCCGGTCTTGGAGAAGC-3'</td>
<td>101-128 (302)</td>
<td>5'-CAGTATGTCGTAGTCTGCTC-3'</td>
<td>311-337 25</td>
</tr>
<tr>
<td></td>
<td>5'-CATCAGCTGCACCCGGGCTTGGGAAAGC-3'</td>
<td>375-402 (300)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-CATGATCTGGGTCATCTTCTCATAGGGTTGC-3'</td>
<td>81-110 (388)</td>
<td>5'-TCAGGGGCCACACGCAGCTCTGTAAGA-3'</td>
<td>348-376 26</td>
</tr>
<tr>
<td></td>
<td>5'-ATGGATGATGATATCGCTGCGCTGGTC-3'</td>
<td>420-449 (388)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>5'-CTCCTTCAAGAACAGGCCGTCGTCTC-3'</td>
<td>349-375 (384)</td>
<td>5'-GAAGATGACCTGACGTTCATAAACC-3'</td>
<td>661-687 27</td>
</tr>
<tr>
<td></td>
<td>5'-GGAGATCTCAGAAAGAGACGGCGTCTC-3'</td>
<td>707-733 (384)</td>
<td></td>
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</tr>
<tr>
<td>IL-6</td>
<td>5'-GCATAACGCATGATGTGCGGAGAGCTAG-3'</td>
<td>111-137 (566)</td>
<td>5'-TTCTGATCTCTCTGAGTTCTGC-3'</td>
<td>446-472 28</td>
</tr>
<tr>
<td></td>
<td>5'-GTCCTACAGCTGCCGCTCAGGGAGACTTCA-3'</td>
<td>651-677 (566)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCF</td>
<td>5'-CAGTATAGGCATCCAAAGCAGCAAGC-3'</td>
<td>506-533 (388)</td>
<td>5'-TGCTACTGCTGCTCCTATAGGGGAC-3'</td>
<td>756-782 29</td>
</tr>
<tr>
<td></td>
<td>5'-GTGGAATGACCTGCTGTTATGCTAGGTA-3'</td>
<td>868-894 (388)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In some experiments, neutralizing concentrations of antibody to IL-1α, IL-3, IL-6, GM-CSF, SCF, and M-CSF were added to YSE- and YSM-conditioned media for 45 minutes at 37°C, followed by the addition of 1 × 10⁶ 5-FU–resistant bone marrow cells. The 5-FU–resistant cells were incubated in conditioned media with or without the antibodies for 2 hours at 37°C and then pelleted, washed, and plated for HPP-CFC determination. Determination of neutralizing antibody concentrations was performed in preliminary experiments. All antibodies were purchased from Genzyme except for antirat SCF, which was a generous gift from Dr Larry Bennett (Amgen). Polyclonal rabbit antimouse IL-1α (diluted 1:5) and antihuman M-CSF (diluted 1:8) inhibited greater than 50% growth of HPP-CFC at concentrations of 500 U and 400 U, respectively (with added SCF), rat antimouse IL-3 (150 ng/mL) inhibited greater than 50% FDCP cell (kindly provided by Dr Yu-Chung Yang, Indiana University School of Medicine, Indianapolis, IN) proliferation induced by 20 U of IL-3, antirat SCF (20 μg/mL) inhibited greater than 75% proliferation of St cells induced by 25 ng/mL SCF, rat antimouse GM-CSF inhibited proliferation of FDCP cells greater than 50% induced by 1 ng/mL GM-CSF, and rat antimouse IL-6 (100 μg/mL) inhibited greater than 50% HPP-CFC growth (with added SCF).

RESULTS

Coculture experiments. The total number of nucleated cells recovered from the cocultures increased fourfold in the U and YSE cultures compared with the input concentration (Fig 1). Significantly more cells (>7-fold increase over input) were recovered from the YSM cocultures. Morphologic examination of the recovered cells showed 93% ± 6% hematopoietic cells with minimal contaminating immortalized stromal cells. The predominant mature blood cells (>90%) present in the cocultures with the bone marrow stromal (U) cells were immature and mature granulocytes with few macrophages, whereas YSE and YSM cocultures supported significant macrophage differentiation (>35%) and a lower percentage of granulocyte differentiation (~70%), as previously described.¹⁸

The concentration of HPP-CFC increased significantly over the input concentration in all three cocultures (Fig 2). The highest concentration of HPP-CFC was present in cells recovered from the YSE cocultures, and both YSE and YSM cocultures supported significantly greater HPP-CFC growth than did the adult bone marrow stromal (U) cocultures (Fig 2).
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To examine the effect of YSE and YSM cell lines on HPP-CFC cycling, we isolated conditioned medium from U, YSE, and YSM cultures and examined the percentage of cycling HPP-CFC after 2 hours of incubation in the various conditioned media. The percentage of 5-FU-resistant HPP-CFC killed by high specific activity \(^{3}H\)-thymidine after incubation in U-conditioned medium was 13.3% ± 5.6%, whereas 21.7% ± 3.1% and 23.9% ± 4.8% of HPP-CFC incubated in YSE- and YSM-conditioned medium, respectively, were killed. These results confirmed a significant increase in cycling HPP-CFC in response to a soluble factor(s) present in the YSE- and YSM-conditioned media.

Additional experiments were performed to determine the effect of YSE- and YSM-conditioned media on HPP-CFC growth. When conditioned media from the YSE, YSM, and U cell lines was directly added (20% by volume final concentration) in the HPP-CFC assay without including any of the standard recombinant growth factors, few HPP-CFC survived during the standard 2 weeks in culture (in 1 experiment, 3 small HPP-CFC colonies were present in each of the YSE- and YSM-conditioned media-containing cultures; however, in 2 subsequent experiments, no HPP-CFC were present in any of these cultures). In contrast, conditioned media alone from the YSE, YSM, and U cell lines did promote low proliferative potential colony-forming cell (LPP-CFC) growth in the cultures (33 ± 34, 34 ± 16, 28 ± 20 LPP-CFC/100,000 cells, respectively). The most marked effect of the YSE- and YSM-conditioned media was evident when the media was added (20% by volume) directly to the HPP-CFC assay along with standard concentrations of IL-1α, CSF-1, SCF, and IL-3 (see Materials and Methods). Significantly higher numbers of HPP-CFC were grown in HPP-CFC assay dishes containing YSE- and YSM-conditioned media plus the recombinant growth factors compared with HPP-CFC cultures containing U cell line conditioned media or unconditioned control culture media plus recombinant growth factors (65 ± 6, 79 ± 8, 37 ± 4, and 33 ± 6 HPP-CFC/100,000 cells, respectively). Thus, YSE- and YSM-conditioned media significantly increased the number of HPP-CFC grown in the presence of recombinant growth factors. Concentrating the YSE-, YSM-, and U cell line-

![Fig 2. The frequency of HPP-CFC present in the fresh 5-FU bone marrow cells (input) and in the nucleated cells recovered at the end of the 2-week coculture period with the adult bone marrow and yolk sac (YS) endoderm- and mesoderm-derived cell lines is indicated as the mean ± SD and represents the results of four experiments. A significant increase in HPP-CFC frequency was determined from the cells recovered from the adult bone marrow cocultures (*) compared with the input HPP-CFC frequency. A significantly higher frequency of HPP-CFC was determined for the cells recovered from the YS endoderm and mesoderm cocultures (**) than from the adult bone marrow cocultures.](#)

\[2\] Combining the results from Fig 1 and 2, the total number of HPP-CFC increased more than 10-fold in the U cocultures and 21- to 28-fold in the YSE and YSM cocultures during the 2-week experiment. These results may have underestimated the total number of HPP-CFC present in the cocultures because numerous "cobblestone areas" in which hematopoietic cells had migrated underneath the immortalized stromal cell monolayers were identified in U, YSE, and YSM cocultures. We observed no difference in the number of cobblestone areas in the cocultures established with the U stromal cell lines compared with the YSE and YSM cell lines (data not shown).

To better assess any differences in the number of nonadherent versus adherent HPP-CFC recovered from the various cocultures, we performed four experiments in which the 5-FU-resistant hematopoietic cells were cocultured for 2 hours with the U, YSE, and YSM cell lines. This period of coculture permits adhesion of hematopoietic cells to the cell monolayers but is not associated with hematopoietic cells migrating beneath the monolayers.\(^{21}\) Essentially all of the HPP-CFC added to the U cell line cocultures were recovered, with 45% of the cells having become adherent to the immortalized cells during the 2 hours of incubation (Table 2). In contrast, significantly more HPP-CFC were recovered from the YSE and YSM cell line-containing wells than were originally plated (Table 2), and significantly more HPP-CFC were recovered in the nonadherent and adherent fractions from the YSE and YSM cultures than from the U stromal cell line-containing cultures. These results suggested that brief incubation of the 5-FU-resistant bone marrow cells with the YSE and YSM cell lines may result in an increase in the number of cycling HPP-CFC or influence the plating efficiency of the hematopoietic cells.

*Effects of conditioned media on HPP-CFC proliferation.*

**Table 2. HPP-CFC Identified After 2 Hours of Coculture of Hematopoietic Cells With Stromal Cell Lines**

<table>
<thead>
<tr>
<th>Stromal Cell Line</th>
<th>Adherent</th>
<th>Nonadherent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>19 ± 9</td>
<td>23 ± 4</td>
<td>42 ± 12</td>
</tr>
<tr>
<td>Yolk sac endoderm</td>
<td>59 ± 121</td>
<td>38 ± 9*</td>
<td>103 ± 16*</td>
</tr>
<tr>
<td>Yolk sac mesoderm</td>
<td>51 ± 171</td>
<td>44 ± 14*</td>
<td>95 ± 19*</td>
</tr>
<tr>
<td>Input</td>
<td>—</td>
<td>—</td>
<td>43 ± 8</td>
</tr>
</tbody>
</table>

In four experiments, the frequency of HPP-CFC identified in 5-FU-resistant bone marrow cells was significantly (\(P < .05\)) higher when comparing the total number of hematopoietic cells cocultured for 2 hours with yolk sac endoderm or mesoderm-derived cells (*) compared with bone marrow stromal cells. Significantly increased numbers of HPP-CFC were present in both the adherent (†) and nonadherent (‡) hematopoietic cells isolated from yolk sac endoderm- and mesoderm-derived cell cocultures compared with adult bone marrow cocultures. Results are expressed as mean ± SD HPP-CFC colonies per 100,000 cells plated.
conditioned media up to 20-fold did not increase or decrease the number of HPP-CFC present at the end of the culture period (data not shown). However, the growth-promoting effects of culturing 5-FU-treated marrow cells with YSE- and YSM-conditioned media was rapidly diminished when the conditioned media was diluted one-half or more (Fig 3). In addition, YSE- and YSM-conditioned media effects on stimulating HPP-CFC proliferation were shown to be restricted to those more primitive HPP-CFC that require a combination of several recombinant growth factors for in vitro growth (Table 3). Essentially no HPP-CFC survived

and formed a colony when plated in the presence of a single recombinant growth factor or when plated in a single growth factor plus YSE- or YSM-conditioned media, but a twofold increase in HPP-CFC growth was shown when hematopoietic cells were plated with a standard combination of four growth factors plus the YSE- or YSM-conditioned media compared with the four growth factor combination without conditioned media (Table 3). Because normal and 5-FU-treated bone marrow cells contain a large population of HPP-CFC that respond to two growth factor combinations, restriction of the additive effect of YSE- and YSM-conditioned media to HPP-CFC responsive to a four growth factor combination and not to single growth factors suggests the YSE- and YSM-conditioned media triggers a more primitive population of hematopoietic cells to form colonies in response to the recombinant growth factors.

Analysis of cell line growth factor expression. Because hematopoietic growth factor requirements for in vitro survival and growth of murine bone marrow HPP-CFC are known, total RNA was isolated from the U, YSE, and YSM cell lines; the RNA was reverse transcribed; and the resulting cDNA were amplified using PCR and oligonucleotide primers specific for six hematopoietic growth factors (Table 1). As summarized in Table 4, mRNA for M-CSF, GM-CSF, IL-6, and SCF were identified in both yolk sac-derived and adult stromal cell lines. In contrast, GM-CSF, IL-3, and IL-1α were detected in RNA isolated from the YSE and YSM but not the U cell lines (Table 3 and Fig 4).

Conditioned media from the U, YSE, and YSM cell lines was analyzed for expression of M-CSF, GM-CSF, IL-6, IL-3, IL-1α, and SCF by ELISA (Table 5). Whereas M-CSF was present at a concentration of 2 to 3 ng/mL in the conditioned media of U, YSE, and YSM cell lines, we were unable to detect the presence of any of the other growth factors (Table 5) even after the concentration of some samples 10-fold. Preincubation of YSE- and YSM-conditioned media with neutralizing antibodies to IL-3, M-CSF, GM-CSF, SCF, and IL-6 did not alter the stimulatory effect of the conditioned media on HPP-CFC growth (Table 6). Furthermore, whereas deleting M-CSF as one of the four standard growth factors (see Materials and Methods) in the HPP-CFC assay diminished the number of HPP-CFC identified (42 ± 8 with M-CSF and 23 ± 9 without M-CSF, HPP-CFC/100,000 cells plated), preincubating hematopoietic cells with 1,600 U M-CSF for 2 hours and then plating the cells in the HPP-CFC
assay in the presence of M-CSF, IL-3, IL-1α, and SCF did not result in an increase in the number of HPP-CFC counted (42 ± 8 no preincubation with M-CSF and 38 ± 13 with M-CSF preincubation, HPP-CFC/100,000 cells plated). These results show that, although M-CSF was present in YSE- and YSM-conditioned media, the stimulatory effects of YSE- and YSM-conditioned media plus added growth factors on HPP-CFC growth cannot be solely attributed to the presence of M-CSF in the conditioned media.

DISCUSSION

Accumulating evidence suggests that the proliferative behavior of hematopoietic stem and progenitor cells decreases with advancing age during human and murine development. Because the yolk sac is the first site of murine hematopoiesis, yolk sac hematopoietic stem cells may have the highest proliferative capacity of any murine hematopoietic stem cells. As an approach to dissecting the cell-to-cell interactions that may influence hematopoietic cell proliferative behavior during embryonic development, we have established cell lines from the embryonic yolk sac. We have hypothesized that the proliferative behavior of yolk sac hematopoietic cells is regulated in part by endoderm and mesoderm cells of the visceral yolk sac.

In this report, we have shown that cell lines established from YSE and YSM promote the in vitro growth of adult bone marrow HPP-CFC to a greater extent than do cell lines similarly established from adult bone marrow stromal cells. The stimulatory effects of the yolk sac-derived cell lines on HPP-CFC growth was also apparent when bone marrow hematopoietic cells were allowed to adhere for 2 hours to

Table 5. Characterization of Cell Line Conditioned Media for Growth Factors Detectable by ELISA

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Sensitivity (pg/mL)</th>
<th>Bone Marrow</th>
<th>YSE</th>
<th>YSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF</td>
<td>300</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>60</td>
<td>–</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>IL-6</td>
<td>40</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IL-3</td>
<td>150</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IL-1α</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SCF</td>
<td>&lt;800</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Conditioned media from the established cell lines was assayed on two occasions for hematopoietic growth factor expression. M-CSF was present (+++) at a concentration ranging from 2 to 3 ng/mL, but all other factors were not detectable (–).
monolayers of the immortalized cells or when the hematopoietic media were preincubated in or directly plated with conditioned media from the YSE and YSM cell lines. Although the conditioned media alone significantly increased the number of cycling HPP-CFC, the media alone did not support HPP-CFC growth in vitro. However, YSE- and YSM-conditioned media significantly increased the number of HPP-CFC grown in vitro in combination with recombinant growth factors. These results suggest that a factor(s) present in YSE- and YSM-conditioned media increases the proliferation of HPP-CFC and recruits these hematopoietic cells to become growth responsive to a combination of recombinant hematopoietic growth factors.

HPP-CFC are a heterogeneous population of hematopoietic progenitor cells with complex growth factor requirements for in vitro growth. The most primitive HPP-CFC are relatively quiescent multipotent cells that are resistant to the in vivo effects of 5-FU, give rise to secondary multipotent colonies, and require at least four hematopoietic growth factors, i.e., IL-3, IL-1α, M-CSF, and SCF (or IL-6), for in vitro survival and growth. According to the recent model proposed by Kriegler et al., primitive HPP-CFC proliferate and differentiate, and HPP-CFC progeny become progressively more responsive to a variety of triple and double growth factor combinations and finally become lineage committed progenitor cells responsive to single hematopoietic growth factors. Ogawa has also recently reviewed the mechanisms regulating proliferation of another class of primitive multipotent hematopoietic progenitor cells, the blast cell colony-forming cell. Similar to requirements for the most primitive HPP-CFC, IL-6, II-11, IL-3, G-CSF, GM-CSF, and SCF are important growth factors that interact to trigger the proliferation of these primitive progenitor cells in vitro.

Several of the hematopoietic growth factors, such as G-CSF, IL-3, GM-CSF, and M-CSF, that stimulate proliferation of the most primitive hematopoietic cells in combination with other factors will function individually to stimulate some HPP-CFC and committed progenitor cell colony growth in vitro. Other hematopoietic growth factors, such as SCF, IL-1α, IL-6, and the recently identified flt3/Flk2 ligand, that synergize to stimulate the proliferation of the most primitive HPP-CFC have limited or no ability as individual growth factors to stimulate HPP-CFC or committed progenitor cell colony growth in vitro. SCF interacts with primitive hematopoietic cells in a unique fashion. This growth factor alone maintains the survival but induces little proliferation and HPP-CFC colony formation of hematopoietic cells in vitro; however, SCF, when combined with 10- to 100-fold below normal concentrations of M-CSF, G-CSF, GM-CSF, IL-1α, and IL-3, stimulates HPP-CFC growth equivalent to cultures initiated with all of the growth factors at 1× concentration.

In the present experiments, YSE- and YSM-derived cell lines produced a soluble factor(s) that also showed novel properties. Because the stimulatory activity present in the YSE- and YSM-conditioned media required the presence of added recombinant growth factors to promote HPP-CFC growth in vitro, but alone was sufficient to stimulate committed progenitor cell colony growth in vitro, the conditioned media factor(s) appears to have some hematopoietic effects similar to the pattern of G-CSF, IL-3, GM-CSF, or M-CSF. Indeed, M-CSF activity was present in both YSE- and YSM-conditioned media by ELISA; however, the addition of neutralizing antibody to M-CSF or adding recombinant M-CSF to YSE- and YSM-conditioned media failed to influence the proliferative effects of YSE- and YSM-conditioned media on HPP-CFC growth. Thus, the M-CSF present in the YSE- and YSM-conditioned media does not appear to account for the stimulatory effect of the conditioned media and recombinant growth factors on HPP-CFC growth. Furthermore, it is unlikely that the HPP-CFC growth-promoting effects of the YSE- and YSM-conditioned media can be attributed to the presence of extremely low (undetectable by ELISA) concentrations of a combination of G-CSF, GM-CSF, IL-3, or M-CSF because the addition of individual recombinant hematopoietic growth factors (at maximal stimulatory concentrations) to the conditioned media failed to result in HPP-CFC growth in vitro. As previously mentioned, SCF alone does not promote HPP-CFC growth in vitro, however, when added to cultures along with a combination of growth factors at very low concentrations, SCF induces HPP-CFC growth nearly equivalent to when all of the growth factors are added at standard concentrations. In the present experiments, 100 ng/mL SCF plus YSE- or YSM-conditioned media failed to promote growth of any HPP-CFC colonies. Finally, we have recently excluded the potential role of flk2/flt3 as the HPP-CFC growth-promoting factor in the YSM-conditioned media because preincubation of post 5-FU-treated bone marrow cells for 2 hours with 100 to 500 U/mL recombinant mouse flt3 ligand failed to promote increased HPP-CFC colony numbers and the addition of a flt3 inhibitory flt3-IgG fusion protein to YSM-conditioned media did not diminish the effect of the YSM-conditioned media on HPP-CFC growth (data not shown).

The finding that YSE- and YSM-conditioned media only promoted increased HPP-CFC colony numbers in combination with multiple hematopoietic growth factors suggests that the YSE- and YSM-conditioned media stimulates a population of primitive HPP-CFC that in the absence of the conditioned media are not responsive to the same combination of hematopoietic growth factors. This growth-promoting activity is novel because the four growth factor combination used in this study (IL-1α, IL-3, M-CSF, and SCF) has previously been determined to be the most optimal in stimulating the maximal number of HPP-CFC colonies from normal or 5-FU–treated murine bone marrow. Metcalf has suggested that the term recruitment may best describe the increased cell proliferation achieved when additional clonogenic cells form colonies in vitro in response to a combination of hematopoietic growth factors as compared with a lower number of colonies formed in response to the growth factors added individually. Recruitment may arise from the combination of hematopoietic growth factors stimulating a different subset of clonogenic cells or because some clonogenic cells may require multiple growth factor signals before becoming competent to proliferate. The present results suggest that YSE- and YSM-conditioned media contains a factor(s) that is involved in the recruitment of primitive adult bone
murrow HPP-CFC to proliferate in vitro when exposed to a combination of recombinant hematopoietic growth factors. Further studies to isolate and characterize the YSE- and YSM-conditioned media factor(s) are underway.

ACKNOWLEDGMENT

The authors thank Natalie Martina and Dr Frank Lee for performing the ELISAs and thankfully acknowledge the gifts of the SCF antibody from Dr Larry Bennett, of the IL-3-dependent cell line from Dr Yu-Chung Yang, and of the flt3-IgG fusion protein and recombinant murine flt3 ligand from Dr Charles Hannum. We also acknowledge the helpful discussions and comments of Dr D. Wade Capp and the secretarial assistance of Patricia Fox in preparing the manuscript.

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