A Murine Stromal Cell Line Allows the Proliferation of Very Primitive Human CD34\(^+\)/CD38\(^-\) Progenitor Cells in Long-Term Cultures and Semisolid Assays

By Cherifa Issaad, Laure Croisille, Andre Katz, William Vainchenker, and Laure Coulombel

Analysis of molecular mechanisms associated with stem cell commitment and differentiation requires an in vitro assay that identifies the most primitive hematopoietic stem cells in human bone marrow. Such primitive stem cells usually do not form colonies in short-term semisolid assays and are best identified by their ability to initiate sustained hematopoiesis when they are cocultured with competent stromal cells. In this study, we investigated whether a murine marrow stromal cell line (MS-5) that supports colony-forming unit-spleen (CFU-S) maintenance would permit, both in short-term colony assays and long-term cultures, the development of primitive human stem cells sorted on the basis of their high expression of CD34 and lack of expression of CD38 antigen. In short-term colony assays, this population included almost exclusively primitive progenitor cells. MS-5 cells synergized with any combination of interleukin-3, Steel factor, granulocyte colony-stimulating factor, agar-leukocyte conditioned medium, and erythropoietin and increased at least twofold both the cloning efficiency of CD34\(^+\)/CD38\(^-\) cells and the size of the colonies. Furthermore, MS-5 cells triggered the development of multipotent blast cell progenitors with a high proliferative potential, which in these conditions represented 1% to 2% of CD34\(^+\)/CD38\(^-\) cells. When MS-5 cells were substituted by human stromal cells or when growth factor combinations were used in the absence of stromal cells, much lower numbers of CFU-blast were detected. This selective action of MS-5 on early progenitors was also observed when MS-5 cells were used as feeders in long-term cultures of CD34\(^+\)/CD38\(^-\) cells. Murine cells promoted the expansion of high proliferative potential primitive progenitor cells up to 3 months, although they did not support their differentiation in mature clonogenic progenitors or terminally differentiated cells. Sustained hematopoiesis in these long-term cultures was accounted for by 2% to 5% of initial CD34\(^+\)/CD38\(^-\) cells as estimated by limiting dilution experiments. Mechanisms by which murine stromal cells act specifically on human primitive stem cells are unclear, but from our data this effect is unlikely to be explained solely by known species cross-reactive growth factors. Further manipulation of this long-term coculture system should prove useful in identifying stromal molecules regulating commitment and differentiation of early human progenitor cells.

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Murine Stromal Cells Support Human Stem Cells

The proliferation and terminal differentiation of grafted human cells remain limited unless human growth factors are provided. However, in most xenogenic settings, the proliferation and terminal differentiation of grafted human cells remain limited unless human growth factors are provided. In this study, we attempted to selectively maintain and expand very primitive human progenitor cells that could be subsequently used for the molecular analysis of the mechanisms associated with their developmental potential. To that purpose, we constructed a long-term culture assay by coculturing primitive human marrow cells selected for their high expression of the CD34 antigen and very low expression of CD38 with a murine clonal stromal cell line (MS-5). MS-5 was derived from noninfected Dexter-type murine long-term marrow cultures and supports colony-forming unit-spleen (CFU-S) maintenance. Our results indicate that MS-5 cells, but not human feeders, allow the expansion of a subpopulation of very early human multipotent progenitor cells for 5 to 10 weeks without the need for exogenously supplied growth factors. Furthermore, we show that MS-5 cells act synergistically with human growth factors to stimulate the formation of blast colonies and macroscopic colonies from high proliferative potential multipotent CD34+/CD38- cells in short-term methylcellulose assays.

Materials and Methods

Marrow Cell Preparation

Bone fragments were obtained after informed consent from patients undergoing hip surgery, and marrow cells were collected by vigorous shaking of bone fragments in a minimal essential medium (αMEM) supplemented with 100 μg/mL of deoxyribonuclease (Sigma Chemical Co, St Louis, MO; DNase type I). Cells were centrifuged once, counted, and separated on Ficoll-Hypaque. Light-density (<1.077 g/mL) cells were suspended at a concentration of 1 × 10^6 cells/mL in αMEM supplemented with 30% fetal calf serum (FCS; JBio Laboratory, Les Ulis, France) and kept at 4°C overnight. The next day, the cells were carefully resuspended in fresh αMEM with 5% FCS and 100 μg/mL DNase (labeling medium) and proceeded for labeling.

Cell Separation

Cells (2 × 10^7 cells/mL) were first incubated with the anti-CD34 monoclonal antibody (MoAb) 8G12 (kindly provided by P. Lansdorp, Terry Fox Laboratory, Vancouver, Canada; purified Ig and used at a concentration of 14 μg/mL), followed by 45 minutes of incubation with a 1/30 dilution of phycoerythrin (PE)-labeled antihuman IgG1 goat Ig (Southern Biotechnology, Birmingham, AL). Remaining free sites on the PE-Ig were blocked by 15 minutes of incubation with mouse serum (1/5 dilution). Cells were subsequently labeled with anti-CD38 MoAb directly coupled with fluorescein (Immunotech, Marseille, France) and used at a concentration of 1/10 dilution. Cells were finally suspended in labeling medium at a concentration of 4 to 5 × 10^5 cells/mL and separated by cell sorting. More recently, cell labeling has been performed in one step by incubating cells simultaneously with the MoAb HPCA-2 (8G12) directly coupled to PE (Becton Dickinson, San Jose, CA) and the CD38-fluorescein isothiocyanate (FITC) MoAb.

Selection of Cells by Cell Sorting

Cells were analyzed and sorted on an ODAM ATC 3000 cell sorter (ODAM/Brucker, Wissembourg, France) equipped with an INNOVA 70-4 Argon ion laser (Coherent Radiation, Palo Alto, CA) tuned at 488 nm and operating at 500 mW. The wide angle light scatter (WALS) was first extracted with a 510 DCLP filter. The two fluorescence signals were then separated with a 550 DCLP filter and finally isolated using a 530 DF 30 filter for FITC and a 575 DF 26 filter for PE (all filters from Omega Optical Inc, Brattleboro, VT). Compensation for double-stained samples was set up with single-stained samples. Data were analyzed on the ATC 3000 computer and all parameters were displayed on a linear scale. Three sorting gates were set to include cells expressing both CD38 and CD34 antigens (referred to as CD34+/CD38+ cells), cells “negative” with respect to CD38 and expressing high levels of CD34 (the 30% CD34 most positive among the CD38 negative/CD34 positive cells referred to as CD34+/-/CD38- cells), and the remaining CD34-/CD38- cells with medium expression of the CD34 antigen. To increase the purity of the sorting, the morphologic two-parameter histograms (WALS v electric measurement of the cell volume) were acquired for the cells falling in each of the three previous gates, and an additional gate was applied to each of these morphologic distributions to retain a well-defined homogeneous population and to reject highly diffusive cells (which may be autofluorescent) and/or too large objects (which may correspond to cell doublets). The four-parameter sorting was run at 3,000 to 3,500 cells per second and the sorted cells were collected in 3.5 mL of αMEM with 10% FCS.

Assessment of Clonogenic Progenitors in the Different CD34/CD38 Cell Fractions

Erythroid (CD34-/CD38-; CFU-E) mature burst-forming unit-erythroid (mBFU-E) and immature BFU-E (iBFU-E), granulocytic (CD34-/CD38-; CFU-granulocyte-macrophage [CFU-GM]), and CFU-granulocyte-erythroid-monocyte-megakaryocyte (CFU-GEMM)) progenitors were quantified using previously described methylcellulose assays. Each of the three fractions selected as described above (CD34+/CD38-, CD34+/CD38+, and CD34-/CD38-) were plated at a concentration of 500 to 1,000 cells/mL of complete methylcellulose medium (0.8% methylcellulose in Iscove’s medium, 30% FCS, 1% deionized bovine serum albumin [BSA], 10 μg/mL 8-mercaptoethanol). Colony-stimulating factors were provided either as 10% of agar-leukocyte conditioned medium (A-LCM [an usual source of hematopoietic growth factors]) or as recombinant growth factors: rhuSteel factor (SF; kindly provided by Angem, Thousand Oaks, CA), recombinant human IL-3 (rhuIL-3), recombinant human granulocyte-CSF (rhu-CSF), and rhuIL-6 (all purchased from Genzyme, Cambridge, MA) used at the concentrations indicated in Results. Human erythropoietin (Epo; purified from human urine and purchased from the Terry Fox Laboratory) was added to every growth factor combination at 3 U/mL. Plates were incubated at 37°C in an air atmosphere supplemented with 5% CO2 and saturated with humidity. Progenitors were scored at day 10 (CFU-E and mBFU-E), days 15 and 16 (primitive iBFU-E), granulocytic (CFU-granulocyte-monocyte-megakaryocyte), erythroid (CFU-erythroid), and megakaryocytic (CFU-megakaryocyte) progenitors, as previously detailed criteria. Parallel experiments were performed to test the ability of different stromal cell populations to alter the clonogenic potential of sorted CD34+/CD38- cells. In these experiments, 10,000 cells of the murine MS-5 cell line or 50,000 irradiated human stromal cells were added per milliliter of the methylcellulose mixture and cells were plated in bacteriologic plastic dishes (Falcon 1008) that allow spreading of stromal cells but slow their proliferation as compared with plating on tissue culture dishes.
Table 1. Hematopoietic Progenitor Cell Content of the Different Fractions Sorted According to the Expression of Both CD34 and CD38 Antigens

<table>
<thead>
<tr>
<th>Cell Fraction and Growth Factors</th>
<th>Progenitor Cells per 1,000 Cells Plated (mean ± SEM)</th>
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<tbody>
<tr>
<td></td>
<td>CFU-E</td>
</tr>
<tr>
<td>CD34+/CD38- (n = 9)</td>
<td>SF + IL-3 + Epo</td>
</tr>
<tr>
<td></td>
<td>A-LCM + Epo</td>
</tr>
<tr>
<td>CD34+/CD38+ (n = 5)</td>
<td>SF + IL-3 + Epo</td>
</tr>
<tr>
<td></td>
<td>A-LCM + Epo</td>
</tr>
<tr>
<td>CD34-/CD38- (n = 3)</td>
<td>SF + IL-3 + Epo</td>
</tr>
<tr>
<td></td>
<td>A-LCM + Epo</td>
</tr>
</tbody>
</table>

One thousand cells from each sorted CD34/CD38 fraction were plated in methylcellulose assays in the presence of either 10% A-LCM or a mixture of rhuSF (50 ng/mL) and rhu-IL-3 (100 U/mL). Epo (3 U/mL) was added in each dish. Colony counts were performed in duplicate or triplicate between days 12 and 22. Each number refers to the mean (±SEM) number of progenitor of each type detected in three to nine experiments.

Abbreviation: ND, not detected.

RESULTS

CD34+/CD38- Cells Are Enriched in Multipotent High Proliferative Potential Primitive Progenitor Cells

The clonogenic progenitor content of the three sorted populations (CD34+/CD38-, CD34+/CD38+, and CD34+/CD38+) was first assayed by plating 500 to 1,000 cells of each population in methylcellulose assays. Maximal numbers of each progenitor type found in each fraction are indicated in Table 1. We first noticed that the level of expression of the CD38 antigen discriminated functionally distinct progenitor cells among CD34+ cells. The CD34+/CD38+ population included 25% to 30% clonogenic progenitors with a mature phenotype, ie, CFU-E, mBFU-E, and small CFU-GM generating colonies of less than 200 cells. In the presence of Epo, these cells could be stimulated by either rhuSF (50 to 100 ng/mL) or rhu-IL-3 (50 to 100 U/mL). In contrast, most of the primitive progenitors, iBFU-E, CFU-GM, and high proliferative potential CFU-GM (generating colonies of more than 10^4 cells) were CD38- and expressed medium to high levels of CD34+. The cloning efficiency of the CD34+/CD38+ fraction was 8% to 16% and that of the CD34+/CD38- fraction was 2% to 3% (Table 1). Colony formation by CD34++/CD38- cells did not occur when cultures contained only one cytokine or A-LCM in addition to Epo, but required at least the combination of SF + IL-3 in addition to Epo, in agreement with the multiple cytokine requirement of early stem cells previously reported.

In contrast to the modest stimulatory effect of soluble recombinant growth factors on CD34++/CD38- cells, addition of murine stromal MS-5 cells to the colony assay dramatically increased the number and size of the colonies detected. MS-5 cells act only in synergy with human growth factors and did not stimulate colony formation by CD34++/CD38- cells when added alone (some <10 cells clusters were observed in the CD34+/CD38- or CD34+/CD38+ fractions). In five experiments, CD34+++/CD38- cells were plated in the presence of MS-5 and either A-LCM + Epo (Fig 1A) or SF + IL-3 + Epo (Fig 1B). In both conditions, the cloning efficiency increased threefold to fourfold over that observed in the pres-
MURINE STROMAL CELLS SUPPORT HUMAN STEM CELLS

Fig 1. Synergistic effect of MS-5 murine stromal cells and growth factors on the cloning efficiency of CD34⁺⁺/CD38⁻⁻ cells. One thousand CD34⁺⁺/CD38⁻⁻ cells were plated in Iscove’s medium methylcellulose assays with different growth factor combinations: (A) Epo (3 U/mL) + 10% A-LCM; (B) Epo (3 U/mL) + IL-3 (100 U/mL) + SF (50 ng/mL). Cells were cultured either without (□) or with (■) 10,000 MS-5 cells. Each column represents the mean (± SEM) number of progenitors observed in five separate experiments. *P < .01 (paired analysis).

ence of growth factors without stromal cells, ie, 10% ± 1.3% versus 2% ± 0.3% for A-LCM + Epo, and 11.2% ± 2.6% versus 3.7% ± 0.8% for SF + IL-3 + Epo with or without MS-5, respectively (Fig 1A and B). Differences expressed in Fig 1A and B were statistically highly significant (P < .01, paired analysis of data, Student’s t-test) for iBFU-E, CFU-GEMM, CFU-GM, and CFU-blast (see below). Most erythroid and granulocytic colonies obtained in colony assays stimulated by MS-5 cells included more than 2 × 10⁵ cells, a macroscopic size that is much higher than that observed in the absence of the stromal cells. This great cell proliferation was predominantly seen in the erythroid lineage, which led to an underestimation of the number of multilineage colonies.

In addition to BFU-E and CFU-GM, MS-5 in synergy with A-LCM or SF + IL-3 + Epo triggered the development of a very primitive progenitor, which shared some of the criteria identifying blast cell progenitors (CFU-blast) (Fig 2). (1) These colonies were undetectable before day 15 and were characterized by their small size (<300 cells) at days 22 and 23 (Fig 2), which contrasted with the macroscopic size of CFU-GM-
and BFU-E-derived colonies. (2) They were composed of cells with an undifferentiated morphology after May-Giemsa staining. (3) They generated high numbers of cells with an undifferentiated morphology after May-Giemsa staining. (4) They were composed of cells with an undifferentiated morphology after May-Giemsa staining. (5) They were composed of cells with an undifferentiated morphology after May-Giemsa staining. (6) They were composed of cells with an undifferentiated morphology after May-Giemsa staining.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of Primary Colonies Repeated</th>
<th>No. of Secondary Colonies Generated</th>
<th>No. of Secondary Colonies per Primary Colony (mean ± SEM)</th>
<th>No. of Secondary Colonies with Erythroid and Granulocytic Lineages</th>
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<tr>
<td>1</td>
<td>36</td>
<td>31 ± 4</td>
<td>21 ± 5</td>
<td>18 (73)</td>
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<tr>
<td>2</td>
<td>30</td>
<td>28 ± 5</td>
<td>25 ± 5</td>
<td>19 (73)</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>22 ± 5</td>
<td>21 ± 5</td>
<td>18 (73)</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>25 ± 5</td>
<td>20 ± 5</td>
<td>21 (60)</td>
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<td>5</td>
<td>12</td>
<td>10 ± 4</td>
<td>9 ± 4</td>
<td>6 (54)</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>12 ± 4</td>
<td>11 ± 4</td>
<td>6 (54)</td>
</tr>
</tbody>
</table>

One thousand CD34+/CD38 cells were grown in methylcellulose colony assays with MS-5, Epo, and A-LCM. At day 22, colonies defined as blast colonies (see text and Fig 2) were individually lifted and replated in secondary methylcellulose plates containing Epo + A-LCM without MS-5. Secondary colonies were scored at days 12 through 15. Percentages are in parentheses.

Analysis of the Proliferative Capacity of CD34+/CD38- Cultured at Limiting Dilutions on MS-5 Feeders

In an attempt to determine whether the production of primitive progenitors observed in these cocultures resulted

![Fig 3. Production of clonogenic progenitor cells from long-term cocultures initiated with 5,000 CD34+/CD38- cells sorted from 4 different marrow samples and cultured in 24-well plates precoated with a confluent layer of MS-5 cells. Wells were sacrificed at regular intervals and their progenitor content quantitated in methylcellulose colony assays. Each point represents the total number of progenitors present in a 24-well culture (ie, per 5,000 initial CD34+/CD38- cells) at the time point indicated. Input (day 0) progenitor values for the four experiments were 185 (Exp 2), 25 (Exp 3), 365 (Exp 4), and 500 (Exp 5).]
from the clonal expansion of a small number of very immature CD34++/CD38− pluripotent cells, we initiated co-
cultures at limiting dilutions by incubating from 2 to 100
CD34++/CD38− cells in 96-well plates precoated with MS-5.
At weeks 5 and 7, microwells initiated with 10, 100, and 5,000 CD34++/CD38− cells were harvested and their progenitor content assessed
in methylcellulose colony assays. Note that MS-5 were therefore
present in each colony assay. Each point represents the mean
(± SEM) number of progenitor cells per positive well (positive wells:
12 of 19 [week 5, 10 cells/well], 7 of 23 [week 5, 10 cells/well],
18 of 18 wells [week 4, 100 cells/well], 10 of 10 wells [week 7,
100 cells/well]). The SEM is not indicated for the 5,000 cells point
as only one well was established at this cell concentration.

Fig 4. Limiting dilution analysis of data from a representative experiment in which decreasing numbers of CD34++/CD38− cells were seeded in 96-well plates precoated with MS-5 cells. At weeks 5 and 7, microwells initiated with 10, 100, and 5,000 CD34++/CD38− cells were harvested and their progenitor content assessed
in methylcellulose colony assays. Note that MS-5 were therefore
present in each colony assay. Each point represents the mean
(± SEM) number of progenitor cells per positive well (positive wells:
12 of 19 [week 5, 10 cells/well], 7 of 23 [week 5, 10 cells/well],
18 of 18 wells [week 4, 100 cells/well], 10 of 10 wells [week 7,
100 cells/well]). The SEM is not indicated for the 5,000 cells point
as only one well was established at this cell concentration.

We first examined the relationship between the clonogenic
output after 5 weeks and the number of CD34++/CD38− cells
initiating the coculture. As shown in Fig 4 for one representa-
tive experiment, the number of clonogenic cells detectable
after 5 and 7 weeks in culture was linearly related to the
number of initial cells. In each of five different experiments,
100% of the wells initiated with 50 or 100 CD34++/CD38−
cells and sacrificed at 4, 6, and 8 weeks (10 wells per time
point) were positive, ie, yielded at least one clonogenic pro-
genitor cell. Eighty percent of wells initiated with 20 cells
and sacrificed in the same conditions as previously were also
positive, but only 65% of those containing 10 cells and 11% of the 180 wells initiated with 2 cells and sacrificed between
weeks 4 and 10 (20 wells per time point). However, there
was a wide variation in the number of progenitor output
measured in individual wells at weeks 5 through 10. Wells
initiated with 100 cells yielded between 4 and 78 progenitor
cells. For wells initiated with 2 CD34++/CD38− cells, the number of progenitors produced ranged from 1 to 29, respec-
tively, with an average of 3.4 progenitor cells per positive
well. As the cloning efficiency of the starting CD34++/CD38−
population is at most 10% (Fig 1A and B), each well initiated
with 2 cells can be assumed to contain less than 0.2 progen-
itors at the initiation of the coculture on MS-5. Our finding
that 4 of the 180 wells initiated with 2 CD34++/CD38− cells
contained more than 4 primitive progenitor cells of both the
erythroid and granulocytic lineages (up to 29 progenitors in
one well) after 4 weeks of coculture on MS-5 favors the hy-
pothesis that a fraction of very immature pluripotent pro-
genitor cells is responsible for the sustained clonogenic output
observed in these culture conditions.

Analysis of the Supportive Capacity of Human Stromal
Cells

In parallel experiments, CD34++/CD38− cells were incu-
bated on 4-week-old irradiated human stromal layers follow-
ing the same procedure as described for cocultures of CD34++/
CD38− on MS-5. As indicated in Table 3, the progenitor
output in cocultures on human stromas was always lower (4-
to 60-fold less) than that measured in cocultures on MS-5 at
any time point during the coculture period. The addition of
MS-5 cells to the colony assays of cells maintained on human
stromas failed to increase progenitor cell recovery, indicating
that the low progenitor output was not due to suboptimal
stimulatory conditions in the clonogenic assay.

Long-Term Cultures of CD34+/CD38− Cells on MS-5
Cells and Human Stromal Cells

In two separate experiments, we assessed the clonogenic
output of CD34+/CD38− cells cocultured with either MS-5
or human stromal cells. As shown in Table 3, cells from this
fraction were unable to maintain a high clonogenic output
when cocultured on either murine or human stromal cells,
a result that was not unexpected considering that the CD38−
fraction is very enriched in mature clonogenic progenitor
cells.

DISCUSSION

Although some primitive pluripotent stem cells will form
colonies7,29,30 in short-term colony assays, their identification
is indirect and usually relies on the measurement of their
clonogenic progeny after 5 to 8 weeks of culture with stromal
cells. These long-term coculture systems do not require the

<table>
<thead>
<tr>
<th>Table 3. Clonogenic Output in Long-Term Cultures of CD34++/CD38− and CD34+/CD38− Cells on Either Murine or Human Stromal Cells</th>
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<tr>
<td>Weeks in Culture</td>
</tr>
<tr>
<td>CD34++/CD38−</td>
</tr>
<tr>
<td>Exp 3</td>
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<tr>
<td>Exp 5</td>
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<tr>
<td>CD34+/CD38−</td>
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<tr>
<td>Exp 3</td>
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<tr>
<td>Exp 5</td>
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Long-term cocultures were initiated by plating 5,000 CD34++/CD38− or CD34+/CD38− cells in 24-well plates precoated with confluent layers of either MS-5 cells or irradiated human stromas. The progenitor content of each well was assessed at weeks 5 and 11 by harvesting the adherent and nonadherent fractions that were plated in methylcellulose assays. Numbers refer to the total progenitor content of each well (adherent and nonadherent fractions).
addition of exogenous growth factors. Because of the lack of species cross-reactivity of most known growth factors, syngeneic stromal cells have usually been preferred to xenogenic feeders. However, recent evidence has accumulated showing that human hematopoiesis can develop in a nonhuman environment. In this study, we show that a murine stromal cell line (MS-5), selected for its ability to support day-12 CFU-S and long-term reconstituting stem cell maintenance (Sainteny et al., in preparation), (1) promotes the expansion of very early human progenitor cells over 8 weeks of culture without the addition of human growth factors and (2) stimulates, in synergy with human growth factors, the development of primitive progenitors in short-term semisolid assays, with some of these progenitors showing a high secondary reconstituting capacity. The first evidence for the selective action of MS-5 on early human progenitor cells was provided by analysis of the colony-forming ability of the different CD34+/CD38- sorted cell populations in response to growth factors when MS-5 cells were included in methyccellulose short-term assays. In our experience, as in that of others, the CD38 was the most powerful antigen to directly separate in one step the clonogenic cells (CD34+/CD38-) from the more primitive pluripotent cells (CD34+/CD38-) contained within the CD34 population. CD34+/CD38- cells, which are free of mature progenitors and included nearly exclusively primitive progenitors, required at least the three-factor combination of SF + IL-3 + Epo to form colonies. This requirement for multiple cytokines is characteristic of primitive cells and has been previously reported for CD34+Lin- or CD34+/HLADR- human cells or Scal-Lin- murine cells. MS-5 cells strikingly synergized with either A-LCM + Epo or IL-3 + SF + Epo to increase fourfold the cloning efficiency of CD34+/CD38- progenitor cells as well as colony size. In contrast, MS-5 cells alone did not stimulate colony formation. Increased cloning efficiency of CD34+/CD38- was almost entirely accounted for by increased numbers of BFU-E and CFU-GEMM and by the growth at days 20 through 22 of small colonies composed of undifferentiated cells. The progenitor cells generating those colonies that could be classified as blast cell colonies represented 1% to 2% of the CD34+/CD38- population and generated high numbers of erythroid and granulopoietic secondary colonies upon retransplantation, although there was no evidence for self-renewal. Very few blast colonies were detected in the absence of MS-5, even when SF, IL-3, and Epo were added to the cultures and their replating potential was very low (Coulombel et al., unpublished observations). In contrast, MS-5 did not increase the cloning efficiency of CD34+/CD38- murine cells, with each of these generating on average 3 to 4 clonogenic progenitor cells. This frequency is very close to that recently reported for CD34+/HLADR+ and CD34+/Thy1+ cells. We (this study) observed, as have others, a wide variation in the number of clonogenic cells generated by CD34+/CD38- cells cultured at 2 cell-limiting dilution (from 1 to 29 progenitors per positive well). This must reflect wide variations in the proliferative capacity of individual primitive stem cells. It is noteworthy that most of the clonogenic cells produced in our long-term cultures exhibited characteristics of primitive progenitors with either high proliferative capacities or multipotent potentialities or both. This was in contrast with the very low output of mature 3- to 8-cluster BFU-E, day-7 to -10 CFU-GM, and terminally differentiated cells, indicating that MS-5 cells did not support human terminal differentiation. Both granulopoietic and erythropoietic progenitors were produced in almost the same proportion, whereas human feeders preferentially amplify granulopoietic progenitor cells. Therefore, these results show that the MS-5 cell line sustains long-term human hematopoiesis by promoting the commitment, proliferation, and differentiation into early granulopoietic and erythroid progenitors of a small population of primitive stem cells. Further studies are required to understand whether MS-5 cells also have the ability to induce the differentiation of CD34+/CD38- cells towards other myeloid and lymphoid lineages, as recently reported for the B lineage with another murine stromal cell line. Little is known on how murine stromal cells stimulate human cell proliferation, but the involvement of yet unidentified growth factors is very likely. Indeed, among hematopoietic growth factors acting on human primitive progenitor cells, murine IL-6, leukemia-inhibitory factor (LIF), IL-3, and GM-CSF had no effect. Only SF and G-CSF are clearly cross-reactive. MS-5 cells do produce SF, but recent data suggest that c-kit and its ligand do not play a major role in the long-term proliferation of primitive murine stem cells on a stromal cell line. However, it cannot be totally excluded that MS-5 acts through membrane-bound growth factors, which may be more active than their soluble counterparts. Sustained expansion of primitive progenitors on MS-5 could also be due to the combined action of growth factors with a peculiar component(s) of the extracellular matrix, as recently shown for thrombospondin. However, our observation that MS-5 clearly synergizes the action of most recombinant human growth factors, including SF, not only in direct close contact, but also in short-term assay (methyccellulose culture) strongly argues for a new presumably diffusible cross-reactive activity.

The chimeric suspension culture described in this study allows for extensive periods of time the expansion of adult human marrow primitive pluripotent hematopoietic progenitor cells without stimulating their terminal differentiation.
As these cocultures are established in the absence of human growth factors they will be of particular interest in identifying new molecules triggering early pluripotent stem cell proliferation and in unraveling the complex interactions between cytokines and extracellular matrix components that might alter differentiation and self-maintenance of early human stem cells. In addition, the synergistic action of MS-5 cells in clonogenic assays may offer a practical and rapid assay for evaluating primitive hematopoietic stem cells.

ACKNOWLEDGMENT

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A murine stromal cell line allows the proliferation of very primitive human CD34++/CD38- progenitor cells in long-term cultures and semisolid assays

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