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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nance and the myeloid and lymphoid differentiation of unfractinated hematopoietic cells, \(^{18}\) CD34\(^+\)/HLA-Dr\(^{low}\),\(^{14}\) or CD34\(^+\)/Thy1\(^{19}\) pluripotent stem cells. Human multipotent cells can also be successfully engrafted in vivo into fetal sheep\(^{20,21}\) or into genetically immunodeficient (SCID) mice.\(^{22,23}\) However, in most xenogenic settings, the proliferation and terminal differentiation of grafted human cells remain limited unless human growth factors are provided.\(^{14,24}\)

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\(^{25}\) 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.\(^{26,27}\) 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 (α-MEM) supplemented with 100 \(\mu\)g/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\(^7\) cells/mL in α-MEM supplemented with 50% fetal calf serum (FCS; JBiolog, Les Ulis, France) and kept at 4°C overnight. The next day, the cells were carefully resuspended in fresh α-MEM with 5% FCS and 100 \(\mu\)g/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, as purified Ig and used at a concentration of 14 \(\mu\)g/mL), followed by 45 minutes of incubation with a 1/30 dilution of phycoerythrin (PE)-labeled antimonooise 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 1/10 dilution. Cells were finally suspended in labeling medium at a concentration of 4 to 5 × 10\(^7\) 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-fluorescin isothiocyanate (FITC) MoAb.

Selection of Cells by Cell Sorting

Cells were analyzed and sorted on an ODAM ATE 3000 cell sorter (ODAM/Bruker, 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 DCSP 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 ATE 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 vs 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 α-MEM with 10% FCS.

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

Erythroid (CFU-erythroid [CFU-E]) mature burst-forming unit-erythroid [BFU-E] and immature BFU-E (iBFU-E), granulocytic (CFU-granulocyte-macrophage [CFU-GM]), and (CFU-granulocyte-erythroid-monocyte-megakaryocyte [CFU-GEMM]) progenitors were quantified using previously described methylcellulose assays.\(^{28}\) Each of the three fractions selected as described above (CD34\(^+\)/CD38\(^-\), CD34\(^+\)/CD38\(^+\), and CD34\(^+\)/CD38\(^+\) cells) 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 \(\mu\)M L-β-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 Amgen, Thousand Oaks, CA), recombinant human IL-3 (rhuIL-3), recombinant human granulocyte-CSF (rhu-G-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 units/mL. Plates were incubated at 37°C in an air atmosphere supplemented with 5% CO\(_2\) and saturated with humidity. Progenitors were scored at day 10 (CFU-E and mBFU-E), days 15 and 16 (primitive iBFU-E and CFU-GM), and days 22 to 24 (CFU-GEMM and blast progenitors) using previously detailed criteria.\(^{28}\)

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>
</tr>
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<tbody>
<tr>
<td></td>
<td>CFU-E</td>
</tr>
<tr>
<td>CD34+/IL-3 + Epo (n = 9)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>A-LCM + Epo</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>CD34+/IL-3 + Epo (n = 5)</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>A-LCM + Epo</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>CD34+/IL-3 + Epo (n = 3)</td>
<td>84 ± 48</td>
</tr>
<tr>
<td>A-LCM + Epo</td>
<td>139 ± 13</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 (60 ng/mL) and rhuIL-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 assessed 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 rhuIL-3 (50 to 100 U/mL). In contrast, most of the primitive progenitors, BFU-E, CFU-GEMM, 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-
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 × 10^5 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). 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-

Fig 2. Photograph of a blast cell progenitor observed at day 22 in methylcellulose colony assays of CD34+ +/CD38- cells cultured in the presence of A-LCM + Epo + 10,000 MS-5 cells. This colony was individually replated and generated 97 secondary colonies (89 GM + 8 BFU-E). (Original magnification × 200.)
Table 2. Replating Potential of Primary Blast Cell Colonies Generated by CD34++/CD38- Cells

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of Primary Colonies Repeated</th>
<th>No. of Primary Colonies Generating Secondary Colonies</th>
<th>No. of Secondary Colonies per Primary Colony (mean ± SEM)</th>
<th>No. of Secondary Plates With Erythroid and Granulocytic Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>26</td>
<td>24 (84)</td>
<td>34 ± 5</td>
<td>19 (73)</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>32 (81)</td>
<td>129 ± 17</td>
<td>21 (60)</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>11 (78)</td>
<td>36 ± 6</td>
<td>6 (54)</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>16 (84)</td>
<td>154 ± 3</td>
<td>9 (53)</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.

and BFU-E-derived colonies. (2) They were composed of cells with an undifferentiated morphology after May-Grünwald Giemsa staining. (3) They generated high numbers of secondary colonies of both erythroid and granulocytic lineages after individual replating; thus, 83 of the 92 primary colonies individually replated gave rise to secondary colonies (Table 2). Each primary colony generated on average 88 (range, 10 to more than 400) secondary colonies and 55 yielded both erythroid and granulocytic colonies, indicating that the parental blast progenitor was pluripotent (Table 2). In 15 colonies in which it has been calculated, the replating efficiency (number of progenitor cells per number of cells in the primary colony) ranged from 10% to 100% (mean ± SEM, 49% ± 9%). The frequency of these progenitor cells in the CD34++/CD38- population was 1% (Fig 1A and B). Irradiation of MS-5 did not abrogate its supportive effect (data not shown). In one experiment that used substitution of MS-5 by human stromal cells, we studied the distribution of progenitor cells in the culture. In both experiments, more than 80% of the progenitor cells were associated with adherent stromal cells independently of their human or murine origin.

Finally, in two experiments in which CD34++/CD38- cells were cocultured 4 to 5 weeks simultaneously on MS-5 and human stromal cells, we studied the distribution of progenitor cells in the culture. In both experiments, more than 80% of the progenitor cells were associated with adherent stromal cells independently of their human or murine origin.

Analysis of the Supportive 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 from different growth conditions, we studied the frequency of committed progenitor cells in the culture. In both experiments, more than 80% of the progenitor cells were associated with adherent stromal cells independently of their human or murine origin.

Analysis of the Supportive Capacity of Murine Stromal MS-5 Cells

To determine whether cells able to initiate long-term hematopoiesis in vitro were present among CD34++/CD38-, we cocultured CD34++/CD38- cells with stromal cells of murine (MS-5) and human origin for 5 to 10 weeks in culture conditions previously shown to support long-term myelopoiesis.6

Analysis of the Proliferative Capacity of CD34++/CD38- Cells Cocultured With Human and Murine Stromal Cells

In a first set of experiments, 5,000 CD34++/CD38- cells sorted from 4 different marrow samples were incubated in 24-well plates precoated with unirradiated MS-5 murine stromal cells and the progenitor content of the wells was determined at regular intervals during the 5- to 10-week coculture period. In each of these four experiments, the number of clonogenic progenitor cells recovered from each culture well increased 3- to 10-fold during the culture period (Fig 3). Interestingly, this amplification was restricted to the compartment of primitive progenitors, ie, iBFU-E, high proliferative potential CFU-GM, and CFU-GEMM. Blast cell progenitors were also detected for several weeks, although there was no clear increase in their numbers. Interestingly, the proportion of iBFU-E and CFU-GEMM detected in the cocultures was always greater than 30% of the total number of progenitors, an observation that might be related to the selective enhancement of erythroid proliferation by MS-5 mentioned earlier. In contrast to its action on early progenitor cells, murine stromal cells did not support terminal differentiation of human cells. This was indicated by two observations: (1) the very low number of mBFU-E and day 7 CFU-GM produced, and (2) even though there was on average a threefold to fourfold increase in the number of nonadherent cells produced at week 5, terminally differentiated granulocytic cells were not observed.

Finally, in two experiments in which CD34++/CD38- cells were cocultured 4 to 5 weeks simultaneously on MS-5 and human stromal cells, we studied the distribution of progenitor cells in the culture. In both experiments, more than 80% of the progenitor cells were associated with adherent stromal cells independently of their human or murine origin.

Analysis of the Supportive 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 cocultures at limiting dilutions by incubating from 2 to 100 CD34+/CD38- cells 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.

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.

Table 3. Clonogenic Output in Long-Term Cultures of CD34+/CD38- and CD34+/CD38+ Cells on Either Murine or Human Stromal Cells

<table>
<thead>
<tr>
<th>Weeks in Culture</th>
<th>Progenitor Cells/Well</th>
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<tbody>
<tr>
<td></td>
<td>MS-5</td>
</tr>
<tr>
<td>CD34+/CD38-</td>
<td></td>
</tr>
<tr>
<td>Exp 3</td>
<td>5</td>
</tr>
<tr>
<td>Exp 5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>11</td>
</tr>
<tr>
<td>CD34+/CD38+</td>
<td></td>
</tr>
<tr>
<td>Exp 3</td>
<td>5</td>
</tr>
<tr>
<td>Exp 5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>11</td>
</tr>
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</table>

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 activity.

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 methycelullose 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 the differentiation of CD34+/CD38− cells towards other lineages, as recently reported for the B lineage with another murine stromal cell line.8

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 (methycelulose 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.

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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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