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The replating capability of human umbilical cord blood (CB) multipotential (CFU-GEMM) progenitors was assessed in vitro as an estimate of self-renewal using erythropoietin (Epo), steel factor (SLF), and either fetal bovine serum (FBS) or CB plasma. This study found a much higher replating efficiency for CB CFU-GEMM than previously reported, in terms of the percentage of colonies that could be replated, the number of secondary colonies per replated primary colony, and the size of secondary colonies. Moreover, the majority of secondary colonies were CFU-GEMM-derived. Although the percentages of bone marrow CFU-GEMM that replate was similar to that for CB CFU-GEMM and the sizes of secondary bone marrow and CB CFU-GEMM were also similar, replated CB CFU-GEMM gave rise to far greater numbers of secondary colonies. No tertiary colonies were observed when secondary CFU-GEMM were replated. Detection of extensive secondary replating potential was enhanced by the addition of CB plasma to the cultures. This activity was not found in either adult blood (PB) plasma, umbilical cord vein endothelial cell-conditioned medium (ECVM), FBS plus ECVM, or FBS plus the combination of interleukin-1 (IL-1), IL-3, IL-6, IL-11, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor. Whether the CB plasma-enhancing activity for CFU-GEMM replating capacity is attributable to a novel factor or factors, or represents effects of other known cytokines, alone or in combination, remains to be determined. Of particular relevance, these studies suggest that human CFU-GEMM have some degree of stemness and perhaps should be classified as a subset of stem cells.

MATERIALS AND METHODS

Cells and cell separation procedures

Cells were obtained from umbilical cord and placental tissues scheduled for discard after the delivery of the infant as described.13 Transected umbilical cords were placed in sterile wide-mouth bottles (No. 25625-200; Corning Glass Works, Corning, NY) containing phosphate-buffered saline supplemented with glucose (15.2 mmol/L Na2HPO4, 2.7 mmol/L KCl, 1.5 mmol/L KH2PO4, 0.14 mmol/L NaCl, 11 mmol/L glucose; pH 7.4). The Institutional Review Board of the Indiana University School of Medicine approved collection of blood and tissue in these circumstances. Bone marrow and peripheral blood (PB) cells were obtained from healthy volunteers who had given informed consent according to the guidelines established by the Institutional Review Board. Bone marrow cells were collected by aspiration from the posterior iliac crest. Ficoll/Hypaque (Pharmacia LKB Biotechnology, Piscataway, NJ) was used to separate low-density (LD) bone marrow cell fractions (1.077 g/mL).

Plasma

Plasma was separated by adding 3 mL of 2% methylcellulose (Sigma Chemical Co, St Louis, MO) to a syringe containing 60 mL of PB or CB. The upper phase was centrifuged at 1,500 rpm for 10 minutes to remove the white blood cells (WBC) from plasma. Aggregates were removed by centrifuging plasma at 3,000 rpm for 10 minutes, followed by filtration through 0.45 μm membranes. PB and CB plasma were stored at 4°C for up to 2 weeks. Because of a limited supply of plasma from individual collections, no two experiments used the same sample or pool of CB plasma.

Cytokines

Purified recombinant human (rhu) Epo (Epogen) was purchased from Amgen Incorporated (Thousand Oaks, CA). Purified rhuSLF (mast cell growth factor), rhu interleukin-3 (IL-3), rhu IL-6, rhu granulocyte colony-stimulating factor (G-CSF), and rhu granulocyte-macrophage CSF (GM-CSF) were obtained from Immuneex Corporation (Seattle, WA). Purified rhuIL-11 was from Genetics In-

Human Hematopoiesis involves life-long production of mature blood cells derived from pluripotent hematopoietic stem cells.1 The phenomenon of stem cell self-renewal is the basis for bone marrow and umbilical cord blood (CB) transplantation.2 Progenitor cell self-renewal in the human and murine systems has been estimated in vitro by secondary colony replating assays,3-10 but this was found to be minimal. We recently showed that human umbilical CB and bone marrow multipotential (CFU-GEMM) progenitors had a high replating efficiency when cultured in the presence of erythropoietin (Epo), steel factor (SLF; the ligand for the c-kit proto-oncogene-encoded receptor protein), and fetal bovine serum (FBS).11 A strict definition of self-renewal would be cell division that resulted in daughter cells with an identical capacity for proliferation and differentiation compared with the parent cell. However, we found that the colonies that grew in secondary cultures were not as large as the primary colony from which they were derived.11 We felt that this phenomenon might result from a lack of hematopoietic growth factors that can enhance replating potential of CFU-GEMM. Growth factors are produced by cells within the hematopoietic microenvironment.12 Because CB is a rich source of stem/progenitor cells at birth,13,14 we reasoned that CB plasma may contain growth factors that influence replating efficiency of progenitor cells. Thus, we investigated replating capacity of human umbilical CB and bone marrow CFU-GEMM in response to Epo, SLF, and hematopoietic growth factors intrinsic to CB plasma. The results show that human CFU-GEMM-derived colonies have high replating capability with generation of numerous large secondary CFU-GEMM-colonies, an effect enhanced by CB plasma. This suggests at least a degree of stemness for human CFU-GEMM.
were incubated at 37°C in a fully humidified atmosphere flushed with 5% ethanol (Fisher Scientific CO, Fair Lawn, NJ), and 2 mmol/L glutamine (GIBCO). Cultures were incubated at 37°C in a fully humidified atmosphere flushed with 5% CO2 for 7 days until the cells were a confluent monolayer. Medium was exchanged on days 2 and 4 of culture and endothelial cell-conditioned medium (ECCM) was harvested on day 7. Cell debris was removed from the ECCM by centrifuging at 1,500 rpm for 10 minutes, followed by filtration through a 0.22 μm pore membrane.

**Experiment**
The ECCM was stored at 4°C.

Endothelial cell cultures. Human endothelial cells were obtained from umbilical cord veins by digestion with collagenase. The endothelial cells were cultured in Medium 199 (GIBCO Life Technologies, Inc, Grand Island, NY) that contained 20% pooled normal human serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 20 mmol/L HEPES, pH 7.3 (GIBCO). Endothelial cell cultures were incubated at 37°C in a fully humidified atmosphere flushed with 5% CO2 for 7 days until the cells were a confluent monolayer. Medium was exchanged on days 2 and 4 of culture and endothelial cell-conditioned medium (ECCM) was harvested on day 7. Cell debris was removed from the ECCM by centrifuging at 1,500 rpm for 10 minutes, followed by filtration through a 0.22 μm pore/L membrane. The ECCM was stored at 4°C.

Culture system. Colony formation by CFU-GEMM was performed as previously described. Unseparated CB cells or LD bone marrow cells were plated at either 1.0 × 10^4, 2.5 × 10^4, or 5.0 × 10^4 cells/mL in standard 35-mm tissue culture dishes (Corning Glass Works). Cultures contained a 1-mL mixture of Iscove's modified Dulbecco’s Medium (IMDM; Whittaker Bioproducts, Inc, Walkersville, MD), 1% methylcellulose (Sigma), 5 × 10^-5 mol/L 2-mercaptoethanol (Fisher Scientific Co, Fair Lawn, NJ), and 2 mmol/L glutamine (GIBCO). Cultures were supplemented with either 30% non-heat-inactivated (NHI) FBS (Hyclone Laboratories, Inc, Logan, UT), 30% NHI CB plasma, 30% NHI PB plasma, 30% ECCM, 30% FBS plus 10% ECCM, or 30% FBS plus rhuIL-3 (10 ng/mL), rhuIL-4, rhuIL-6 (10 ng/mL), and rhuIL-11. The final concentration of the components in the 1.2 mL secondary culture medium was added and the culture was mixed by vortexing. Colony transfer experiments. On day 14 of primary culture, single, well-isolated CFU-GEMM colonies were removed from primary cultures under sterile conditions using a micropipettor (Gilson P-200; Rainin Instrument Co, Emeryville, CA). Each colony in a volume of primary culture not greater than 30 μL, was transferred to an individual tube containing 0.64 mL of culture medium supplemented with either 30% FBS, 30% CB plasma, 30% PB plasma, 30% ECCM, 30% FBS plus 10% ECCM, or 30% FBS plus 10 ng/mL rhuIL-1α, 100 U/mL rhuIL-3, 10 ng/mL rhuIL-6, 10 ng/mL rhuIL-11, and 100 U/mL rhuG-CSF. Colonies were dispersed into single-cell suspensions by gentle pipetting. To each tube, 0.56 mL of methylcellulose culture medium was added and the culture was mixed by vortexing. (The final concentration of the components in the 1.2 mL secondary culture was as described for primary cultures.) A 1 mL syringe was used to place the entire volume of the tube into a single 35-mm dish containing 1 U/mL rhuEpo, 50 ng/mL rhuSLF, and 100 U/mL rhuGM-CSF. Secondary cultures were examined with an inverted microscope immediately after plating for the presence of any cellular aggregates that remained. The few plates containing such aggregates were discarded. Secondary cultures were incubated for 14 days as described above and scored for colonies.

**Cell counts.** On day 14 of incubation, some colonies were removed individually from primary and secondary cultures with a micropipettor and each was gently dispersed in 10 mL of isotone buffer (Coulter Electronics, Hialeah, FL). The number of cells per primary or secondary colony was then determined by analyzing 2 mL of each sample with a Coulter cell counter (Coulter Electronics). In some cases, the entire contents of the methylcellulose culture medium was harvested from individual day 14 secondary cultures.

**Statistics.** Because of variation between, but not within samples, replating data is shown for individual experiments. Other results are averaged and are expressed as the mean ± 1 SEM. The level of significance between cases was determined using Student’s independent t distribution (two-tailed test).

**RESULTS**

**Influence of initial cell concentration on replating potential.** In previous studies, we saw little or no difference in either the replating efficiency or numbers of secondary colonies per replate when primary CB cultures were initiated at 2.5 × 10^4 to 5.0 × 10^4 cells/mL. To determine if lower cell concentrations would influence the replating potential of human CB CFU-GEMM, we plated unseparated CB at either 1.0 × 10^4, 2.5 × 10^4, or 5.0 × 10^4 cells/mL in primary cultures containing Epo, SLF, and FBS (Table 1). Individual, well-isolated CFU-GEMM colonies were removed by hand and replated into secondary cultures that contained Epo, SLF, GM-CSF, and FBS. Day 1 secondary cultures contained evenly-dispersed single cells (Fig 1A). The replating efficiency in Table 1 is shown as the percentage of primary colonies that gave rise to at least one secondary colony. In two of the
Effect of CB plasma on the replating capacity of human CB CFU-GEMM. Primary cultures of unseparated CB cells were initiated in Epo, SLF, and either CB plasma or FBS. Resultant CFU-GEMM colonies picked from each point were reseeded into secondary cultures containing Epo, SLF, GM-CSF, and either FBS or CB plasma. Figure 2 summarizes the individual results of six experiments in which a total of 625 primary CFU-GEMM colonies were replated. Although there was variation between experiments, within individual experiments the percent of CFU-GEMM colonies that replated was higher when at least secondary cultures contained CB plasma, compared with when primary and secondary cultures contained FBS (Fig 2A). More importantly, the number of secondary colonies per replated primary CFU-GEMM colony was greatest when both primary and secondary cultures contained CB plasma, although large numbers of secondary colonies were seen when CB plasma was present in secondary cultures only (Fig 2B). In the previous study, we found that replated CB CFU-GEMM gave rise to CFU-GM, BFU-E, and CFU-GEMM colonies in secondary cultures. Here, when

three experiments, we noticed an increase in replating efficiency that correlated with the number of cells plated in primary cultures (Table 1). However, in all experiments, the total number of secondary colonies per replated primary CFU-GEMM colony was much lower for primary cultures initiated with 1.0 × 10⁴ cells/mL, compared with cultures initiated with 2.5 × 10⁴ or 5.0 × 10⁴ cells/mL. Therefore, to increase the sensitivity of the replating assay for the evaluation of growth factors that might influence replating capacity, CB cultures were initiated at 1.0 × 10⁴ cells/mL in subsequent experiments.
CB plasma was added to either primary or secondary cultures, 96% to 100% of the secondary colonies were CFU-GEMM.

To further evaluate replating potential, secondary CFU-GEMM derived from primary colonies grown in Epo, SLF, and CB plasma were replated into tertiary cultures. Secondary and tertiary cultures contained Epo, SLF, GM-CSF, and CB plasma. In three experiments, no colonies were observed in the tertiary cultures, suggesting that, under the above conditions, there may be a limit to the replating capability of CB-GEMM.

**Effect of adult PB plasma or cytokines on the replating potential of human CB CFU-GEMM.** To compare the effects of CB plasma and adult human PB plasma on replating, unseparated CB cells were grown in the presence of Epo, SLF, and either CB or PB plasma (Fig 3). In two separate experiments, primary CFU-GEMM colonies (149 total) were replated into the same combination of factors as the primary culture, plus GM-CSF. In both experiments, the replating efficiency of CB CFU-GEMM was higher when cultures contained CB plasma (Fig 3A). In addition, the number of secondary colonies per replated CFU-GEMM colony was 3 to 10 times higher when cultures were initiated in CB plasma, compared with PB plasma (Fig 3B). Because both CB and PB samples were collected into heparin, and plasma was prepared in an identical manner, the enhancing effect of CB plasma on replating potential was not attributable to heparin.

**Effect of growth factors on the replating capacity of human CB CFU-GEMM.** To determine whether a combination of cytokines that act on early hematopoietic cells could mimic the effect of CB plasma on the replating capacity of CB CFU-GEMM, unseparated CB cells were cultured in the presence of Epo, SLF, and either CB plasma or the combination of FBS plus IL-1, IL-3, IL-6, IL-11, G-CSF, and GM-CSF (Fig 4). In the two experiments shown, CFU-GEMM colonies (210 total) picked from each point were replated into secondary cultures that contained Epo, SLF, GM-CSF,
and either CB plasma or the combination of FBS plus cytokines (Fig 4). The percent of CFU-GEMM colonies that replated was greatest when at least secondary cultures contained CB plasma, compared with when both primary and secondary cultures contained FBS plus the cytokine combination (Fig 4A). When both primary and secondary cultures contained CB plasma, there were 665 to 885 times the number of secondary colonies per replate observed compared with when both cultures contained the combination of FBS plus cytokines (Fig 4B).

To evaluate the cytokines in plasma, the concentrations of cytokines in 11 samples of CB plasma and 2 samples of PB plasma (diluted 1:2) were measured using immunoassays.20 The growth factors analyzed and the assay sensitivities were as follows: IL-3 (40 pg/mL), IL-4 (40 pg/mL), IL-5 (20 pg/mL), IL-6 (20 pg/mL), G-CSF (200 pg/mL), and GM-CSF (20 pg/mL). Neither CB plasma nor PB plasma contained these cytokines.

**Effect of umbilical vein endothelial cell-conditioned medium on the replating capability of human CB CFU-GEMM.** First, we compared primary colony-formation by CB cells grown in Epo, SLF, and either CB plasma, FBS, PB plasma, ECCM, FBS plus cytokines, or FBS plus ECCM. As shown in Table 2, colony formation by \(1 \times 10^6\) CB CFU-GM and CFU-GEMM was greatest when cultures contained ECCM alone or FBS plus ECCM. CB plasma did not increase numbers of CFU-GM or CFU-GEMM colonies above the level seen with FBS alone. However, CB plasma did not significantly increase the number of cells per colony (\(P < .003;\) Table 3). Primary CFU-GEMM colonies cultured in CB plasma ranged in size from 6,630 to 921,230 cells per colony and measured up to 6 mm in diameter in situ.

Next, we wanted to determine if factors constitutively released from umbilical cord vein endothelial cells (EC) could substitute for the enhanced replating activity of CB plasma. Unseparated CB cells were plated in primary cultures that contained Epo, SLF, and either CB plasma, FBS, ECCM, or FBS plus ECCM. In two separate experiments, primary CFU-GEMM colonies (306 total) were replated into secondary cultures that contained the same combination of factors, plus GM-CSF (Fig 5). The replating efficiency and the number of secondary colonies per replate observed when cultures contained FBS plus ECCM were similar to those seen with FBS alone (Fig 5A and B). The replating efficiency of CFU-GEMM grown in ECCM alone was low (Fig 5A).

**Secondary CB CFU-GEMM colony size.** CB plasma was effective in increasing the size of secondary CFU-GEMM (Fig 1B). These ranged in size up to 192,625 cells per colony, compared with 39,225, 28,800, 25,690, 0, and 28,265 cells per colony when cultures contained, respectively, either FBS or PB plasma or FBS plus rhuIL-1, IL-3, IL-6, IL-11, G-CSF, and GM-CSF, or ECCM or PB plus primary and secondary plates.

As a means of estimating the expansion of cells from a single primary CFU-GEMM colony grown in CB plasma, the entire methylenecellose culture medium was harvested from 10 individual secondary plates and the total cell number was determined. The total number of cells in each secondary culture ranged from \(0.22 \times 10^6\) to \(2.31 \times 10^6\), which represents up to a 10-fold expansion in cell number when compared with the mean of \(0.20 \times 10^6\) cells per primary CB CFU-GEMM colony (range, 6,600 to 921,230 cells/colony).

**Effect of CB plasma on the replating potential of human bone marrow CFU-GEMM.** In our previous study,11 replated CB CFU-GEMM gave rise to CFU-GM, BFU-E, and CFU-GEMM in secondary cultures, but replated bone marrow CFU-GEMM gave rise mainly to CFU-GM. Therefore, we evaluated whether addition of CB plasma to bone marrow cultures would lead to increased numbers of secondary CFU-GEMM. LD bone marrow cells were not plated at \(1.0 \times 10^4\) cells/mL, as were CB cells, because the frequency of CB progenitors is greater than that of bone marrow and few colonies developed from \(1.0 \times 10^4\) LD bone marrow cells/mL. In two separate experiments, primary cultures of LD bone marrow cells were initiated at \(2.5 \times 10^5\) cells/mL in Epo, SLF, and either FBS or CB plasma (Table 4). Primary CFU-GEMM colonies were replated into secondary cultures that contained Epo, SLF, GM-CSF, and either FBS or CB plasma. The number of replates and colonies per replate of bone marrow CFU-GEMM was enhanced by CB plasma, and when CB plasma was added to either primary or secondary cultures, secondary colonies were 95% to 100% CFU-GEMM and contained up to 179,500 cells (Fig 1C).

### Table 2. Number of Primary Colonies per 10^5 Unseparated CB Cells

<table>
<thead>
<tr>
<th>Primary Colonies Grown in rhuEpo (1 U/mL) + rhuSLF (50 ng/mL) Plus:</th>
<th>No. Plates Evaluated</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-GEMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB plasma</td>
<td>104</td>
<td>1.7 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>64</td>
<td>4.9 ± 0.5</td>
<td>6.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>PB plasma</td>
<td>26</td>
<td>0.9 ± 0.2</td>
<td>2.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>FBS/cytokines</td>
<td>24</td>
<td>9.4 ± 1.2</td>
<td>8.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>ECCM</td>
<td>29</td>
<td>12.2 ± 1.2</td>
<td>8.0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>FBS/ECCM</td>
<td>29</td>
<td>12.9 ± 1.0</td>
<td>8.4 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

* Compared with: FBS, \(P < .001;\) PB plasma, \(P < .01;\) FBS/cytokines, \(P < .001;\) ECCM, \(P < .001;\) FBS/ECCM, \(P < .001.\)

+ Compared with: FBS, \(P < .001;\) PB plasma, \(P < .001;\) FBS/cytokines, \(P < .001;\) ECCM, \(P < .001;\) FBS/ECCM, \(P < .001.\)

### Table 3. Number of Cells per Individual Primary CB CFU-GEMM Colony

<table>
<thead>
<tr>
<th>Primary Colonies Grown in rhuEpo (1 U/mL) + rhuSLF (50 ng/mL) Plus:</th>
<th>No. Colonies Evaluated</th>
<th>No. Cells/Primary CFU-GEMM Colony</th>
<th>Range in No. of Cells/Primary CFU-GEMM Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB plasma</td>
<td>54</td>
<td>202,925 ± 21,460*</td>
<td>6,630-921,230</td>
</tr>
<tr>
<td>FBS</td>
<td>65</td>
<td>93,920 ± 9,433</td>
<td>3,475-340,905</td>
</tr>
<tr>
<td>PB plasma</td>
<td>35</td>
<td>63,977 ± 7,410</td>
<td>8,300-171,555</td>
</tr>
<tr>
<td>FBS/cytokines</td>
<td>35</td>
<td>117,370 ± 10,860</td>
<td>12,737-141,144</td>
</tr>
<tr>
<td>ECCM</td>
<td>27</td>
<td>64,793 ± 7,162</td>
<td>11,780-180,120</td>
</tr>
<tr>
<td>FBS/ECCM</td>
<td>21</td>
<td>95,586 ± 10,346</td>
<td>34,850-214,205</td>
</tr>
</tbody>
</table>

* Compared with: FBS, \(P < .001;\) PB plasma, \(P < .001;\) FBS/cytokines, \(P < .001;\) ECCM, \(P < .001;\) FBS/ECCM, \(P < .001.\)
There was no significant difference in primary colony formation by BFU-E or CFU-GEMM grown in CB plasma or FBS in either experiment (Table 5). However, there were significantly greater numbers of CFU-GM in cultures containing FBS in both experiments ($P < .052$ and .021). There was no significant difference in the number of cells per primary CFU-GEMM colony grown in FBS or CB plasma (Table 6).

Primary bone marrow CFU-GEMM colonies grown in CB plasma were smaller on average than primary cord blood CFU-GEMM colonies grown in CB plasma. Primary bone marrow colonies grown in CB plasma contained a mean of 29,960 cells compared with a mean of 202,925 cells per primary CB CFU-GEMM colony.

**DISCUSSION**

The ability of an individual progenitor cell colony to replate in an in vitro colony-forming assay has been used as an indicator of the self-renewal potential of that progenitor cell.\(^1\)\(^1\) We have shown in a previous study that the use of SLF in combination with Epo in colony replating assays allowed detection of human CB and bone marrow CFU-GEMM with a high replating efficiency in terms of the percentage of colonies that could be replated and the number of secondary colonies that formed per replated primary colony.\(^1\)\(^1\) CB CFU-GEMM, unlike bone marrow CFU-GEMM, gave rise to many secondary CFU-GEMM when replated. However, the data showed that the secondary CFU-GEMM colonies were much smaller than the primary CFU-GEMM colony from which they were derived.\(^1\)\(^1\) We could not rule out the possibility that the secondary CFU-GEMM colonies were derived from a more mature progenitor than that which gave rise to primary CFU-GEMM. Because normal hematopoiesis takes place within a microenvironment,\(^2\)\(^3\) we felt that soluble growth factors present within the microenvironment of an umbilical cord that were capable of influencing the replating potential of CB CFU-GEMM should be present in CB plasma.

By plating CB cells at a lower initial concentration in the current study ($1.0 \times 10^4$ cells/mL), we increased the sensitivity of the replating assay to the effects of added factors. CB plasma in combination with Epo and SLF did not increase primary cloning efficiency of CB but did enhance the size of primary CFU-GEMM colonies. More importantly, the combination of Epo, SLF, and CB plasma also increased the size of secondary colonies such that these were now within the range

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**Table 4. Replating Efficiency of Adult Human Bone Marrow Multipotential (CFU-GEMM) Hematopoietic Progenitor Cells Grown in CB Plasma v FBS**

<table>
<thead>
<tr>
<th>Primary Colonies Grown in</th>
<th>Secondary Colonies Grown in</th>
<th>No. Primary Colonies Replated</th>
<th>% Replaces With at Least One Colony</th>
<th>CFU-GM/10^5 Replated 1^st Colony</th>
<th>BFU-E/10^5 Replated 1^st Colony</th>
<th>CFU-GEMM/10^5 Replated 1^st Colony</th>
<th>Total No. of 2^nd Colonies/10^5 Replated Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhEpo (1 U/mL) + rhSLF (50 ng/mL) Plus</td>
<td>CB plasma</td>
<td>30</td>
<td>10.0</td>
<td>0.8</td>
<td>0</td>
<td>21.8</td>
<td>22.6</td>
</tr>
<tr>
<td>FBS</td>
<td>CB plasma</td>
<td>29</td>
<td>24.1</td>
<td>0.8</td>
<td>0</td>
<td>16.0</td>
<td>16.8</td>
</tr>
<tr>
<td>FBS</td>
<td>CB plasma</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FBS</td>
<td>FBS</td>
<td>27</td>
<td>14.8</td>
<td>1.1</td>
<td>0</td>
<td>0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

| Experiment 2 | | | | | | | |
| CB plasma | CB plasma | 30 | 50.0 | 0 | 0 | 25.0 | 25.0 |
| CB plasma | FBS | 30 | 33.3 | 0.3 | 0 | 17.1 | 17.4 |
| FBS | CB plasma | 29 | 13.8 | 0 | 0 | 9.8 | 9.8 |
| FBS | FBS | 30 | 13.3 | 0.2 | 0.6 | 1.0 | 1.8 |
of sizes observed for primary colonies. This included maintenance of the differentiation capacity of CFU-GEMM grown in CB plasma, as secondary colonies were almost entirely CFU-GEMM. A single CB CFU-GEMM colony, containing an average of 202,925 cells, has the capacity to give rise to as many as 1,012 secondary CFU-GEMM colonies. Each of these secondary colonies can contain up to 192,625 cells. This represents a tremendous cellular expansion of multiple cell types, derived from a single CFU-GEMM progenitor cell.

The replating capacity of bone marrow CFU-GEMM was also enhanced by CB plasma. We showed previously that replated bone marrow CFU-GEMM only rarely gave rise to secondary CFU-GEMM. Secondary colonies derived from replated bone marrow CFU-GEMM were mainly CFU-GM. In the current study, when bone marrow CFU-GEMM cultured with Epo, SLF, and CB plasma were replated, secondary cultures contained mainly CFU-GEMM colonies that were equal to, or greater than the size of the primary colony from which they were derived. Additionally, bone marrow CFU-GEMM that were grown in Epo, SLF, and CB plasma in primary and secondary cultures gave rise to greater numbers of secondary colonies than those that were grown in Epo, SLF, and FBS. Whereas the percentages of CB and bone marrow CFU-GEMM that replated and the sizes of CB and bone marrow secondary CFU-GEMM colonies are similar, the number of secondary colonies per replate was greater for CB CFU-GEMM than for bone marrow CFU-GEMM. Therefore, the replating potential of bone marrow CFU-GEMM may be more limited than that of CB CFU-GEMM.

These results suggest that human CFU-GEMM have some degree of stemness and perhaps should be classified as a subset of stem cells. The inability to replate secondary CFU-GEMM colonies into tertiary plates suggests a limited degree of stemness. Alternatively, we are still missing something to detect further replating, such as an integral stimulus from other cells.

Neither PB plasma, the combination of the early acting cytokines IL-3, IL-4, IL-5, IL-6, G-CSF, and GM-CSF plus Epo, SLF and FBS, nor ECCM substituted for CB plasma as an enhancer of CFU-GEMM replating capacity. The identity of the factor or factors present in CB plasma that are responsible for the enhanced replating efficiency of CFU-GEMM is currently unknown. Depending on the type of delivery of the infant, human umbilical CB plasma has been shown to contain varying concentrations of IL-1β and also contains IL-6 and tumor necrosis factor. Our present data suggests CB plasma does not contain significant levels of IL-3, IL-4, IL-5, IL-6, G-CSF, or GM-CSF. It is possible that we did not detect IL-6 because the level of sensitivity of the IL-6 immunoassay was close to the previously reported CB plasma concentration of about 50 pg/mL. Whether the enhancing activity of CB plasma in colony replating assays for CB and bone marrow CFU-GEMM is attributable to a novel factor or factors, or represents effects of other known cytokines, alone or in combination, remains to be determined.

This will no doubt be a source of intense future investigation.

ACKNOWLEDGMENT

The authors thank Drs John Abrams and Jon Silver (DNAX Research Institute, Palo Alto, CA) for performing the immunoassays; Dr Sharon Andreoli and Colleen Mallet (Department of Pediatrics, Riley Hospital for Children, Indianapolis, IN) for helping with the preparation of ECCM; and Rebecca Robling for helping with the preparation of the manuscript.

REFERENCES


Table 5. Number of Primary Colonies per 2.5 × 10⁴ LDBM Cells

<table>
<thead>
<tr>
<th>Colonies Grown in rhEpo (1 U/mL)</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-GEMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(60 ng/mL) Plus:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB plasma</td>
<td>34.5 ± 5.5*</td>
<td>40.5 ± 2.4</td>
<td>60.0 ± 5.7</td>
</tr>
<tr>
<td>FBS</td>
<td>58.5 ± 6.5</td>
<td>49.0 ± 4.1</td>
<td>54.5 ± 4.3</td>
</tr>
</tbody>
</table>

| Experiment 2                     |        |       |          |
| CB plasma                       | 24.8 ± 3.21 | 14.8 ± 2.5 | 23.8 ± 2.5 |
| FBS                             | 37.0 ± 2.3  | 11.3 ± 0.8  | 26.5 ± 2.1  |

Data is represented as the mean ± 1 SEM colony number scored for 8 cultures.

* Compared with FBS, P < .052.
† Compared with FBS, P < .021.

Table 6. Number of Cells per Primary Bone Marrow CFU-GEMM Colony

<table>
<thead>
<tr>
<th>Colonies Grown in rhEpo (1 U/mL)</th>
<th>+ rhSLF (50 ng/mL) Plus:</th>
<th>No. Colonies Evaluated</th>
<th>No. Cells/Primary CFU-GEMM Colony</th>
<th>Range in No. of Cells/Primary CFU-GEMM Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB plasma</td>
<td>24</td>
<td>29.960 ± 5.045</td>
<td>1.035-76,810</td>
<td></td>
</tr>
</tbody>
</table>

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Human multipotential progenitor cells (CFU-GEMM) have extensive replating capacity for secondary CFU-GEMM: an effect enhanced by cord blood plasma

CE Carow, G Hangoc and HE Broxmeyer