Alternative Mechanisms With and Without Steel Factor Support Primitive Human Hematopoiesis

By H.J. Sutherland, D.E. Hogge, D. Cook, and C.J. Eaves

As a first approach to defining the molecular requirements for supporting human hematopoietic stem cell maintenance and differentiation in vitro, we have analyzed and compared the ability of various factors to support the maintenance and initial differentiation of human long-term culture-initiating cells (LTC-ICs), a distinct, rare primitive hematopoietic cell type whose progeny after 5 weeks include cells detectable as colony-forming cells. Normal human marrow cells highly enriched in LTC-ICs (~1% pure) were placed in cultures containing either preestablished, irradiated human marrow adherent feeder layers, or feeders consisting of Steel factor-deficient S/S or, normal +/- murine fibroblasts, or no feeders. In some groups, either Steel factor alone, granulocyte colony-stimulating factor (G-CSF) and interleukin-3 (IL-3), or all three factors combined were also added repeatedly. S/S murine fibroblasts were equivalent to +/- controls and to normal human marrow feeders in supporting both LTC-IC maintenance and clonogenic cell output over a 5-week period. Soluble Steel factor alone could, however, effectively substitute for human marrow feeders to support LTC-IC maintenance, although clonogenic cell output was markedly reduced under these conditions. Conversely, soluble Steel factor with G-CSF and IL-3 or with feeders (or all together) did not further enhance (or depress) LTC-IC maintenance, although under these conditions clonogenic cell output was markedly increased. These findings confirm previous evidence that LTC-IC maintenance and clonogenic cell production are differentially regulated and show for the first time that LTC-IC maintenance can be supported by different nonsynergizing factors that may, but need not, include Steel factor. © 1993 by The American Society of Hematology.
cells in normal human marrow to be separated from one another. This is consistent with the view that transplantable hematopoietic stem cells and LTC-ICs are closely related, if not identical, populations.  

Moreover, at least the initial preservation of transplantable human hematopoietic stem cells in LTC is suggested by the rapid and sustained recovery of hematopoiesis observed in patients with chronic myelogenous leukemia (CML) transplanted with autologous marrow cells previously maintained in LTC for 10 days. Nevertheless, no culture conditions are known that can match the efficiency of hematopoietic stem cell self-renewal that occurs during ontogeny or during hematopoietic reconstitution following myeloablative treatments. Identification of specific factors that mediate this process thus continues to pose a major challenge to many basic and applied studies, including those focused on leukemogenesis, cancer treatment, and gene therapy.

**MATERIALS AND METHODS**

**Bone marrow cells.** Aliquots of normal human bone marrow cells were obtained from informed and consenting allogeneic bone marrow transplant donors at the time of marrow harvests or from thawed cadaver specimens. Light-density (<1.077 g/cm³) cells were sorted using a FACStar® (Becton Dickinson, Mountain View, CA) to isolate cells with low to intermediate forward and low 90° light scattering properties, expressing high levels of CD34 and low levels of HLA-DR (referred to as CD34⁺DR⁻) cells to obtain a highly enriched LTC-IC population as previously described.

**Stromal feeders.** Irradiated (15 Gy of 250-kVp x-rays) normal human marrow adherent cells were subcultured at 3 × 10⁶ cells/cm² from 2- to 6-week-old primary LTCs. Murine SI/SI and +/+ or +/+ (or +/+ WC/Re) parents. Whole embryos that were either pale SI/SI or healthy appearing +/+ or +/+, for simplicity referred to henceforth as +/+ were trypsinized after removal of the liver, spleen, and heart and the adherent fibroblasts were then propagated by multiple serial passages. The presence (+/+ and absence (SI/SI) of the wild-type Steel gene were confirmed by both Northern and Southern analyses (data not shown). SI/SI and +/+ fibroblasts were irradiated with 80 Gy of 250-kVp x-rays prior to their use as feeders at 3 × 10⁴ cells/cm².

**Cultures.** Primary LTCs were initiated by seeding from 900 to 20,000 sorted human marrow cells into 35-mm tissue culture dishes (with or without feeders, as indicated) and then performing weekly half-medium changes as previously described. In cultures without feeders, all nonadherent cells were returned at the time of each weekly half-medium change to prevent artificial depopulation of the culture. After 10 days or 5 weeks, the number of nonadherent cells was determined and the adherent cells suspended by trypsinization. An aliquot of the combined nonadherent and adherent cells were then assayed for erythropoietic (burst-forming unit-erythroid [BFU-E]), granulopoietic (colony-forming unit-granulocyte and/or macrophage [CFU-GM]), and multilineage (colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte [CFU-GEMM]) clonogenic progenitors using standard procedures. The remaining cells were assayed for LTC-ICs by seeding them into secondary LTC-containing preestablished, irradiated human marrow feeder layers and then assessing the total number of clonogenic cells (BFU-E plus CFU-GM plus CFU-GEMM) present 5 weeks later. Division by 4 of the total clonogenic progenitor content at the 5-week time point gives the number of LTC-ICs present in the initial population assayed.

**Growth factors.** Recombinant human Steel factor (Amgen Corporation, Thousand Oaks, CA) was added to co-cultures (as indicated) to achieve a final concentration of 100 ng/mL three times per week. G-CSF and IL-3 were obtained as crude supernatants from COS cells (ATCC, Rockville, MD) that had been transfected 48 hours previously with an expression vector containing the appropriate cDNAs and added to cocultures (as indicated). Three times per week to achieve a final concentration of 20 ng/mL.

**RESULTS**

**General experimental design.** Primary cultures were initiated with highly enriched suspensions of small, light-density CD34⁺DR⁻ cells isolated from normal human marrow as described in the Materials and Methods section. In the absence of an exogenous source of factors, the LTC-ICs initially present in such suspensions are not self-sustaining (Fig 1 and Table 1). Using this condition as a baseline, the effect of Steel factor (with or without the repeated addition of G-CSF and IL-3) on both LTC-IC maintenance and production of clonogenic cells was then determined, first by assessment of cultures where Steel factor was neither produced nor added (ie, cultures containing SI/SI fibroblast feeders), and second by assessment of cultures to which large amounts of Steel factor and/or G-CSF plus IL-3 were added repeatedly (with or without feeders). The effect of adding G-CSF and IL-3 was studied because this combination had previously been shown to enhance the in vitro maintenance and differentiation of LTC-ICs. The number of clonogenic cells present after 10 days was also measured, as were the total number of nonadherent cells present after 5 weeks in order additionally to evaluate the relative importance of each of the conditions studied on later stages of granulopoiesis. Our experimental design thus employed a series of sequential cultures. In the first of these, the input cells were maintained for up to 5 weeks under a first set of test conditions. The cells in these primary cultures were then harvested and, in addition to nonadherent cell counts and clonogenic cell assays being performed, an aliquot was seeded into standard secondary LTC-IC assay cultures (containing human marrow feeders). The number of clonogenic progenitors produced another 5 weeks later (divided by 4, see Materials and Methods) then provided a measure of the number of LTC-ICs that had been maintained in the primary cultures until the time they were harvested.

**Steel factor is not essential for the maintenance and early differentiation of LTC-ICs.** The number of both LTC-ICs and clonogenic cells detected in 5-week-old LTCs containing either SI/SI or +/+ feeders was the same, and both were equivalent in this respect to primary human marrow feeders (Fig 1). In the absence of feeders, both LTC-ICs and clonogenic cell numbers rapidly declined. LTC-IC maintenance after the first 10 days also was equivalent for cultures con-
Fig 1. Changes in the clonogenic and LTC-IC content of cultures initiated with sorted marrow cells on irradiated Sl/Sl feeders (○) or +/+ feeders (□) as compared with cultures containing irradiated human marrow feeders (positive control, upper dotted line) or no feeders (negative control, lower dotted line). The progenitor content of cultures with Sl/Sl feeders did not differ from those with +/+ feeders and neither differed from those with human marrow feeders at week 5 (P > .05). The LTC-IC contents at 10 days were also not different (P > .05), but the clonogenic cell content at 10 days was significantly lower in the +/+ and Sl/Sl co-cultures as compared with human marrow controls (P < .05).

Table 1. Differential Ability of Various Growth Factors to Support Different Stages of Hemopoiesis in Suspension Cultures of Highly Enriched Populations of Human Marrow LTC-ICs

<table>
<thead>
<tr>
<th>Factors(s) Added</th>
<th>No. of Experiments</th>
<th>No. of Cells/100 Initial Nucleated Cells</th>
<th>LTC-IC</th>
<th>Clonogenic Cells</th>
<th>Total Nonadherent Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Start +10 d</td>
<td>Start +10 d</td>
<td>Start +5 wk</td>
</tr>
<tr>
<td>Steel</td>
<td>8</td>
<td></td>
<td>1.1 (0.7-1.7)</td>
<td>0.73 (0.56-0.96)</td>
<td>0.16 (0.13-0.20)</td>
</tr>
<tr>
<td>G-CSF + IL-3</td>
<td>7</td>
<td></td>
<td>1.1 (0.7-1.7)</td>
<td>0.44 (0.26-0.76)</td>
<td>0.18 (0.11-0.30)</td>
</tr>
<tr>
<td>Steel + G-CSF + IL-3</td>
<td>8</td>
<td></td>
<td>1.1 (0.7-1.7)</td>
<td>0.68 (0.35-1.35)</td>
<td>0.16 (0.11-0.26)</td>
</tr>
<tr>
<td>Human marrow feeders</td>
<td>16</td>
<td></td>
<td>1.0 (0.8-1.3)</td>
<td>0.67 (0.44-0.73)</td>
<td>0.11 (0.079-0.15)</td>
</tr>
<tr>
<td>No addition</td>
<td>12</td>
<td></td>
<td>1.0 (0.8-1.4)</td>
<td>0.08 (0.07-0.10)</td>
<td>0.04 (0.03-0.06)</td>
</tr>
</tbody>
</table>

Cultures were initiated with a few thousand highly purified CD34+, HLA-DR+ normal human marrow cells and maintained as described. Cultures did not contain feeders unless specified. Results shown are the means of logarithmically transformed data after normalization of each result per 100 sorted cells initially seeded into each type of culture. Total nonadherent cells were assessed only after 5 weeks.

* Indicates values that are significantly different from the results obtained in experimentally paired (positive) control cultures containing human marrow feeders (P < .05; two-tailed t test).

† Indicates values that are significantly higher than the results obtained in experimentally paired (negative) control cultures to which no factors were added (P < .05; one-tailed t test).
Table 2. Differential Ability of Various Growth Factors to Enhance the Ability of Human Marrow Feeders to Support Different Levels of Hemopoiesis in Cultures Initiated With Highly Enriched Populations of Human Marrow LTC-ICs

<table>
<thead>
<tr>
<th>Factors(s) Added</th>
<th>No. of Experiments</th>
<th>No. of Cells/100 Initial Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LTC-IC</td>
<td>Clonogenic Cells</td>
</tr>
<tr>
<td></td>
<td>Start</td>
<td>+10 d</td>
</tr>
<tr>
<td>Steel</td>
<td>5</td>
<td>0.9</td>
</tr>
<tr>
<td>G-CSF + IL-3</td>
<td>5</td>
<td>0.9</td>
</tr>
<tr>
<td>Steel + G-CSF + IL-3</td>
<td>8</td>
<td>1.4</td>
</tr>
<tr>
<td>None</td>
<td>16</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Indicates values that are significantly higher than the corresponding results obtained in control cultures containing human marrow feeders but to which no factors were added (P < .05, one-tailed t-test).

Cultures were initiated and maintained as described. Note that all cultures in these series of experiments contained preestablished, irradiated human marrow feeders. Growth factor addition, data normalization and analysis were the same as for the experiments in Table 1. Under none of these conditions were the numbers of LTC-ICs maintained significantly different than the values obtained in parallel standard cultures containing human marrow feeders but to which no factors were added and all values were significantly increased by comparison to the no-addition control. In contrast, by 5 weeks, the production of clonogenic cells from input LTC-ICs was significantly higher (fivefold) but not altered qualitatively (% BFU-E not significantly different from controls paired t-test) when Steel factor plus G-CSF and IL-3 were used to replace human marrow feeders and significantly decreased (fourfold) when only Steel factor was added. The addition of Steel factor alone did not support terminal granulopoiesis (measured as the 5-week output of total nonadherent cells or the 10-day output of clonogenic cells) as seen by the similarity of these groups to controls without feeders or factor supplements. This confirms the previously observed inability of Steel factor on its own to support the terminal stages of human granulopoiesis and now shows that alone it is capable of supporting LTC-IC maintenance.

**Steel factor and G-CSF plus IL-3 synergize with feeders in promoting intermediate and later stage of hematopoiesis but not LTC-IC maintenance.**

Steel factor alone or G-CSF plus IL-3 (or all 3 factors combined) were added. Under none of these conditions were the numbers of LTC-ICs maintained significantly different than the values obtained in parallel standard cultures containing human marrow feeders but to which no factors were added and all values were significantly increased by comparison to the no-addition control. In contrast, by 5 weeks, the production of clonogenic cells from input LTC-ICs was significantly higher (fivefold) but not altered qualitatively (% BFU-E not significantly different from controls paired t-test) when Steel factor plus G-CSF and IL-3 were used to replace human marrow feeders and significantly decreased (fourfold) when only Steel factor was added. The addition of Steel factor alone did not support terminal granulopoiesis (measured as the 5-week output of total nonadherent cells or the 10-day output of clonogenic cells) as seen by the similarity of these groups to controls without feeders or factor supplements. This confirms the previously observed inability of Steel factor on its own to support the terminal stages of human granulopoiesis and now shows that alone it is capable of supporting LTC-IC maintenance.

Steel factor and G-CSF plus IL-3 synergize with feeders in promoting intermediate and later stage of hematopoiesis but not LTC-IC maintenance. Because Steel factor as well as G-CSF plus IL-3 and another unidentified factor produced by S1/S1 fibroblasts were all able to support LTC-IC maintenance, it was of interest to determine whether the latter would synergize with Steel factor alone or in combination with G-CSF plus IL-3. All combinations enhanced markedly the output of nonadherent cells and clonogenic cells detected at day 10, with the maximum effect achieved by all three factors (a 15-fold increase in nonadherent cells and a twofold increase in day 10 clonogenic cells) (Table 2). Steel factor alone enhanced the 5-week clonogenic cell output. However, no enhancement of LTC-IC maintenance was achieved by any combination of factor addition to cultures also containing feeders.

**DISCUSSION**

In this study, we present the first analysis of the factor requirements for LTC-IC maintenance in vitro. The results (summarized in Fig 2) show that equivalent LTC-IC maintenance can be achieved by Steel factor alone, G-CSF plus IL-3 alone, or by co-culture of the target cells with murine fibroblasts that are genetically incapable of producing Steel factor. These findings are thus indicative of at least three alternative ways that can enhance LTC-IC maintenance. Whether these involve similar mechanisms at the cellular level in terms of effects on proliferation versus survival and/or in terms of intracellular signaling remains to be elucidated. Steel factor, although unnecessary for LTC-IC maintenance, is alone sufficient but not synergistic with either G-CSF plus...
IL-3 or the factors produced by human marrow adherent cells to support these cells. In contrast, Steel factor plus G-CSF and IL-3 or human marrow adherent cells were clearly synergistic in stimulating clonogenic cell production from LTC-ICs, but this enhancement of clonogenic cell output did not occur at the expense of LTC-IC maintenance. The present study thus extends previous data from our group indicating that these two closely related responses of LTC-ICs can be dissociated at the molecular level.5,25

The competence of murine Sf/Sf fibroblasts to support human LTC-IC maintenance as effectively as +/+ fibroblasts or human marrow feeders is consistent with recent evidence for the existence of additional factors that may act on the most primitive hematopoietic cells. One of these is IL-11. IL-11 is produced by fibroblasts and appears to have profound effects on early hematopoietic cells when combined with other hematopoietic growth factors.31,32 Another candidate factor is the as yet unidentified ligand for flk-2. flk-2 is a tyrosine kinase cell surface receptor belonging to the c-met, platelet-derived growth factor receptor family and its expression on murine hematopoietic cells is restricted to very primitive subpopulations.33 It will, therefore, be important to determine whether either IL-11 or the flk-2 ligand can explain or enhance the supportive function of murine and human fibroblasts (or whether additional factors are involved) toward the future goal of obtaining amplified human stem cell populations for therapeutic as well as investigative applications.

ACKNOWLEDGMENT

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