Effect of Recombinant Erythropoietin in Interaction With Stromal Factors on Cord Blood Hematopoiesis

By Takanori Abe, Yoichi Takaue, Yoshifumi Kawano, and Yasuhiro Kuroda

To investigate the effect of recombinant erythropoietin (Epo) on primitive human hematopoietic progenitor cells, we cultured cord blood mononuclear cells (CBMNC) and CB CD34+ cells in a Dexter-type long-term culture system (LTC), to which various concentrations of Epo were added at day 0 or 7, with or without direct contact with irradiated allogeneic human marrow stromal layers. In regular stroma-contact cultures, when CBMNC were inoculated, the addition of Epo at 1 to 10 U/mL induced a significant increase in LTC-initiating cells (LTC-IC), particularly in the myeloid component, compared with the control without Epo. Significantly LTC-IC were generated by the delayed addition of Epo on day 7 than on day 0. On the other hand, when CD34+ cells were inoculated, physiologic concentrations of Epo (0.1 U/mL) induced a more than twofold increase in LTC-IC, which was attributed equally to both the myeloid and erythroid lineages, only when added on day 0. In stroma-noncontact cultures, which were created using a Transwell 0.4-μm microporous membrane filter, dose-dependent suppression of the myeloid component of LTC-IC was observed with a higher concentration of Epo (1 to 100 U/mL) when CBMNC was inoculated. On the other hand, without Epo, fourfold more LTC-IC was generated from CD34+ cells in stroma-noncontact than in stroma-contact cultures, which was then significantly augmented by the addition of Epo (0.1 or 10 U/mL) on day 0. This increase was due to both the myeloid and erythroid lineages. A higher concentration of Epo (100 U/mL) resulted in a decrease in LTC-IC, mainly in myeloid progeny, in all of the culture conditions. Hence, Epo may play an important physiologic role in the maintenance and proliferation of immature stem/progenitor cells, in close interaction with factors from narrow stromal cells.

© 1996 by The American Society of Hematology.
were released from the beads by incubation with 200 \( \mu \text{L} \) of 1 \% papain (Baxter) for 15 minutes at 37°C. In this study, the purity of CD34\(^+\) cells ranged from 60\% to 70\%.

**Hematopoietic growth factors.** Human recombinant interleukin-3 (IL-3; 1 \( \times \) 10\(^3\) \( \mu \text{g} \) protein), granulocyte colony-stimulating factor (G-CSF; 1 \( \times \) 10\(^3\) \( \mu \text{g} \)), stem cell factor (SCF; 2 \( \times \) 10\(^3\) \( \mu \text{g} \)), and Epo (2 \( \times \) 10\(^3\) \( \mu \text{g} \)) were kindly provided by Kirin Brewery Co (Tokyo, Japan).

**Long-term hematopoietic cultures.** LTC assay was performed according to methods previously described by other researchers,\(^{11,12}\) with minor modifications.\(^{13}\) Briefly, LTC medium (LTCM) was prepared by supplementing Iscove’s modified Dulbecco’s medium (IMDM; Flow Laboratories, Irvine, UK) with 12.5\% fetal bovine serum (FBS; GIBCO, Grand Island, NY), 12.5\% horse serum (GIBCO), 10\(^{–6}\) \( \text{mol/L} \) hydrocortisone (Sigma, St Louis, MO), and 2 \( \text{mmol/L} \) l-glutamine (GIBCO). The frozen BMMNC were thawed with Dulbecco’s modified Eagle’s medium (DMEM; Flow Laboratories) containing 30\% FBS and 100 \( \mu \text{L} \) DNase (Sigma) and washed three times with PBS.

The cells were plated in 25-cm\(^2\) tissue culture flasks with a phenolic style cap (Corning, New York, NY) in 6 mL LTCM at 10\(^5\) cells/cm\(^2\). Cells were incubated in a fully humidified atmosphere of 5\% O\(_2\), and 5\% CO\(_2\), and fed weekly by replacing the medium containing nonadherent cells with freshly prepared LTCM. After 1 or 2 weeks, the stromal layers were removed by the addition of 2 mL trypsin (GIBCO) for 5 minutes at 37°C and washed with PBS. Stromal cells were then inoculated into collagen-coated 24-well plates (Corning 25820 COIL) at 10\(^7\) cells/well in 1 mL LTCM. Two days later, plates were irradiated (15 Gy) to eliminate the endogenous production of hematopoietic cells before inoculation of the target cells.

CBMNC or CB CD34\(^+\) cells were then thawed by the same method as used for BMMNC and inoculated directly to the plates at 2 \( \times \) 10\(^3\) or 5 \( \times \) 10\(^3\) cells/well in 1 mL LTCM after the old medium was removed (stroma-contact cultures). Human Epo was added to the wells at day 0 or 7 to give a final concentration of 0.05, 0.1, 1, 5, 10, or 100 \( \mu \text{g/mL} \). In some experiments, to examine the effect of further delayed addition of Epo, Epo (10 \( \mu \text{g/mL} \)) was added to the culture at day 10 or 14. In other experiments, a collagen-coated 0.4-\( \mu \text{m} \) microporous membrane filter (Transwell; Costar, Cambridge, MA) was hung on top of the stromal layers after removing the old LTCM, as described by Verfaillie et al.\(^{14,15}\) Thawed CBMNC or CB CD34\(^+\) cells were plated in the upper wells at 2 \( \times \) 10\(^3\) or 5 \( \times \) 10\(^3\) cells/0.2 \( \mu \text{L} \) of LTCM, respectively. LTCM (0.8 \( \mu \text{L} \)) was then added to the bottom wells to bring the total volume of the medium to 1 \( \mu \text{L} \). As a result, the stromal layers that adhered to the bottom well were completely separated from CBMNC or CD34\(^+\) cells on the microporous membrane filters throughout the culture period. Because the filter allowed the free passage of diffusible factors, such as stroma-derived soluble cytokines, but prevented direct cell-to-cell contact, the system created stroma-noncontact cultures. Human Epo was added to the bottom wells as in the stroma-contact cultures. Wells that did not receive Epo were evaluated as a control.

All plates were kept at 37°C in a fully humidified atmosphere of 5\% O\(_2\), 5\% CO\(_2\), and 90\% N\(_2\). At weekly intervals, cultures were fed by removing half of the cell-free supernatant (0.5 \( \mu \text{L} \)) and replacing it with fresh complete LTCM containing a determined dose of Epo (0 to 100 \( \mu \text{g/mL} \)). When the cultures were evaluated for LTC-IC, both adherent and nonadherent cells were harvested at week 5 from a single well of each plate by collecting medium and adding 0.5 \( \mu \text{L} \) trypsin for 5 minutes at 37°C.

**Hematopoietic progenitor cell assay in methylcellulose.** Cells harvested from LTC were inoculated in methylcellulose cultures supplemented with 20\% FBS, 450 \( \mu \text{g} \)/mL of human transferrin, and 1\% of deionized bovine serum albumin, as previously reported.\(^{16}\) Quadruplicate cultures were plated in volumes of 0.4 \( \mu \text{L} \) in 24-well tissue culture plates (Corning). Recombinant cytokines were added to the cultures at various prescreened concentrations (200 \( \mu \text{g/mL} \) for SCF, 200 \( \mu \text{g/mL} \) for IL-3, and 200 \( \mu \text{g/mL} \) for G-CSF). The plates were incubated at 37°C in a humidified atmosphere of 5\% CO\(_2\), 5\% O\(_2\), and 90\% N\(_2\) for 2 weeks. Colonies were scored for colony-forming unit for granulocyte-macrophage (CFU-GM) and BFU-E at 14 days of culture using an inverted microscope, but colony-forming unit for mixed lineage (CFU-mix) was not evaluated because of its very small number.

Results concerning cellularity and the number of progenitors per culture at week 5 are expressed as the mean ± standard error of the mean (SEM) and as a percentage of the control cultures. Significance was determined using a two-sided nonpaired Student’s \( t \)-test analysis.

**RESULTS**

Figure 1 shows LTC-IC as a percentage of the values for control cells without Epo in stroma-contact cultures. Recovery of the absolute number of LTC-IC from 10\(^5\) CBMNC or
The recovery of LTC-IC from 10^6 CBMNC or 10^5 CD34+ cells in stroma-contact and stroma-noncontact cultures is shown. Data are the mean ± SEM.

Abbreviation: NS, not significant.

* Significance levels were determined by a two-sided nonpaired Student’s t-test analysis.

### Table 1. Number of LTC-IC Recovered From Cord Blood MNC and CD34+ Cells in Stroma-Contact and Stroma-Noncontact Cultures

<table>
<thead>
<tr>
<th>Concentration of Epo (U/mL)</th>
<th>Day of Addition</th>
<th>Stroma-Contact</th>
<th>Stroma-Noncontact</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBMNC (1 x 10^6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51 ± 10 (n = 25) (range, 40-65)</td>
<td>50 ± 11 (n = 22) (38-66)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>48 ± 7 (39-57)</td>
<td>51 ± 9 (38-63)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>47 ± 12 (36-66)</td>
<td>47 ± 10 (38-64)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>51 ± 7 (43-63)</td>
<td>50 ± 9 (40-65)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>49 ± 10 (37-67)</td>
<td>48 ± 9 (40-82)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>57 ± 8 (47-68)</td>
<td>40 ± 6 (33-50)</td>
<td>&lt;.02</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>60 ± 8 (50-72)</td>
<td>42 ± 6 (31-62)</td>
<td>&lt;.02</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>64 ± 8 (54-75)</td>
<td>36 ± 10 (22-48)</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>73 ± 7 (66-84)</td>
<td>38 ± 9 (24-62)</td>
<td>&lt;.005</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>69 ± 10 (54-85)</td>
<td>30 ± 12 (16-60)</td>
<td>&lt;.005</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>81 ± 9 (64-96)</td>
<td>36 ± 10 (20-52)</td>
<td>&lt;.002</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>33 ± 4 (29-38)</td>
<td>20 ± 4 (14-28)</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>41 ± 3 (38-45)</td>
<td>24 ± 3 (16-26)</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>CB CD34+ cells (1 x 10^5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10 ± 3 (n = 6) (range, 6-16)</td>
<td>40 ± 7 (n = 6) (35-52)</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>25 ± 9 (9-36)</td>
<td>57 ± 12 (43-80)</td>
<td>&lt;.005</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>19 ± 2 (7-13)</td>
<td>41 ± 7 (29-51)</td>
<td>&lt;.002</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16 ± 3 (11-20)</td>
<td>65 ± 7 (57-73)</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>9 ± 2 (7-12)</td>
<td>36 ± 6 (28-45)</td>
<td>&lt;.002</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2 ± 1 (1-3)</td>
<td>28 ± 10 (1-10)</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2 ± 1 (1-3)</td>
<td>26 ± 9 (10-34)</td>
<td>&lt;.05</td>
<td></td>
</tr>
</tbody>
</table>

The delayed addition of Epo on day 7 was significantly greater than that with the addition of Epo on day 0 (P < .05) or in the control (P < .05 to P < .005). This increase was primarily the result of an increase in CFU-GM. No further increase in LTC-IC was observed with the addition of 10 U/mL of Epo at day 10 or 14 (results not shown). LTC-IC formation was suppressed at 100 U/mL of Epo compared with the control (P < .01), primarily due to a decrease in CFU-GM.

Generation of LTC-IC from CB CD34+ cells in the stroma-contact culture contrasted with that from CBMNC. At physiologic concentrations of Epo (0.1 U/mL), significantly more LTC-IC were formed with Epo than in the control (P < .002), but only when Epo was added on day 0. CFU-GM and BFU-E increased equally. Delayed addition of Epo, which exerted a stimulatory effect on LTC-IC in cultures with CBMNC, had no effect on LTC-IC. LTC-IC formation was stimulated by the addition of 10 U/mL on day 0 (P < .01), but was suppressed at 100 U/mL of Epo, compared with the control (P < .001). This suppression is mainly attributed to a decrease in CFU-GM.

Figure 2 shows LTC-IC as a percentage of control colonies in stroma-noncontact cultures. When CBMNC were inoculated, there was no significant difference in the number of LTC-IC at physiologic concentrations of Epo (0.05 or 0.1 U/mL) compared with the control without Epo. Regardless of whether Epo was added on day 0 or 7, LTC-IC significantly decreased at higher concentrations of Epo (1, 5, 10, or 100 U/mL) compared with the control (P < .05 to P < .001). This was primarily attributed to a decrease in CFU-GM. In contrast, the generation of LTC-IC from CD34+ cells was significantly stimulated by Epo (0.1 or 10 U/mL), but only when added on day 0. Generally, both CFU-GM and BFU-E increased. The delayed addition of Epo on day 7 had no effect on LTC-IC. LTC-IC formation was suppressed at 100 U/mL of Epo, mainly due to a decrease in CFU-GM, compared with the control (P < .01).

In the inoculation of CBMNC, when Epo was not added to the culture or was added at a physiologic concentration (0.05 or 0.1 U/mL), there was no significant difference in the number of colonies between stroma-contact and stroma-noncontact cultures (Table 1). However, at higher concentrations of Epo (1, 5, 10, or 100 U/mL), significantly more LTC-IC were observed in stroma-contact than in stroma-noncontact cultures (P < .02 to P < .001), regardless of when Epo was added. These results were in sharp contrast to those with CD34+ cells. Without Epo, fourfold more LTC-IC was generated from stroma-noncontact than from stroma-contact cultures (P < .001). CFU-GM and BFU-E increased equally. With Epo, the number of LTC-IC was significantly greater in stroma-noncontact than in stroma-contact cultures (P < .05 to P < .001), regardless of the concentration of Epo or when it was added. Delayed addition of Epo at 0.1 or 10 U/mL had no effect on LTC-IC recovery, whereas a higher concentration of Epo (100 U/mL) resulted in a decrease in the number of LTC-IC under all of the culture conditions.
The control was added on day 0 or 7. Data are presented as in Fig 1. When CBMNC were inoculated, regardless of whether Epo was added on day 0 or 7, LTC-IC was significantly lower with Epo (1, 5, 10, or 100 U/mL) than in the control (P < .05 to P < .001). The number of LTC-IC from CB CD34+ cells with Epo (0.1 or 10 U/mL) added on day 0 was significantly greater than that with Epo added on day 7 (P < .05) or in the control (P < .01 or P < .005). LTC-IC formation was suppressed by the addition of Epo at 100 U/mL in both culture conditions compared with the control (P < .01 or P < .001), regardless of whether Epo was added on day 0 or 7.

**DISCUSSION**

Although human Epo is considered to be a physiologic regulator of erythropoiesis that preferentially acts on the late stage of erythroid maturation, there have been some reports that Epo also plays a role in the division and differentiation of immature erythroid cells in the embryo. This has not been fully tested in human hematopoiesis during development. In normal hematopoiesis, stem/progenitor cells are, for the most part, in close association with stromal cells in the bone marrow, forming a highly interactive system. Regulation of stroma-dependent hematopoiesis requires both direct cell-to-stroma interaction and soluble factors secreted from stromal layers. An LTC technique that can maintain hematopoiesis in vitro over several months originated from direct cell-to-stroma interaction and soluble factors secreted from stromal layers. Finally, we analyzed the different in the response of different cell types to Epo in LTC.

In this study, when CBMNC were used as a target, there was no difference in the number of LTC-IC recoverd without Epo between stroma-noncontact and stroma-contact cultures. In stroma-contact cultures with CBMNC, our results suggest that the addition of Epo at higher concentrations (1, 5, or 10 U/mL) on day 7 provides a better condition for maintaining LTC-IC than addition on day 0. Addition of physiologic concentrations of Epo (0.05 to 0.1 U/mL) did not affect the proliferation of LTC-IC, regardless of when Epo was added. On the other hand, LTC-IC decreased with the addition of Epo (1, 5, 10, or 100 U/mL) in stroma-noncontact culture. The results with CD34+ cells were quite different from those with CBMNC. Without Epo, fourfold more LTC-IC was generated from stroma-noncontact than from stroma-contact cultures. This was further augmented by the addition of a physiologic concentration of Epo (0.1 U/mL) on day 0. Interestingly, delayed addition of Epo, which had a stimulatory effect on LTC-IC in cultures with CBMNC, had no effect on LTC-IC, regardless of which culture conditions (contact or noncontact) were used. LTC-IC decreased with a high concentration of Epo (100 U/mL) regardless of the culture conditions.

When HSC are cultured in direct contact with stromal cells with a mixed population of CB containing accessory cells, generation of LTC-IC will be determined by the net balance of the stimulatory and inhibitory effects of stromal cells, ECM, and cytokines produced by both the stroma and accessory cells. Manabe et al reported that direct contact with stroma was required for the survival of normal B lymphoblasts. Rowley et al showed that the generation of colony-forming cells from 4HC-resistant subpopulations of CD34+lin−precursors required an as yet undefined interaction with marrow stroma. On the other hand, Verfaillie et al previously reported that direct contact between HSC and stromal cells is not required for either differentiation or conservation of marrow-derived CD34+/HLA-DR− cells, but is essential for the regulated production of mature blood elements. The differences in the reported data are likely due to differences in the cell source and culture conditions. We used CB as a source of HSC, and this may have different properties than cells at postnatal ontogeny. The fetus develops in a hypoxic environment, which we maintained during LTC. In this study, the results of LTC with CBMNC and CD34+ cells were in sharp contrast, which is likely due to the presence or absence of an accessory cell population in the inoculum. With the use of purified CD34+ cells, the effects of cytokines from accessory cells become minimal. Under these conditions, stroma-noncontact culture generated more LTC-IC than did the stroma-contact condition, a result that agrees with the data reported by Verfaillie et al.
Our working hypothesis to explain the findings in this study is that an adequate concentration of Epo by itself may lack the potential to stimulate uncommitted and multipotent HSC or to prevent their differentiation. In stroma-noncontact cultures with purified CD34+ cells, a negative regulator in stroma or the effect of excreted inhibitory cytokines, present locally at low concentration, was eliminated and either the stimulatory effect of excreted cytokines from stroma became predominant or terminal differentiation was suspended. In the early phase of cell activation, the addition of a physiologic concentration of Epo may act in synergy with these stimulatory cytokines to drive very immature HSC to generate more LTC-IC. Furthermore, stimulation of the terminal differentiation of activated cells may be the primary effect of the late addition of Epo, which would therefore have no ultimate effect on LTC-IC. Although similar Epo kinetics were also observed in stroma-contact cultures with CD34+ cells, the degree of stimulation became weaker due to the presence of an inhibitor in stroma. On the other hand, in stroma-noncontact cultures with unpurified CBMNC, the inhibitory effect of cytokines excreted from the accessory cell population may overwhelm the stimulatory effect of cytokines excreted from stroma. However, after priming with stromal cells, possibly in synergy with the stimulatory effect of cytokines from accessory cells, lineage-uncommitted HSC may become sensitive to high doses of Epo to yield an increased number of LTC-IC. Early exposure to Epo in LTC may make the cell more vulnerable to the inhibitory effect of cytokines from accessory cells or enhance terminal cell differentiation.

Whereas both the erythroid and myeloid components of LTC-IC increased in some culture conditions with Epo, a higher concentration of Epo (100 U/mL) inhibited the generation of LTC-IC, mainly in the myeloid lineage. Although the reason for this finding is unclear, the occurrence of neutropenia of less than 1 × 10^9/L has been reported in premature infants who were treated with Epo for anemia. High concentrations of Epo have a negative effect on the formation of CFU-GM and the possibility of competitive interaction between erythroid and myeloid progenitor cells has been suggested in murine hematopoiesis. Our observation is in general agreement with these reports, although further investigation is required.

In conclusion, our data suggest that Epo may play an important physiologic role in the maintenance and proliferation of immature HSC that differentiate into both erythroid and myeloid progenies, in close interaction with factors from marrow stromal cells. Based on these findings, further study is warranted to evaluate the clinical potential of adding Epo to enhance hematopoiesis of lineages other than erythroid lineage.

ACKNOWLEDGMENT

We thank Kirin Brewery Co for providing recombinant human IL-3, G-CSF, SCF, and Epo.

REFERENCES


14. Verfaillie CM: Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood 82:2045, 1993


19. Eliason JF, Testa NG, Dexter TM: Erythropoietin-stimulated


Effect of recombinant erythropoietin in interaction with stromal factors on cord blood hematopoiesis

T Abe, Y Takaue, Y Kawano and Y Kuroda