Differential and Synergistic Effects of Human Granulocyte-Macrophage Colony-Stimulating Factor and Human Granulocyte Colony-Stimulating Factor on Hematopoiesis in Human Long-Term Marrow Cultures

By Donna E. Hogge, Johanne D. Cashman, R. Keith Humphries, and Connie J. Eaves

The ability of granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF to influence hematopoiesis in long-term cultures (LTC) of human marrow was studied by cocultivating light density normal human marrow cells with human marrow fibroblast feeders engineered by retroviral infection to constitutively produce one of these growth factors. Feeders producing stable levels of 4 ng/mL GM-CSF or 20 ng/mL G-CSF doubled the output of mature nonadherent cells. The numbers of both colony forming unit-GM (CFU-GM) and erythroid burst forming unit (BFU-E) in the G-CSF LTC were also increased (twofold and fourfold, respectively, after 5 weeks in culture), but this effect was not seen with the GM-CSF feeders. At the time of the weekly half medium change 3H-thymidine suicide assays showed primitive adherent layer progenitors in LTC to be quiescent in both the control and GM-CSF cultures. In contrast, in the G-CSF cultures, a high proportion of primitive progenitors were in S-phase. A single addition of either recombinant GM-CSF or G-CSF to LTC in doses as high as 80 ng/mL and 150 ng/mL, respectively, failed to induce primitive progenitor cycling. However, three sequential daily additions of 150 ng/mL G-CSF did stimulate primitive progenitors to enter S-phase and a single addition of 5 or 12.5 ng/mL of G-CSF together with 10 ng/mL GM-CSF was able to elicit the same effect. Thus, selective elevation of G-CSF in human LTC stimulates proliferation of primitive clonogenic progenitors, which may then proceed through to the terminal stages of granulopoiesis. In contrast, the effects of GM-CSF in this system appear limited to terminally differentiating granulopoietic cells. However, when both GM-CSF and G-CSF are provided together, otherwise biologically inactive doses show strong stimulatory activity. These findings suggest that the production of both of these growth factors by normal stromal cells may contribute to the support and proliferation of hematopoietic cells, not only in LTC, but also in the microenvironment of the marrow in vivo.

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The growth of normal hematopoietic cells in colony assays in vitro requires the addition of specific glycoprotein regulators commonly referred to as hematopoietic growth factors. Two of these are granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). The cloning of the genes for these molecules and the availability of recombinant proteins has allowed confirmation of the activity of these materials as stimulators of the growth and differentiation of both granulocytes and macrophages. In addition, the ability of these two factors to directly stimulate erythroid and multilineage progenitors, and synergy both with each other and with other factors such as interleukin-3 (IL-3) and IL-6 has been documented. Both GM-CSF and G-CSF cause granulocytosis when administered in pharmacologic doses in primates and humans, and GM-CSF may also stimulate production of monocytes and platelets. However, the role of these molecules in regulating normal hematopoiesis in vivo within the marrow microenvironment is unknown.

Long-term marrow cultures (LTC) have been found to mimic many of the features of marrow hematopoiesis. In this system a complex mesenchymal cellular environment forms that is essential for the sustained output of hematopoietic cells. Primitive hematopoietic cells are selectively retained in this adherent layer and proliferate and differentiate releasing their mature progeny into the culture medium. A regulatory role for the adherent stromal cell population has been demonstrated by the fact that the most primitive clonogenic progenitors in the adherent layer of unstimulated, normal LTC are quiescent, as are such cells in the bone marrow, whereas progenitors of the same type but in suspension and thus lacking contact with the stroma proliferate continuously. The addition of mesenchymal cell activators such as IL-1β and platelet-derived growth factor to LTC triggers the adherent layer progenitors into S-phase. Concomitant with this response is the stimulation of production of a variety of hematopoietic growth factors, including IL-1, IL-6, G-CSF, and GM-CSF, as seen in mRNA from LTC adherent layers and by detection of bioactivity in the culture medium. Based in part on these observations, we have proposed a model of hematopoietic cell regulation in the LTC system in which the pulsed activation of one or all of these factors plays an important role in allowing new cohorts of primitive cells to be sequentially recruited from a quiescent pool to result in sustained granulopoiesis for many weeks.

According to such a model, one might then anticipate that modifications to the LTC system allowing elevated growth factor levels to be achieved might lead to enhancement of the hematopoietic activity of the primitive cells initially added. To test this hypothesis with respect to GM-CSF and G-CSF, we have analyzed the effects of providing marrow cells in LTC with fibroblasts from normal
human LTC adherent layers genetically engineered by retroviral infection to constitutively produce one of these factors. The results obtained indicate that either of these factors alone can enhance hematopoiesis, but differently, and together they may have important synergy.

**MATERIALS AND METHODS**

**Retroviral constructs and viral producer cell lines.** The cDNA for human G-CSF was obtained from Immunex Corp (Seattle, WA) while that for GM-CSF was cloned from a phytohemagglutinin (PHA)-stimulated human leukocyte cDNA library (D.E.H., unpublished data). The 3’ untranslated region from both cDNAs was truncated to remove AT-rich sequences thought to destabilize mRNA transcripts.\(^\text{(18)}\) The herpes simplex thymidine kinase (tk) promoter (contained in a 250-bp \(Pvu\) II, \(Bgl\) II fragment from the \(pX1\) vector) was ligated to the 5’ end of each cDNA.\(^\text{(19)}\)

The tk-cDNA units were then subcloned into the \(Xho\) I site 3’ of the neo gene in the retroviral vector N2, from Dr E. Gilboa (Princeton University, Princeton, NJ)\(^\text{(20)}\) (Fig 1). The resulting plasmids were used to create retroviral producer cell lines as outlined by Miller et al.\(^\text{(21)}\) Briefly, plasmids were transfected into the ecotropic retroviral packaging cell line, \(\Psi2.\)\(^\text{(22)}\) Conditioned medium from the \(\Psi2\) cells was used to infect the amphotropic packaging line, PA317.\(^\text{(23)}\) G418-resistant clones of PA317 cells were isolated and expanded and tested for neo+ virus titer on NIH-3T3 cells and for production of growth factor bioactivity (see below). The presence of the expected proviral sequences in producer cell DNA was verified by Southern blotting. Northern blotting of total cellular RNA from producer cells was used to demonstrate the expected retroviral RNA transcripts. Southern and Northern blots were prepared using standard techniques and hybridized to \(^{32}\text{P}\)-oligo-labeled GM-CSF or G-CSF cDNA probes.\(^\text{(24,25)}\)

**Growth factors.** Recombinant human GM-CSF (rGM-CSF) and IL-1β were kindly provided by Biogen (Geneva, Switzerland). Human G-CSF was purchased from Amersham (Oakville, Ontario, Canada). The rGM-CSF and rG-CSF were used as standards for bioactivity measurements in assays quantitating \(^{3}\text{H}\)-thymidine incorporation into responsive cells (AML-193 cells for GM-CSF and NFS-60 cells for G-CSF).\(^\text{(26,27)}\) They were also tested for their ability to stimulate colony forming unit-GM (CFU-GM) growth by nonadherent human marrow cells plated in standard methylcellulose assays. Fifty percent maximal CFU-GM stimulation was seen with approximately 0.1 ng/mL rGM-CSF and 10 ng/mL rG-CSF. For LTC experiments where G-CSF was used at 150 ng/mL, a crude supernate was harvested from COS cells that had been transfected 48 hours previously with an expression vector containing the cDNA for hG-CSF. (The COS cell expression vector was constructed by R. Kay and one of the investigators [R.K.H.]). The undiluted supernate contained 190 ng/mL bioactive G-CSF. Supernate from COS cells transfected with the expression vector lacking the G-CSF cDNA did not stimulate a response from either the NSF 60 cell line or normal marrow CFU-GM.

**Cultures.** All tissue culture work involving amphotropic retrovirus was performed at level III biohazard containment. Most cell lines were cultured in Dulbecco’s modified Eagle medium with high glucose (4.5 g/L) and 10% heat-inactivated calf serum (for \(2\) cells) or 10% fetal calf serum (FCS) (for PA317 or NIH-3T3 cells). AML 193 cells were grown in Iscove’s medium with 20% FCS and 10% medium conditioned by the 5637 cell line as a source of hGM-CSF.\(^\text{(28)}\) NFS 60 cells were grown in RPMI 1640 with 20% FCS and 5% pokeweed-stimulated mouse spleen cell conditioned medium.

Leftover human marrow cells were obtained from consenting adults undergoing diagnostic marrow aspiration or marrow harvest before transplantation after approval of the Clinical Screening Committee for Research Involving Human subjects of the University of British Columbia. Light density (< 1.068 g/mL) marrow cells were isolated by passage over a discontinuous Percoll density gradient (Pharmacia Canada, Baie d’Orfle, Quebec, Canada). Adherent layers of primary human marrow fibroblasts were established from unprocessed human marrow as previously described.\(^\text{(19)}\) Briefly, cultures were established as for standard LTCs but were maintained at 37°C with weekly complete medium changes until the adherent layer had reached confluency (approximately 2 weeks). To generate hG- or GM-CSF-producing marrow feeders, tissue culture dishes containing these confluent, unpassaged marrow adherent layers were trypsinized and the cells replated at 2 x 10^6 cells/60-mm tissue culture dish in \(alpha\) medium with 20% FCS or 20% FCS. After overnight incubation at 37°C the growth medium was removed and replaced with 2 mL of medium conditioned by high titer viral producer cells and containing 8 µg/mL polybrene. After 4 hours of incubation at 37°C, the virus-containing medium was replaced with \(alpha\) 20% FCS. Two days later the infected cells were retrypsinized and the cells from each dish split into three dishes in \(alpha\) 20% FCS containing 0.2 mg/mL active concentration G418 (GIBCO/BRL, Burlington, Ontario, Canada). When the cells had expanded in G418-containing medium to produce a confluent layer of fibroblasts, their growth medium was tested for growth factor bioactivity. Some cells were harvested and processed

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**Fig 1.** Retroviral vectors containing cDNAs for human GM-CSF and G-CSF. The cDNAs were truncated at the 3’ end to remove AT-rich mRNA destabilizing sequences. They were then ligated to the tk promoter and inserted in the \(Xho\) I site in the N2 vector as described in Materials and Methods. The constructs used for the experiments described here have tk GM-CSF in the 5’-3’ orientation or tk G-CSF in the 3’-5’ orientation in the vector. LTR, long terminal repeat; SD, splice donor; SA, splice acceptor; neo, neomycin phosphotransferase; tk, herpes simplex thymidine kinase promoter; *, the splice acceptor site in N2 is a cryptic splice site.
to obtain RNA for Northern analysis. Uninfected control marrow fibroblasts were generated in the same way, except that they were never exposed to virus or G418. For feeders producing both CSFs, dishes of GM-CSF and G-CSF producing fibroblasts were trypsinized, mixed 1:1, and replated at the same cell density. LTC experiments with CSF-producing feeders were initiated by seeding 5 x 10^6 light density normal human marrow cells in 8 mL of modified α medium containing 12.5% FCS; 12.5% horse serum, 10^-4 mol/L hydrocortisone, and 10^-4 mol/L 2-mercaptoethanol (LTC medium) onto preestablished, confluent (but unirradiated) feeders, as specified. For LTC where soluble growth factor was added to the cultures after their establishment (ie, > 3 weeks), 2.5 x 10^6 unprocessed nucleated marrow cells were suspended in 8 mL LTC medium in a 60-mm tissue culture dish and placed at 37°C for the first 3 days. All LTC were maintained at 33°C with weekly half-medium changes and semi-depopulation of the nonadherent cells. Nonadherent cells from representative cultures were cytospun onto glass slides, stained with Wright-Giemsa stain, and examined morphologically.

When recombinant growth factors were added to LTC this was performed 7 days after the regular medium change. In this case, each LTC received a “mock medium change” in which all of the old medium and cells were removed and then returned to the culture with or without the addition of growth factor. When LTC were sacrificed, nonadherent cells were harvested using either collagenase or trypsin. To determine the cycling status of primitive adherent layer progenitors, these cells were washed twice in nucleoside-free medium and then incubated with or without high specific activity ^3H-thymidine as described. Erythroid and GM colonies achieving different sizes were scored separately using established criteria that allow quiescent and cycling subpopulations to be distinguished.

**RESULTS**

**Generation of human CSF-producing feeders.** Titers of recombinant retrovirus were measured as the number of G418 resistant (G418') colonies of NIH-3T3 cells generated by 1 mL of growth medium obtained from the viral producer cells. Titers were greater than 10^6 cfu/mL for PA317 cells producing either the GM-CSF or G-CSF viruses (Table 1). Growth factor bioactivity generated by the highest titer producer cells was 10 ng/mL for the GM-CSF virus and 100 ng/mL for the G-CSF virus. N2 viral producers showed comparable viral titers, but no growth factor bioactivity. Confluent primary human marrow fibroblasts (obtained by subculturing normal LTC adherent layers) that were infected with the G-CSF virus released 20 ng/mL G-CSF into the growth medium but no detectable GM-CSF activity. Conversely, similarly isolated cells infected with the GM-CSF virus produced 4 ng/mL GM-CSF bioactivity but no G-CSF (Table 2). This level of growth factor production remained stable for in excess of 8 weeks in culture without G418 selection. Unstimulated cells infected with the parent N2 virus never showed any evidence of production of either growth factor.

Figure 2 is a Northern blot of total cellular RNA from either uninfected or GM-CSF producing feeders, after hybridization with a GM-CSF cDNA probe. Uninfected cells showed no GM-CSF RNA while infected cells showed the three expected RNA species. The two larger and more abundant forms are the full-length and spliced transcripts initiated from the retroviral LTR while the smaller, fainter band is initiated from the tk promoter attached to the GM-CSF cDNA. The far right lane shows the expected 1.2-kb GM-CSF transcript seen in PHA-stimulated lymphocyte RNA. Similar evidence of correctly initiated G-CSF transcripts was obtained when RNA from the G-CSF-infected cells was hybridized with a G-CSF cDNA probe (data not shown).

**LTC with human CSF-producing feeders.** GM-CSF or G-CSF virus-infected human marrow fibroblasts were then used as adherent feeder layers in LTC seeded with normal, light density primary marrow cells as a source of primitive hematopoietic cells. Cultures with uninfected or N2-infected fibroblast feeders served as controls. There was no difference between the cultures with N2-infected and uninfected feeders according to any of the parameters measured.

Figure 3 shows the number of nonadherent cells (ie, continuously produced mature granulocytes and macrophages) maintained in LTC with CSF-producing feeder cells as compared with controls. The total number of nonadherent cells approximately doubled when either GM-CSF- or G-CSF–producing feeders were present. This difference became readily apparent by week 3 of culture and persisted to at least week 5. There was no apparent change in the morphologic types of cells in the nonadherent fraction of any of the cultures.

Figure 4 shows the effect of CSF-producing feeders on the total progenitor content in the same LTC. The majority (> 60% by 5 weeks) of all progenitors were in the adherent layer and effects of the feeders were the same on both nonadherent and adherent progenitor numbers. Thus, the

### Table 1. Viral Titers and Growth Factor Bioactivity Generated by PA317 Viral Producer Cell Lines

<table>
<thead>
<tr>
<th>Retroviral Vector</th>
<th>Titer* (G418 cfu/mL)</th>
<th>Bioactivity† (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2-tkGM-CSF</td>
<td>2 x 10^6</td>
<td>10</td>
</tr>
<tr>
<td>N2-tkG-CSF</td>
<td>1 x 10^6</td>
<td>100</td>
</tr>
<tr>
<td>N2</td>
<td>1.5 x 10^6</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, none detected.

* Determined by infection of NIH-3T3 cells.
† Determined by comparison with recombinant growth factor standards of the ability of growth medium from producer cells to stimulate ^3H-thymidine incorporation on responsive cell lines.
$^3$H-thymidine suicide assays were performed to determine whether the presence of CSF-producing feeders could alter the regulation of proliferation of primitive progenitors located in the adherent layer of these LTC (Table 3). Cells were harvested at the time of the regular weekly half-medium change. At this time primitive BFU-E and CFU-GM in the adherent layer of standard LTC are quiescent.$^{14,15}$ This finding was confirmed in the present study where no significant progenitor kill was seen in either standard LTC (Table 4), or LTC containing control (non-CSF-producing) feeders (Table 3). Analysis of primitive BFU-E and CFU-GM from the GM-CSF cultures showed that the presence of these feeders had had no effect on the proliferative status of these cells. In contrast, in LTC with feeders producing G-CSF either alone or in combination with GM-CSF both types of primitive progenitors were in S-phase (Table 3).

Addition of soluble growth factor to LTC (Table 4). Because it had been previously shown$^{14}$ that primitive adherent layer progenitors in established ($\geq$ 3 weeks old) standard LTC initiated with unprocessed marrow without a preestablished feeder exhibit the same cycling characteristics as adherent layer progenitors in LTC with uninfected human marrow feeders regardless of whether the source of hematopoietic cells in the cocultures was T-cell–depleted peripheral blood mononuclear cells,$^{9}$ light density ($< 1.068$ g/mL) marrow cells, or unprocessed marrow (data not shown), the experiments described in this section were performed using the former, simpler design without feeders. Thus, to determine whether the effects seen with the CSF-producing feeders could also be achieved by adding soluble growth factor to LTC, standard LTC were initiated and maintained for 4 weeks. At the time of the regular

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**Fig 2.** Northern blot of RNA from primary human bone marrow fibroblasts, either uninfected, or infected with virus containing the tk-promoted GM-CSF cDNA in the 5'–3' orientation with respect to the retroviral genome, and RNA from normal human peripheral blood mononuclear cells stimulated for 24 hours with PHA. Each lane contains 10 $\mu$g of total cellular RNA. Numbers to the left of the photograph are size markers in kilobases. To the right are indicated the expected sizes for transcripts initiated within the proviral DNA. The probe was a $^{32}$P-oligolabeled cDNA for human GM-CSF.

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**Fig 3.** Nonadherent cells (NAC) in LTC with CSF-producing marrow fibroblast feeders as compared with control cultures with uninfected feeders. $\bar{x} \pm$ SEM absolute values for the control LTC represented as 100% on the figure were $2.6 \pm 0.5 \times 10^5$ NAC at 3 weeks and $0.82 \pm 0.09 \times 10^5$ NAC at 5 weeks per $5 \times 10^5$ light density marrow cells initially seeded. Each bar indicates the mean of normalized values for the number of experiments indicated in brackets above the bar. Lines above the bars indicate SEM.

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data for both cell fractions have been combined for the comparisons shown. There was no change in either erythroid burst forming unit (BFU-E) or GM progenitor (CFU-GM) numbers in the LTC with CSF-producing feeders at 3 weeks. However, at 5 weeks there was a trend toward a decrease in both CFU-GM and BFU-E in the GM-CSF cultures. In contrast, a significant increase in the number of both of these progenitor types in the G-CSF cultures was seen. Neither the size nor the morphology of the colonies subsequently formed in the methylcellulose assays was demonstrably affected by the type of LTC from which the progenitors had been generated.
GM-CSF AND G-CSF IN LONG-TERM MARROW CULTURES

![Graph showing percentage of control over weeks 3 and 5 for CFU-GM and BFU-E](image)

Fig 4. Total clonogenic progenitor content (both adherent and nonadherent layer cell populations) of LTC with CSF-producing feeders as compared with LTC with normal, uninfected feeders. x SEM absolute progenitor numbers for control LTC represented as 100% on the figure were 1,334 ± 121 and 520 ± 197 at week 3 and 246 ± 93 and 51 ± 16 at week 5 for CFU-GM and BFU-E, respectively, for 5 × 10⁴ light density marrow cells initially seeded. The mean progenitor content of the input cells was 5,633 ± 1,313 for CFU-GM and 7,968 ± 1,244 for BFU-E. Each bar indicates the mean of normalized values for the number of experiments indicated in brackets above the bar. The lines above the bar indicate SEM.

weekly half-medium change some cultures received a mock medium change with addition of various amounts of recombinant growth factor(s), and then after 2 or 3 days of further incubation, 3H-thymidine suicide assays were performed. Table 4 shows that the one-time addition of sufficient GM-CSF to achieve a concentration in the medium as high as 80 ng/mL did not induce the proliferation of primitive adherent layer progenitors. Identical results were obtained with G-CSF where concentrations of up to 150 ng/mL were tested. Bioactivity levels in the medium of these G-CSF cultures exceeded 35 ng/mL throughout the 2- to 3-day interval before assessing progenitor cycling status. Despite the fact that this level of G-CSF activity was higher than that achieved in the LTC containing G-CSF-producing feeders, progenitor cycling was not triggered in any of the five G-CSF addition experiments performed. G-CSF (150 ng/mL) was then added to LTC on 2 or 3 consecutive days. Primitive progenitor cycling was then assessed on the day after the last growth factor addition, at which time the G-CSF bioactivity level in the culture medium always exceeded 120 ng/mL. Only in the five experiments where the growth factor was added daily for 3 days were the primitive progenitors found to be in S-phase.

In contrast to the results obtained with addition of even high doses of G- or GM-CSF alone, a single addition of as little as 5 ng/mL G-CSF in combination with 10 ng/mL GM-CSF consistently induced progenitor proliferation.

DISCUSSION

G-CSF and GM-CSF are likely candidates for regulation of hematopoiesis both in LTC and in the marrow microenvironment. A variety of cell types including fibroblasts, endothelial cells, macrophages, and T lymphocytes are capable of producing these growth factors, and all of these cell types are found in LTC as well as in the marrow in vivo. In the adherent layer of normal LTC, as in the marrow microenvironment of the bone marrow, most of the very primitive hematopoietic cells are quiescent. However, the ongoing production of functional, mature end cells in both

### Table 3. 3H-Thymidine Suicide Measurements of Hematopoietic Progenitors From the Adherent Fraction of Long-Term Cultures With CSF-Producing Feeder Layers

<table>
<thead>
<tr>
<th>Feeder</th>
<th>No. of Experiments</th>
<th>Progenitor*</th>
<th>% Kill (x ± SE)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>BFU-E</td>
<td>-5 ± 12</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>CFU-GM</td>
<td>2 ± 11</td>
<td>---</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5</td>
<td>BFU-E</td>
<td>-12 ± 21</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CFU-GM</td>
<td>2 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>4</td>
<td>BFU-E</td>
<td>35 ± 15</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CFU-GM</td>
<td>42 ± 9</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>GM-CSF + G-CSF</td>
<td>2</td>
<td>BFU-E</td>
<td>65 ± 6</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CFU-GM</td>
<td>47 ± 26</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

*Primitive BFU-E, greater than 8 clusters or CFU-GM, greater than 500 cells.
†T-Test comparison of CSF feeder LTCs to controls.

### Table 4. 3H-Thymidine Suicide Values of Primitive Adherent Layer Progenitors in Standard LTC Following the Addition of Growth Factor(s)

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Dose</th>
<th>No. of Experiments</th>
<th>% Kill, (x ± SE)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>---</td>
<td>7</td>
<td>1 ± 2</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>G-CSF</td>
<td>5 ng/mL</td>
<td>3</td>
<td>-3 ± 2</td>
<td>-6 ± 3  NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>20 ng/mL</td>
<td>11</td>
<td>2 ± 2</td>
<td>2 ± 3  NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>150 ng/mL</td>
<td>5</td>
<td>-6 ± 1</td>
<td>4 ± 3  NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>150 ng/mL</td>
<td>25</td>
<td>-1 ± 1</td>
<td>5 ± 1  NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>150 ng/mL</td>
<td>3</td>
<td>46 ± 7</td>
<td>57 ± 5  &lt;.001</td>
</tr>
<tr>
<td>G-CSF</td>
<td>10 ng/mL</td>
<td>3</td>
<td>-2 ± 2</td>
<td>3 ± 2  NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>80 ng/mL</td>
<td>5</td>
<td>3 ± 3</td>
<td>4 ± 4  NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>5 ng/mL</td>
<td>3</td>
<td>34 ± 6</td>
<td>35 ± 7  &lt;.001</td>
</tr>
<tr>
<td>G-CSF</td>
<td>10 ng/mL</td>
<td>3</td>
<td>41 ± 10</td>
<td>31 ± 9  &lt;.02</td>
</tr>
<tr>
<td>G-CSF</td>
<td>12.5 ng/mL</td>
<td>3</td>
<td>41 ± 10</td>
<td>31 ± 9  &lt;.02</td>
</tr>
<tr>
<td>G-CSF</td>
<td>10 ng/mL</td>
<td>3</td>
<td>34 ± 6</td>
<td>35 ± 7  &lt;.001</td>
</tr>
<tr>
<td>IL-1β</td>
<td>12 U/mL</td>
<td>8</td>
<td>48 ± 3</td>
<td>48 ± 5  &lt;.001</td>
</tr>
</tbody>
</table>

Growth factors were added to 3- or 4-week-old LTC beginning 7 days after a previous half-medium change. Harvest of cells for 3H-thymidine suicide studies was performed 2 or 3 days after a single addition of growth factor and the day following 2 or 3 sequential daily additions of G-CSF.

Abbreviation: NS, not significant.

t-Test comparison of various additions relative to no addition.
†Primitive BFU-E greater than 8 clusters.
‡Primitive CFU-GM greater than 500 cells.
§150 ng/mL × 2, ×3—Sequential daily additions of G-CSF for 2 or 3 days.
systems requires that such cells must occasionally divide to allow the continuous production of differentiated progeny. In a complex, multicellular environment where many positive and negative regulators may be present at any one time it is difficult to determine whether or how a particular molecule contributes to an observed effect. In LTC, weekly half-medium changes involving the addition of fresh horse serum induce primitive progenitors in the adherent layer to enter S-phase. Northern analysis of RNA extracted from the adherent cells in these cultures has shown that the presence of horse serum in the fresh medium will also induce the production of both IL-1β and IL-6 mRNA. Added IL-1β, in turn, will also induce progenitor cycling, as well as production of its own message and mRNA for IL-6, G-CSF, and GM-CSF. Because it has been difficult to show that IL-1β acts directly on human hematopoietic progenitors, it may be that the effects observed following its addition to LTC are due to the secondary release of these known regulators. However, it is also possible that unknown stimulatory factors are being induced or inhibitors downregulated. For example, transforming growth factor β (TGF-β), a direct-acting negative regulator of primitive hematopoietic progenitors, will inhibit the cycling that is induced by IL-1β or other mesenchymal cell activators and appropriately timed addition of neutralizing antibodies to TGF-β will allow normally quiescent progenitors to remain continuously in cycle. Thus, although cells of the marrow microenvironment can be induced to produce G-CSF and GM-CSF mRNA and increased levels of bioactive growth factor can be detected in LTC medium after stimulation with agents that induce progenitor cycling, it was not clear from previous studies what, if any, effect these latter factors would have on hematopoiesis in LTC.

In this study we have used feeders engineered to produce GM-CSF and G-CSF to selectively elevate the level of each of these factors in the LTC system and have evaluated the effects of these modifications at multiple levels of hematopoietic cell activity, i.e., activation of very primitive progenitor cycling, amplification of the total clonogenic progenitor population, and the total final output of terminally differentiating granulopoietic cells. The results have clearly shown the ability of both of these growth factors to modulate hematopoietic activity in this complex system. They also demonstrate their distinct, albeit overlapping, activities. It appears that G-CSF can act on very primitive as well as later hematopoietic progenitors in the LTC system, whereas the action of GM-CSF as a single agent may be restricted to the terminally differentiating cells. In addition, the continuous provision of G-CSF by endogenous feeder production results in effects that are not seen with a single short exposure to even very high doses of this factor. This result may explain why Coutinho et al also found that rG-CSF, when added to human LTC (in weekly pulses to give concentrations of 50 to 100 ng/mL), was a relatively weak hematopoietic stimulus. It is also possible that the functional concentration of G-CSF seen by primitive hematopoietic cells in the adherent layer of cultures containing G-CSF producing feeders may be much higher (or more effective) than that suggested by levels of G-CSF in the medium. It has been shown that growth factors may be compartmentalized within the stromal layers by binding to glycoproteins or cell surfaces. The presentation of growth factors in a cellular context, perhaps in association with a cell surface molecule that has regulatory activity, could lead to effects not readily achieved with the soluble growth factor addition. While these are intriguing possibilities, experiments designed to look at the effects of continuous addition of growth factor throughout the culture period or to test the effect of feeders engineered to express a cell surface bound form of G-CSF will ultimately be required to ascertain the importance of microenvironmental presentation of growth factors.

Another interesting finding shown by the present study was the ability of GM-CSF and G-CSF to synergize with each other to stimulate the activation of very primitive clonogenic cells in LTC. Synergy of activity between many growth factors has been documented repeatedly in semisolid assays of hematopoietic colony-forming cells. Evidence of a similar synergy in the LTC system, given the complex mixture of cellular and molecular species present, provides considerable support for the view that these factors might have synergistic effects in vivo. Further analysis of the effects of additional growth factors in LTC (both alone and in combination) using the strategies outlined here may thus offer an important approach to predicting how to modulate hematopoiesis in vivo by growth factor therapy.

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