Differential Regulation of Primitive Human Hematopoietic Cells in Long-Term Cultures Maintained on Genetically Engineered Murine Stromal Cells

By H.J. Sutherland, C.J. Eaves, P.M. Lansdorp, J.D. Thacker, and D.E. Hogge

Various growth factors are known to stimulate both early and late stages of human hematopoietic cell development in semisolid assay systems, but their role as microenvironmental regulators is poorly understood. To address this problem, we developed a novel coculture system in which highly purified primitive human hematopoietic cells were seeded onto an irradiated feeder layer of cells from a murine marrow-derived stromal cell line (M2-10B4) previously engineered by retroviral-mediated gene transfer to produce specific human factors. Effects on cells at very early, intermediate, and late stages of hematopoiesis were then evaluated by assessing the number of clonogenic cell precursors (long-term culture initiating cells [LTC-IC]), clonogenic cells, and mature granulocyte and macrophage progeny present in the cultures after 5 weeks. In the absence of any feeders, cells at all stages of hematopoiesis decreased to very low levels. In contrast, maintenance of LTC-IC was found to be supported by control murine stromal cells as effectively as by standard human marrow adherent layers. The presence of granulocyte colony-stimulating factor (G-CSF) and interleukin-3–producing M2-10B4 cells in combination was able to further enhance the maintenance and early differentiation of these cells without a decline in their proliferative potential as measured by the clonogenic output per LTC-IC. However, this effect was lost if granulocyte-macrophage CSF (GM-CSF)–producing feeders were also present. On the other hand, in the presence of GM-CSF–producing feeders, the output of mature granulocytes and macrophages increased 20-fold. These findings show that it is possible to selectively improve the maintenance of very primitive human hematopoietic cells in vitro or their output of mature progeny by appropriate manipulation of the long-term marrow culture system. Further exploitation of this approach should facilitate investigation of the mechanisms operative within the human marrow microenvironment in vivo and the design of protocols for in vitro manipulation of human marrow for future therapeutic applications.

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LTC-IC and devoid of stromal elements or their precursors was used to initiate hematopoiesis in the present studies. These cells were seeded onto preformed feeder layers that had been previously derived from a cloned, stromal cell line (M2-10B4) of mouse marrow origin and infected with ecotropic retrovirus capable of the transfer and expression of cDNAs for human G-CSF, GM-CSF, or IL-3. Through the use of such genetically engineered growth factor-producing feeders it was hoped to reproduce the way in which regulators may be localized in the adherent layer and/or presented to adjacent hematopoietic cells on the assumption that this might influence the nature and magnitude of their effects. All cultures were maintained for 5 weeks and the contributions of GM-CSF-, G-CSF-, and IL-3-producing feeders (alone or in combination) to LTC-IC maintenance (self-renewal), clonogenic cell production (LTC-IC differentiation), and production of nonadherent cells (terminal cell output from intermediate clonogenic progenitor cell types) were then assessed and compared.

**MATERIALS AND METHODS**

**Bone marrow cells.** Aliquots of normal human marrow cells were obtained from informed and consenting allogeneic bone marrow transplant donors at the time of marrow harvests and with approval of the Clinical Screening Committee for Research Involving Human Subjects of the University of British Columbia (Vancouver, Canada). Percolated low-density cells (<1.068 g/mL) were stained with anti-CD34 antibody directly conjugated to fluorescein isothiocyanate (8G12-FITC), and HLA-DR directly conjugated to phycoerythrin (HLA-DR-PE; Becton Dickinson, Mountain View, CA), and then sorted on a FACStar (BD FACS Systems; Becton Dickinson). Cells were sorted within low to intermediate forward light scatter and low 90° light scatter gates to include cells with properties similar to small lymphocytes. Cells were additionally sorted for high CD34 expression and very low or negative HLA-DR expression. This sorting allowed isolation of a subpopulation representing ~0.4% of total bone marrow cells that was enriched ~400-fold in cells that produce clonogenic cells detected after 5 weeks in LTC.5

**Cell lines.** ¥2 and NIH-3T3 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (4.5 g/L) and 10% heat-inactivated fetal calf serum (FCS). M2-10B4 cells, a cloned murine (B6C3F1) marrow fibroblast cell line, were maintained in RPMI medium plus 10% FCS. AML-193 cells (American Type Culture Collection [ATCC], Rockville, MD) were grown in Iscove’s medium with 20% FCS and 10% medium conditioned by the 5637 cell line (ATCC). NFS-60 cells, obtained from Dr J. Ihle (National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD), were grown in RPMI with 20% FCS and 5% pokeweed-stimulated mouse spleen cell conditioned medium. B9 cells were grown in DMEM with 10% FCS and 100 U/mL IL-6.13

**Retroviral vectors and viral producer cell lines.** The retroviral vectors for human GM-CSF and G-CSF have been described previously. Similar principles were used to construct a vector containing a human IL-3 cDNA. Briefly, an IL-3 cDNA (from Genetics Institute, Boston, MA), was truncated at the 3’ end to remove A-T rich sequences thought to be responsible for destabilizing mRNA transcripts. This 632-bp cDNA fragment was linked to a 250-bp PvuII-BglIII fragment from the pX1 vector containing the promoter from the herpes simplex thymidine kinase (tk) gene and the tk-IL-3 cassette inserted into the XhoI site in the N2 retroviral vector, which is 3’ of the neo’ gene. Retroviral constructs were transfected into the ¥2 ecotropic packaging cell line. Individual clones of G418 resistant (G418+) transfected cells were isolated, expanded, and assessed both for viral titer by the ability of their growth medium to generate G418’ NIH-3T3 cells, and for the production of growth factor bioactivity on growth factor-responsive cell lines. Clones producing viral titers greater than 10⁶ colony-forming units/mL were used to infect M2-10B4 cells.

**Stromal feeders.** Irradiated (15 Gy of 250-kV peak x-rays) normal human marrow adherent layer feeders (MF) were prepared as previously described. To generate human growth factor-producing M2-10B4 feeders, cell-free growth medium was harvested from viral producer cells and, together with 8 μg/mL polybrene, added to subconfluent cultures of M210-B4 cells. After 4 hours of incubation at 37°C the virus-containing medium was replaced with standard growth medium. Forty-eight hours post-infection the cells were trypsinized, replated in growth medium containing 0.4 μg/mL G418, and the cells grown to confluence, at which time the growth medium was tested for growth factor bioactivity. Mass cultures of these retrovirally infected, G418+, growth factor-producing M2-10B4 cells were subsequently maintained and passaged as continuous cell lines. Using standard techniques and hybridization of blots to 32P-oligolabeled GM-CSF, G-CSF, or IL-3 cDNA probes, Southern and Northern analysis showed grossly intact proviral DNA and the expected full-length and spliced retroviral transcripts in the infected M2-10B4 cells (data not shown). M2-10B4 feeders were prepared before the initiation of cocultures by seeding 3 × 10⁷ M2-10B4 cells into 35-mm Corning tissue culture dishes (Corning Glassworks, Corning, NY) or into Nunc 96-well plates (A/S Nunc, Roskilde, Denmark) at 10⁴ cells per well. In cultures containing cells from more than one growth factor-producing cell line (to test the effect of specific combinations of growth factors), equal numbers of each of the types of cells were used, keeping the total cells plated constant at the values given above. All M2-10B4 feeders were irradiated with 80 Gy of x-rays.

**Cocultures.** In a total of 16 experiments, 800 to 11,000 sorted normal human bone marrow cells were placed in cultures with or without feeders (as indicated) in LTC medium. Cultures were then maintained at 33°C for 5 weeks with weekly half-medium changes as previously described. At the end of 5 weeks, all nonadherent cells were removed and counted, and the adherent cells were then suspended by trypsinization. Aliquots equal to ½ to ½ of total cells adherent and nonadherent cells were plated in standard methylcellulose cultures for assessment of total erythropoietic (BFU-E), granulopoietic (CFU-GM), and multilineage (CFU-GEMM) progenitors detected 20 days after initiation. Aliquots were also reseeded on top of new irradiated normal human MF in 96-well plates for assessment of LTC-IC content by limiting dilution analysis and measurement of total clonogenic cell content after an additional 5 weeks.

**Growth factor bioactivity.** Growth factor bioactivity was measured in growth media collected 2 days after a complete change of the medium in confluent cultures of viral producer cells or feeders, and in media removed from cocultures at weekly intervals. Bioactivity was measured by comparing the stimulation of [H]-thymidine incorporation into appropriate growth factor-responsive cell lines to that obtained with recombinant growth factor standards. Recombinant GM-CSF and IL-3 were gifts from Biogen (Genova, Switzerland) and Behring (Frankfurt, Germany), and recombinant IL-6 was purchased from R & D Systems, Inc (Minneapolis, MN). Recombinant G-CSF was purchased from Amersham (Oakville, Canada). GM-CSF and IL-3 levels were measured on human AML 193 cells, G-CSF on NFS 60 cells, and IL-6 on B9 cells.
RESULTS

Growth factor production by engineered M2-10B4 cells. Human growth factor-producing M2-10B4 cells were generated by infection of the cells with ecotropic retrovirus capable of the transfer and expression of both the neo gene, which renders eukaryotic cells resistant to the neomycin analogue G418, and the cDNAs for either human GM-CSF, G-CSF, or IL-3. When retrovirally infected M2-10B4 cells had grown to confluence under G418 selection, samples of their growth medium were tested for growth factor bioactivity (Table 1). Bioactivity was detected only from cells infected with the appropriate virus and the levels measured ranged from ~1 to 20 ng/mL. Bioactivity from cultures containing two or three types of growth factor secreting M2-10B4 cells was twofold to threefold lower, consistent with the lower number of each type of cell in these cultures. These remained stable for at least 2 months in the absence of G418 selection, even after the cells were irradiated. Bioactivity at levels approximately equal to the levels from the feeders alone was also detected in media removed from cocultures of M2-10B4 cells with purified human marrow cells, and levels remained unchanged throughout the period of the experiments. In cocultures with uninfected M2-10B4 feeders no bioactivity could be detected (data not shown). Assays for IL-6 were also performed on media conditioned by M2-10B4 cells and media removed weekly from cocultures. These assays showed IL-6 levels to be consistently less than 0.01 ng/mL. In previous experiments the concentration of purified recombinant growth factor required to stimulate half-maximal hematopoietic colony growth from nonadherent marrow cells placed in short-term methylcellulose assays has been shown to be 0.1 ng/mL for GM-CSF, 10 ng/mL for G-CSF, and 1 ng/mL for IL-3,13 suggesting that growth factor production by the retrovirally infected M2-10B4 cells was sufficient to warrant testing these cells as feeders in LTC.

Capacity of M2-10B4 cells to support human hematopoiesis. Total numbers of nonadherent cells, clonogenic cells, and LTC-IC in cocultures 5 weeks old were measured to assess the ability of control (uninfected and/or N2-infected) M2-10B4 cells to support hematopoiesis at these three levels of hematopoietic cell development (Table 2). Results obtained in each case were compared with those obtained from cultures containing normal human MF or no feeders (ie, hematopoietic cells seeded directly onto plastic). Despite the lack of detectable G-CSF, GM-CSF, IL-3, or IL-6 in cultures containing control M2-10B4 cells, significant support for all levels of hematopoiesis was evident by comparison to results for cultures without feeders. For LTC-IC maintenance and production of clonogenic cells, M2-10B4 cells were almost as effective as normal human MF. However, human MF did offer a significant improvement over M2-10B4 cells when effects on terminal cell numbers (nonadherent cell production) were assessed.

Specific growth factor effects on terminal hematopoiesis. Nonadherent cell numbers in cultures 5 weeks old containing growth factor-producing M2-10B4 cells were compared in a paired t-test to cultures with control M2-10B4 cells (Fig 1) and to cultures with human MF. IL-3–producing M2-10B4 cells alone were not different than control M2-10B4 cells (P = .4). However, all other types of growth factor-producing feeders, either alone or in combination, increased nonadherent cell output above that seen with control M2-10B4 feeders (P < .005), GM-CSF–producing M2-10B4 cells with or without other growth factor-producing M2-10B4 cells were most effective in this regard. Alone, they supported the production of ~20 times more nonadherent cells than cultures containing control M2-10B4 cells, and ~4 times more nonadherent cells even than cultures containing human MF (P < .005). Although the

<table>
<thead>
<tr>
<th>Table 1. Growth Factor Production by Retrovirally Infected M2-10B4 Cells</th>
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<tr>
<td><strong>Retroviral Vector</strong></td>
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<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Uninfected or N2</td>
</tr>
<tr>
<td>N2-hGM-CSF</td>
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<tr>
<td>N2-hG-CSF</td>
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<tr>
<td>N2-tkIL-3</td>
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*Using a paired t-test a significant difference from M2-10B4 was observed.

Fig 1. The number of nonadherent (NA) cells in 5-week-old cocultures initiated with equal numbers of sorted (light density, CD34<sup>–</sup>, HLA-DR<sup>+</sup>, FL<sup>+</sup>) cells seeded onto M2-10B4 feeders producing human growth factors or human MF is compared with NA cells in 5-week-old cocultures containing control M2-10B4 cells. A paired t-test was applied to log transformed data from n experimental pairs to identify groups that were significantly different (*, P < .05) from controls. Control values for all cocultures on M2-10B4 feeders are presented in Table 2. In this and all subsequent analyses a value that was at the limit of detection of the assay as determined by the number of cells assessed was substituted for all zero values.
combination of IL-3- and G-CSF-producing M2-10B4 cells was less effective for the promotion of terminal cell amplification than any feeder producing GM-CSF, it was equivalent to human MF in this regard.

Specific growth factor effects on clonogenic cell output. Production of clonogenic cells was analyzed in the same experiments by comparison of numbers of clonogenic cells in 5-week-old cocultures to controls using a paired t-test (Fig 2). G-CSF feeders alone and G-CSF plus IL-3-producing feeders provided more support than control M2-10B4 feeders \( (P \leq .05) \) and G-CSF plus IL-3 feeders were twice as supportive as human MF, although this latter difference did not quite reach statistical significance \( (P = .12) \). GM-CSF feeders either alone or in combination with G-CSF or IL-3 feeders resulted in clonogenic cell output values at 5 weeks that were close to those obtained in cultures with control M2-10B4 cells. To distinguish whether the IL-3- plus G-CSF-producing feeder combination increases clonogenic cells by increasing the number of LTC-IC recruited to differentiate, or by increasing the proliferative ability displayed by the LTC-IC originally present, an additional series of experiments was undertaken. In these experiments, sorted normal bone marrow cells were seeded at a limiting dilution onto either IL-3- plus G-CSF–producing M2-10B4 cells or human MF, and the frequency and average clonogenic cell output by individual LTC-IC was then determined from a knowledge of the clonogenic content of wells measured 5 weeks later. The results from five such experiments suggest a slight but not statistically significant advantage for the IL-3–plus G-CSF–secreting M2-10B4 cells for both parameters assessed, ie, the proportion of initially seeded cells detected as LTC-IC was 1.4% and 1.1% and the average number of clonogenic cells produced per LTC-IC detected was 5.5 and 4.6 for the IL-3 + G-CSF feeders and human MF, respectively.

Specific growth factor effects on LTC-IC maintenance. By plating cells at limiting dilution on human MF, the absolute number of LTC-IC in a population can be quantitated.\(^9\) This analysis can be performed on cells removed at various time points from a culture to provide a measure of the ability of the conditions prevailing in the cultures to promote the maintenance and/or self-renewal of LTC-IC. Approximately 25% of the number of input LTC-IC were detected after 5 weeks in LTC initiated by seeding sorted marrow onto human MF. (Mean ± SEM LTC-IC per 1,000 sorted cells originally plated = 16.7 ± 4.0 on day 0, and = 4.3 ± 1.0 at 5 weeks, in six experiments.) The ability of control and growth factor-producing M2-10B4 cells to maintain LTC-IC was similarly assessed by quantitating the number of LTC-IC remaining after 5 weeks in primary cultures containing various feeders. As shown in Fig 3, the combination of IL-3 plus G-CSF feeders in the primary cultures allowed better maintenance of LTC-IC than control M2-10B4 feeders \( (P < .05) \) and was even somewhat better than human MF, GM-CSF–producing feeders alone, or together with G-CSF–producing feeders, provided less LTC-IC maintenance than human MF \( (P \leq .05) \) and any culture that contained GM-CSF–producing feeders appeared worse than control M2-10B4 cells for LTC-IC maintenance \( (P = .14 \text{ to } .18) \). The other feeder combinations tested provided support of LTC-IC maintenance that did not differ significantly from that obtained with human MF or M2-10B4 cells.

Lack of any growth factor effect on the proliferative potential displayed by LTC-IC present after 5 weeks in culture. In addition to determining the number of LTC-IC maintained under various coculture conditions, the proliferative potential of these cells, as indicated by the average number of clonogenic progenitors produced per LTC-IC (CFU/LTC-IC) before and after culture, was measured by limiting
dilution analysis. CFU/LTC-IC was the same for LTC-IC maintained on human MF for 5 weeks as for the LTC-IC in the original purified marrow sample (4.0 ± 0.7 vs 4.3 ± 0.4). Moreover, despite the fact that the number of LTC-IC maintained in primary cocultures with various types of growth-factor producing M2-10B4 cells varied from twofold higher to threefold lower than the number of LTC-IC maintained in cultures containing human MF, the proliferative potential of the LTC-IC present after 5 weeks was not influenced by the type of feeder used in the primary culture (analysis of variance, P = .46) (Table 3).

**DISCUSSION**

Analysis of specific growth factor effects on different stages of hematopoiesis requires a system where the factors of interest can be tested individually, or in combination, as desired. In addition, separate assays must be available for the direct quantitation of primitive hematopoietic cells and their more differentiated progeny. In this report an experimental strategy that meets both of these requirements has been described. This strategy was then used to evaluate the role of three growth factors with unique and distinct effects on different types of human hematopoietic cells. The strategy involved establishing cocultures of primitive human hematopoietic target cells with feeder cells from a murine marrow-derived stromal cell line that was not known to secrete growth factors capable of stimulating human hematopoietic cells. The murine cells had been genetically engineered by retroviral-mediated gene transfer to allow elevated levels of endogenously produced human G-CSF, GM-CSF, and/or IL-3 to be sustained in the coculture system. Because normal human marrow aspirate samples contain many cells that can also release both G-CSF and GM-CSF in culture, an attempt was made to minimize the presence of such cells. Accordingly, cocultures were initiated with a subpopulation of human marrow cells that had been highly enriched in LTC-IC (and some clonogenic cells) and depleted of human marrow stromal cells or their precursors, and of monocytes and T cells. Measurements of the number of LTC-IC, clonogenic cells, and nonadherent cells (representing mature granulocytes and macrophages almost exclusively) present in the cultures 5 weeks later then allowed quantitative comparisons of the effect of each growth factor tested on primitive hematopoietic cell self-renewal, generation of clonogenic progeny, and final amplification and differentiation into mature end cells.

The least-anticipated finding was that none of the three human growth factors evaluated was required for the sustained output of cells at any of the three levels of hematopoiesis examined. Particularly at the level of the maintenance of primitive LTC-IC and their differentiation into clonogenic cells, M2-10B4 cells alone provided effective support not achieved in their absence. Thus, these murine stromal cells have an as yet uncharacterized growth supporting ability that, like G-CSF, crosses species barriers. We have recently found that M2-10B4 cells produce the recently reported factor that binds to the c-kit gene product (data not presented), like most stromal cell types thus far examined. Because this molecule stimulates primitive hematopoietic cells, its production by M2-10B4 cells may explain the support provided by these cells in LTC. The present findings are also consistent with recent reports suggesting that human myeloid cells can be maintained in vivo in murine recipients without the provision of human growth factors.

The ability of GM-CSF to greatly amplify the output of mature (nonadherent) cells in vitro above that obtained with standard human MF extends the results of previous experiments in which soluble GM-CSF was added to standard LTC or when it was provided by human MF engineered to constitutively produce GM-CSF. The design of the present experiments, which allowed the effects of GM-CSF to be examined in the virtual absence of G-CSF or IL-3, suggests that the terminal amplification of granulopoiesis by GM-CSF does not depend on synergy with either of these two other factors. Although the combined production of both IL-3 and G-CSF in the absence of GM-CSF was able to stimulate terminal granulopoiesis as effectively as human MF and was clearly superior to control M2-10B4 feeders, the effect was still markedly less than that achieved by GM-CSF feeders alone.

**Table 3. Number of Clonogenic Progenitors per LTC-IC Harvested From Five-Week-Old Cocultures Containing Different Types of Feeder**

<table>
<thead>
<tr>
<th>Feeder</th>
<th>No. of Clonogenic Progenitors per LTC-IC</th>
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<tr>
<td>Human MF</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>M2-10B4</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>M2-10B4 + G</td>
<td>5.5 ± 1.0</td>
</tr>
<tr>
<td>M2-10B4 + GM</td>
<td>6.4 ± 2.1</td>
</tr>
<tr>
<td>M2-10B4 + IL-3</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>M2-10B4 + G + GM</td>
<td>3.7 ± 1.1</td>
</tr>
<tr>
<td>M2-10B4 + IL-3 + G</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td>M2-10B4 + IL-3 + G+ GM</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>M2-10B4 + IL-3 + G + GM</td>
<td>6.0 ± 2.4</td>
</tr>
</tbody>
</table>

Abbreviations: G, G-CSF; GM, GM-CSF.
points should help to explain the basis of the negative effect of GM-CSF on primitive human hematopoietic cells seen here.

It is interesting to note that the effects of G-CSF plus IL-3 on LTC-IC maintenance correlate well with the ability of both of these factors to stimulate primitive (high proliferative potential) clonogenic cells to enter S-phase either when added repeatedly to standard LTC of unsorted marrow or when presented by human MF engineered to produce increased levels of G-CSF or IL-3.13,15 These results also correlate with the ability of these factors to stimulate blast colony formation.15 Additional evidence favoring the very primitive nature of the cell detected by the LTC-IC assay was provided by the lack of decline in their proliferative capacity after 5 weeks in culture, even under conditions that enhanced their self-renewal/maintenance during that period.

The ability to analyze the differentiation and the maintenance/self-renewal of LTC-IC independently should facilitate future elucidation of the mechanisms that couple these processes to prevent depletion of the stem cell pool when stem cell differentiation is required. It may also permit the identification of mechanisms that allow these processes to be uncoupled either in disease states or for therapeutic benefit. For example, a factor or combination of factors that could be manipulated to alternatively promote self-renewal at the expense of differentiation and vice versa could replace the need for large marrow transplants, create new opportunities for gene therapy, and provide the beginnings for the creation in vitro of a variety of blood cell products.

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