Bone marrow (BM) stromal fibroblasts produce hematopoietic growth factors (HGFs) in response to inflammatory mediators such as tumor necrosis factor-α or interleukin-1α (IL-1α). In the absence of such inflammatory stimuli, production of HGFs by BM stromal cells has been problematic and controversial. In vivo, however, basal hematopoiesis maintains blood counts within a normal homeostatic range even in the absence of inflammation, and HGFs are required for progenitor cell differentiation in vitro. To better ascertain the contribution of BM stromal fibroblasts to basal hematopoiesis, we therefore studied HGF production in quiescent BM stromal fibroblasts by three sensitive assays: serum-free bioassay, enzyme-linked immunosorbent assay, and reverse transcriptase polymerase chain reaction. Stromal fibroblasts were cultured in the presence or absence of normal human serum to determine if serum factor(s) present in the noninflammatory (basal) state induce secretion of HGFs. Human serum was found to induce or enhance transcription and secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) and enhance secretion of constitutively expressed IL-6. In contrast, no secretion of either granulocyte-CSF (G-CSF) or IL-3 was found. These data indicate that factors in normal human serum are active in enhancing GM-CSF and IL-6 production by stromal fibroblasts and suggest that these growth factors contribute to the maintenance of normal, basal hematopoiesis in vivo.

**MATERIALS AND METHODS**

**BM samples.** BM cells were obtained from healthy adult volunteer donors, after obtaining informed consent, by aspiration into preservative-free heparin. The procedure was performed...
under a protocol approved by the University of Michigan Human Investigations Committee.

**Stromal layer preparation.** The mononuclear cells were separated by density centrifugation over Ficoll-Hypaque (specific gravity 1.077) and the stromal cells separated by adherence to plastic. The cells were grown to confluence in Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL Laboratories, Grand Island, NY) with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 10% equine serum (ES; Hyclone), with nonadherent cells removed after 48 hours. For most experiments, the adherent layers were passaged at confluence.

Immunohistochemical analyses of multiply passaged monolayers were performed on acetone-fixed slides. Antibodies used included anti-CD2 (pan T cell); CD14, KPI, Mac 387 (monocyte/macrophage); CD19 (pan B cell); CD34 (hematopoietic progenitor cells, monocytes); DRC, S100 (dendritic reticulum cells); and factor VII (endothelial cell, megakaryocytes). Immunostaining was accomplished using a three-step avidin-biotin-peroxidase method, with hematoxylin used as a counterstain.14

**Stromal cell conditioned medium (CM).** After several passages, the adherent stromal layers were cultured in either IMDM or AIM-V (GIBCO-BRL Laboratories) in the absence of serum for 96 hours. Individual flasks of the stromal layers were then cultured in medium supplemented with either 10% to 20% human serum, 10% to 20% FCS, or 10 ng/mL recombinant human IL-la (Amgen, Thousand Oaks, CA), G-CSF (40 to 5,000 pg/mL; IL-1, 30 to 2,000 pg/mL; G-CSF, 4 pg/mL; and GM-CSF, 200 pg/mL). Used in conjunction with concentrated CM, these assays were able to detect concentrations as low as: IL-3, 3 pg/mL; IL-6, 3 pg/mL; G-CSF, 4 pg/mL; and GM-CSF, 200 pg/mL. To determine whether supernatant concentration substantially altered ELISA results, control experiments were performed in which known standards were concentrated and reassayed. In these experiments, preconcentration and postconcentration sample assays gave similar results ±15%.

**RNA preparation.** After appropriate culture, total cellular RNA was recovered from BM stromal cells.19 Cells were suspended in GITC solution (final concentrations are 4 mol/L guanidine isothiocyanate, 5% sarcosyl, 25 mmol/L citric acid, pH 7.0, and 0.007% b-mercaptoethanol), then sequentially mixed with 0.2 mol/L sodium acetate, 0.25 mol/L calcium acetate, and 0.1% bovine serum albumin (BSA) for 43°C for 4 hours.20 Alternatively, conditioned medium collected after stimulation of stromal layers was diluted 1:2 in heparitinase buffer and then digested for 4 hours at 43°C. In other cases, 3 hours after stimulation the stromal layers were sacrificed and total cellular RNA prepared, as described below.

**Concentration of CM.** Aliquots of CM were concentrated 4- to 10-fold by centrifugation at 1,000 g at 25°C fixed angle JA-17 rotor (Beckman, Palo Alto, CA) in Centricon-10 concentrators (Amicon Division of W.R. Grace and Co, Danvers, MA) until the desired volume had been reached.

**Progenitor cell enrichment.** The nonadherent BM mononuclear cells were enriched for progenitor cells as previously described.19 Briefly, the cells were incubated with saturating quantities of a panel of eight murine monoclonal antibodies (MoAbs) as follows: anti-Leu-1 (with specificities for T cells), anti-Leu 5b (T cells and natural killer [NK] cells), anti-Leu-10 (HLA-DQ), anti-Leu-M1 (natural and precursor myeloid and monocytic cells), anti-Leu-12 (B cells), anti-CALLA (pre-B cells), TG-1 (granulocytes, myeloid precursors, and some monocytes), and glycoporphin A (erythroid cells) for 1 hour at 4°C. The cells were then thrice washed to remove excess antibody. Antibody-negative cells were isolated by panning by incubating anti-lg-coated plastic dishes with the labeled cells for 1 hour at 4°C. The nonadherent antibody-negative cells, enriched 9- to 20-fold for progenitor cells, were then recovered and subsequently used in proliferation assays.

**Proliferation assays.** BM mononuclear cells enriched for progenitor cells were plated in AIM-V at a concentration of 1 × 106 cells/well in triplicate. Cultures were incubated in 96-well plates (Costar, Cambridge, MA). The cells were either unstimulated or stimulated with 1%, 10%, or 20% CM collected as described. The cells were incubated for 96 hours at 37°C under 5% CO₂. During the last 6 hours of the incubation, 1 μCi ¹³¹H-TdR (Dupont, NEN Products, Boston, MA) was added to each well. The cells were harvested using a PHD cell harvester (Cambridge Technology, Inc, Cambridge, MA) and ³H-TdR uptake measured by a Wallac 1410 liquid scintillation counter (Pharmacia, Gaithersburg, MD).18 In some experiments, cells were cultured in the presence of either neutralizing anti-IL-1α or anti–IL-1β antibody (Genzyme), added at quantities sufficient to neutralize 500 U/mL IL-1α or IL-1β, respectively. ELISA. ELISAs were performed using the double-antibody sandwich method, using commercially available kits. The cytokines tested were IL-3, IL-6, and IL-1 (R & D Systems, Minneapolis, MN), G-CSF (Amgen, Thousand Oaks, CA), and GM-CSF (Genzyme). Based on parallel assays of known diluted standards, the sensitivities of the assays using unconcentrated CM were as follows: IL-3, 30 to 2,000 pg/mL; IL-6, 30 to 2,000 pg/mL; G-CSF, 4 to 5,000 pg/mL; IL-1, 30 to 2,000 pg/mL; and GM-CSF, 2 to 500 pg/mL. Used in conjunction with concentrated CM, these assays were able to detect concentrations as low as: IL-3, 3 pg/mL; IL-6, 3 pg/mL; G-CSF, 4 pg/mL; and GM-CSF, 200 pg/mL. To determine whether supernatant concentration substantially altered ELISA results, control experiments were performed in which known standards were concentrated and reassayed. In these experiments, preconcentration and postconcentration sample assays gave similar results ±15%.

**Oligonucleotide primers.** Sense and antisense primers were prepared in the oligonucleotide synthesis core at the University of Michigan. The primers used were as follows: GM-CSF, (nucleotides 100 to 124, sense) GACAGATGAGCACTGCTGCAAGATCAAA23; G-CSF, (nucleotides 95 to 115, sense) CACAGTGTCACCTGACAGTCCAGAGGA and (nucleotides 480 to 510, antisense) CATTCCCAGTCTTCCATC and (nucleotides 421 to 450, antisense) CACGCAGCTTCAATCCTACTACGAGTCTAAGTCTTCTTCTCTCAGTCA; IL-3, (nucleotides 90 to 120, sense) GATCTCCCGAAGACAGTGGTCTGGCAC and (nucleotides 339 to 368, antisense) AAGCTTCTCCCTCCTACTACGAGTCTAAGTCTTCTTCTCTCAGTCA; and (nucleotides 121 to 144, sense) GGGCACAATCCATGAAGGCCTGAT and (nucleotides 766 to 789, antisense) GTCAGTGTAAGGGTGGCCGGCCCGC25; IL-1β, (nucleotides 121 to 144, sense) CTTGCGTCTGTGATGTCGCT and (nucleotides 541 to 564, antisense) CAGCGACGACAGTTCACTACTACGAGTCTAAGTCTTCTTCTCTCAGTCA. All oligonucleotide primer pairs spanned intron-exon splice sites, thus PCR products generated from any DNA present in the RNA preparations could be distinguished from those generated after RT-PCR.

**RT-PCR.** RNA, 96 μg, 20X RT buffer (1X RT buffer consists of 50 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 8.0 mmol/L MgCl₂, and 10 mmol/L dithiorthreitol), 25 mmol/L dXTP mix (25 mmol/L of each dXTP [dCTG], 3.0 μg oligo d(T), and 2.5 U reverse transcriptase (AMV-Reverse Transcriptase, GIBCO-BRL, Gaithersburg, MD) were incubated at 41°C for 1 hour. One-fifth of the double-stranded product was mixed with 10X Taq/RT buffer (1X

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Taq/RT buffer consists of 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.01% gelatin, and 2.0 mmol/L dithiothreitol), 1 mmol/L dNTP mix, 500 ng each of the sense and antisense oligonucleotides of interest, and 2.5 U Taq polymerase (AmpliTaq DNA polymerase; Perkin Elmer Cetus, Norwalk, CT). The reaction mixture underwent thermal cycling at 94°C for 1 minute and 72°C for 3 minutes for 35 cycles, then finally a 10-minute extension at 72°C (Perkin Elmer Cetus DNA Thermal Cycler). PCR products were electrophoresed in agarose visualized using ethidium bromide.

DNA probes. The cDNA probes used for IL-3, GM-CSF, and G-CSF used resulted from random hexanucleotide labeling with [32P]dCTP of gene-specific inserts (25 ng). The IL-3 probe is a 1.0-kb XhoI cDNA fragment, the GM-CSF probe is a 700-bp EcoRI-HindIII fragment and the G-CSF probe is a 900-bp XhoI cDNA fragment. A synthetic IL-6 oligonucleotide was end-labeled using polynucleotide kinase and [32P]gATP.

Alkaline Southern blot analysis. The identity of PCR products (GM-CSF and IL-6) present on agarose gels was confirmed and the absence of signal (G-CSF and IL-3) on agarose gels was evaluated by alkaline Southern blot analysis. The agarose gel samples were transferred to nylon (Zeta-Probe blotting membranes; Biorad, Richmond, CA) in 0.4 mol/L NaOH, and then the membranes were hybridized with the appropriate [32P]-labeled probe (Amersham, Arlington Heights, IL) probe. Hybridization to cDNA probes (GM-CSF, G-CSF, and IL-3) was performed in final concentrations of 6× standard saline citrate (SSC; 1× SSC is 0.15 mol/L NaCl and 0.015 mol/L sodium citrate), 0.5% blotto, 1.0% sodium dodecyl sulfate (SDS), 0.01 mol/L EDTA, and 100 mg/mL denatured sonicated salmon sperm DNA and probed with the appropriate [32P]-labeled probe. Alternatively, hybridization to an IL-6 oligonucleotide probe was performed in final concentrations of 6× NET (1× NET is 0.15 mol/L NaCl, 15 mmol/L Tris, pH 8.0, and 1.0 mmol/L EDTA), 10× Denhardt’s solution, and 1% SDS. Filters were further analyzed by scanning densitometry using the Betascope 603 blot analyzer (Betagen Corporation, Waltham, MA).

RESULTS

To determine if confluent, multiply passaged stromal fibroblasts were capable of producing any HGFs under conditions emulating basal state, the ability of stromal CM to stimulate progenitor cell proliferation in serum-free bioassays was measured. Confluent BM stromal cells were prepared by multiple (at least 3) passages of BM adherent layers. Whereas primary adherent stromal cells consisted largely of fibroblasts, but also included endothelial cells and monocytes, multiply passaged stromal layers were essentially homogeneous fibroblasts, apparently free of contaminating monocytes, endothelial cells, and T and B cells, as measured by indirect immunofluorescence.

Confluent, multiply passaged BM stromal cells were cultured in 20% FCS for 7 days, and the CM collected. Highly enriched BM progenitor cells were then cocultured in the presence of either serum-free medium alone or 1%, 10%, or 20% CM, and proliferation of the progenitor cells in response to these stimuli was assayed by [3H]-TdR incorporation. Under these conditions, stromal CM stimulated the proliferation of highly enriched progenitor cells (Fig 1). To control for the serum present in the CM, FCS was added in the appropriate final concentrations to control wells. The serum in these final concentrations itself had no effect on progenitor cell proliferation. When the stromal cells were cultured in the absence of serum, this CM did not induce progenitor cell proliferation (data not shown).

These results suggested that multiply passaged BM stromal fibroblasts cultured in the presence of serum stimulate the proliferation of hematopoietic progenitor cells.

To confirm the suggestion that HGFs were produced by these stromal layers, total cellular RNA was prepared from layers cultured either serum-free or with or without IL-1α. The RNA was amplified by rtPCR with sense and antisense oligonucleotides for GM-CSF, G-CSF, IL-3, or IL-6 and for the proto-oncogene abl as a positive control for the PCR procedure. A positive control for each specific oligonucleotide was performed by stimulating stromal layers with IL-1α, known to induce the genes for GM-CSF, G-CSF, and IL-3. In addition, RNA prepared by stimulation of peripheral blood T cells with phorbol myristate acetate (PMA) and ionomycin served as the positive control for IL-3. As a negative control, the PCR reaction was performed with all reagents except the RNA. This blank (B) was negative in all cases.

The results showed the presence of message for both GM-CSF and IL-6. In preliminary studies comparing cytokine messenger RNA (mRNA) expression between 1 and 24 hours, we found that maximal message levels were found between 3 and 6 hours after initiation of serum-replete cultures. Unless otherwise specified, all data presented here derive from cultures after 3 hours of incubation in serum. Specifically, message for GM-CSF was either found to be stimulated (in 9 of 17 stromal layers studied) or augmented (in 8 of 17 stromal layers studied) after stimulation with human serum. Two such samples, one representative serum-stimulated message induction and the other serum-stimulated amplification, are shown in Fig 2. These
data suggest that GM-CSF gene transcription may be constitutive, but at a very low level, and thus not always detected. Alternatively, induction of the GM-CSF gene may be variably responsive to other factors in the culture. Nonetheless, the addition of human serum to the culture results in increased expression of the GM-CSF transcript compared with the unstimulated state.

To verify that apparent differences in GM-CSF mRNA levels detected by rtPCR from basal and human serum-stimulated conditions were reflective of real differences in mRNA levels, serial twofold dilutions of mRNA containing known GM-CSF message were isolated and amplified for GM-CSF mRNA by rtPCR. The results demonstrated that this rtPCR assay is able to distinguish approximately fourfold or greater differences in mRNA levels, over a range of 31.3 ng to 2.0 μg total RNA, resulting in comparable signals to those obtained in the basal stromal cell rtPCR studies (Fig 3). IL-6 message was detected at similar levels in RNA isolated from stromal cells cultured both in the presence and absence of serum. Two representative samples are shown (Fig 2). The identity of both the GM-CSF and IL-6 transcripts was confirmed by alkaline Southern blot hybridization with radiolabeled specific cDNA or oligonucleotide probes, as described in Materials and Methods (Fig 4).

Although these stromal fibroblast monolayers were apparently free of monocytes as assayed by immunocytochemistry, we were concerned that endogenous IL-1 production in the monolayer cultures might have contributed to the expression of IL-6 in unstimulated cultures. Assays were therefore performed at both RNA and protein levels to

![Fig 2. Stromal fibroblasts accumulate GM-CSF and IL-6 mRNAs. Multiply passaged stromal layers were cultured in the absence (−) or presence (+) of 20% human serum for 3 hours. The stromal layers were sacrificed and total cellular RNA prepared by the acid phenol method. Oligo d(T) RNA was amplified by PCR using sense and antisense oligonucleotides for abl, GM-CSF, G-CSF, IL-3, and IL-6. The results for two different normal donors are shown in their respective panels. Markers (M) are shown. As a negative control, blanks (B) were prepared by mixing all PCR reagents except the RNA in the initial reverse transcriptase step. Two positive controls were performed. The proto-oncogene abl is constitutively expressed, and appropriately present in all samples except the blank. The positive control (C) corresponds to use of oligo d(T) RNA known to be positive for the growth factor of interest (see text).](image)

![Fig 3. HGF rtPCR measurements discriminate specific mRNA levels. Multiply passaged stromal layers or peripheral blood T cells were stimulated with 10 ng/mL IL-1α for 3 hours. Total cellular RNA was prepared by the acid phenol method and amplified for abl, GM-CSF, G-CSF, and IL-6 in the case of the stromal cell RNA and for IL-3 for the T-cell RNA. Markers (FC × HaeIII) and blanks (B) are shown. Results represent serial dilutions of total cellular RNA that then undergo rtPCR. In the case of GM-CSF, G-CSF, IL-3, and abl, the undiluted sample (1) represents an rtPCR reaction that started with 2 μg total cellular RNA. In the case of IL-3, the undiluted sample (1) represents 500 ng total cellular RNA.](image)
IL-3 and G-CSF agarose gels were prepared. The G-CSF alkaline Southern blot demonstrates no G-CSF message at a 110-hour exposure, confirming that stromal fibroblasts do not transcribe G-CSF under unstimulated or human serum-stimulated conditions (Fig 4). Signal for IL-6 mRNA was generally absent, and only appeared to be detected once, after film exposure for 1.5 hours, in contrast to the T cell RNA control, in which IL-6 message was clearly discernable at 1.5 minutes. Even in this one case, when the alkaline Southern blots for both G-CSF and IL-3 were additionally analyzed by scanning densitometry, no message above background was detected in either the unstimulated or serum-stimulated case, for either cytokine. This photograph is shown in Fig 4.

Because the stromal fibroblasts studied in these assays had been enriched by repetitive passaging from a mixed population of stromal endothelial cells, macrophages, and adherent lymphocytes as well as fibroblasts, we then asked whether G-CSF mRNA might be detected from unpasaged, primary stromal monolayers. In these studies, G-CSF message was clearly detected from primary stromal cultures.
in the presence of serum, both in the presence and absence of IL-1α. However, G-CSF message was not detectable in stromal layers cultured under serum-free conditions, even from these primary monolayers containing multiple cell types (Fig 6).

Specific ELISAs were performed for GM-CSF, G-CSF, IL-6, and IL-3 to quantitate the individual growth factors present in human serum-stimulated stromal layer CM. GM-CSF was measured in control (unstimulated), human serum-stimulated, and IL-1α-stimulated CM, 72 hours poststimulation (Fig 7). Two (of 20) representative experiments are shown using two different normal donors. GM-CSF is present in the unstimulated samples at concentrations of 0 and 380 fg/mL, in the human serum-stimulated samples at concentrations of 670 and 700 fg/mL, and in the IL-1α-stimulated samples at 1.45 and 137 pg/mL. These low levels of GM-CSF were detected by ELISAs performed on 10-fold concentrated CMs and these values are corrected for the concentration factor of the CM. As demonstrated for the GM-CSF transcript, the GM-CSF protein was sometimes found to be present in unstimulated CM, but the concentration of the protein always increased after stimulation with human serum. The low level of GM-CSF protein observed in our experiments was not due to binding of the protein to stromal layer glycosaminoglycans such as heparan sulfate because digestion of the stromal layers with heparitinase, as described in Materials and Methods, did not result in elaboration of increased amounts of GM-CSF (data not shown).

IL-6 was also detected by ELISA in supernatants conditioned by stromal fibroblasts. Data from two (of 20) representative donors are shown (Fig 8). IL-6 levels in the unstimulated CM were 1,000 and 2,240 pg/mL, and in the human serum stimulated CM were 2,100 and 2,680 pg/mL.
As was found with GM-CSF, stimulation of stromal fibroblasts by human serum increased the amount of IL-6 protein detected in the CM, although the amount of stimulation varied among donor samples studied. ELISAs for both IL-3 and G-CSF, performed on concentrated CM and consistent with the rtPCR data, demonstrated neither IL-3 nor G-CSF to be present in the unstimulated or human serum-stimulated CM. In primary stromal monolayer cultures, however, G-CSF was detectable at between 50 and 400 pg/mL.

**DISCUSSION**

HGFs are potent molecules that are active in very low concentrations. Sensitive analysis of stromal cells by PCR amplification of cytokine messages and CM by ELISA demonstrates that stromal fibroblasts do transcribe and secrete CSF at low levels in the femtomolar to picomolar range. This emphasizes the importance of the BM microenvironment. HGFs are produced and used locally, where low cell-cell contact may serve to maximize the effective concentration and effects of these HGFs.

Recent studies suggest that one critical HGF produced by stromal cells is the c-kit ligand (also termed stem cell factor [SCF] or mast cell factor [MCGF]). Normal human fibroblasts constitutively secrete this protein, which although inactive by itself, synergizes with other differentiation-inducing HGFs such as GM-CSF, IL-3, and G-CSF. This synergy between c-kit ligand and other more specialized HGFs appears to permit low levels of these hormones to be functionally active. The factors that normally regulate the low level, constitutive, in vivo secretion of these unilineage and multilineage HGFs are therefore critical in the basal regulation of hematopoiesis in vivo.

The present study demonstrates that normal human BM fibroblasts do indeed secrete both GM-CSF and IL-6 in low quantities, even under conditions of basal culture. Stromal fibroblast production of GM-CSF requires the presence of human serum for either induction or augmentation of both gene expression and protein secretion. Expression of the GM-CSF gene was variable from person to person in our samples. In some cases assayed by rtPCR, human serum stimulation appeared to be required for expression of the GM-CSF gene, while in others human serum stimulation augmented gene expression. No differences were identified in the way cells were cultured to explain this variable GM-CSF expression. For example, all cultures were grown using the same lots of serum. Furthermore, neither the number of passages of the stromal layer nor the medium used to starve the cells prestimulation (IMDM v AIM-V) correlated with quiescence or expression of the gene in the absence of serum. Nonetheless, in all cases cultures in the presence of serum amplified the expression and/or secretion of GM-CSF.

IL-6 appears to be constitutively expressed by stromal layers because it was present in all unstimulated samples tested. However, addition of human serum to cultures did increase the amount of IL-6 secreted. Although it is formally possible that the presence of IL-6 mRNA and protein was entirely due to stimulation by IL-1α or IL-1β, we could not find evidence for this interpretation. No IL-1 was detectable in culture supernatants, and no IL-1 mRNA was detectable in fibroblast RNA. While the inclusion of anti-IL-1β appeared to diminish somewhat the expression of serum-induced IL-6 message, it had no effect on IL-6 expression in serum-deprived stromal cells. Moreover, inclusion of either anti-IL-1α or anti-IL-1β antibody in stromal cultures had no effect on measured protein secretion of IL-6. These data suggest an important role for IL-6 in basal hematopoiesis. This role may range from maintenance of the integrity of the stromal layer itself to synergism with GM-CSF for precursor cell development. Alternatively, IL-6 may play a permissive role in induction of other growth factor genes.

Quantitation of IL-6 and GM-CSF protein secreted into the CM demonstrates that GM-CSF is present in very low concentration (femtograms per milliliter). While one possibility is that GM-CSF is effective at this concentration, another is that this concentration is the result of the specific experimental procedure used. In our experimental protocol, the stromal layers were pulsed only once with human serum or IL-1α. The concentration of GM-CSF detected in IL-1α-stimulated cultures from 24 to 84 hours was constant (data not shown), suggesting that either GM-CSF is produced and consumed at equivalent rates or, alternatively, is secreted in response to the initial stimulation, but additional GM-CSF is not produced over time. Caldwell et al have reported that stromal cells pulsed with fresh medium transiently secrete GM-CSF at picograms per milliliter levels. As discussed by Caldwell et al, diffusion times across micron distances for large proteins (10 to 100 Kd) are on the order of hours. If some critical stimulatory proteins are rapidly consumed at the boundary layer of stromal fibroblasts, these cells will rapidly become starved in a static culture. Thus, one function of the significant fraction (approximately 2% to 5%) of cardiac output that is delivered to BM may be to provide a continuous supply of the requisite stromal cell stimulants.

In contrast to IL-6 and GM-CSF, no G-CSF message was detected in multiply cultured marrow stromal fibroblasts by alkaline Southern blot analysis and no G-CSF was detected by ELISA. One possible interpretation of these data is that G-CSF may not play a role in the support of basal hematopoiesis by stromal fibroblasts, but rather is more important under inflammatory conditions. Alternatively, G-CSF may be important in basal hematopoiesis but may be secreted by stromal monocytes, a known source of G-CSF, or via the interaction of fibroblasts and monocytes within the stromal milieu. This latter interpretation is in fact supported by our data documenting the presence of G-CSF mRNA in complex stromal monolayers, and the presence of small quantities of G-CSF secreted by such monolayers. Similar support is provided by the studies of Migliaccio et al demonstrating that complex stromal monolayers containing both fibroblasts and monocytes secrete a bioactivity consistent with G-CSF. Alternatively, however, the bioassay used by Migliaccio et al could have been detecting the activity of a cross-reacting molecule.
Very little if any IL-3 message was detected by alkaline Southern blot, consistent with previously reported data. Barge et al reported that IL-3 transcript and protein was detectable in normal BM stroma. However, they reported that the IL-3 was only detected in stromal layers containing CD2+CD3ε+ or CD2-CD3ε+ cells, corresponding to mature or immature T cells. In CD2-CD3ε- stromal layers, no IL-3 message was detected. In the present studies with multiply passaged normal human BM fibroblasts, no densitometry signal above background was detected, and no secreted IL-3 protein was detected. Thus, if IL-3 is involved in basal hematopoiesis, it appears likely that it must be derived from T-lymphoid lineage cells that either circulate into the marrow microenvironment or secrete IL-3 at a distance. Of note, however, two lines of evidence suggest that such T-cell signals are unlikely to be essential for basal hematopoiesis. First, hematopoietic recovery after allo-geneic BM transplantation occurs despite the fact that the recovering T cells are extremely defective in their ability to secrete HGFs and stimulate progenitor cell differentiation. Second, neonatal T cells likewise fail to secrete HGFs to normal stimuli, apparently due to their developmental immaturity.

In summary, we have shown that normal human BM stromal fibroblasts secrete GM-CSF and IL-6 when cultured in the presence of serum, in the absence of inflammatory stimuli. Of note, two lines of evidence suggest that such T-cell signals are unlikely to be essential for basal hematopoiesis. First, hematopoietic recovery after allo- geneic BM transplantation occurs despite the fact that the recovering T cells are extremely defective in their ability to secrete HGFs and stimulate progenitor cell differentiation. Second, neonatal T cells likewise fail to secrete HGFs to normal stimuli, apparently due to their developmental immaturity.

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Bone marrow stromal fibroblasts secrete interleukin-6 and granulocyte-macrophage colony-stimulating factor in the absence of inflammatory stimulation: demonstration by serum-free bioassay, enzyme-linked immunosorbent assay, and reverse transcriptase polymerase chain reaction

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