Peptide Growth Factors Stimulate Macrophage Colony-Stimulating Factor in Murine Stromal Cells

By Sherry L. Abboud and Massimo Pinzani

Bone marrow stromal cells influence hematopoiesis through cell-cell interaction and release of hematopoietic growth factors. Macrophage colony-stimulating factor (M-CSF) is constitutively produced by several murine and human stromal cell lines and is induced by inflammatory mediators such as interleukin-1 α or tumor necrosis factor-α (TNF-α) in a variety of mesenchymal cells. Other potentially important regulatory molecules such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), released by activated monocytes in response to inflammation, stimulate the growth of human stromal cells. However, the effect of these peptide mitogens on M-CSF expression in stromal cells has not been explored. In this study, we used TC-1 murine bone marrow-derived stromal cells that constitutively secrete M-CSF to determine the effect of PDGF and bFGF on cell proliferation and M-CSF gene expression. PDGF and bFGF, but not TNF-α, were potent mitogens for the TC-1 cells. Similar to mouse L cells, TC-1 murine stromal cells constitutively expressed two major mRNA transcripts of 4.4 and 2.2 kb that hybridized to a murine M-CSF cDNA. PDGF, bFGF, and TNF-α markedly stimulated the steady-state expression of M-CSF mRNA with different time-course kinetics. The increased expression of M-CSF mRNA was associated with enhanced secretion of M-CSF as determined by radioimmunoassay. These findings suggest that PDGF, bFGF, and TNF-α may regulate hematopoiesis indirectly through release of M-CSF by stromal cells and may modulate, at least in part, the hematopoietic response to inflammation.

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MATERIALS AND METHODS

Peptide growth factors and M-CSF probe. Recombinant PDGF (PDGF BB homodimer c-cis), recombinant human bFGF, and TNF-α were purchased from Amgen Biologicals (Thousand Oaks, CA). The specific activity of the TNF preparation was greater than 10^5 U/mg. Each unit represents the concentration of TNF required to yield 50% lysis of mitomycin C-treated L929 mouse fibroblasts. The M-CSF cDNA probe is a 2.4-kb fragment inserted into the EcoRI site of SP65 and was a generous gift of Dr S. Clark (Genetics Institute, Cambridge, MA).3

Stromal cell line. The TC-1 stromal cells (kindly provided by Dr Peter Quesenberry, University of Virginia, Charlottesville) are adherent cells isolated from murine long-term marrow culture. Their phenotypic characterization has been previously described.5 The cells were maintained in Fischer's medium (GIBCO, Grand Island, NY) supplemented with 10 mmol/L HEPES, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, penicillin 100 U/mL, streptomycin 100 μg/mL, nystatin 25 ng/mL, and 17% fetal calf serum. At confluency, cells were made quiescent by incubation in serum-free Fischer's medium for 24 hours. Cells were then replaced by an equal volume of undiluted 3:1 methanol:acetic acid fixative inserted into the EcoRI site of SP65 and was a generous gift of Dr S. Clark (Genetics Institute, Cambridge, MA).3

DNA synthesis. [3H]-thymidine (Tdr) incorporation into the TC-1 cells was used as a measure of DNA synthesis. In brief, 5 X 10^4 cells suspended in 1 mL of Fischer's medium with 17% FCS were seeded into each of 24-well flat-bottomed dishes and incubated at 37°C in a humidified atmosphere in 5% CO2. Confluent cells were allowed to become quiescent by placing them in Fischer's medium with 1% serum for 48 hours. Cells were incubated with or without various growth factors for 20 hours and then pulsed for 4 hours with 1.0 μCi/mL of [3H]-Tdr (6.7 Ci/mmol; New England Nuclear, Boston, MA). In some experiments, as specified, cells were pulsed with [3H]-Tdr at the time growth factors were added. The assay was terminated by gently aspirating the medium and washing the cells three times with ice-cold 5% trichloroacetic acid to precipitate proteins and nucleic acids and remove unincorporated [3H]-Tdr. Cells were then neutralized and isotope uptake was determined by liquid scintillation counting.

 Autoradiography. Stromal cells were plated onto 4-chamber LabTek slides (Miles Scientific, Naperville, IL) at a density of 1 X 10^5 cells per chamber in Fischer's medium supplemented with serum. Confluent cells were made quiescent as described previously and then incubated with various growth factors and 1 μCi/mL of [3H]-Tdr for 24 hours. At the end of the pulsing period, an equal volume of freshly prepared 3:1 methanol:acetic acid fixative was added to the medium for 10 minutes. This half-strength fixative was then replaced by an equal volume of undiluted 3:1 methanol:acetic acid fixative. After 10 minutes, cells were air dried and exposed to NTB-2 nuclear emulsion (Kodak, Rochester, NY) for 3 days at 4°C. The slides were then developed and fixed with Kodak D19 developer and Kodak fixer, respectively, and stained with Giemsa.4 Four hundred cells per each incubation condition were counted and the percent of labeled nuclei (labeling index) was determined.

 Stromal cell proliferation. Stromal cells were seeded into 12-well dishes at a density of 1 X 10^5 cells/well in Fischer's medium with 17% FCS. After 24 hours, media was aspirated and replaced by Fischer's medium containing 1% serum. At this time, test conditions were added to each well (time 0). Cells in each well were trypsinized and cell counts were performed on triplicate wells at time 0 and after 3 and 7 days. Results were expressed as the mean ± SE.

 RNA purification and Northern analysis. Cells, 5 X 10^6 to 1 X 10^7, suspended in complete medium were seeded into 100-mm Petri dishes. At confluence, cells were made quiescent by incubation in 1% serum overnight. Cells were then incubated in the absence or presence of growth factors. At specified time intervals, stromal cells were washed twice in phosphate-buffered saline (PBS), lysed with 5 mol/L guanidium thiocyanate, and the RNA recovered after centrifugation through 5.7 mol/L cesium chloride step gradient.27 Samples were enriched for poly A-containing RNA by chromatography over oligo(dt) cellulose.25 Aliquots were size-fractionated by electrophoresis through 1% agarose-formaldehyde gels. The RNA was transferred to GeneScreen (New England Nuclear) and prehybridized at 42°C for 1 hour in 50% deionized formamide, 0.5% SDS, 2X PIPES-NaC1-EDTA buffer, and 0.1 mg/mL salmon sperm DNA. The M-CSF probe was nick-translated and labeled with [3P]-dCTP (Amersham, Arlington Heights, IL) to a specific activity of 1 X 10^6 cpm/μg DNA. Probe, 2 X 10^6 cpm, was added to 20 mL of prehybridization solution and the blot was hybridized for 16 hours at 42°C. Blots were washed sequentially four times each in 2X SSC (1X SSC = 0.15 mol/L NaCl/0.015 mol/L sodium citrate, pH 7.4), 0.1% SDS at 22°C and 65°C, and 0.1X SSC, 0.1% SDS at 22°C for 15 minutes. Autoradiography was performed with x-ray film and intensifying screens at −70°C.

 Preparation of conditioned medium. Cells were seeded into flasks and allowed to reach confluence in complete medium. Serum containing medium was removed, and cells were washed once and incubated in serum-free Fischer's medium for 24 hours to eliminate residual contaminating serum. This medium was discarded and replaced with fresh serum-free medium with or without PDGF and bFGF. After a 3-hour incubation period at 37°C, the media were removed, cells washed, and fresh serum-free medium added. Cell-free supernatants were collected after an additional 8 hours, sterile filtered (0.45 μm), and stored at −20°C. In an additional experiment, the effect of TNF on M-CSF secretion was examined after 8 and 24 hours. Assays for CSF activity were performed on aliquots of unconcentrated supernatants.

 Radioimmunoassay for M-CSF. M-CSF activity was quantitated using a competitive radioimmunoassay developed by Stanley.36 Units are defined by an in vitro murine clonal assay, where 1 U (0.44 fmol of M-CSF protein) is the amount of M-CSF required to produce one colony from 7.5 X 10^4 marrow cells plated in agar culture. Results are expressed in units per 10^6 cells.

RESULTS

Growth factor stimulation of [3H]-thymidine incorporation and cell growth. Quiescent TC-1 stromal cells in culture were assayed for their ability to incorporate [3H]-Tdr into DNA after exposure to PDGF or bFGF. As shown in Fig 1, both PDGF and bFGF stimulated DNA synthesis in a concentration-dependent manner. PDGF was a slightly more potent mitogen than bFGF. Maximum stimulation of [3H]-Tdr incorporation into DNA of TC-1 cells occurred in response to 10 ng/mL of PDGF or bFGF. In several experiments, the fold stimulation varied from twofold to fivefold with 10 ng/mL of PDGF or bFGF. In contrast to PDGF and bFGF, incubation with TNF (1 to 25 ng/mL) did not increase DNA synthesis. Time-course experiments (Fig 2) demonstrate that the addition of PDGF or bFGF induced a progressive increase in [3H]-Tdr incorporation into DNA at 12 hours, reaching a peak effect at 24 hours for PDGF and 32 hours for bFGF. DNA synthesis did not increase over time in cultures treated with 10 ng/mL of TNF (Fig 2). The stimulation of DNA synthesis by PDGF and bFGF was confirmed by autoradiographic analysis and determination of the labeling index as shown in Table 1. There was a significant increase in the labeling index
GROWTH FACTORS AND MURINE STROMA

Table 1. Effect of Peptide Growth Factors on DNA Synthesis of TC-1 Cells Measured by Autoradiography

<table>
<thead>
<tr>
<th>Condition</th>
<th>Labeling Index</th>
<th>% of Labeled Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>PDGF 1 ng/mL</td>
<td>7.9 (11)</td>
<td></td>
</tr>
<tr>
<td>PDGF 10 ng/mL</td>
<td>69.8 (116)</td>
<td></td>
</tr>
<tr>
<td>bFGF 1 ng/mL</td>
<td>14.8 (25)</td>
<td></td>
</tr>
<tr>
<td>bFGF 10 ng/mL</td>
<td>42.2 (70)</td>
<td></td>
</tr>
<tr>
<td>10% FCS</td>
<td>19.7 (33)</td>
<td></td>
</tr>
</tbody>
</table>

Cells were plated onto 4-chamber Lab-Tek slides at a density of 1 x 10^4 cells/chamber in Fischer's medium with 17% serum. Confluent cells were made quiescent in medium containing 1% serum for 48 hours and then incubated with PDGF or bFGF for 24 hours. [3H]-thymidine, 1.0 μCi/mL, was added at the time of growth factor addition. At the end of the 24-hour incubation period, cells were fixed and developed. Four hundred cells per each incubation condition were counted and the percent of labeled nuclei (labeling index) was determined. Numbers in brackets represent fold stimulation.

Fig 1. Dose-response curve for the effect of peptide growth factors, PDGF and bFGF, on TC-1 stromal cell DNA synthesis. Cells were plated in 24-well dishes at 5 x 10^4 cells/well in Fischer's medium with 17% serum. At confluence, cells were made quiescent by incubation in Fischer's medium with 1% serum for 48 hours. Growth factors were then added and cells were simultaneously pulsed with [3H]-thymidine (1.0 μCi/mL) for 24 hours. Control wells were incubated with medium containing 1% serum alone. [3H]-thymidine incorporation into DNA was measured as trichloroacetic acid (TCA)-precipitable material. Each point represents the mean of data tested in duplicate or triplicate wells.

Table 2. Effect of Peptide Growth Factors on the Growth of TC-1 Stromal Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell Number x 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td>7.8 ± .3</td>
</tr>
<tr>
<td>PDGF</td>
<td>10.9 ± .4</td>
</tr>
<tr>
<td>bFGF</td>
<td>10.1 ± .4</td>
</tr>
</tbody>
</table>

Cells were plated in medium with 17% serum and after 24 hours they were placed in Fischer's medium with 1% serum and incubated with 10 ng/mL PDGF or 10 ng/mL bFGF. On day 3, triplicate wells from each condition were trypsinized and counted using a Coulter counter (Coulter, Hialeah, FL). Remaining wells had their media replaced with fresh medium without (control) or with growth factors. Cell count at day 0 was 7.3 ± .2 x 10^4/well (mean ± SE). Data from two separate experiments, each performed in triplicate wells.

Fig 2. Time course for the effect of PDGF and bFGF on TC-1 stromal cell DNA synthesis. Cells were plated and made quiescent as described in the legend to Fig 1. PDGF (10 ng/mL) or bFGF (10 ng/mL) was then added and cells were pulsed with [3H]-thymidine (1.0 μCi/mL) for 4 hours before harvesting at the indicated time points. Data are expressed as percent change from control wells incubated without growth factors at each time point. Each condition was tested in triplicate. Representative of two separate experiments.

Constitutive expression of mRNA encoding M-CSF gene in TC-1 stromal cell line. Because TC-1 cells have been shown to constitutively secrete M-CSF into conditioned medium, we evaluated basal M-CSF gene expression in cells grown in complete medium. TC-1-C-11 cells, which are similar to TC-1, were also evaluated. As shown in Fig 3, a Northern blot containing total and poly(A)+RNA isolated from each cell line demonstrates two major hybridizing species of about 4.4 and 2.2 kb and a less-abundant species of about 1.4 kb. A similar pattern of transcript hybridization was observed in mouse L cells that were used as a control.

Stimulation of M-CSF mRNA by peptide growth factors. To determine if PDGF and bFGF stimulate the expression of mRNA encoding M-CSF, time-course experiments were performed.
performed using quiescent and PDGF- or bFGF-treated cells. Figure 4 shows a clear, low-intensity signal in unstimulated cells. Exposure of TC-1 cells to an optimal dose of PDGF (10 ng/mL) markedly increased the steady-state levels of M-CSF mRNA by 1 hour, with a peak effect at 3 hours subsiding to near-basal levels by 16 hours. In response to 10 ng/mL of bFGF, induction of M-CSF mRNA levels was seen within 2 hours, reaching a peak effect by 6 to 12 hours, and the increased mRNA levels persisted for 12 to 24 hours. TNF-α also increased the level of M-CSF mRNA in TC-1 cells with a peak effect occurring by 1 to 2 hours. In all experiments, the relative abundance of the three M-CSF mRNA transcripts did not change after induction. To determine the specificity of the effects of these peptides for M-CSF mRNA, blots were boiled to remove M-CSF probe and rehybridized to an α-tubulin cDNA probe. There is little if any variation in tubulin mRNA levels, suggesting that the response in M-CSF mRNA is specific and does not simply reflect a global increase in total RNA.

To determine if the increased expression of M-CSF mRNA is associated with enhanced secretion of the corresponding protein, we used a sensitive radioimmunoassay to detect M-CSF activity in both quiescent and PDGF- or bFGF-stimulated stromal cells. Conditioned medium collected from quiescent stromal cells contained 379 U/10⁶ cells of M-CSF. When cells were incubated with 10 ng/mL of either PDGF or bFGF, M-CSF secretion increased at 8 hours to 621 and 644 U/10⁶ cells, respectively. Incubation of TC-1 cells with 10 ng/mL of TNF also increased M-CSF secretion from 447 to 697 U/10⁶ cells after 8 hours and from 1,104 to 2,872 U/10⁶ cells after 24 hours.

**DISCUSSION**

The present study demonstrates that PDGF and bFGF, but not TNF-α, are potent mitogens for murine stromal cells and that these peptides markedly stimulate the expression of mRNA encoding for M-CSF and secretion of the protein. The mitogenic effect of PDGF and bFGF on the murine TC-1 stromal cells was documented by enhanced DNA synthesis, increase in the labeling index by autoradiography, and increase in cell growth in response to each peptide. It is well known that the major source of PDGF is the α granules of platelets and that activated monocytes, endothelial cells, and fibroblasts release PDGF and express PDGF mRNAs that encode the A and/or B chains of the PDGF molecule. Although the precise role of PDGF and bFGF in normal hematopoiesis remains to be determined, recent studies have shown that PDGF stimulates the growth of erythroid progenitors. Delwiche et al suggested that this effect of PDGF is mediated through two accessory cell populations, fibroblasts and smooth muscle cells, but not endothelial cells or macrophages. Michalevitz et al using a highly enriched early bone marrow population, found that PDGF directly stimulated mixed erythroid-myeloid colony-forming units in this fraction, although an indirect effect through accessory cells was not excluded. Previous studies have shown that PDGF is a potent mitogen and that it increases IL-1, IL-6, and G-CSF mRNA transcripts in human stromal cells. Recently, bFGF has shown to enhance the growth of human stromal cells in long-term culture. It also interacts synergistically with other CSFs, such as IL-3 and GM-CSF, to stimulate the growth of early human hematopoietic progenitors. Our finding raises the possibility that bFGF released by activated macrophages may influence progenitor cell growth indirectly via accessory cells. It is also likely that PDGF and bFGF participate in the hematopoietic response to inflammation.

The expression of murine M-CSF specific transcripts in TC-1 cells using total or poly A-enriched cellular RNA and M-CSF activity is in agreement with a previous report demonstrating the constitutive release of M-CSF protein into medium by these cells. The hybridization pattern
showing three clear bands of 4.4, 2.2, and 1.4 kb is similar to that found in mouse L cells. Recent studies have addressed the regulation of M-CSF in bone marrow stromal cells, but with conflicting results. IL-1 has been shown to induce the production of M-CSF by human long-term stromal cell cultures. However, Gimble et al., using murine stromal cells, were unable to show a significant change in the expression of M-CSF mRNA by a variety of cytokines, including IL-1 and TNF. Induction of M-CSF mRNA has also recently been reported in 3T3 fibroblasts using high concentrations of the purified AB isofrom of PDGF.

Because both PDGF and bFGF were mitogenic to TC-1, an important issue raised by these findings is whether the effect of these two peptides on M-CSF mRNA represents a specific signaling pathway or is related to DNA synthesis and cell growth. TNF-α at a concentration that markedly increased M-CSF levels had no effect on DNA synthesis, suggesting that the stimulatory effect of this cytokine on M-CSF mRNA is not related to cell growth. The underlying mechanism for the effect of PDGF and bFGF on M-CSF mRNA is not known. While this work was in progress, Falkenburg et al. reported that PDGF, bFGF, or combinations of peptide growth factors that stimulate proliferation of murine 10T1/2 fibroblast cells also induce M-CSF mRNA expression. The investigators suggested that M-CSF expression correlates with the proliferative state of the cells. IL-1, on the other hand, which does not appear to have a mitogenic effect in those cells, also stimulated M-CSF mRNA expression. The precise role and association of cell-cycle events to the regulation of M-CSF mRNA remains to be determined.

It is likely that the release of M-CSF from stromal cells may act as a positive feedback mechanism to further enhance the production of monocyte PDGF, bFGF, and other cytokines providing a positive signal that amplifies the hematopoietic response to inflammation. The recent observation that murine erythroid cells release PDGF activity in vitro and in vivo, taken together with our present findings, expands the role of locally produced PDGF and perhaps other polypeptide growth factors in regulating hematopoiesis. Moreover, the potent stimulatory effect of PDGF and bFGF on M-CSF mRNA in TC-1 stromal cells suggests that these cells may provide a useful model to study the cellular mechanisms of M-CSF gene regulation.

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