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_J Immunol_ 2003; 171:6846-6855; doi: 10.4049/jimmunol.171.12.6846
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Granulocyte-Macrophage Colony-Stimulating Factor Is a Chemoattractant Cytokine for Human Neutrophils: Involvement of the Ribosomal p70 S6 Kinase Signaling Pathway

Julian Gomez-Cambronero,* Jeffrey Horn,* Cassandra C. Paul,†‡ and Michael A. Baumann†‡

GM-CSF stimulates proliferation of myeloid precursors in bone marrow and primes mature leukocytes for enhanced functionality. We demonstrate that GM-CSF is a powerful chemotactic and chemokinetic agent for human neutrophils. GM-CSF-induced chemotaxis is time dependent and is specifically neutralized with Abs directed to either the ligand itself or its receptor. Maximal chemotactic response was achieved at ~7 nM GM-CSF, and the EC50 was ~0.9 nM. Both concentrations are similar to the effective concentrations of IL-8 and less than the effective concentrations of other neutrophil chemoattractants such as neutrophil-activating peptide-78, granulocyte chemotactic protein-2, leukotriene B4, and FMLP. GM-CSF also acts as a chemoattractant for native cells bearing the GM-CSF receptor, such as monocytes, as well as for GM-CSF receptor-bearing myeloid cell lines, HL60 (promyelomonocyte leukemic cell line) and MPD (myeloproliferative disorder cell line), following differentiation induction. GM-CSF induced a rapid, transient increase in F-actin polymerization and the formation of focal contact rings in neutrophils, which are prerequisites for cell migration. The mechanism of GM-CSF-induced chemotaxis appears to involve the cell signaling molecule, ribosomal p70 S6 kinase (p70S6K). Both p70S6K enzymatic activity and T421/S424 and T489 phosphorylation are markedly increased with GM-CSF. In addition, the p70S6K inhibitor hamartin transduced into cells as active protein, interfered with GM-CSF-dependent migration, and attenuated p70S6K phosphorylation. These data indicate that GM-CSF exhibits chemotactic functionality and suggest new avenues for the investigation of the molecular basis of chemotaxis as it relates to inflammation and tissue injury. The Journal of Immunology, 2003, 171: 6846–6855.

Granulocyte-macrophage colony-stimulating factor is a hemopoietic cytokine capable of supporting the proliferation and survival of granulocytic precursors in the bone marrow. Released as a paracrine hormone principally by activated T cells, macrophages, fibroblasts, and endothelial cells (1–3), GM-CSF also enhances the functionality of mature cells, such as neutrophils. In neutrophils, GM-CSF potentiates degranulation, the release of oxygen and nitrogen radical ions, phagocytosis (4–9), and inhibits apoptosis (10, 11). Recently, GM-CSF has been found to have a potent effect in inducing the expression of neutrophil receptors for CC chemokines (12).

In a classical inflammatory response, neutrophils are recruited to sites of inflammation by chemoattractants (i.e., IL-8, C5a, and leukotriene B4 (LTB4))1 that are secreted by several tissue cells in response to a physical or chemical insult, or by certain bacterial products, such as LPS or FMLP, during infection (13, 14). The first step in phagocytosis involves neutrophil migration toward the in-sulting stimulus (chemotaxis). A role for the signaling molecules phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B in regulating neutrophil migration has been demonstrated (15–18), although the PI3K inhibitor LY-294002 did not inhibit FMLP-triggered migration (18). Despite this evidence implicating the involvement of the upstream members of the PI3K pathway, the role of downstream links such as mammalian target of rapamycin (mTOR) and ribosomal p70 S6 kinase (p70S6K) has not been examined directly in hemopoietic cells. S6Ks comprise two kinases, products of two different genes. Both enzymes phosphorylate ribosomal S6 protein, and both are highly homologous, but they differ in their N- and C-terminal regulatory domains. The S6K isoforms are termed p70S6Kα2 (p70) and p85S6Kα1 (p85), the latter being a longer form with nuclear localization. Ribosomal S6K1 has at least 12 sites of phosphorylation, the most critical residues being Thr249, S241, Thr421, Ser489, and Ser604 (19–21).

We report that GM-CSF elicits chemotaxis and chemokinesis of neutrophils. This process is correlated with the activity of the p70S6K pathway, and inhibition of p70S6K activity inhibits neutrophil locomotion.

Materials and Methods

GM-CSF and IL-8 were from R&D Systems (Minneapolis, MN); neutrophil-activating peptide-78 (ENA-78) and granulocyte chemotactic protein-2 (GCP-2) were from Cell Sciences (Norwood, MA); 20-trifluoro-LTB4 was from Cayman Chemicals (Ann Arbor, MI); 24-well Transwell membrane tissue plates (with 6.5 mm diameter, 5-μm-pore inserts) were from Corning Costar (Cambridge, MA); trypsin blue, phalloidin-FITC conjugate, phalloidin-tetramethylrhodamine isothiocyanate (TRITC) conjugate, Amanita phalloides, 4′,6′-diamidino-2-phenylindole (DAPI), FMLP,
protein kinase A inhibitor, calphostin, anti-rabbit IgG (agarose beads), the cAMP-dependent kinase inhibitor TYYDAFISARGTRRRNAIHD, anti-p70S6 kinase polyclonal Ab used for immunoblotting, and pertussis toxin were from Sigma-Aldrich (St. Louis, MO); electrophoresis chemicals were from Bio-Rad (Richmond, CA); [γ-32P]ATP (30 Ci/mmol) and Percoll (density 1.13 g/ml) were from Amersham Pharmacia Biotech (Arlington Heights, IL); GelMount was from Bio-Media-Fisher (Pittsburgh, PA); FACS FLOW buffer was from Fisher (Hanover Park, IL); rapsamycin, LY294002, and wortmannin were from Calbiochem (San Diego, CA); anti-GM-CSF receptor, common β (CD131)-chain, anti-GM-CSF, and anti-fibroblast growth factor (FGF) Abs were from BD Biosciences (Franklin Lakes, NJ); p70S6 kinase substrate KRRNTT LTKR was from Upstate Biotechnology (Lake Placid, NY); hamartin (60-kDa purified tagged fusion protein corresponding to aa 1–300 of hamartin of human origin) was from Santa Cruz Biotechnology (Santa Cruz, CA); and the Bio Porter protein delivery reagent was purchased from Gene Therapy Systems (San Diego, CA).

Cells

**Neutrophils.** Human peripheral blood neutrophils were isolated based on a protocol described by English and Andersen (22). Between 50 and 55 ml of blood was collected from the antecubital vein of healthy individuals (who signed an Institutional Review Board-approved consent form) using sodium citrate as anticoagulant. Blood was mixed with 15 ml of 6% dextran and allowed to settle, and the plasma and buffy coat were removed and spun down at 1000×g for 5 min. The pellet was resuspended in 0.5 ml of saline and centrifuged again for 15 min at 10°C in a Ficoll-Histopaque discontinuous gradient. Neutrophils were recovered, and contaminating erythrocytes were lysed by hypotonic shock. Cells were washed, and the pellet was resuspended in HBSS. Our experience has indicated that using this protocol, neutrophil aggregation (i.e., the hallmark for neutrophil activation) does not occur. Viability is usually >98 ± 2% as per trypan blue exclusion. Cells were resuspended at the concentration of 5 × 10⁶ cells/ml in fresh HBSS or in RPMI 1640 at 2 × 10⁶ cells/ml at the time of the experiment, and used within 5 h after isolation.

**Monocytes and lymphocytes.** Human peripheral blood mononuclear cells (MNCs) were isolated from blood using first a Ficoll-Histopaque discontinuous gradient, as above. The interface containing MNCs (lymphocyte:monocyte ratio of 2:1) was removed (23) containing 8 × 10⁶ cells was washed twice in PBS at low speed (200 × g for 5 min) to reduce platelet contamination (24), and cells were subjected to a second gradient centrifugation in Percoll according to Ref. 25 with some modifications, as follows. MNCs were resuspended in 7.5 ml of Percoll adjusted to a density of 1.094 g/ml with 0.5% BSA to a concentration of 5 × 10⁶/ml and placed on ice. The MNCs were centrifuged at 2000 × g for 30 min at 4°C, as confirmed by Wright-stained cytopreparations and cytological examination. The resulting population had the following differential: 95% neutrophils, segmented neutrophils, 4% monocytes, and 1% lymphocytes. The mixture was added in 0.5 ml of chemotaxis buffer to the upper chambers or inserts of Transwell plates that are separated by a 3 μm-pore polycarbonate membrane. For the study of chemokinesis, GM-CSF or IL-8 was added in 0.5 ml of chemotaxis buffer to the lower wells of Transwell plates (26). For the study of chemotaxis, GM-CSF or IL-8 was added in 0.5 ml of chemotaxis buffer to the lower chambers and in 200 μl of chemotaxis buffer to the upper chambers. In either case, the Transwell plates were incubated for 45 min at 37°C under a 5% CO₂ atmosphere. The number of cells that migrated to the lower wells was calculated by counting four fields of duplicate 20-μl aliquots on a microscope using a hemocytometer. Viability (by trypan blue exclusion) at the end of the assay in both chambers was maintained >95%, even in the presence of the inhibitors rapsamycin, wortmannin, or LY294002, ruling out a toxic effect. We have also observed that RPMI 1640 as the medium to resuspend the neutrophil cells and conduct the assay (i.e., the chemotaxis buffer) is preferable over HBSS with regard to being migration permissive: with the latter buffer, a large percentage of cells pass the 5-μm-pore polycarbonate membrane, but remained attached to the filters. The number of cells counted in the lower chamber is lower than expected.

**Chemotaxis functional assay in Boyden chamber**

Freshly isolated neutrophils were resuspended in RPMI 1640 medium plus 0.5% BSA to a concentration of 5 × 10⁶ cells/ml and placed on ice. The 24-well minichamber was disassembled to provide access to the bottom wells, and a final volume of 27 μl of RPMI 1640 only or RPMI 1640 + agonist was added to each bottom well, as indicated. Next, one polycarbonate polystyrene-lypdroiodine-free filter (5 μm pore size) was placed directly over the bottom wells, and the chamber was mounted. Next, 50 μl of cells (25 × 10⁶ cells) was added to each top well. The chamber was then incubated at 37°C, 5% CO₂ for 30 min. Following this incubation, the filter was then gently peeled away from the chamber and was rinsed cell side up using sterile PBS. Following each rinse, the Into and gag side of the filter was run against the blunt end of a clean razor blade and rinsed again three times. The filter was dipped in methanol, allowed to dry, stained with Wright stain and photographed.

**Microscopy of TRITC-conjugated phallolidin**

To measure F-actin polymerization in neutrophils, cells were added to glass coverslips that were placed in each well of a six-well tissue culture plate. Cells were seeded into the wells at a concentration of 5 × 10⁶ cells/ml without coverslips and allowed to equilibrate in HBSS for 15 min in a CO₂ incubator. The medium was aspirated from the coverslips, and the cells were washed with PBS. The cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 10 min. After one wash with PBS, cells were stained for 40 min at room temperature with 25 μg/ml TRITC-conjugated phallolidin for actin staining and 2 μg/ml DAPI for DNA staining in 1 ml/well. The coverslips were washed three times with PBS for 10 min to remove excess stain and mounted on glass slides using GelMount. Stained samples were examined on a Nikon Labophot fluorescent microscope and photographed (SPOT camera; Diagnostic Instruments, Sterling Heights, MI) by consecutive exposure using red and blue filters, or were examined on an Olympus Fluoview laser-scanning confocal microscope with a ×60 numerical aperture 0.9 objective.

**F-actin measurement by flow cytometry**

Neutrophils were stained with phallolidin-FITC, as described (27), with some modifications. Briefly, F-actin polymerization was initiated in vivo by the addition of GM-CSF to a neutrophil cell suspension (5 × 10⁶ cells/ml) for 5 min at 37°C. After this, 0.2-ml aliquots were taken and mixed with 1 ml of prechilled fixing solution (two parts of double-concentrated phosphate buffer, pH 7.4, one part of 20% formaldehyde, and one part of 75% glycerol in water). Samples were stored at −80°C until ready for flow cytometry. At that time, samples were thawed and spun down for 5 min at 600 × g in a refrigerated Eppendorf microfuge. Pellets were resuspended in freshly prepared F-actin-staining solution (35 μl of a 3.3 μg/ml methanol FITC-phallolidin stock plus 315 μl H₂O), and stained in the dark for 30 min at room temperature. Samples were centrifuged as above, and pellets were resuspended in 1 ml of FACScan FLOW. They were then analyzed by flow cytometry on a FACScan BD Biosciences (ImmuneCytometry Systems, San Jose, CA) flow cytometer at 488 nm excitation wavelength. Data were analyzed using CellQuest software. The relative F-actin content was expressed in comparison with resting cells that received no treatment. Results of fluorescence intensities were shown in a logarithmic scale, as described previously (27, 28).

p70S6K 764valid/S424 and T389 phosphorylation

Immunoprecipitation with specific anti-p70S6K Abs and immunoblotting with anti-p70S6K and anti-phospho-p70S6K were performed, as indicated previously (29), with some modifications, as described in this work. Stimulation of neutrophils (5 × 10⁶ cells/ml in RPMI 1640) with appropriate agents was terminated by centrifugation (14,000 × g, 15 s) and resuspension of pellets in bolting SDS solution (1% SDS in 10 mM Tris, pH 7.4).
Samples were boiled in a heat block for 10 min with frequent vortexing to achieve complete dissolution, taken to an ice bucket, and mixed with 0.3 ml cold ddH2O and 0.4 ml of cold Triton X-100-based, lysis buffer (12 mM Tris-HCl, pH 7.2; 0.75 mM NaCl; 100 μM sodium orthovanadate; 10 mM PMSF; 0.2 mM β-glycerophosphate; 5 μg/ml each of aprotinin, pepstatin A, and leupeptin; and 0.12% Triton X-100). The resulting 1 ml total cell lysates were spun down (14,000 × g, 1 min, 4°C) to remove any insoluble material and then used for immunoprecipitation. For this, the primary Ab (anti-p70S6K) was previously mixed at a final concentration of 2 μg/ml with anti-rabbit (IgG, whole molecule) Ab conjugated to agarose beads in lysis buffer for 4 h at 4°C. The beads were then thoroughly washed and mixed with total cell lysates, prepared as indicated above, at a ratio agarose beads/cell lysates 1:8 (v/v). After a 2-h incubation period at 4°C, immune complexes were recovered by centrifugation (7000 × g, 1 min, 4°C). Pellets were washed twice with lysis buffer, twice with buffer A (100 mM Tris-HCl, pH 7.4, 400 mM LiCl), and twice with buffer B (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). Immune complex beads were resuspended in a final volume of 60 μl with lysis buffer and mixed with 2× SDS sample buffer (1:1 v/v) for subsequent protein gel electrophoresis/immunoblotting. Resulting gels were transferred onto polyvinylidene difluoride membranes and used for immunoblots that were developed with anti-T421/S424 or anti-T389 Abs and visualized by the ECL method. For fluorochrome membranes and used for immunoblots that were developed with SDS sample buffer (1:1 v/v) for subsequent protein gel electrophoresis, ribosomal p70S6K enzymatic activity was quantified by using an immunocellulose kinase assay, as reported previously (30–32), tailored to measure this particular kinase activity in human neutrophils (33). Neutrophil lysates were obtained after centrifugation (7000 × g, 1 min, 4°C) in supernatants mixed with anti-p70S6K Ab conjugated to agarose beads, as indicated above. Immune complex beads were resuspended in a final volume of 40 μl of ice-cold lysis buffer (diluted 1/10) and used in an in vitro kinase assay. For this, the phosphoacceptor peptide substrate for this assay was 75 μM of the S6 kinase substrate peptide KRRKRTLTK prepared in freshly prepared kinase buffer (13.4 mM HEPES, pH 7.3, 25 mM MgCl₂, 30 mM NaCl, 1 mM Na₂VO₄, 5 mM p-nitrophenyl phosphate, 2 mM EGTA, 2 μM cAMP-dependent kinase inhibitor TTYADFIASGRGTRRNAIHD, 0.420 μM ATP (7 nM), and 68 μM unlabeled ATP). One microgram of cAMP-dependent kinase inhibits 2000–6000 phosphorylating units of protein kinase A (equivalent to the transference of 2–6 nmol of phosphate from ATP). To initiate the phosphotransferase reaction, aliquots (20 μl of kinase buffer containing the appropriate substrates were mixed 1:3 (v/v) with the cell lysates or immunocomplex beads. The reaction was conducted at 37°C for 20 min in a rotator and terminated by blotting 40 μl of the reaction mixture onto P81 ion exchange chromatography cellulose phosphate papers. Filter squares were washed, dried, and counted for radioactivity. Controls were run in parallel with no S6 kinase substrate peptide. Counts were subtracted from samples.

Transduction of proteins into living neutrophils

Delivery of proteins into neutrophils has been successfully accomplished using BioPorter, a lipid-based delivery reagent (34). We used the same reagent for the delivery (transduction) of hamartin into neutrophils, with the following protocol: per each experimental condition, an aliquot of 3.7 μl of delivery lipid reagent BioPorter (from dried powder, resuspended in 250 μl of methanol) was pipetted into the bottom of 1.4-ml Eppendorf tubes and evaporated under a N₂ stream. Hamartin protein, resuspended in 20 μl PBS, was added to the lipid delivery reagent film, then supplemented with 230 μl of serum- and BSA-free RPMI 1640, and this complex was mixed gently with 5 × 10⁶ cells. The suspension was incubated at 37°C for 1 h with slow rocking, then an aliquot of RPMI 1640 containing BSA was added to achieve 0.5% final BSA concentration, and the cells were incubated for an additional period of 30 min. Next, the transduced cells were used for either biochemical analyses or chemotaxis in Transwell. The transduction control was an approximately equimolar concentration of β-galactosidase delivered to neutrophils under the same experimental conditions.

Statistical analysis

Data are presented as the mean ± SEM. The difference between means was assessed by the Single Factor ANOVA test. Probability of less than 0.05 (p < 0.05) was considered to indicate a significant difference.

Results

GM-CSF is a chemoattractant agent for human neutrophils

We demonstrate that GM-CSF is able to induce human neutrophil chemotaxis (migration toward a stimulus) and chemokinesis (random migration) in a time-dependent manner (Fig. 1A). Maximal chemokinesis (at 4.2 × 10⁴ total cells) accounts for slightly more than one-half of the level observed for chemotaxis (7.7 × 10⁴ total cells). As indicated in Fig. 1B, the rate of migration for chemotaxis at early time points (i.e., the first 5 min) is higher than that for chemokinesis (5 × 10⁴ vs 2.2 × 10⁴ cells/min), and both are in excess of spontaneous or control migration (1 × 10⁴ cells/min). This demonstrates that: 1) neutrophils move directionally in higher numbers and at a faster rate when confronted with a GM-CSF concentration gradient, and 2) GM-CSF enhances migration capability above spontaneous movement. To fully distinguish between chemotaxis and chemokinesis, we performed the checkerboard analysis presented in Table I. These data confirm that GM-CSF is
both a chemotactic and a chemokinetic agent for neutrophils. As seen in values along the diagonal of the table, GM-CSF enhances chemokinesis at concentrations up to 10 nM, but higher concentrations do not result in further enhancement. Fig. 2 shows that the effect is specific, because it was abolished by anti-GM-CSF receptor and by anti-GM-CSF (ligand) Abs. As a negative control, Fig. 2 also shows that chemotaxis is not inhibited with an Ab against a related growth factor (FGF), further demonstrating that GM-CSF-induced chemotaxis is real and highly specific.

Accurate dose-response curves have been obtained for GM-CSF and five other neutrophil chemoattractants (Fig. 3). The concentration of GM-CSF needed to achieve maximal chemotaxis (3.3-fold over controls) is ~5–9 nM (median, 80 ng/ml), and the EC50 is ~0.9 nM (Fig. 3A). The latter is ~15 times the value of the $K_d$ for the binding of GM-CSF to its receptors on neutrophils (37–85 pM) (35, 36), and represents a value that is well expected for a whole cell physiological response (as cell migration is) vs the usually lower concentration needed to elicit a molecular event (e.g., receptor activation or Ca$^{2+}$ rise) (37). The dose-response plot results in a bell-shaped curve, in agreement with other known leukocyte chemoattractants. Effective GM-CSF concentrations are quite similar to those reported for the classical neutrophil chemokine IL-8 (~5 nM or 60 ng/ml for maximal response) (Fig. 3B) and less than those of the other chemoattractants tested: FMLP and LTB4 (Fig. 3A) and other known neutrophil chemokines: ENA-78 and GCP-2 (Fig. 3B). The potency of GM-CSF as a chemoattractant agent for neutrophils is as high as ~56% of LTB4/FMLP, and ~41% of IL-8/ENA-78/GCP-2. The chemoattractant nature of GM-CSF was also confirmed using another technical setting: the Boyden chamber (Fig. 4). Clear accumulation of migrated cells in stained filters is seen with concentrations of GM-CSF as low as 0.7 nM and peaks at values of 7 nM. The potency of GM-CSF can also be ascertained visually when compared with IL-8 in Fig. 4. All of these data indicate that GM-CSF is a bona fide chemoattractant.

**Figure 2.** Neutralization of chemotaxis by ligand and receptor Abs. For the anti-receptor experiments, freshly isolated neutrophils were incubated with anti-GM-CSF receptor (common $\beta$-chain, CDw131) before being exposed to GM-CSF and placed on the Transwell inserts. For the anti-ligand experiments, either anti-GM-CSF (clone GM4.1.9) or anti-FGF (Ab-2 polyclonal) Abs were added to the lower wells of Transwell plates in conjunction with GM-CSF. In all cases, final Ab concentrations were 15 $\mu$g/ml (or 1 $\mu$g per 6.6 ng GM-CSF). Results are the mean ± SE of three to four independent experiments. Activation by GM-CSF over control ($*$) and inhibition of GM-CSF activation by anti-GM-CSF receptor and anti-GM-CSF (**) were significant ($p < 0.05$).

**Figure 3.** Dose-response curves for several cytokines and chemoattractants. GM-CSF is compared against the neutrophil chemoattractants LTB4 and FMLP (A) and against neutrophil chemokines IL-8, ENA-78, and GCP-2 (B). Treatments are similar to those described in Fig. 1 for chemotaxis. Activation by all chemoattractants at their respective efficacy maxima over controls (no addition) was significant ($p < 0.05$).
To determine whether GM-CSF would act as a chemoattractant in GM-CSF receptor-bearing cells other than normal mature neutrophils, we performed cell migration experiments using the promyelomonocytic cell line HL60 (Fig. 5A). Exponentially cycling, immature HL60 cells that have receptors for GM-CSF do not migrate significantly upon GM-CSF exposure. However, following 3 days of differentiation induction in DMSO (DMSO-HL60 cells), GM-CSF becomes capable of stimulating migration. The spontaneous (unstimulated) mobility of HL60 cells was similar to the spontaneous mobility of neutrophils (Fig. 1). Similar results were obtained with a second myeloid cell line, MPD, that was established in the laboratories of the authors (38). These cells were induced to maturation (reaching a population of up to 95% neutrophils and segmented cells) with G-CSF for 5 days, and then showed a 2.5-fold increase in chemotaxis in Transwell plates when stimulated by GM-CSF (Fig. 5A, inset). We have also tested GM-CSF on peripheral blood monocytes, another native GM-CSF receptor-expressing cell type. As indicated in Fig. 5B, monocytes respond to GM-CSF, albeit with a lower affinity than neutrophils do. The concentration of GM-CSF needed to achieve maximal chemotaxis (2.5-fold over controls) is ≈40 nM (median, 500 ng/ml), and the EC₅₀ is ≈10 nM, about an order of magnitude higher than the neutrophil response. Conversely, PBLs, as they do not express receptors for GM-CSF, did not respond to the cytokine.

**GM-CSF has a robust effect on actin polymerization**

We next studied F-actin polymerization, a hallmark of cell migration in agonist-stimulated neutrophils. Changes in actin polymerization occurred quickly (~5 min) after the addition of GM-CSF to the cells (Fig. 6), as analyzed by flow cytometry, a technique that allowed for precise quantification of cytoskeletal changes upon cell stimulation. The figure shows the time course profile of GM-CSF-induced F-actin polymerization, and it can be seen that a maximum effect is reached at 5 min. Quantitatively, the extent of GM-CSF stimulation is ~66% of that observed with FMLP. The next series of experiments was intended to show the effect of GM-CSF on actin polymerization during cell adhesion by immunofluorescence microscopy. This was examined because actin polymerization has been observed in neutrophils adherent to solid surfaces, and cell adhesion is a prerequisite for chemotaxis (39). Normal peripheral blood neutrophils were preincubated in the presence or absence of GM-CSF on coverslips before fixing and staining with TRITC-phalloidin/DAPI to observe actin fibers and the cell nucleus under fluorescence. Fig. 7, A–D, shows that GM-CSF caused an increase in the F-actin polymerization signal, and a polarized concentration of actin around the membrane (focal contacts) was observed in confocal microscopy images. Thus, GM-CSF causes an enhancement of focal adhesion points that is a prerequisite for cell movement.

**FIGURE 5.** A, Differentiated myeloid cell lines migrate in response to GM-CSF. To ascertain whether GM-CSF elicited a chemotactic response in myeloid cells other than normal neutrophils, either exponentially growing HL60 cells or HL60 cells exposed to 1.25% DMSO for 3 days (DMSO-HL60) were placed on the upper or insert chambers of Transwell plates at the density of 2 × 10⁶ per well. The lower wells received either buffer (control), 7 nM GM-CSF, or 10 nM IL-8. The plates were incubated for 1 h at 37°C in a 5% CO₂ atmosphere. Inset, Shows a similar experiment using MPD that had been induced to maturation. Activation by GM-CSF or IL-8 over controls (+) was significant (p < 0.05) for DMSO-HL60 and Diff-MPD lines. B, Effect of GM-CSF on the migration of peripheral blood monocytes and lymphocytes. Freshly isolated mononuclear cells were further separated into the monocyte and the lymphocyte components, resuspended in RPMI 1640-based chemotaxis buffer, and placed on the upper or insert chambers of 6.5-mm Transwell plates at a density of 2 × 10⁶ per well. The lower wells received the indicated concentrations of GM-CSF. Cells were allowed to migrate, as indicated in the legend of Fig. 1. Shown is the mean ± SE of three independent experiments done in duplicate.

**FIGURE 6.** GM-CSF induces F-actin polymerization. Neutrophils were incubated with either buffer (control), 50 nM FMLP, or 10 nM GM-CSF at 37°C for the indicated lengths of time. The stimulation was stopped by fixing cells in formaldehyde. Samples (200 μl) of 1 × 10⁶ cells were incubated with phalloidin-FITC and analyzed by flow cytometry. Values on the y-axis represent relative actin content and were calculated as follows: the log fluorescence at zero time in each experimental set was normalized to a value of 100, and fluorescence intensity at different times was calculated in comparison. Inset, Linearly scaled dual-parameter light scatter diagram of nonstimulated cells showing the neutrophil population.
On the mechanism of GM-CSF-induced neutrophil migration

As known, a number of neutrophil chemoattractants exert their action through mechanisms involving receptor-coupled G proteins. However, the majority of those act through receptors that are coupled to membrane G proteins, as is the case of FMLP, LTB₄, and platelet-activating factor. We sought to investigate whether the chemotactic effect induced by GM-CSF was dependent on pertussis toxin-sensitive G proteins. Fig. 8 shows that the GM-CSF-stimulated cell migration was partially prevented by treatment with the toxin, especially at concentrations of 0.5 and 1 μg/ml. However, the control (spontaneous migration) was also affected by the treatment. The ratio GM-CSF/control expressed as percentage in the figure serves to demonstrate that the GM-CSF-induced component of neutrophil migration is only marginally (25% at best) abolished by preincubation of neutrophils with pertussis toxin. Greater inhibition was observed at concentrations >1 μg/ml, but the viability of cells decreased below 80% and, thus, were not considered.

A strong correlation exists between activation of p70S6K and GM-CSF-induced cell migration

Next, we designed experiments to explore the molecular mechanism underlying the GM-CSF receptor and membrane-related proteins. The first series of experiments assessed the effect of known inhibitors of the PI3K pathway on cell chemotaxis. It has been shown previously that wortmannin and LY294002 have an inhibitory effect on leukocyte migration (15–18). Fig. 9 corroborates these findings in GM-CSF- and IL-8-stimulated neutrophil migration. LY294002 preferentially inhibited IL-8-stimulated chemotaxis, while wortmannin was very effective in reducing cell migration elicited by either cytokine. Unexpectedly, rapamycin, at a subnanomolar concentration (the lowest of all three inhibitors used), effectively abolished GM-CSF- (and IL-8)-stimulated chemotaxis (Fig. 9). Viability of cells (assessed by trypan blue exclusion) after exposure to wortmannin and LY294002 was >95% and for rapamycin <94%, thus excluding cytotoxicity in these assays.

The second series of experiments was aimed at studying the role of p70S6K (a rapamycin-inhibitable signaling molecule) in GM-CSF-induced chemotaxis. We analyzed the phosphorylation status of three key amino acid residues in anti-p70S6K immunoprecipitates and performed studies of enzyme activity, also in immune precipitates. Immunoprecipitation with specific anti-p70S6K Abs and immunocomplex assays with the specific p70S6K peptide substrate, KKRNRRTLTK (that bears the consensus phosphorylation...
site), accurately describes p70S6K, setting it apart from similar kinases (particularly p90rsk) (40). Fig. 10 shows the results of all these analyses. First, GM-CSF caused strong phosphorylation of ribosomal p70S6K on T389/S424 residues that was dependent on the time of stimulation with GM-CSF (Fig. 10A). Next, we measured p70S6K enzymatic activity in vitro in immunoprecipitates derived from cells that had been stimulated with GM-CSF. Fig. 10B shows that GM-CSF increased p70S6K enzymatic activity. We also studied the effect of the chemokines and chemoattractants IL-8, ENA-78, GCP-2, LTB4, and FMLP. All increased phosphorylation of p70S6K activity to varying degree. ENA-78 and GCP-2 in particular, which were potent chemottractants, were also able to stimulate kinase activity. Finally, we measured phosphorylation of T389 because this has been used by several authors as a marker of activity. As Fig. 10C shows, GM-CSF also caused a robust phosphorylation of T389 and, in comparison, G-CSF induced only a marginal increase in phosphorylation.

To further illustrate the strong correlation between p70S6K and GM-CSF-induced cell migration, we demonstrated that kinase inhibitors that interfere with GM-CSF-dependent migration also attenuate GM-CSF-induced p70S6K phosphorylation. In addition to rapamycin, that inhibited cell migration (Fig. 9), we have chosen to study in depth a direct inhibitor of p70S6K, hamartin, the protein product of the tuberous sclerosis complex-1 gene. Previous reports have established a negative effect of hamartin on ribosomal p70S6K function (41–43) using mammalian cell transfection of plasmids containing the cDNAs encoding full-length hamartin and mutants. We used the approach of transducing hamartin protein into cells with a lipid transduction reagent. As shown in Fig. 11A, transduced hamartin inhibited cell migration with an IC50 of 5 nM. The inhibitory effect of hamartin was not due to toxicity of either the protein or the transduction reagent used to deliver it into the cell, because a control protein (β-galactosidase) used at equal or greater molar concentration than hamartin failed to inhibit cell migration (Fig. 11A). Fig. 11B confirms that the target of hamartin in neutrophils is the signaling kinase p70S6K, because the phosphorylation level of this protein in response to GM-CSF is diminished when cells are pretransduced with hamartin. It is believed that hamartin exerts its action on S6K in cooperation with tuberin, forming a complex (41–43). In summary, the results presented in Figs. 8–10, taken together, strongly suggest that GM-CSF-induced leukocyte migration is dependent on the activation of the mTOR/S6K pathway.

**Discussion**

These studies demonstrate that GM-CSF is a neutrophil chemotactic agent. The concentration of GM-CSF needed to achieve...
Results reported by Harakawa et al. (44) agreed with our finding that GM-CSF produces an early stimulation of neutrophil chemotaxis (32). Other earlier studies provided contradictory data on the effect of GM-CSF on neutrophil migration. In a checkerboard assay using polycarbonate filters, Wang et al. (45) demonstrated that GM-CSF induced chemotaxis in neutrophils, while Kharazmi et al. (46) were unable to detect any migration. The explanation for this might lie in the fact that the former authors used doses of GM-CSF up to 10,000 U/ml, while the latter authors reported their data using concentrations much lower (up to 100 U/ml). Results in the present study would be in agreement with those indicated previously (45). Our demonstration that GM-CSF induces F-actin polymerization in neutrophils supports the conclusion that GM-CSF is a true enhancer of neutrophil migration, because such cytoskeletal reorganization is a general prerequisite of cell locomotion and phagocytosis (39). Further support for this contention is given by the fact that other authors have demonstrated GM-CSF being a chemotactant for endothelial (47) and mesenchymal cells (48), thus effectively involving GM-CSF in tissue remodeling in addition to inflammation. The cell specificity can be summarized as GM-CSF having been a chemotactant for neutrophils and monocytes (45) (present study), endothelial and mesenchymal cells (47, 48), differentiated myeloproliferative disorder (MPD), and promyelocytic HL60 cell lines (present study), all of which express GM-CSF receptors; and GM-CSF failing to be a chemotactant in large granular lymphocytes (45) and peripheral lymphocytes (present study) that do not express the GM-CSF receptor.

Our present study has demonstrated for the first time the mechanism involved in GM-CSF-induced neutrophil migration. Traditionally, the initial mechanisms of a chemotaxant action involve a pertussis toxin-sensitive G protein-linked receptor (as is the case for FMLP, LTB₄, and platelet-activating factor, among others) and intracellular Ca²⁺ concentration transients. As indicated in Fig. 8, the effect of pertussis toxin in GM-CSF-induced migration is only minimal. A full inhibition of neutrophil migration was not expected, because it is known that the GM-CSF receptor is not a G protein-linked receptor. A marginal inhibition of both activated and random migration, as indicated in the experiment mentioned above, should lie further down the signal transduction pathways that are indeed dependent on G proteins. The full understanding of this phenomenon will require future work. Regarding calcium signaling, other authors have reported that GM-CSF does not affect the resting levels of intracellular calcium, but a large response in intracellular Ca²⁺ concentration transients is seen after preincubation of neutrophils with GM-CSF, followed by stimulation with a neutrophil agonist, such as FMLP. The simple physical stimulus of adhesion to a solid surface could also suffice for neutrophil prestimulation. In this vein, we have demonstrated earlier (49) that GM-CSF is able to activate the enzyme phospholipase D in neutrophils adherent to extracellular matrix-coated protein surfaces, while it cannot do so in neutrophils maintained in suspension.

In attempting to further elucidate the mechanism by which GM-CSF influences neutrophil locomotion, and concentrating now on signal transduction links downstream the receptor, we report in this study first evidence for a role of the mTOR/p70S6K signaling system. There was a strong positive correlation of p70S6K enzymatic activity and phosphorylation status with migration induced by GM-CSF that was abrogated by specific inhibitors of the PI3K-S6K pathway at subnanomolar concentrations. The inhibition observed with the p70S6K-specific inhibitor hamartin (Fig. 11) suggests that this downstream signaling link may be of particular importance. These findings add to previous observations that rapamycin prevents neutrophil Ca²⁺-associated stress fiber formation in neutrophils (50) and that p70S6K colocalizes with actin stress fibers near the forward edge of migrating 3T3 fibroblasts (51). We have recently shown that rapamycin inhibits GM-CSF-induced actin polymerization (52) and that it has a direct inhibitory effect on p70S6K T⁴⁴⁷/S⁴⁲⁴ and T⁸⁹⁹ phosphorylation, which can be rescued by the immunophillin FK506 (33).

Stimulation of neutrophils with other chemotaxants (IL-8, IL-1β, LTB₄, and FMLP) led to variable activation of p70S6K (Fig. 11). GM-CSF was one of the most potent stimulators of p70S6K when compared with the other chemotaxants. This could be potentially important, considering the evidence suggesting that the signaling pathways governing locomotion vary depending on the stimulus. Previous studies (53–57) showed that FMLP and C5a activate chemotaxis through p38MAPK, while IL-8 uses the PI3K pathway. IL-8 activates ERK2 (p42MAPK), but this is apparently unrelated to its effect on chemotaxis. In contrast, our laboratory (58) has shown that MEK inhibition hampers GM-CSF-induced chemotaxis. It has been suggested that individual steps of cell locomotion such as adhesion, cell polarization, and pseudopodia formation might be influenced by a series of cooperating chemokines using differing signaling pathways (59). The data in this study point to p70S6K as having a key signaling role in GM-CSF-induced neutrophil chemotaxis.
Our observations suggest a possible role for GM-CSF in the recruitment of neutrophils to areas of inflammation. Although the potency (EC₅₀) of GM-CSF is relatively high, the efficacy (maximum migration rate) seems less than that seen with other known chemotactants (Fig. 3). This may have some physiologic benefit because the influx of neutrophils would be limited in rate and might actually be inhibited once the local GM-CSF concentration exceeds 20 nM (Fig. 3). This could limit undesirable consequences of inflammation, such as collateral tissue injury. These findings suggest new avenues for the study of the mechanisms of inflammation that could be important in improving our understanding of beneficial inflammatory responses and also the dysregulated inflammation that contributes to diseases such as arthritis, asthma, and even atherosclerosis. Our findings that suggest a possible important role for p70S6K in neutrophil chemotaxis could lead to new strategies for potential treatments.

Acknowledgments
We thank Dr. Dan R. Halm for helpful insights in Discussion, Dr. Mauricio Di Fulvio for the preparation of the figures, Nick Lehman for technical assistance, and the Center for Brain Research (Wright State University) for the use of imaging facilities.

References


