Involvement of Tyrosine Kinases in the Activation of Human Peripheral Blood Neutrophils by Granulocyte-Macrophage Colony-Stimulating Factor

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The aim of the present study is to evaluate the involvement of human neutrophil tyrosine kinase(s) in the signal transduction mechanism of granulocyte-macrophage colony-stimulating factor (GM-CSF). Stimulation of neutrophils with GM-CSF resulted in a time- and dose-dependent phosphorylation of several proteins having estimated molecular weights of approximately 40, 55, 74, 97, 118, and 155 Kd, detected by immunoblot using a monoclonal antibody directed against phosphotyrosine. GM-CSF-induced tyrosine phosphorylation was inhibited in a dose- and time-dependent manner by the tyrosine kinase inhibitor erbstatin. Using this inhibitor, we were able to correlate tyrosine phosphorylation with several functional effects of GM-CSF on human neutrophils. Pretreatment of neutrophils with erbstatin before incubation with GM-CSF completely inhibited the GM-CSF-induced intracellular alkalization, downregulation of the leukotriene B4 receptor, enhancement of fMet-Leu-Phe-induced intracellular calcium mobilization, as well as the accumulation of mRNA for the proto-oncogene c-fos. Taken together, these data suggest that tyrosine kinase activation in human neutrophils plays a critical regulatory role in both the stimulation and priming of neutrophil function by GM-CSF.

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the Limulus amebocyte assay for lipopolysaccharide were negative. All products used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were reagent grade and purchased from Sigma. 

Cell separation. Whole blood was obtained by venepuncture, collected into tubes containing heparin, and after dextran sedimentation neutrophils were purified by centrifugation on Ficoll-Paque cushions. Contaminating erythrocytes were removed by hypotonic lysis and the cells were resuspended in HBSS containing Ca²⁺ (1.6 mmol/L) at a final concentration of 5 × 10⁶ cells/mL. The percentage of neutrophils in the cell preparations used in this study exceeded 97% and cell viability as determined by trypan blue exclusion was greater than 98%. The entire separation was performed at room temperature.

Measurement of intracellular calcium. Intracellular free calcium was monitored using the fluorescent probe fura-2 as described by Naccache et al. Briefly, neutrophil suspensions (1 × 10⁶ cells/mL) were incubated with 10 μg/mL erbastin for 1 hour at 37°C followed by a further incubation at 37°C with GM-CSF and 1 μmol/L FURA-2/AM for 30 minutes. The cells were then washed free of the extracellular probe, resuspended at 5 × 10⁶ cells/mL, and allowed to re-equilibrate for 10 minutes at 37°C. They were then transferred to the thermostatted cuvette compartment (37°C) of the fluorimeter and the fluorescence was monitored (excitation and emission wavelengths, 340 and 510 nm, respectively), and calibrated as described previously.

c-fos mRNA expression. Neutrophils were prepared as described previously, resuspended at 2 × 10⁶ cells/mL in HBSS and treated with either erbastin or dimethyl sulfoxide (DMSO) for 1 hour at 37°C followed by treatment with either GM-CSF or diluent control for a further 30 minutes at 37°C. The cells were then rapidly centrifuged and either frozen in a solution of dry ice/ethanol and stored at -70°C until used or processed directly. Total RNA was purified using a solution of guanidium isothiocyanate (6 mol/L guanidium isothiocyanate, 20 mmol/L sodium acetate pH 5.2, 0.1 mmol/L dithiothreitol, 0.5% sarkosyl, and 1% mercaptoethanol) and centrifugation through a cesium chloride gradient essentially as previously described. Electrophoresis was then performed on a 1% agarose gel. Integrity of RNA and equal loading was verified by staining with ethidium bromide and by hybridization with a cDNA probe that nonspecifically anneals with ribosomal RNA. The RNA was transferred onto Hybond-N membranes (Millipore Corporation, Bedford, MA) using a vacuGene apparatus (Bio-Rad, Mont-real, Quebec). Hybridization and posthybridization washes were performed under stringent conditions as previously described. A c-fos cDNA clone (a generous gift from Dr Raymond Vincent, Centre Hospitalier Robert Giffard, Quebec, Canada) labeled using the “Random Primer Method” (BRL Canada, Burlington, Ontario) was used for all hybridizations. The Northern blots were visualized by autoradiography with Kodak X-Omat films (Picker, Montreal, Quebec, Canada).

[32P]-GM-CSF binding to neutrophils. Recombinant human GM-CSF was iodinated to a specific activity of 4.8 × 10⁶ dpm/mol by the Bolton Hunter method. The iodinated GM-CSF remained biologically active. Cells used for the binding experiments were washed once and resuspended in binding buffer (Iscove’s modified Dulbecco’s medium containing 25 mmol/L HEPES and 2 mg/mL BSA, pH 7.4) at a concentration of 10⁶ cells/mL. For the experiments studying the effect of erbastin on the binding of GM-CSF to neutrophils, 200 μL of the cells was preincubated with 10 μg/mL erbastin or DMSO control for 1 hour at 37°C. The cells were incubated with [32P]-labeled GM-CSF at various concentrations in the presence or absence of a 50-fold excess of cold GM-CSF for 2 hours at 23°C. After the incubation, the cells were resuspended and transferred onto 0.75 mL of an ice-cold solution of 75% fetal bovine serum (FBS) in binding buffer. The cells were then centrifuged for 2 minutes in a microcentrifuge, the supernatant was aspirated, and the pellets were sliced off with a razor blade for the determination of their radioactivity. Specific binding was defined as the amount of binding blocked by a 50-fold excess of unlabeled GM-CSF.

Data from these binding experiments were analyzed by weighted nonlinear least squares curve fitting developed by Munson and Rodbard. Objective statistical criteria (F-test, extra sum squares principle) were used to evaluate goodness-of-fit and for discriminating between models. Curves from multiple experiments were analyzed both individually and simultaneously using constrained curve fitting to obtain precision of parameter estimates. Nonspecific binding was treated as a parameter subject to error and was fitted simultaneously with other parameters.

[3H]-leukotriene B₄, binding to neutrophils. Neutrophils were purified as described previously and resuspended in binding buffer at 5 × 10⁶ cells/mL. After incubation of the cells with erbastin as described previously, the cells were treated with either GM-CSF (1 nmol/L) or diluent control for a further 45 minutes at 37°C followed by a final incubation for 60 minutes at 4°C with an equal volume of 4 nmol/L [3H]-leukotriene B₄, (specific activity 191 to 210 Ci/mmol) and increasing concentrations of unlabeled leukotriene B₄. After the 60-minute incubation, bound [3H]-leukotriene B₄ was separated from free [3H]-leukotriene B₄ by rapidly filtering over Whatman GF/C filters, followed by a 10-mL wash with phosphate-buffered saline (PBS) at 4°C. Percent inhibition of [3H]-leukotriene B₄ binding was determined according to the following formula:

\[
\frac{\text{cpm Bound With Dilotent}}{\text{cpm Bound With GM-CSF}} - \frac{\text{cpm Bound With Dilotent Control}}{\text{cpm Bound With 100X Excess LTB₄}}
\]

Intracellular pH measurement. Intracellular pH was monitored using the fluorescent probe BCECF essentially as previously described. Briefly, neutrophil suspensions (10⁶ cells/mL) were incubated with either 10 μg/mL erbastin or the equivalent volume of DMSO for 1 hour at 37°C. BCECF (4 μmol/L) was added during the final 30 minutes of the incubation. The cells were then washed free of the probe which remained in the extracellular milieu, resuspended at 3 × 10⁶ cells/mL, incubated for 10 minutes at 37°C, transferred to a thermostatically controlled, magnetically stirred cuvette compartment of a fluorometer (SLM 8000; SLM Instruments, Inc, Urbana, IL), and the fluorescence intensity was monitored (emission and excitation wavelengths of 500 and 530 nm, respectively). The fluorescent signals were calibrated by lysing the cells with 0.1% Triton X-100 (Sigma) and recording the fluorescence of the signals at varying pH values. A correction value of 0.15 pH units was applied to the calculated values of intracellular pH as described previously and the spectra were normalized with respect to the slope of the relationship between the changes in fluorescence and the pH to account for variations in the amounts of cell-incorporated BCECF and for quenching.

Western blots for phosphotyrosine. Neutrophils were incubated at 5 × 10⁶ cells/mL with 1.0 mmol/L DFP for 5 minutes at 37°C before stimulation for varying periods of time with either DMSO, fMet-Leu-Phe (10⁻⁵ mol/L), diluent control (0.01% BSA), or GM-CSF. The reactions were terminated by rapid centrifugation,
the supernatants were discarded, and the pellets were resuspended in 70 μL of an ice-cold stopping solution (Ca²⁺ free HBSS, 10 mmol/L HEPEs at pH 7.4, 2 mmol/L Na orthovanadate, 10 mmol/L NaF, 10 mmol/L nitrophosphate, 10 mmol/L Na pyrophosphate, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Seventy microliters of the sample buffer (10% mercaptoethanol, 10% glycerol, 8% SDS in Tris-HCl, pH 6.8) was then added and the samples were denatured by boiling for 5 minutes and loaded onto a 5% to 15% SDS polyacrylamide gel. Electrophoretic transfer cells (Hoeffer Scientific Instruments, Canberra Packard Canada, Mississauga, Ontario) were used to transfer proteins from the polyacrylamide gels to Immobilon PDVF membranes (Millipore). Nonspecific sites were blocked using 3% BSA for 1 hour at 37°C. The monoclonal antiphosphotyrosine antibody PY20 (ICN Biomedical, Montréal, Québec, Canada) was then incubated with the membranes overnight at 4°C at a final dilution of 1:1,000. The membranes were washed at room temperature six times in TBS-Tween 20 solution (25 mmol/L Tris HCl, pH 8.0, 190 mmol/L NaCl, 0.05% vol/vol Tween 20) for 10 minutes each. The washed membranes were then incubated with a [³²P]-labeled goat antiserum IgG (labeled to a specific activity of 7 to 10 μCi/μg) for 1 hour at 37°C and washed six times as described previously. The phosphotyrosine bands were then visualized by autoradiography by exposing the membranes to Kodak X-Omat films overnight at -70°C.

RESULTS

Effect of GM-CSF on neutrophil tyrosine kinase phosphorylation. Neutrophils were incubated with different concentrations of GM-CSF (or diluent) for varying periods of time, or with fMet-Leu-Phe or DMSO (the diluent for fMet-Leu-Phe) for 1 minute, and the incubations were stopped by rapid centrifugation. The pellets were then resuspended in sample buffer, processed for SDS-PAGE, transferred onto Immobilon-P membranes, and the tyrosine phosphoproteins were identified by Western blot using a specific antiphosphotyrosine monoclonal antibody. In each experiment, we stimulated the cells for 1 minute at 37°C with 10⁻⁷ mol/L fMet-Leu-Phe as a “positive control” because we and others have previously shown that fMet-Leu-Phe stimulates tyrosine phosphorylation in neutrophils, and we have recently demonstrated that erbstatin inhibits this effect of fMet-Leu-Phe. In the present study, incubation of neutrophils with fMet-Leu-Phe stimulated the phosphorylation of two proteins having estimated molecular weights of approximately 40 and 118 Kd (Fig 1). Using fMet-Leu-Phe as an agonist, phosphorylation of the 40-Kd band was the most strongly and consistently enhanced over an additional 30 experiments.

Exposure of neutrophils to GM-CSF resulted in the dose-dependent tyrosine phosphorylation of neutrophil proteins. A dose-response curve to GM-CSF (20 minutes at 37°C) from one representative experiment is shown in Fig 1. Similar results were seen in eight additional experiments. Visual assessment of autoradiographs indicated that the effect of GM-CSF on neutrophil tyrosine phosphorylation was maximal at concentrations between 1 and 3 nmol/L. Over the entire study, GM-CSF most consistently stimulated the tyrosine phosphorylation of several proteins having molecular weights of approximately 40, 55, 74, 97, 118, and 155 Kd, although at times the tyrosine phosphorylation of up to 10 different bands was observed (data not shown). Densitometric analysis of the phosphotyrosine bands in the autoradiograph shown in Fig 1 provides a quantitative assessment of the effect of GM-CSF on the tyrosine phosphorylation of each of these proteins (Fig 2). The amounts of all six phosphoproteins increased relative to the control (ie, cells incubated with 0.01% BSA) in a dose-dependent manner after stimulation with GM-CSF. Tyrosine phosphorylation in response to GM-CSF was observed when the cells were stimulated with concentrations of GM-CSF between 100 and 300 pmol/L. Maximal tyrosine phosphorylation occurred upon stimulation of the

![Fig 1. Dose-dependence of the stimulation of phosphotyrosine proteins by GM-CSF in human neutrophils. Neutrophils were incubated with either fMet-Leu-Phe (10⁻⁷ mol/L) or the equivalent volume of DMSO for 1 minute at 37°C, or with increasing concentrations of GM-CSF for 20 minutes at 37°C. Western blotting using a monoclonal antibody directed against phosphotyrosine proteins was then performed as described in Materials and Methods.](image-url)
GM-CSF STIMULATES TYROSINE PHOSPHORYLATION

Fig 2. Densitometric analysis of the effects of concentration of GM-CSF on levels of tyrosine phosphorylation. The autoradiograph from the experiment shown in Fig 1 was analyzed using a Research Analysis Systems densitometer (Amersham Canada, Oakville, Ontario) and the values were corrected for background, and are expressed as percent maximal response.

Fig 3. Time-dependence of the stimulation of phosphotyrosine proteins in human neutrophils. Neutrophils were incubated with either diluent control for 1 minute (0.01% BSA) or with 1 nmol/L GM-CSF for increasing periods of time. Western blotting using a monoclonal antibody directed against phosphotyrosine proteins was then performed as described in Materials and Methods.

Fig 4. Densitometric analysis of the levels of phosphotyrosine proteins stimulated by GM-CSF. The autoradiograph from the experiment shown in Fig 3 was analyzed by densitometry as described in the legend to Fig 2.

cells with between 1 and 3 nmol/L GM-CSF. It should be noted that some variability with respect to the relative increase in tyrosine phosphorylation of each band was also observed between experiments; however, over the entire study the tyrosine phosphorylation of all six of these proteins was consistently enhanced.

Neutrophils were stimulated with 1 nmol/L GM-CSF at 37°C for various periods of time and blotted for phosphotyrosine proteins as described previously. An autoradiograph from a representative experiment is shown in Fig 3, indicating that the effect of GM-CSF was time-dependent. Densitometric analysis of this experiment is shown in Fig 4. Similar results were seen in five other experiments. Control studies showed that the level of tyrosine phosphorylation in nonstimulated cells (cells incubated with 0.01% BSA) did not significantly change over a period of 120 minutes (data not shown). In contrast, an effect of GM-CSF on tyrosine phosphorylation was consistently observed after a minimum stimulation time of between 1 and 5 minutes. The tyrosine phosphorylation induced by GM-CSF followed two different temporal patterns, one where the maximal effect was reached with between 15 and 30 minutes of stimulation after which the tyrosine phosphorylation rapidly decreased (this was apparent for the 74-, 97-, 118-, and 155-Kd proteins), and the second group (the 40- and 55-Kd phosphoproteins) whose level of phosphorylation continued to increase with up to 120 minutes of stimulation.

Effect of erbstatin on GM-CSF-induced tyrosine phosphorylation. To determine whether the tyrosine phosphorylation observed with GM-CSF was inhibitable with erbstatin, neutrophils were preincubated for 1 hour at 37°C with either DMSO or 5 or 10 µg/mL erbstatin. The cells were then stimulated with 10 nmol/L GM-CSF for 20 minutes and the samples were immunoblotted using the antiphosphotyrosine antibody as described previously. The phosphorylation of all the phosphotyrosine bands was dose-dependently inhibited after pretreatment of the neutrophils with erbsta-
Fig 5. The effect of the tyrosine kinase inhibitor erbstatin on the stimulation of the levels of phosphotyrosine proteins by GM-CSF. Neutrophils were incubated for 1 hour at 37°C with either DMSO or 5 μg/ml or 10 μg/ml of erbstatin and stimulated either for a further 20 minutes with 10 nmol/L GM-CSF or diluent for GM-CSF (0.01% BSA). Western blotting using a monoclonal antibody directed against phosphotyrosine proteins was then performed as described in Materials and Methods.

Inhibition of the 40-, 55-, 74-, and 155-Kd bands and inhibited phosphorylation of the 97- and 118-Kd bands by more than 60%. As a control measure for these studies, the effect of erbstatin on fMet-Leu-Phe-induced (10⁻⁷ mol/L fMet-Leu-Phe, 1 minute) tyrosine phosphorylation was examined in each experiment. Under these circumstances, the tyrosine phosphorylation induced by fMet-Leu-Phe was consistently inhibited (data not shown).

Effect of erbstatin on the binding of GM-CSF to neutrophils. Previous reports have demonstrated inhibition of fMet-Leu-Phe-induced tyrosine phosphorylation in neutrophils by ST638. However, this compound also inhibited the binding of fMet-Leu-Phe to its cell surface receptor, thereby rendering it impossible to draw meaningful conclusions concerning the point at which activation of a tyrosine kinase may have been involved in the fMet-Leu-Phe signal transduction pathway. Therefore, we conducted experiments to determine whether or not erbstatin was inhibiting GM-CSF-induced tyrosine phosphorylation by interfering with the binding of GM-CSF to its receptors. Neutrophils were therefore incubated for 1 hour with 10 μg/ml erbstatin as described (see Materials and Methods). Pretreatment of neutrophils with 10 μg/ml erbstatin did not alter the kd or the number of GM-CSF receptors measured on human neutrophils. A scatchard plot from a representative experiment is shown in Fig 7. Similar results were obtained in three other experiments. The relevant data are summarized in Table 1.

Effect of erbstatin on the alteration in intracellular pH induced by GM-CSF. Neutrophils were pretreated with the fluorescent probe BCECF as described in Materials and Methods and the effect of GM-CSF on intracellular pH was studied. Within 5 minutes, GM-CSF directly induced an intracellular alkalinization (Fig 8). This effect was dose-dependent and the maximal response was seen using concentrations of GM-CSF of between 100 and 1,000 pmol/L. This response was consistent with previously reported results. The minor acidification observed in this particular experiment was not consistently observed and
Neutrophils were incubated with 10 μg/mL erbstatin or diluent control (the equivalent volume of DMSO) for 1 hour at 37°C. The binding of GM-CSF to its cell surface receptor was then measured as described (see Materials and Methods). Data represent the mean ± SD from three separate experiments.

Effect of erbstatin on the GM-CSF-induced decrease in the surface receptor expression of the leukotriene B₄ receptor. Neutrophils were preincubated with GM-CSF for 45 minutes at 37°C and assayed for leukotriene B₄ receptor expression. GM-CSF dramatically reduced the binding of [³H]-leukotriene B₄ to its receptor on human neutrophils (Fig 9A). The specific binding of 4 nmol/L [³H]-leukotriene B₄ was reduced by 92.5% ± 7% (n = 7) when neutrophils were pretreated with 1 nmol/L GM-CSF for 45 minutes at 37°C (Fig 9B). Pretreatment of the neutrophils with erbstatin completely reversed the downregulation of the leukotriene B₄ receptor (Fig 9B). Erbstatin by itself had no effect on the binding of [³H]-leukotriene B₄ to untreated neutrophils (data not shown).

Effect of erbstatin on the increase in intracellular calcium mobilization induced by GM-CSF. We have previously observed that while GM-CSF by itself does not significantly alter the resting level of intracellular calcium, preincubation of neutrophils with GM-CSF leads to an increased calcium mobilization in response to subsequent stimulation.

Table 1. Lack of Effect of Erbstatin on the Binding of GM-CSF to Its Cell Surface Receptor

<table>
<thead>
<tr>
<th>Receptors/cell</th>
<th>633 ± 27</th>
<th>640 ± 37</th>
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<tr>
<td>kd (×10⁻⁹)</td>
<td>76 ± 5</td>
<td>84 ± 7</td>
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Fig 8. The inhibition by erbstatin of the GM-CSF–induced cytoplasmic alkalinization in human neutrophils. The cells were pretreated with either DMSO or 10 μg/mL erbstatin for 1 hour at 37°C. The fluorescent probe BCECF/AM (4 μmol/L) was added 30 minutes before the end of the incubation period. The cells were then washed free of the extracellular probe and intracellular pH was monitored as described. This figure is representative of four other experiments that yielded similar results.

Fig 9. The inhibition by erbstatin of the downregulation of the leukotriene B₄ receptor stimulated by GM-CSF. (A) Neutrophils were preincubated with 10 μg/mL erbstatin or DMSO for 1 hour at 37°C and incubated with GM-CSF or diluent for 45 minutes at 37°C. The expression of the leukotriene B₄ receptor was then determined in the presence of increasing concentrations of leukotriene B₄ thereby generating a displacement curve. This curve is representative of three similar experiments. (□), Control; (■), GM-CSF; (○), GM-CSF + erbstatin. (B) Neutrophils were pretreated with erbstatin or DMSO followed by stimulation with GM-CSF for 45 minutes at 37°C. The specific binding of 4 nmol/L [³H]-leukotriene B₄ was then determined as described. Data are expressed as percent inhibition of specific leukotriene B₄ binding and is the mean ± SD from seven separate experiments. (□), GM-CSF; (■), GM-CSF + erbstatin.
by chemotactic factors such as fMet-Leu-Phe. Furthermore, we have recently shown that the intracellular calcium mobilization induced by chemotactic factors is not mediated by tyrosine kinase activity because this response was not inhibited by erbstatin.\(^4\) Therefore, we conducted experiments to determine whether the priming action of GM-CSF on chemotactic factor-induced calcium fluxes could be inhibited by erbstatin. Neutrophils were incubated as described with or without erbstatin followed by a further incubation with the intracellular calcium probe FURA-2/AM with or without GM-CSF for 30 minutes at 37°C. Intracellular calcium mobilization studies were then performed as described (see Materials and Methods). As reported previously, erbstatin had no effect on the mobilization of calcium induced by fMet-Leu-Phe, and GM-CSF significantly enhanced the ability of fMet-Leu-Phe to increase intracellular calcium levels (Fig 10). In contrast, pretreatment of the cells with erbstatin before incubation with GM-CSF completely abrogated the priming by GM-CSF of fMet-Leu-Phe–induced calcium mobilization. The same results were observed using either leukotriene B\(_4\), or PAF in place of fMet-Leu-Phe as the second signal (data not shown).

**Effect of erbstatin on the expression of c-fos mRNA induced by GM-CSF.** To determine whether the stimulatory effect of GM-CSF on neutrophil gene expression is under tyrosine kinase control, neutrophils were pretreated with erbstatin as described, and stimulated with either GM-CSF or diluent control (0.01% BSA) for a further 30 minutes. Total RNA was then prepared and processed for analysis of the expression of mRNA for the proto-oncogene c-fos. As previously reported,\(^4\) control neutrophils exhibited a minor constitutive expression of c-fos mRNA (Fig 11, lane A) that was enhanced after stimulation of the cells with GM-CSF for 30 minutes (Fig 11, lane B). This enhanced expression of c-fos mRNA induced by GM-CSF was significantly inhibited by pretreatment with erbstatin (Fig 11, lane C). In these experiments, erbstatin also exhibited an inhibitory effect on the nonstimulated levels of c-fos mRNA (Fig 11, lane D), most likely indicating that the constitutive expression of c-fos is also under tyrosine kinase control. Densitometric analysis of each of three experiments showed the same overall trend (Fig 12). GM-CSF consistently stimulated a threefold increase in c-fos mRNA levels and this enhancement was significantly inhibited by pretreatment with erbstatin.

**DISCUSSION**

The activities of many growth factors and other cytokines appear to be very closely associated with stimulation of a variety of tyrosine kinases. For example, the receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulinlike growth factor (IGF), fibroblast growth factor (FGF), and macrophage-CSF (M-CSF) contain tyrosine kinase domains and tyrosine-phosphorylate specific substrates.\(^6\) Furthermore, alterations in tyrosine kinase domains of such receptors results in an altered growth and transforming ability of these receptor-ligand systems.\(^6\) In addition, it has recently come to light that protein tyrosine kinases may also play an important role in the activation pathways of terminally differentiated cells. Recent studies have shown that myeloid cells such as neutrophils possess tyrosine kinases such as hck, c-fes, and...
and that these tyrosine kinases may play important roles in the cellular activation mechanisms within both hematopoietic and nonhematopoietic cells. Indeed, it is clear at this time that several “classical” neutrophil agonists such as the chemotactic factors fMet-Leu-Phe, leukotriene B₄, and C5a stimulate neutrophil tyrosine kinases⁴¹,⁴² that are critical for superoxide production and alteration of intracellular pH, but do not appear to be involved in others such as calcium mobilization, actin polymerization, and degranulation.⁴¹

In the current study, we have extended the observation that tyrosine kinase(s) is/are involved in certain intracellular pathways of neutrophil activation by chemotactic factors to include both direct and priming effects GM-CSF on human neutrophils. Three of the direct effects of GM-CSF on neutrophils were abrogated by the selective tyrosine kinase inhibitor erbstatin. These included alteration of intracellular pH, downregulation of the leukotriene B₄ receptor, and accumulation of mRNA for the proto-oncogene c-fos. In addition, we have shown that erbstatin inhibited GM-CSF-induced tyrosine phosphorylation of at least six substrates in human neutrophils. These effects of erbstatin could not be explained by an attenuation of GM-CSF binding to its cell surface receptor because multiple experiments failed to demonstrate an effect of erbstatin on ¹²⁵I-GM-CSF binding to human neutrophils.

While it is possible that the inhibition of the effects of GM-CSF by erbstatin is due to interference of as yet unknown signal transduction pathways in neutrophils, our results indicate that the inhibition of GM-CSF-induced neutrophil activation by erbstatin closely correlated the inhibitory effects of erbstatin on GM-CSF-induced tyrosine kinase activation in both a temporal and dose-dependent manner. Therefore, it is quite likely that erbstatin inhibits the stimulation of human neutrophils by GM-CSF by interfering with the activation of one or more tyrosine kinases. Moreover, previous studies with this compound have indicated that its inhibitory effects are highly selective for tyrosine kinases. For instance, it has been established that erbstatin inhibits semi-purified EGF receptor autophosphorylation in A431 cells,⁳⁰ and inhibits tyrosine kinase activation by competing with peptide substrates for the active site of the EGF receptor kinase.⁴³ In addition, erbstatin has been shown to be without effect on purified protein kinase C (PKC) or protein kinase A (PKA),⁴³ although it has recently been shown that high concentrations of erbstatin (approximately twofold to fivefold greater than those used in the present study) inhibited PKC activity in a cell-free system. However, in the same report erbstatin was without effect on the PKC-mediated phosphorylation in intact cells.⁴³ Apart from these previous demonstrations attesting to the specificity of erbstatin for tyrosine kinases (at least in intact cells), it has also repeatedly been shown that the effect of GM-CSF in neutrophils is not mediated by PKC.⁴⁴,⁴⁵ In addition, we have recently shown that erbstatin blocks fMet-Leu-Phe–induced tyrosine phosphorylation in neutrophils without inhibiting phospholipase C activation or degranulation induced by fMet-Leu-Phe.⁴⁵ These latter results indicate that erbstatin is not toxic to neutrophils.

Human peripheral blood neutrophils appear to express only one class of GM-CSF receptor, a high-affinity receptor with a kd of approximately 100 to 300 pmol/L.⁴⁶ At these concentrations, the effect of GM-CSF on tyrosine phosphorylation was observed most dramatically on the 155-, 118-, 97-, and 74-Kd substrates. In fact, the effect of GM-CSF on the 55- and 40-Kd substrates was not usually apparent unless the cells were stimulated with concentrations of GM-CSF of 1 nmol/L or greater. In addition, the time course experiments with GM-CSF indicated the presence of at least two temporally distinct groups of substrates. In one group, the proteins were rapidly phosphorylated after stimulation by GM-CSF (155-, 118-, 97-, and 74-Kd substrates). Maximal phosphorylation of these proteins occurred within 5 to 15 minutes after stimulation and declined rapidly, approaching control levels after 60 minutes of stimulation. The second group (40- and 55-Kd substrates) were phosphorylated more slowly and reached maximal levels after 60 to 120 minutes of stimulation. These observations indicate that the effect of GM-CSF on the first four substrates may have more physiologic relevance than its effect on the 40- and 55-Kd substrates, or alternatively, that these two sets of substrates may be involved in different actions of GM-CSF. Evidence in support of the latter theory derives from previous studies indicating differences in the preincubation times required for the maximal priming effect of GM-CSF on distinct neutrophil functions such as calcium mobilization, leukotriene synthesis, and superoxide production.⁴,¹⁵,¹⁷,²³

Several recent studies using a variety of different cell types ranging from interleukin-3 (IL-3)– and GM-CSF–dependent human and murine cell lines to fully differentiated cells such as human neutrophils have demonstrated a large number of tyrosine phosphoproteins of varying molecular weights after stimulation with GM-CSF. Our present results are best related to those of Gomez-Cambronero et
al., who showed in two separate studies that GM-CSF stimulates tyrosine phosphorylation of proteins having molecular weights of 40, 54, 78, 92, and 118 Kd (although the 118-Kd phosphoprotein was only observed in one of the studies). However, in our study we found that the major effect of GM-CSF was on the tyrosine phosphorylation of a 155-Kd protein (see Fig 1, 3, and 5). The tyrosine phosphorylation of this protein was enhanced up to 50-fold by GM-CSF. Furthermore, in our study, phosphorylation of the 74- and 119-Kd proteins were enhanced up to threefold and fourfold after stimulation by GM-CSF. These findings are in contrast to those of Gomez-Cambronero et al., who did not report finding a 155-Kd phosphotyrosine protein and indicated that the 40- and 55-Kd proteins were most consistently and strongly phosphorylated by GM-CSF. The reasons for these variations are presently unclear but may be related to the fact that we used a monoclonal antiphosphotyrosine antibody that may have provided greater (or different) sensitivity and specificity than the polyclonal antibody used in their study.

In the current report, we have presented data that indicate at least four of the functional consequences of tyrosine kinase activation by GM-CSF in human neutrophils. However, the specific mechanism by which GM-CSF receptor-binding activates cellular tyrosine kinases remains unknown. Studies with pertussis toxin have indicated that the GM-CSF receptor is functionally linked to a G protein, and it has been reported that pertussis toxin inhibits the ability of GM-CSF to tyrosine phosphorylate at least some of the phosphoproteins detected in neutrophils. In addition, the recent cloning and sequencing of both low and high-affinity human GM-CSF receptor subunits has indicated a lack of tyrosine kinase domains. These results strongly suggest that the stimulation of neutrophil tyrosine kinase activity by GM-CSF occurs at a step distal to a G protein. Further studies are presently being performed in an attempt to better define this pathway.

While the precise mechanism by which GM-CSF both directly stimulates and primes neutrophils for increased responses to second signals remains unknown, the key to understanding the details of the GM-CSF signal transduction pathways in mature neutrophils is likely to lie in the identification of one or all of the proteins whose tyrosine phosphorylation is enhanced when neutrophils are stimulated with GM-CSF and of the specific tyrosine kinases whose activities are altered by GM-CSF. Several recent studies may provide an indication of the possible identity of some of these phosphotyrosine proteins. Of particular relevance may be the fact that stimulation of IL-3-dependent murine and human myeloid cell lines with either IL-3 or GM-CSF stimulates tyrosine phosphorylation of c-raf, a 74-Kd protein. Thus, it is tempting to speculate that the 74-Kd protein documented in the present study may be c-raf. In addition, studies in PDGF-stimulated Swiss 3T3 fibroblasts have shown that several tyrosine phosphoproteins of similar molecular weights to those reported in the present study coimmunoprecipitate with the PDGF receptor. These proteins have been identified as PLC-γ (140 Kd), GAP (120 Kd), PI-3 kinase (85 Kd), and c-raf (74 Kd). While the precise nature and function of this PDGF receptor signaling complex requires clarification, it has further been postulated that the association of these proteins with the PDGF receptor is vital to the function of the PDGF signal transduction pathway. The determination of an equivalent GM-CSF receptor signaling complex in neutrophils as well as the identification of the phosphotyrosine proteins documented in the present report awaits the availability of antihuman GM-CSF receptor antibodies for coimmunoprecipitation studies.

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Involvement of tyrosine kinases in the activation of human peripheral blood neutrophils by granulocyte-macrophage colony-stimulating factor

SR McColl, JF DiPersio, AC Caon, P Ho and PH Naccache