Regulation of Stimulated Integrin Surface Expression in Human Neutrophils by Tyrosine Phosphorylation


The control of the adhesive properties of human neutrophils is an essential element of their defense function. One level at which this control is exerted involves the upregulation of the surface expression of β2-integrins. In this study, we have examined the potential involvement of tyrosine phosphorylation in the latter process. Two inhibitors of tyrosine kinases with differing modes of action, arsstatin and herbimycin A, were found to inhibit the expression of CD11b and CD18 stimulated by chemotactic factors (fMet-Leu-Phe or leukotriene B4) or growth factors (tumor necrosis factor α). This inhibition was not shared by an inactive analog of arsstatin or by the protein kinase C inhibitor Ro 31-8230. Erbstatin also inhibited the unrolling of activation-specific neoepitopes detected by antibody CBRM1/5. Pretreatment of neutrophils (but not of endothelial cells) with erbstatin inhibited the stimulation of neutrophils’ adherence to endothelial cells induced by fMet-Leu-Phe. Augmentation of tyrosine phosphorylation by inhibiting tyrosine phosphatases using hydroperoxyxidylate led to an increased surface expression of CD11b and CD18 and enhanced the adhesion of neutrophils to endothelial cells. Finally, the leumedin NPC 15689, which had previously been shown to inhibit stimulated CD11b expression and neutrophil adherence to endothelial cells and to exhibit anti-inflammatory properties in various in vivo models of inflammation, inhibited the stimulation of tyrosine phosphorylation induced by fMet-Leu-Phe. Taken together, these data establish a strong correlation between tyrosine phosphorylation and integrin upregulation in stimulated human neutrophils.

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sine phosphorylation. Taken together, these results strongly implicate a tyrosine phosphorylation-dependent event in the regulation of the surface expression of the β2-integrins in human neutrophils and therefore in the control of the adhesive properties of these cells.

MATERIALS AND METHODS

Compounds and reagents. Ficoll-Paque and Dextran T-500 were from Pharmacia (Dorval, Québec, Canada). Hank's Buffered Salt Solution (HBSS), RPMI 1640, and fetal calf serum (FCS) were from Gibco Labs (Grand Island, NY). Formyl-Methionyl-Leucyl-Phenylalanine (fMet-Leu-Phe) and phorbol 12-myristate, 13-acetate (PMA) were obtained from Sigma Chemical Company (St Louis, MO). Leukotriene B4 was a generous gift from Dr. R. Young (Merck-Frosst, Dorval, Québec, Canada). NPC 15669 and NPC 14692 were prepared as previously described. All stock solutions were prepared in low-endotoxin HBSS or dimethyl sulfoxide (Sigma) and diluted in the incubation medium. The monoclonal antiphosphotyrosine antibody UB 05-321 and the horseradish peroxidase-labeled sheep anti-mouse IgG were purchased from Upstate Biotechnology Inc (Lake Placid, NY). Monoclonal antibody CBRM1/5 was generously provided by The Center for Blood Research (Boston, MA). The enhanced chemiluminescence (ECL) Western blotting system was provided by The Center for Blood Research (Boston, MA). The enhanced chemiluminescence (ECL) Western blotting system was acquired from Amersham Corp (Arlington, IL). The monoclonal anti-CD11b (OKM1) and anti-CD18 (TS1/18.1.2.11) antibodies were generated from their respective hybridomas (ATCC CRL 8026 and ATCC HB 203, respectively; American Type Culture Collection, Bethesda, MD). Fluorescein-isothiocyanate (FITC)-conjugated goat antitmouse IgG (GAM-IgG) was purchased from Coulter Electronic (Montréal, Quebec, Canada). Vanadyl hydroperoxide (V₄'OOH) was prepared by 15 minutes of incubation of equimolar concentrations of sodium orthovanadate and hydrogen peroxide (10 mmol/L) as described in Bourgoin and Grinstein. Recombinant tumor necrosis factor α (TNFα) was a generous gift from Knoll Pharmaceuticals (Whippany, NJ).

Purification of neutrophils. Venous blood from healthy volunteers was steriley collected on citrate/phosphate/dextrose/adenine anticoagulant solution. Neutrophils were isolated by means of 2% dextran sedimentation followed by standard techniques of Ficoll-Paque gradients and hypotonic lysis of erythrocytes. Neutrophils were resuspended in HBSS, pH 7.4, at a final concentration of 2.5 x 10⁷ cells/mL. Final cell preparations contained at least 98% neutrophils and cell viability exceeded 97% as measured by the release of lactate dehydrogenase activity.

Flow cytometric analysis of integrin expression. Neutrophils isolated as described above were resuspended in HBSS at 2.5 x 10⁷ cells/mL. They were then incubated for the indicated lengths of time with the desired compounds at 37°C. The cells were then washed twice, resuspended in RPMI medium containing 10% decomplemented FCS at a concentration of 5 x 10⁶ cells/mL and stimulated with the indicated agonists. The reactions were stopped by 15 seconds of centrifugation in a microcentrifuge and the supernatants were discarded. The first antibody (anti-CD11b, anti-CD11b, or anti-CD18) was then added in RPMI medium containing 10% FCS and the cells were incubated for 30 minutes at 4°C. The cells were washed twice in the same medium and GAM-FITC was added for a 30-minute period in the dark at 4°C. After two washes in RPMI + FCS, the fluorescence of the cells was evaluated by flow cytometry, and the mean fluorescence values were linearized. The data are expressed as the percentage of the value obtained with control, unstimulated cells. Each experiment was repeated at least three times.

Immunoblotting with antiphosphotyrosine antibodies. Freshly purified human neutrophils were incubated at 15 x 10⁶ cells/mL with the leumeds at 37°C before stimulation with fMet-Leu-Phe (10⁻⁶ mol/L for 1 minute). The reactions were terminated by adding 100 μL of cell suspensions to 100 μL of boiling sample buffer (Tris-HCl, pH 6.8, 2 mmol/L NaCl, 0.5% Nonidet P-40, 10 mmol/L NaF, 10 mmol/L pyrophosphate, 1 mmol/L phosphate, 10 mmol/L NaF, 10 mmol/L Na3PO4, 10 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 20% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol, 17.5% glycerol, and 0.1% bromophenol blue). The samples were immediately denatured by boiling for 7 minutes at 100°C and loaded onto a 7.5% to 20% SDS polyacrylamide gel. After electrophoresis, the samples, together with the molecular weight markers (Sigma), were transferred onto Immobilon PVDF membranes (Millipore Corp., Bedford, MA) with the use of electrophoretic transfer cells ( Hoeffer Scientific Instruments, Canberra Packard, Ontario, Canada). The blots were then stained with Ponceau S and subsequently destained in distilled water to locate the molecular weight markers on the blots. Nonspecific sites were blocked using 2% gelatin in TBS-Tween 0.1% (25 mmol/L Tris-HCl, pH 8.0, 190 mmol/L NaCl, 0.1% vol/vol Tween 20) for 1 hour at 37°C. The monoclonal antibody UB 05-321 was then incubated with the membranes for 1 hour at 37°C at a final dilution of 1:4,000 in 2% gelatin, 0.1% TBS-Tween. The membranes were washed at room temperature three times in TBS-Tween 0.1% and further incubated with horseradish peroxidase-labeled sheep antirabbit IgG at 45 minutes at 37°C at a final dilution of 1:20,000 in 2% gelatin, 0.1% TBS-Tween. The membranes were then washed three times as described above and covered with the ECL detection solution according to the manufacturer’s instructions. The phosphotyrosine bands were visualized by autoradiography after exposure to Kodak X-Omat films (Eastman Kodak, Rochester, NY). Selected autoradiograms were analyzed using a Research Analysis System densitometer (Amersham Canada, Oakville, Ontario, Canada) and the values were corrected for background and expressed as arbitrary units.

Adherence assay. Human umbilical vein endothelial cells (HUVEC) were grown to confluence and neutrophil adhesion to this biologic substratum was tested as previously described. Briefly, umbilical cord veins were rinsed of formed blood elements with phosphate-buffered saline (PBS) containing antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B). Collagen (2.5 mg/mL; 149 U/mg) was instilled into the vein and that cord incubated for 20 minutes at 37°C. The cords were gently massaged to ensure detachment of endothelial cells from the vessel wall. The digest was collected into centrifuge tubes and the collagenase was inactivated with FCS and centrifuged (400 g for 10 minutes at 25°C). The pellet was resuspended in M199 containing 10% FCS and antibiotics and plated in 25-cm² flasks. The cultures were incubated in 5% CO₂ at 37°C and 96% humidity, expanded by trypsinization, and grown to confluence in 48-well plates.

Neutrophils from healthy donors were purified by dextran sedimentation followed by hypotonic lysis and Histopaque centrifugation as previously described. Except for the dextran sedimentation step, which was performed at room temperature, the cells were kept at 4°C throughout the isolation procedure. Cell preparations contained greater than 95% neutrophils with greater than 99% viability using Trypan Blue dye exclusion. After isolation, neutrophils were resuspended at a final concentration of 2 x 10⁶ cells/mL in PBS. Neutrophil adherence assay was a modification of the method of Fehr and Duhinden. Briefly, neutrophils were labeled by incubating neutrophils (2 x 10⁶ cells/mL) with 30 μCi/mL of ³¹NaClO₃ at 37°C for 60 minutes. The cells were washed three times and resuspended in PBS. Neutrophils were allowed to adhere to the endothelial monolayers for 60 minutes under control conditions and in the presence of fMet-Leu-Phe (10⁻⁶ mol/L), in another set of experiments, neutrophils (2 x 10⁶ cells/mL) were preincubated with erbstatin (10 μg/mL) for 60 minutes at 37°C, washed three times,
Inhibition of integrin expression by tyrosine kinase inhibitors. The ability of the chemotactic factor fMet-Leu-Phe to stimulate the expression of the integrin CD11b in control, untreated, human neutrophils and in erbastatin-treated cells was first investigated. In these experiments, the cells were treated with 5 μg/mL erbastatin or its inactive analog 5-O-methyl erbastatin for 1 hour before being stimulated with 10^{-7} mol/L for 10 minutes. The effects of the inhibitor and the chemotactic peptide were assayed for the level of expression of CD18 and inhibited the Met-Leu-Phe-induced upregulation of CD18 in much the same way it affected that of CD11b. In preliminary experiments, 5-O-methyl erbastatin was without effect on the basal or fMet-Leu-Phe-stimulated levels of CD18 (data not shown). On the other hand, no significant effects of fMet-Leu-Phe or erbastatin were noted on the levels of expression of CD11a (results not shown).

The inhibitory effects of erbastatin were not limited to the level of expression of CD11b. CBM115 is a newly characterized antibody that recognizes epibodies on CD11b expressed only after activation of the cells. Its use allows the monitoring of the “activation state” of CD11b. As shown in Fig 3, erbastatin, but not its inactive analog 5-O-methyl erbastatin, inhibited the stimulation of the expression of the activation epitope on human neutrophils stimulated by fMet-Leu-Phe or PMA.

The inhibition of the stimulation of integrin expression by erbastatin was shared by another tyrosine kinase inhibitor, herbimycin A. In these experiments, the cells were treated with 10 μmol/L herbimycin for 10 minutes at 37°C. Conditions that were found to be optimal for the inhibition of the stimulation of tyrosine phosphorylation by fMet-Leu-Phe (results not shown). Under these conditions, herbimycin A had no effect on the basal levels of CD11b or CD18 expression, but significantly inhibited the increased expression induced by fMet-Leu-Phe, but not by PMA (results not shown).

Inhibition by erbastatin of agonists other than fMet-Leu-Phe. The ability of erbastatin to inhibit the increased expression of integrin by stimuli other than fMet-Leu-Phe was tested next. Erbastatin inhibited the increased expression of CD11b and CD18 induced by the lipid mediator, leukotriene B_4. The effect of the tyrosine kinase inhibitor was observed at the same concentrations that affected the responses to fMet-Leu-Phe (results not shown). The effects of erbastatin were not limited to interference with chemotactic factors because the increased expression of CD11b induced by the tyrosine kinase inhibitor was not caused by a decrease of the upregulation rate. Whereas the stimulation of CD11b expression by fMet-Leu-Phe reached a plateau after about 10 minutes in control cells, no increase in CD11b was evident for up to 20 minutes after the addition of the chemotactic factor to erbastatin-treated neutrophils (results not shown).

The effects of erbastatin on the fMet-Leu-Phe-stimulated expression of CD11b as concentration-dependent (Fig 2, left panel). Increasing concentrations of erbastatin (1 to 5 μg/mL) progressively reduced the unstimulated level of expression of CD11b as well as the ability of fMet-Leu-Phe to increase this parameter of neutrophil activation.

The effects of fMet-Leu-Phe on the expression of CD11a and CD18 in the absence or presence of erbastatin were investigated next. As shown in the right panel of Fig 2, erbastatin (1 to 5 μg/mL) decreased the unstimulated levels of expression of CD18 and inhibited the fMet-Leu-Phe-induced upregulation of CD18 in much the same way it affected that of CD11b. In preliminary experiments, 5-O-methyl erbastatin was without effect on the basal or fMet-Leu-Phe-stimulated levels of CD18 (data not shown). On the other hand, no significant effects of fMet-Leu-Phe or erbastatin were noted on the levels of expression of CD11a (results not shown).
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Fig 2. Concentration-dependence of the effects of erbastatin in the basal and fMet-Leu-Phe-stimulated levels of CD11b and CD18 in human neutrophils. The cells were incubated with the indicated concentrations of erbastatin for 1 hour at 37°C before being challenged, or not, with 10^{-7} mol/L fMet-Leu-Phe for 10 minutes. *P < .01 versus unstimulated cells; **P < .01 versus untreated cells. (A) CD11b; (B) CD18. Mean ± SEM of three experiments, each performed in duplicates. (□) Control; (□) fMet-Leu-Phe.

cytokine TNFα was also abrogated by the tyrosine kinase inhibitor (results not shown).

Effects of tyrosine phosphatase inhibitors on tyrosine phosphorylation, integrin expression, and neutrophil adherence to endothelial cells. Hydroperoxyvanadate has been shown to inhibit tyrosine phosphatases. Its addition to human neutrophils resulted in a time- and concentration-dependent increase in the level of tyrosine phosphorylation (results not shown). Concomitantly, the addition of 100 μmol/L hydroperoxyvanadate for 15 minutes at 37°C to a suspension of human neutrophils caused an upregulation of CD11b and CD18 by 25% and 30%, respectively (Fig 4, left panel). In addition, 15 minutes of incubation of neutrophils with 100 μmol/L hydroperoxyvanadate also stimulated the adherence of the cells to a monolayer of cultured endothelial cells (Fig 4, right panel).

Fig 3. Effect of erbastatin and 5-O-methyl erbastatin on the staining of human neutrophils with CBRM1/5. The cells were incubated with 5 μg/ml erbastatin (ERB) or 5-O-methyl erbastatin (5M-ERB) for 1 hour at 37°C. They were then stimulated with (A) 10^{-7} mol/L fMet-Leu-Phe or (B) PMA for 10 minutes. The intensity of staining with CBRM1/5 was determined as described in Materials and Methods. *P < .05 versus untreated cells. Mean ± SEM of three experiments, each performed in duplicates.

Lack of effect of protein kinase C inhibitors. The potential involvement of protein kinase C in the upmodulation of integrin expression stimulated by fMet-Leu-Phe was tested next using the recently described inhibitor Ro 31-8330. As shown in Fig 5, 5 minutes of incubation with 0.33 μmol/L Ro 31-8330 (conditions optimal for the inhibition of PMA-induced superoxide production [data not shown]) did not inhibit to any significant degree the effects of fMet-Leu-Phe on the surface levels of CD11b or CD18. A small, but statistically significant, increase in the basal integrin levels was noted in Ro 31-8330-treated cells. At the concentrations used, Ro 31-8330 did not affect the stimulation of tyrosine phosphorylation induced by fMet-Leu-Phe (data not shown).

Effect of leumedin on tyrosine phosphorylation. Leumedin represents a family of recently described anti-inflammatory compounds that have been shown to inhibit...
The cells were treated for 15 minutes with 100 pmol/L expression of CD11b and CD18 and on the adherence determined and compared with that of control, untreated cells. Mean ± SEM of three (integrin expression) or four (adherence assays) experiments, each performed in duplicates.

Effect of erbstatin on neutrophil adherence to endothelial cells. The effects of erbstatin on the Met-Leu-Phe-stimulated adherence of human neutrophils to cultured HUVEC monolayers were tested next. In these experiments, neutrophils or endothelial cells were pretreated with erbstatin, the excess inhibitor was removed by washing, and the percentage of neutrophils that adhered to endothelial cells in the absence or presence of a stimulation by Met-Leu-Phe was measured. The addition of the chemotactic factor increased the percentage of adherent cells from 5.2% ± 0.8% to 38.2% ± 4.8% (mean ± SEM of 8 separate determinations). The addition of erbstatin slightly, but significantly, increased the basal level of adherence up to 8.8% ± 1.3% and 10.1% ± 2.2%, depending on whether neutrophils or endothelial cells were pretreated. More significantly, pretreatment of neutrophils with erbstatin significantly decreased the stimulation of their adherence response to Met-Leu-Phe by nearly 40%, whereas pretreatment of endothelial cells had no effect (Fig 7). Erbstatin was also found to inhibit leukotriene B_4-stimulated adherence to endothelial cells. On the other hand, the responses to phorbol esters such as PMA were unaffected (results not shown).

DISCUSSION

The results of the experiments described above provide evidence implicating tyrosine phosphorylation in the regulation of the surface expression and conformation of physiologically relevant antigens, namely the CD11b and CD18 members of the β2-integrin family. Inhibition of chemotactic factor-stimulated tyrosine phosphorylation (but not of pro-
analog of erbstatin, 5-O-methyl erbstatin, which lacks inhibi-

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Firstly, two unrelated tyrosine kinase inhibitors, erbstatin

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Secondly, the Leu-

Cells Treated with Erbstatin

Fig 7. Effect of erbstatin on the stimulation of the adherence of human neutrophils to endothelial cells induced by fMet-Leu-Phe. In these experiments, neutrophils or endothelial cells were pretreated with erbstatin (5 µg/mL) for 1 hour at 37°C. The cells were then washed and allowed to adhere to endothelial cells in the presence of 10−7 mol/L fMet-Leu-Phe for 60 minutes, and the percentage of adherent cells was measured as described in Materials and Methods. Inhibition was measured with respect to the respective controls. Mean ± SEM of eight separate determinations.

tein kinase C-mediated serine/threonine phosphorylation) was shown to abrogate the increase in surface expression of CD11b and CD18 as well as the appearance of an activation-specific neo-epitope and to inhibit the chemotactic factor-stimulated adherence of neutrophils to endothelial cells. Conversely, inhibition of tyrosine phosphatases (resulting in increases in tyrosine phosphorylation) led to increases in the levels of membrane-bound integrins and to an increased spontaneous adherence to endothelial cells. Finally, the leu-

The relationship between tyrosine phosphorylation and integrin expression induced by chemotactic factors. Taken together, these results indicate that tyrosine phosphorylation, and not serine/threonine phosphorylation (as mediated by protein kinase C), plays a major regulatory role in the fusion of integrin-containing granules with the plasma membrane and in the regulation of integrin function.

The critical role of tyrosine phosphorylation in integrin-dependent events is further highlighted by the recent report of stimulation of tyrosine phosphorylation in fibroblasts and of p125FAK, a cytosolic tyrosine kinase, in platelets after exposure of surface integrins to their physiological ligands. We have similarly observed that the addition of anti-CD11b (but not of anti-CD18) antibodies induces the phosphorylation of a 120-kD substrate in human neutrophils that is, however, immunologically distinct from α5 (unpublished observations). Fuertes et al. have also recently reported that the stimulation of human neutrophils adhered to fibronectin-coated surfaces by TNFα leads to a stimulation of the tyrosine phosphorylation of a 120-kD substrate that is not recognized by anti-p125FAK antibodies. The constitutive and stimulated serine/threonine phosphorylation of β2-integrin components has previously been reported and a relationship to stimulated adhesion has been suggested. The lack of effect of a potent and relatively specific protein kinase C inhibitor Ro 31-8330 on the chemotactic factor-stimulated upmodulation of CD11b/CD18 indicates, however, that the signalling pathways dependent on protein kinase C are not critical to the enhancement of integrin expression induced by chemotactic factors. This interpretation is consistent with previous observations that inhibition of protein kinase C did not affect C5a-induced and only modestly reduced phorbol ester-stimulated CD11b upregulation or fMet-Leu-Phe–stimulated neutrophil adhesion. Intriguingly, Ro 31-8330 increased to a small extent the basal and stimulated levels of CD11b and CD18, and staurosporine (a potent but nonspecific protein kinase C inhibitor) prolonged the stimulation of adherence induced by fMet-Leu-Phe. However, it should be pointed out that the phorbol ester-induced upmodulation of integrins was unaf-
fected by erbstatin or herbimycin A and inhibited to a partial but significant degree by Ro 31-8330 (unpublished observations). Thus, protein kinase C-mediated integrin upmodulation is indeed possible, but appears to be unlikely after stimulation by chemotactic factors.

Stimulation of human neutrophils with chemotactic factors leads to increases in the levels of tyrosine phosphorylation of several substrates. With the exception of MAP kinase, the function of which is presently unknown, none of the tyrosine phosphorylated substrates have been identified. It is presently unclear which, if any, of the tyrosine phosphorylated substrates is directly related to the fusion of the integrin-containing granules. It is tempting to suggest a potential, but as yet unproven, role for calpain(s) in this process because these proteins have been shown to play roles in granule fusion and because some of their functions are modulated by tyrosine phosphorylation.

It is equally intriguing to note that the stimulation of tyrosine phosphorylation has been correlated to that of the lipid-modifying phospholipase D. These observations suggest a relationship between the generation of phosphatidate and/or lysophosphatidate and the upregulation of integrins that is worthy of investigation.

In summary, the above data provide strong evidence for a new mechanism of regulation of the adhesive properties of human neutrophils. Specifically, these data establish a correlation between tyrosine phosphorylation and integrin upregulation in stimulated human neutrophils. Additionally, they raise several questions. Firstly, these data indicate that studies into the nature of the tyrosine phosphorylated substrates are called for, and should focus, in part at least, on the identification of proteins capable of modulating membrane fusion. Secondly, the potential role of tyrosine phosphorylation in the stimulated changes in affinity of integrins remains to be investigated. Thirdly, these data raise the possibility of specifically modulating the adhesion-dependent in vivo responses of neutrophils by interfering with the upregulation of integrins. The parallel inhibitory effects of the leukocidins on integrin upmodulation, on tyrosine phosphorylation, and on animal models of inflammation strongly support such a link.

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