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, erbstatin 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 erbstatin or by the protein kinase C inhibitor Ro 31-8230. Erbstatin also inhibited the upregulation 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 hydroperoxynitrate led to an increased surface expression of CD11b and CD18 and enhanced the adhesion of neutrophils to endothelial cells. Finally, the leumedin NPC 15669, 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. © 1994 by The American Society of Hematology.

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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 adhe-
sive 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 anti-
body 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 acquired from Amersham Corp (Arlington, IL). The monoclonal
anti-CD11b (OKM1) and anti-CD18 (TS1/18.12.11) 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
antimouse IgG (GAM-IgG) was purchased from Coulter Electronic
(Montréal, Québec, Canada). Vanadyl hydroperoxide (V4'-OOH)
was prepared by 15 minutes of incubation of equimolar concen-
trations of sodium orthovanadate and hydrogen peroxide (10 mmol/L)
as described in Bourgoin and Grinstein. Recombinant tumor necro-
sis factor α (TNFα) was a generous gift from Knoll Pharmaceuticals
(Whippany, NJ).

Purification of neutrophils. Venous blood from healthy volun-
teers was sterilely 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
× 10^6 cells/mL. Final cell preparations contained at least 98% neu-
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Flow cytometric analysis of integrin expression. Neutrophils
isolated as described above were resuspended in HBSS at 2.5 × 10^6
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% decomple-
tented FCS at a concentration of 5 × 10^6 cells/mL and stimulated
with the indicated agonists. The reactions were stopped by 15 sec-
onds of centrifugation in a microcentrifuge and the supernatants
were discarded. The first antibody (anti-CD11a, 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 cytome-
try, 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 × 10^6 cells/mL
with the leuomedins 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, 0.1% sodium dodecyl sulfate (SDS), 10% 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 PDVF membranes (Millipore Corp, Bed-
ford, MA) with the use of electrophoretic transfer cells ( Hoeffer
Scientific Instruments, Canberra Packard, Ontario, Canada). The
membranes were then stained with Ponceau S and subsequently
dstained 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-

Adherence assay. Human umbilical vein endothelial cells (HU-
VECs) 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 µ/mL
penicillin, 100 µg/mL streptomycin, and 1 µg/mL amphotericin B).
Collagenase (2.5 mg/mL; 149 U/mg) was instilled into the vein

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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 erbstatin-treated cells was first investigated. In these experiments, the cells were treated with 5 µg/mL erbstatin or its inactive analog 5-O-methyl erbstatin for 1 hour before being stimulated with 10^{-7} mol/L for 10 minutes. The effects of the inhibitor and the chemotactic peptide. The results of these experiments are summarized in Fig 1. In the absence of erbstatin, fMet-Leu-Phe increased the level of CD11b expression by close to 50%. The level of expression of CD11b was found to be 26% lower in cells preincubated with erbstatin for 1 hour at 37°C than in the controls. In addition, erbstatin abrogated the stimulatory effects of fMet-Leu-Phe on CD11b surface expression. The effects of erbstatin on the levels of CD11b expression in unstimulated cells may be related to an inhibition of the spontaneous upregulation of integrins observed by several investigators on incubation of human neutrophils at 37°C. The inhibitory effects of erbstatin were not shared by its inactive analog 5-O-methyl erbstatin, which did not affect the stimulation of tyrosine phosphorylation by chemotactic factors (data not shown). The decreased expression of CD11b by erbstatin was not caused by a decrease in the upregulation rate. Whereas the stimulation of CD11b expression by fMet-Leu-Phe reached a plateau after about 20 minutes in control cells, no increase in CD11b was evident for up to 20 minutes after the addition of the chemotactic factor to erbstatin-treated neutrophils (results not shown).

The effects of erbstatin on the fMet-Leu-Phe-stimulated expression of CD11b were concentration-dependent (Fig 2, left panel). Increasing concentrations of erbstatin (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 erbstatin were investigated next. As shown in the right panel of Fig 2, erbstatin (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 that it affected that of CD11b. In preliminary experiments, 5-O-methyl erbstatin 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 erbstatin were noted on the levels of expression of CD11a (results not shown).

The inhibitory effects of erbstatin were not limited to the level of expression of CD11b. CBRM1/5 is a newly characterized antibody that recognizes epitopes 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, erbstatin, but not its inactive analog 5-O-methyl erbstatin, 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 erbstatin 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 erbstatin of agonists other than fMet-Leu-Phe. The ability of erbstatin to inhibit the increased expression of integrin by stimuli other than fMet-Leu-Phe was tested next. Erbstatin inhibited the increased expression of CD11b and CD18 induced by the lipid mediator, leukotriene B_4. This 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 erbstatin were not limited to interference with chemotactic factors because the increased expression of CD11b induced by the
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.45-46 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).

**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 31-8330.47-49 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 leumedins on tyrosine phosphorylation.** Leumedins represent a family of recently described anti-inflammatory compounds that have been shown to inhibit
specifically the recruitment of leukocytes in a variety of animal models of inflammation such as dermatitis, sepsis, and inflammatory bowel diseases. Their mechanism of action involves the inhibition of the upregulation of integrins in chemotactic factor-stimulated human neutrophils. A link between tyrosine phosphorylation and the increase in surface expression of integrin is implied by the above-described effects of tyrosine kinase inhibitors. This suggestion prompted the examination of the effects of one leumedin, NPC 15669, and of its inactive analog NPC 14692, on the stimulation of tyrosine phosphorylation induced by fMet-Leu-Phe. The results of a representative immunoblot are shown in Fig 6. These data clearly show that NPC 15669, but not NPC 14692, inhibited in a concentration-dependent manner the stimulation of tyrosine phosphorylation induced by fMet-Leu-Phe. These effects of NPC 15669 occurred at the same concentrations that affected integrin upmodulation. NPC 15669 was also found to inhibit the tyrosine phosphorylation induced by leukotriene B4 and interleukin-8 (results not shown).

Effect of erbstatin on neutrophil adherence to endothelial cells. The effects of erbstatin on the fMet-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 fMet-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 fMet-Leu-Phe by nearly 40%, whereas pretreatment of endothelial cells had no effect (Fig 7). Erbstatin was also found to inhibit leukotriene B4-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-
The relationship between tyrosine phosphorylation and integrin expression is supported by various lines of evidence. Firstly, two unrelated tyrosine kinase inhibitors, erbsatin and herbimycin A, which differ in their mechanism of action (erbsatin competing with the kinase substrates, herbimycin A with the ATP binding site), essentially abrogated the stimulated expression of CD11b and CD18. Secondly, the concentrations at which these inhibitors affected integrin up-modulation closely corresponded to those that inhibited the stimulation of tyrosine phosphorylation by these same agonists (unpublished observations). Thirdly, a closely related analog of erbsatin, 5-O-methyl erbsatin, which lacks inhibitory activity towards tyrosine kinases, was similarly without effect on the increased expression of CD11b and CD18.

On the other hand, tyrosine kinase inhibitors only partially inhibited the stimulated adherence of neutrophils, a function that is essentially entirely mediated by CD18 integrins (P. Kubes, data not shown). A likely explanation of these results is that the stimulation of adherence depends not only on integrin surface numbers but also on their affinity for the opposing ligands and that the chemotactic factor-stimulated modulation of the affinity of CD11b/CD18 is not controlled by tyrosine phosphorylation.

The ability of the tyrosine kinase inhibitors to affect the responses to two unrelated chemotactic factors (fMet-Leu-Phe and leukotriene B4) and to a cytokine (TNFa) indicates that the steps affected are central and critical to the events regulating integrin upmodulation, ie, the fusion of the integrin-containing granule subpopulation with the plasma membrane. It is of some relevance to point out that the degranulation of specific and azurophil granules observed in cytochalasin B-treated human neutrophils on the addition of chemotactic factors was previously found to be unaffected by the same concentrations of erbsatin. These results are consistent with the hypothesis that the integrin-containing granule subpopulation, although it may represent a subset of specific granules, is nevertheless functionally distinct and subject to separate control mechanisms.

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 physiologic 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 p125FAK (unpublished observations). Fuortes et al have also recently reported that the stimulation of human neutrophils adhered to fibronectin-coated surfaces by TNFa 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 p2 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 erbastin 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 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 leukemids on integrin upmodulation, on tyrosine phosphorylation, and on animal models of inflammation strongly support such a link.

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Regulation of stimulated integrin surface expression in human neutrophils by tyrosine phosphorylation

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