Activation of the β integrin Mac-1 (CD11b/CD18) by an endogenous lipid mediator of human neutrophils and HL-60 cells

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SUMMARY

β integrins (CD11/CD18) play a key role in the adhesion, activation, migration and phagocytosis of human neutrophils. In order to exert their functions, β integrins require activation, which results in an enhancement of ligand affinity. This functional up-regulation is probably due to a conformational change of the β integrins, but the mechanisms of inside-out signaling that trigger this activation are still under investigation. In the present study, the effect of cellular lipids on the affinity state of β integrins was investigated. Lipids were extracted from human neutrophils and HL-60 cells after stimulation with IL-8 or phorbol ester, respectively. The extracts were purified by anion exchange chromatography and/or HPLC fractionation.

INTRODUCTION

The recruitment of human neutrophils to sites of inflammation requires adhesive interactions with their environment (endothelium, extracellular matrix). A major part of these cell-cell- and cell-matrix-interactions is mediated by binding of the conformationally activated β integrin Mac-1 (CD11b/CD18) to specific ligands (Hynes, 1992; Diamond and Springer, 1993; Walzog et al., 1995). The enhancement of the ligand binding affinity of Mac-1 is induced by cellular activation, which leads to expression of new epitopes on Mac-1 (Diamond and Springer, 1993). This process is a prerequisite for efficient adhesion, since Mac-1 on resting neutrophils exerts only poor ligand-binding activity. Although the functional up-regulation upon cellular activation is accompanied by recruitment of additional Mac-1 from intracellular storage sites to the cell surface, this is not required for effective adhesion (Vedder and Harlan, 1988). Together this shows that activation of Mac-1 represents a key event during neutrophil recruitment to sites of inflammation.

Although it is well known that Mac-1 is an activation-dependent molecule (Diamond and Springer, 1994), the molecular mechanisms that underly functional up-regulation of β integrin-binding activity are still under investigation. Previous studies on Mac-1 and LFA-1 have shown that phosphorylation of the integrin α subunit (Buyon et al., 1990), interaction of the integrin cytoplasmatic tails with cytoskeletal proteins (Pavalko and LaRoche, 1993), divalent cations (Graham and Brown, 1991; Dransfield and Hogg, 1989; Dransfield et al., 1992; Lee et al., 1995) or involvement of the small guanosine triphosphate-binding protein RhoA, may be crucial for the inside-out signaling that leads to activation (Laudanna et al., 1996). Recently, a protein called cytohesin-1 was identified, which up-regulates β integrin-mediated ligand binding by interaction with the intracellular domain of CD18 (Kolanus et al., 1996).

In one report, a small anionic lipid mediator called integrin modulating factor 1 (IMF-1) was found upon neutrophil stimulation by phorbol ester (PMA), formyl-norleucyl-leucyl-phenylalanine (fNLLP) or tumor necrosis factor α, which enhances β integrin-dependent neutrophil adhesion as well as ligand binding activity of the isolated β integrin CD11b/CD18 (Hermanowski-Vosatka et al., 1992). However, the molecular nature of IMF-1 has not been identified. In addition, a lipid activity that enhanced LFA-1-dependent adhesion to IL-1β-stimulated HUVEC was described in lymphoid and myeloid cell lines. The activity, termed leukocyte adhesion lipid (LAL), was found in these cell lines upon stimulation with phorbol ester (Lee et al., 1994). It is not clear whether LAL is identical to IMF-1.
This study was undertaken to investigate whether endogenous lipid mediator(s) in human neutrophils and HL-60 cells can enhance ligand affinity of \( \beta \) integrins. Furthermore, the ability of the chemokine interleukin 8 (IL-8) to induce such activity was studied, since IL-8 is a very potent and physiologically important activator of human neutrophils and is generated by various cells (Cassatella et al., 1992; Hébert and Baker, 1993).

Lipids were extracted from PMA- or IL-8-treated human neutrophils as well as from PMA-treated HL-60 cells and partially purified by anion exchange chromatography and HPLC fractionation. Effects of these lipid preparations on \( \beta \) integrin ligand-binding activity were studied in cellular and cell-free adhesion assays. The integrin-up-regulating activity that was found in activated neutrophils and HL-60 cells was further characterized by its sensitivity to esterase hydrolysis and by ultraviolet spectrometry.

**MATERIALS AND METHODS**

**Reagents, column materials and antibodies**

Antiproteases, fibrinogen, N-formyl-Met-Leu-Phe (fMLP), Percoll, PMA (phorbol 12-myristate 13-acetate), Protein A-Sepharose CL-4B, Sepharose CL 4B 200 and zymosan were obtained from Sigma (Deisenhofen, Germany). Ficoll was purchased from Biochrom (Berlin, Germany). IL-8 was a gift from Dr J. Baker (Genentech, CA, USA). DEAE-Sephadex A25 was purchased from Pharmacia (Freiburg, Germany). SepPak C18 Plus cartridges were from Waters-Millipore (Eschborn, Germany). Eurosystem columns (5 mm, 250 mm x 4.6 mm) were obtained from Knauer (Berlin, Germany) and actonitrite (HPLC grade) was from Rathburn (Edinburgh, UK). Buffers and and cell culture media were obtained from Biochrom KG, Berlin, Germany. The monoclonal anti-CD18 antibody IB4 was provided by Dr S. D. Wright (Rockefeller University, NY, USA) through Dr C. Lundberg, Pharmacia AB, Uppsala Sweden. The FITC-conjugated goat anti-mouse IgG was obtained from DAKO (Hamburg, Germany).

**Cells**

Neutrophils were isolated from freshly heparinized venous human blood. For lipid extraction, neutrophils were isolated by dextran T500 sedimentation followed by Ficoll-hypaque centrifugation and hypotonic lysis of erythrocytes, as described (Böyum, 1968). For adhesion assays, neutrophils were isolated after spontaneous erythrocyte sedimentation on a discontinuous Percoll gradient, as described (Hjort et al., 1981). Neutrophils were suspended in PBS with 0.1% glucose and 2% human serum albumin. The myeloid cell line HL-60 was grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) in 5% CO2 at 37°C.

**Cell stimulation**

Neutrophils or HL-60 cells (10⁶) were suspended at a cell density of 10⁷/ml in PBS in the presence of 1.2 mM Ca²⁺ and 1 mM Mg²⁺ and stimulated for 30 minutes at 37°C with 100 nM PMA or 1 μg/ml IL-8, respectively. Subsequently, cells were washed twice and the pellets were subjected to lipid extraction. As a negative control, unstimulated neutrophils or HL-60 were prepared as described above without addition of activators. In addition, an amount of PMA equal to that used for cell stimulation was subjected to the lipid extraction protocol to investigate whether residual PMA activity could be recovered.

**Lipid extraction**

Lipids were extracted as described (Hermanowski-Vosatka et al., 1992). Briefly, cell pellets were resuspended in chloroform/methanol/water (10:10:1). After constant stirring for 36 hours and three solvent changes, extracts were dried and resuspended in 1 ml chloroform/methanol (2:1). The preparation was centrifuged at 100,000 g (Kontron, Eching, Germany) for 20 minutes and dried under nitrogen. The extract from 10⁶ neutrophils or HL-60 cells was dissolved in 1 ml chloroform/methanol (2:1). For adhesion assays with crude lipid extracts, 100 μl samples of the preparations were dried under nitrogen and resuspended in 100 μl PBS supplemented with 0.1% glucose and 2% human serum albumin.

**Neutrophil adhesion assay**

Neutrophils (5000 per well) were seeded onto 96-well microtiter plates (precoated with 20 μg/ml fibrinogen) in the presence of divalent cations (1 mM Mg²⁺ and 1.2 mM Ca²⁺). Samples of different dilutions of the lipid extracts (10 μl) or PMA (as a positive control) were added and incubated for 20 minutes at 37°C. After washing twice, the fraction of adherent neutrophils was quantified by a myeloperoxidase assay (Suzuki et al., 1983). Adherent neutrophils were lysed by 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer (pH 6). Myeloperoxidase content of the samples was determined by incubation of 50 μl samples with 0.16 mM tetramethylbenzidine and 0.5 mM hydrogen peroxide in 80 mM phosphate buffer (pH 5.4) for 20 minutes at 37°C. The difference of optical densities at 620 nm and 492 nm was measured with a microtiter plate reader (Flow Laboratories, Meckenheim, Germany).

**Cell-free adhesion assay**

The assay was performed with C3bi-coated zymosan beads (Ross et al., 1985). Mac-1 (CD11b/CD18) was isolated from neutrophils lysed in n-octyl-β-D-glucopyranoside by ligand-affinity chromatography, as described (Van Strijp et al., 1993). Terasaki plates were coated with 1:50 dilutions of Mac-1 preparations, which contained material derived from 10⁶ neutrophils per 100 μl. Prior to each experiment, coating efficiency was tested by immunofluorescence staining with mAb IB4 and a secondary FITC-labeled antibody.

**Purification of lipid extracts and HPLC**

Lipid extracts were purified on an anion-exchange column (Fredman et al., 1980) and desalted by a Sep-Pak C18-cartridge (Hermanowski-Vosatka et al., 1992). Recovery of activity was approximately 53%. For HPLC application, 100 μl samples of extracts were dried under nitrogen, resuspended in acetone/ethanol/water (1:1:0.05) and centrifuged, and the supernatant was loaded onto a 300 μl injection loop. The C18-reversed-phase column was preequilibrated with 20% acetonitrile and run with a 1% /minute increment of acetonitrile over water dest. starting at 20% acetonitrile. After 80 minutes, the column was equilibrated with acetonitrile for 30 minutes. Absorbance was measured with a Kontron spectrophotometer (Eching, Germany) at 196 nm and 1 ml fractions were collected. For measurement of neutrophil adhesion to fibrinogen, a 400 μl sample of each fraction was dried under nitrogen and suspended in 50 μl PBS supplemented with 0.1% glucose and 2% human serum albumin. For UV spectrometry, a 600 μl sample of the activity-bearing fraction was dried under nitrogen and resuspended in 100% acetonitrile.

**RESULTS**

**Effect of crude lipid extracts on neutrophil adhesion**

Crude lipid extracts from unstimulated human neutrophils were able to moderately (twofold) enhance adhesion of neutrophils to fibrinogen-coated surfaces (Fig. 1). Lipid extracts (1:100 dilution) from IL-8 (1 μg/ml)-stimulated neutrophils enhanced binding of neutrophils more than 17-fold over background. Extracts derived from cells treated with PMA (100 nM) had a similar effect. Alkaline hydrolysis of crude lipid extracts derived from IL-8-stimulated neutrophils revealed that...
the major portion of the activity was not sensitive to the cleavage of ester bonds because the activity was only moderately affected (about 30%). In one separate experiment, neutrophils were stimulated with 100 nM fMLP and similar results were obtained (data not shown). By contrast, the activity of the extract derived from IL-8-activated PMN only slightly. Data represent -fold increase of adhesion. Background was defined as neutrophil adhesion in the presence of vehicle (dashed line). Mean ± s.d. of quadruplicates, data are representative of three independent experiments.

**Effect of crude lipid extracts on adhesiveness of purified Mac-1**

The effect of hydrolyzed crude lipid extracts derived from PMA-activated neutrophils on the affinity of Mac-1 was tested in a cell-free assay system (Fig. 2). Preparations of affinity purified Mac-1 were coated on Terasaki plates. The binding avidity of Mac-1 was measured as its ability to bind C3bi-bearing zymosan beads. Crude lipid extracts were able to enhance ligand binding by Mac-1 dose-dependently. A 1:100 dilution of the extracts showed a maximal effect and induced a twofold increase of Mac-1 binding to C3bi-coated beads. PMA alone had no effect in this cell-free assay system (data not shown). Furthermore, the observed binding was specific, because it was completely abolished by pre-clearing Mac-1 preparations with the anti-CD18 mAb IB4 and Protein A-Sepharose prior to coating.

**Effects of purified lipid extracts on neutrophil adhesion**

Crude lipid extracts from activated neutrophils were further purified on an anion exchange column. At a dilution of 1:100,
the purified extract from PMA-activated neutrophils increased neutrophil adhesion to fibrinogen more than tenfold (Fig. 3). Ester hydrolysis reduced this activity only slightly. In contrast, the activity of a solution consisting of 10 nM PMA (without neutrophil-derived lipid extracts) increased neutrophil adhesion by more than 100-fold, but was almost completely abolished by ester hydrolysis. The flow-through from the anion exchange column also stimulated neutrophil adhesion, but this was largely due to contamination with residual PMA, as shown by the effect of ester hydrolysis.

**Effects of HPLC-purified lipid extracts on neutrophil adhesion**

The lipid extracts of neutrophils or HL-60 cells were analysed spectrophotometrically by HPLC at a wavelength of 196 nm and subsequently fractionated (Fig. 4). The extracts from PMA-stimulated HL-60 cells and neutrophils as well as from untreated HL-60 cells shared several absorption peaks. One prominent peak eluted at 58 minutes from both HL-60 cells and neutrophils. Strikingly, this absorption peak correlated with a peak in biological activity, as determined by using the neutrophil-fibrinogen adhesion assay (about 30-fold increase in neutrophil adhesion). In contrast, the fraction that originated from unstimulated HL-60 cells showed no significant activity. These results indicate that the lipid extracts derived from activated neutrophils and HL-60 cells contain an endogenous integrin up-regulating activity. Furthermore, adhesion was also markedly enhanced by fractions eluting at 78/79 minutes. However, this adhesion peak was only obtained in samples to which PMA was added and not in unstimulated cells, strongly suggesting that this is the result of residual PMA. Thus, the endogenous activities that originated from cells stimulated by PMA and from PMA directly were separated by HPLC fractionation.

**Absorption spectrum of the activity bearing HPLC fraction**

The activity-bearing HPLC fraction 58 derived from human neutrophils was subjected to ultraviolet spectrometry (Fig. 5). The spectrum revealed an absorption maximum at 194±2 nm. As a control, the spectrum of PMA (50 μg) was recorded, which revealed completely different absorption features.

![Fig. 5. Ultraviolet absorption spectrum of the activity-bearing HPLC fraction. Absorption spectra were measured in a double-beam spectrophotometer (Lambda 5 UV/Vis spectrophotometer, Perkin Elmer) in quartz cuvettes. The activity-bearing HPLC fraction was measured in 100% acetonitrile against 100% acetonitrile. The spectrum showed an absorption maximum at 194±2 nm (solid line). As a control, the spectrum of PMA (50 μg) was determined (dashed line).](image)

**DISCUSSION**

This study provides evidence that activated HL-60 cells and human neutrophils generate a lipid mediator(s), which is able to enhance β integrin-mediated neutrophil adhesion to fibrinogen and the binding of purified Mac-1 to C3bi. Similar findings have been described in two previous publications (Hermanowski-Vosatka et al., 1992; Lee et al., 1994). Hermanowski and co-workers proposed the existence of a lipid termed integrin-modulating factor (IMF-1) in human neutrophils, which was able to up-regulate the ligand-binding activity of Mac-1. Lee et al. reported a LFA-1 up-regulating activity in HL-60 cells, which was called leukocyte adhesion lipid (LAL). Our data show that both PMN and HL-60 cells generate similar biological activities under exactly the same experimental conditions. Furthermore, the absorption spectrum of the activity-bearing lipid preparation was identical to that of IMF-1 as described earlier (Hermanowski-Vosatka et al., 1992).

We extend the previous findings by showing that production of the mediator is not only induced by PMA and fNLLP (Hermanowski-Vosatka et al., 1992) but also by the endogenous chemokine IL-8. The fact that the lipid extracts were able to enhance the affinity of isolated Mac-1 obtained from resting neutrophils suggests that modifications of the intracellular domains of the β-integrins, such as phosphorylation or association with intracellular proteins, are not necessary for functional regulation of ligand-binding affinity. Furthermore, the enhancement of Mac-1 affinity in the cell-free assay suggests that the activation is due to an allosteric effect of the lipid mediator on Mac-1.

We show that even unstimulated neutrophils contain some activity, which suggests that the activity-bearing lipid is ubiquitous and synthesis is up-regulated upon cellular activation. The major fraction of the activity was found to be resistant to alkaline hydrolysis, which excludes contamination with PMA and suggests that the active compound does not contain...
ester bonds. The purification of the crude extracts on the DEAE-Sephadex column greatly reduced the residual PMA effect, concentrated the specific activity and provided evidence that the mediator may be anionic. The finding of Hermanowski-Vosatka et al. (1992) that the integrin-modulating factor IMF-1 eluted as one single peak of activity was verified in our experiments. In addition, the activity was reported to bear chemical features that correspond to an unsaturated fatty acid or isoprenoid. The identity of the active compound remains unknown and further investigations regarding the pathway of its synthesis and the enzymes involved will be necessary to understand this mechanism of inside-out signaling.

Taken together, our data lend support to the concept that endogenous lipid mediator(s) can allosterically activate Mac-1 to bind its ligands. This regulatory pathway may be important for neutrophil adhesion and recruitment under in vivo conditions, since activation can be induced by the endogenous chemokine IL-8, one of the most potent neutrophil activators known. A recent report provided evidence that a protein called cytohesin-1 is involved in the up-regulation of the adhesive functions of β integrins by direct interaction with the intracellular domain of CD18 (Kolanus et al., 1996). This finding is not contradictory to our results, but shows that regulation of the functional activity of β integrins in situ may be a multi-step process involving both intracellular protein and lipid factors, which directly interact with the integrin.

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