β2-Integrins and Acquired Glycoprotein IIb/IIIa (GPIIb/IIIa) Receptors Cooperate in NF-κB Activation of Human Neutrophils

Received for publication, May 16, 2007, and in revised form, July 2, 2007 Published, JBC Papers in Press, July 20, 2007, DOI 10.1074/jbc.M704039200

Birgit Salanova, Mira Choi, Susanne Rolle, Maren Wellner, Friedrich C. Luft, and Ralph Kettritz

From the Franz Volhard Clinic and Max Delbrueck Center for Molecular Medicine, Medical Faculty of the Charité, HELIOS Kliniken, 13125 Berlin, Germany

Microparticles from various cells are generated during inflammation. Platelet-derived microparticles (PMPs) harbor receptors that are not genuinely expressed by neutrophils. We tested whether or not functional glycoprotein IIb/IIIa (GPIIb/IIIa) receptors can be acquired by neutrophils via PMPs and whether these receptors participate in pro-inflammatory signaling. Surface expression was analyzed by flow cytometry and confocal microscopy. NF-κB activation was analyzed by Western blot experiments, electrophoretic mobility shift assays, and reverse transcription-PCR. Cell adhesion and spreading were estimated by myeloperoxidase assay and light microscopy. We found that PMPs transfer GPIIb/IIIa receptors to isolated and whole blood neutrophils via PMPs. We used specific antibodies in granulocyte macrophage colony-stimulating factor-treated neutrophils and observed that acquired GPIIb/IIIa receptors colocalized with β2-integrins and cooperated in NF-κB activation. We show that Src and Syk non-receptor tyrosine kinases, as well as the actin cytoskeleton, control NF-κB activation. In contrast to NF-κB, acquisition of GPIIb/IIIa receptors was not necessary to induce adhesion to fibronectin or phosphatidylinositol 3-kinase/Akt signaling. When granulocyte macrophage colony-stimulating factor-stimulated neutrophils were incubated on fibronectin, strong NF-κB activation was observed, but only after loading with PMPs. Blocking either β2-integrins or GPIIb/IIIa receptors abrogated this effect. Therapeutic GPIIb/IIIa inhibitors were similarly effective. The compounds also inhibited NF-κB-dependent tumor necrosis factor-α mRNA up-regulation. The data implicate GPIIb/IIIa receptors as new therapeutic targets in neutrophil-induced inflammation.

Cells send and receive signals to communicate with their environment. In addition to soluble mediators such as cytokines and growth factors, cells may also generate membrane-derived microparticles. Conditions that lead to release of microparticles include cell activation, apoptosis, oxidative stress, and shear stress (1). The composition of these microparticles varies from cell type to cell type. The microparticle cargo determines the biological consequences. For example, microparticles are able to transfer receptors to cells that do not normally express a particular receptor. The acquired receptors may be functional, as demonstrated for the CCR5 chemokine receptor released by lymphocytes. Acquisition of CCR5 receptors by endothelial cells rendered these cells susceptible to human immunodeficiency virus infection (2). Another example for the generation of microparticles is inflammatory vascular injury, including acute coronary syndromes and acute vasculitis. Both conditions are associated with an increase in circulating PMPs (3, 4). Circulating PMPs can be acquired by components of the vessel wall and by blood-borne cells, such as neutrophils (5).

Neutrophils participate in non-infectious inflammatory processes including the acute coronary syndrome and systemic necrotizing vasculitis (6). The nuclear factor κB (NF-κB) is important for neutrophil survival and the generation of numerous inflammatory mediators (7). We tested the hypothesis that GPIIb/IIIa receptors can be transferred to human neutrophils via PMPs and that these receptors participate in the NF-κB activation when neutrophils are challenged by cytokines. We found that acquired GPIIb/IIIa receptors cooperate with β2-integrins, allowing for NF-κB activation when GM-CSF-treated neutrophils interact with fibronectin, but not in suspension cells. The specific GPIIb/IIIa blockers abciximab, eptifibatide, and tirofiban abrogated NF-κB activation. We suggest that GPIIb/IIIa receptors provide a new therapeutic target in neutrophil-induced inflammation.

EXPERIMENTAL PROCEDURES

GM-CSF was obtained from R&D Systems (Wiesbaden-Nordenstedt, Germany). Ficoll-Hypaque and cytochalasin B were from Sigma (Deisenhofen, Germany). Dextran was purchased from Amersham Biosciences (Amsterdam, Netherlands); the α-actin antibody (C-2), the polyclonal rabbit antibodies against IκBα (C-21), and the monoclonal antibodies to CD41/61 (GPIIb/IIIa receptor) and to CD51/61 (vitronectin receptor) were obtained from Santa Cruz Biotechnology (Santa Cruz,

2 The abbreviations used are: PMP, platelet microparticle; PRP, platelet-rich plasma; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte macrophage colony-stimulating factor; GPIIb/IIIa, glycoprotein IIb/IIIa; PI3K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor κB; RT, reverse transcription; HBSS ++, Hanks’ balanced salt solution with calcium and magnesium; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; DAPI, 4’,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; Fam, carboxyfluorescein; Tamra, 6-carboxy-tetramethylrhodamine.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Division of Nephrology, Franz Volhard Clinic, Schwanebecker Chaussee 50, 13125 Berlin, Germany. Tel.: 49-30-940152801; Fax: 49-30-940152809; E-mail: kettritz@charite.de.
CA). Horseradish peroxidase-labeled donkey anti-rabbit IgG was from and Amersham Biosciences (Braunschweig, Germany), Hanks’ balanced salt solution with calcium and magnesium (HBSS+), phosphate-buffered saline, and trypsin blue were from Biochrom (Berlin, Germany). The PI3K inhibitor LY294002, the p38 MAPK blocker SB202190, the Src inhibitor PP2, and the Syk inhibitor piceatannol were from Calbiochem (Schwalbach, Germany). The antibody to Akt and the phosphospecific antibodies to p38 and Akt were from Cell Signaling (Frankfurt am Main, Germany). The FITC-labeled antibodies to CD18, the isotype control, and the blocking monoclonal antibodies to CD18 (7E4), CD41/61 (P2), and CD42b (SZ2) were purchased from Immunotectnique (Marseille, France). The CD18-activating clone KIM 127 was described elsewhere (8) and was a generous gift from Dr. Mark Ginsberg (University of California, San Diego, CA) and was described previously (9). Fibronectin was from Roche Applied Science (Mannheim, Germany). Abciximab (ReoPro®) was obtained from Centocor (Leiden, The Netherlands) and Lilly (Giessen, Germany), respectively (10, 11). Eptifibatide (Integrilin®) was from SP Europe (Brussels, Belgium) and Essex Pharma (Munich, Germany). Tirofiban hydrochloride (Aggrastat®) was obtained from MSD Sharp & Dohme (Haar, Germany). We used abciximab at final concentrations of 100 ng/ml, eptifibatide at 1500 ng/ml, and tirofiban at 40 ng/ml. These concentrations are at the lower range of the in vivo levels found with treatment (12–14).

In experiments where we saw no effect with these low concentrations, we used 1000 ng/ml abciximab, 2500 ng/ml eptifibatide, and 60 ng/ml tirofiban, respectively. These concentrations are at the upper limit of therapeutic concentrations used in vivo. Endotoxin-free reagents and plastic disposables were used in all experiments.

Preparation and Culture of Human Neutrophils—Heparinized whole blood was drawn from healthy human donors after written approval (EA 3/011/06, Charité Berlin). Neutrophils were isolated using a double gradient method. The gradient was formed by layering an equal volume of Histopaque 1077 over Histopaque 1119. Whole blood was carefully layered onto the upper gradient and centrifuged for 30 min. Cells of the granulocytic series were found at the Histopaque 1077/1119 interphase, whereas lymphocytes, other mononuclear cells, and platelets were found at the plasma/Histopaque 1077 interphase. The granulocytic cells were carefully aspirated and washed once with phosphate-buffered saline. Remaining erythrocytes were lysed by incubation with hypotonic saline for 15 s. PMN were spun down (1050 rpm, 10 min) and reconstituted in HBSS with calcium and magnesium. The final cell concentration was 5 x 10⁶ cells/ ml. The cell viability was determined in every cell preparation by trypan blue exclusion and found to be >99%. With this method, we were able to isolate neutrophils with a very low amount of CD41/61-positive cells.

Preparation of Platelet-rich Plasma (PRP), PMPs, and Platelet-free Plasma—PRP was obtained by centrifugation of heparinized whole blood at 200 x g for 10 min at room temperature. PMPs were generated using previously reported protocols with minor modifications (15, 16). Briefly, PRP was activated with 10 µM ADP for 20 min at 37 °C, and whole platelets were removed by centrifugation (twice at 4600 rpm, 13 min) and filtration through a 0.8-µm filter. To remove ADP, this solution was centrifuged for 60 min at 20,000 x g. The pellet containing PMPs was resuspended in HBSS++. As a negative control, we also prepared platelet-free plasma that was ADP-treated, filtered, and centrifuged in the same way as described above.

Preparation of PMP-loaded Neutrophils in Vitro—PMP-loaded neutrophils were prepared by co-incubation of isolated human neutrophils with PMPs for 45 min at 37 °C. PMN were spun down (200 x g, 7 min) and resuspended in HBSS++ with a final cell concentration of 5 x 10⁶ cells/ml.

Western Blot—Samples were incubated for 5 min at 95 °C in loading buffer (250 mm Tris-HCl, pH 6.8, with 4% SDS, 20% glycerol, 0.01% bromphenol blue, 10% β-mercaptoethanol). 10–20 µg of protein was loaded per lane, electrophoresed on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline with Tween, 5% skim milk for 1 h and incubated overnight with the indicated antibodies. Membranes were washed and incubated with a horseradish peroxidase-labeled secondary antibody. The blot was developed by incubation in a chemiluminescence substrate (ECL, Amersham Biosciences) and exposed to an x-ray film. We confirmed equal loading of protein in parallel experiments using α-actin or total Akt antibodies.

Flow Cytometry—CD41/61 receptor expression on freshly isolated neutrophils was determined by incubation with saturating concentration of the antibody (Clone A2A9/6, Santa Cruz Biotechnology) for 25 min on ice. A mouse IgG2a antibody was used in the same concentration as a control (Santa Cruz Biotechnology). Cells were washed and resuspended in HBSS++, and phycoerythrin-conjugated F(ab')₂ fragments of goat-anti mouse IgG (DAKO, Hamburg, Germany) were added in saturating concentration for a further 25 min. After washing, samples were assayed using a FACSScan (BD Biosciences, Heidelberg, Germany).

CD41/61 receptor expression on whole blood neutrophils was determined by stimulation of whole blood for 20 min with 10 µM ADP or phosphate-buffered saline, respectively, and further incubation with saturating concentration of the antibody for 20 min at room temperature. Erythrocytes were lysed by incubation with FACS lysing solution (BD Biosciences) for 10 min at room temperature. After centrifugation and washing, samples were assayed using a FACSScan.

In experiments where CD61 receptor expression was determined on PMP-loaded neutrophils after incubation with the indicated inhibitory antibodies, we first incubated PMP-loaded neutrophils with 20 µg/ml of the antibodies for 30 min at 37 °C. The cells were washed and incubated with saturating concentration of a CD61-FITC antibody (Clone Y2/51, DAKO, Glostrup, Denmark) for a further 25 min on ice. After washing, samples were assayed using a FACSScan (BD Biosciences).

Fluorescence Microscopy—After incubation with the respective antibody as described for flow cytometry, cells were spun down on poly-L-lysine coated coverslips and incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma;
Acquired GPIIb/IIIa by Neutrophils and NF-κB

0.25 μg/ml, 30 s) for nuclei counterstaining. Immunostained specimen were analyzed with a Leica DMRB light microscope (Leica Microsystems, Bensheim, Germany). Images were acquired using a digital camera (SPOT) and processed with MetaVue software.

Confocal Analysis—For double-staining experiments, cells were immunolabeled with a monoclonal antibody against CD41/61, washed, and incubated with a Alexa Fluor® 555 donkey anti-mouse IgG antibody (Molecular Probes, Eugene, OR). Cells were washed again and incubated with a direct-conjugated CD18-FITC antibody (Clone 7e4, Immunotech, Mar seille, France). After incubation and washing, cells were spun down on coverslips. Immunofluorescence images were scanned with a three-channel confocal laser scanning microscope with a ×40/1.25 NA oil immersion objective (Leica TCS SP-2, Leica Microsystems). All digitized images were analyzed using the Leica confocal software.

Nuclear Extract Preparation—Nuclear extracts were prepared as described previously (7). The reaction was stopped with ice-cold HBSS++ supplemented with diisopropyl fluorophosphate (2 mM final concentration). Cells were centrifuged at 2000 × g for 2 min at 4 °C, and pellets were resuspended with the hypotonic buffer A (HEPES 10 mM, pH 7.5, KCl 10 mM, EGTA 0.1 mM, EDTA 0.1 mM, pH 8.0) containing an anti-protease mixture (2 mM diisopropyl fluorophosphate, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 0.5 mM benzamidine, 1 mM dithiothreitol). Cells were kept on ice for 10 min. Cells were vortexed briefly and centrifuged (1000 × g, 10 min, 4 °C) to pellet the nuclei and to remove intracellular granules that may cause nuclear degradation. The pellet was washed again with buffer A, and the hypertonic, high salt buffer C was added (HEPES 20 mM, pH 7.5, NaCl 0.4 M, EDTA 1 mM, EGTA 1 mM, glycercol 20%) together with the above mentioned protease inhibitor mixture. After centrifugation (10 min at 14,000 × g, 4 °C), the supernatant was collected and referred to as the nuclear extract.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA were performed as described previously (7). Briefly, nuclear extracts (5 mg of protein) were incubated with 20,000 cpm of a 32P-labeled H3K probe. Incubations were performed for 30 min at room temperature in the presence of poly(dI-dC) and 20 mM HEPES, containing 60 mM KCl, 4% Ficoll, 5 mM dithiothreitol, and 0.5 mg/ml nuclease-free bovine serum albumin. Probes were subjected to electrophoresis on native 5% polyacrylamide gels and autoradiographed.

Quantitative RT-PCR—Total RNAs were isolated according to a Qiagen protocol including DNase treatment. Quantitative RT-PCR was performed using TaqMan technology (Applied Biosystems, Weiterstadt, Germany). Reverse transcription was carried out according to the SuperScript protocol (Invitrogen, De Schelp, NL). TaqMan RT-PCR was performed using the Master Mix (Applied Biosystems). The quantification was checked for each sample using probes for gyceraldehyde-3-phosphate dehydrogenase mRNA. Primers and probes were designed using the primer express program (Applied Biosys tems). The following oligonucleotides were used for IkBα: forward primer, 5′-CCCTGTAATGGCCGGACTG-3′, reverse primer, 5′-AGGAGTGAACACCAGGTCAGGA-3′, and the probe FAM 5′-CCTTACCTCGAGTGACCTGC-3′ Tamra. RT-PCR and quantification were performed using the TaqMan 5700 (Applied Biosystems). The following oligonucleotides were used for TNF-α: forward primer 5′-GGTGCTTGTCC-TCAGCCTC-3′, and reverse primer, 5′-CAGGCCAGAAGAGCGTGGTG-3′.

For quantification of the amount of RNA present in the various samples, the fluorescence signal was measured at each PCR cycle, and the increase in the fluorescence normalized reporter signal (Rn) was documented in an amplification plot. Using non-template controls, the threshold was set in the log phase to subtract unspecific fluorescence signals. Cycle threshold (Ct) values were determined for each sample. In short, the Ct-value difference (∆Ct) was used to calculate the factor of differential expression (2ΔCt). Results were imported in an Excel spreadsheet and analyzed according to the standard curve method.

Scatting and Adhesion Assay—We used 96-well plates coated with fibronectin (10 μg/cm²) for the adhesion assay. 1 × 10⁶ neutrophils in 100 μl of HBSS++ were either left untreated or were treated with 20 ng/ml GM-CSF. Plates were incubated at 37 °C in 5% CO₂ for 60 min. Wells were flicked dry and washed three times with phosphate-buffered saline, and adherent cells were estimated using the myeloperoxidase assay.

Briefly, adherent cells were lysed in 100 μl of 0.5% Triton-X-100 for 10 min. 100 μl of substrate (2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid, Sigma) was added, and OD was read after 10 min at 450 nm with a microtiter plate reader. Each experiment was done in triplicate. OD of the experimental sample was compared with a standard curve that showed an excellent correlation between OD and cell number. The standard curve was established over a range of 1 × 10⁴–1 × 10⁵ cells and showed an R² value of 0.96.

For estimation of cell spreading, neutrophils were cultured as described above. At the indicated time point, non-adherent cells were discarded, and the percentage of spread cells was assessed. At least 100 cells were counted using phase contrast microscopy, and those cells that were phase dark, enlarged, and irregular were considered as spread.

Statistical Analysis—Results are given as mean ± S.E. Comparisons between two groups were done using paired t tests. Comparisons between multiple groups were done using one-way analysis of variance. Specific differences between multiple groups were then determined by use of a Bonferroni post hoc test. Differences were considered significant if p < 0.05 and are indicated by asterisks in the figure legends.

RESULTS

Neutrophils Acquire GPIIb/IIIa Receptors via Transfer of PMPs—We first tested whether or not GPIIb/IIIa receptors can be acquired by the neutrophil via PMPs. We stimulated whole blood with ADP to activate platelets and to generate PMPs. By flow cytometry, we observed that whole-blood neutrophils displayed very low amounts of GPIIb/IIIa receptors on their membranes and that this amount was significantly up-regulated after ADP whole blood treatment (Fig. 1A). Using fluorescence
microscopy in DAPI-treated cells, we could detect a high amount of neutrophils with very small CD41/61-positive particles on the cell surface in the ADP-treated sample (Fig. 1B). In addition, we observed whole platelets (see inserted box) and platelet-aggregates (not shown) that were larger and showed much higher fluorescence intensity when compared with PMPs. Epinephrine treatment gave similar results to those observed with ADP (data not shown).

We next confirmed the whole blood data under defined in vitro conditions. We particularly wanted to exclude whole platelets and platelet aggregates, ensuring that platelet-derived microparticles caused the GPIIb/IIIa transfer to neutrophils. This setting would also allow us to study functional consequences of such a GPIIb/IIIa receptor transfer. Thus, we generated PMPs from PRP in vitro and transferred these particles to isolated neutrophils. Filtration through 0.8-μm filters assured complete removal of whole platelets. We observed that incubation of neutrophils with PMPs resulted in acquisition of GPIIb/IIIa receptor staining (Fig. 2). By flow cytometry, both the percentage of GPIIb/IIIa β3-integrin receptor-positive neutrophils, as well as the mean fluorescence intensity for GPIIb/IIIa staining, increased significantly (p < 0.05, Fig. 2A). We also observed significant transfer of GPIIb (from 14.2 ± 4.1 to 39.1 ± 8.1% of the neutrophils; n = 5, p < 0.05 and from 14.3 ± 1.1 to 16.2 ± 2.8 for mean fluorescence intensity, respectively, not significant). However, the amount of receptor acquisition was lower when compared with GPIIb/IIIa receptors. In contrast, the other known β3-integrin receptor, vitronectin (avβ3), was not detected on neutrophils, neither without nor with PMP loading (data not shown). Fluorescence microscopy clearly visualizes acquisition of GPIIb/IIIa receptors in isolated neutrophils after PMP incubation (Fig. 2B).

Acquired GPIIb/IIIa Receptors Are Functional and Cooperate with β2-Integrins to Activate NF-κB after GM-CSF Stimulation—We next studied whether or not GPIIb/IIIa receptors acquired by the neutrophil were functional. We reported previously that β2-integrins provide co-stimulation, allowing cytokines such as GM-CSF and interleukin-8 to activate NF-κB signaling (17). However, in our earlier study, we had...
not investigated the contribution of PMPs to this process. Importantly, the isolation method used in these earlier experiments yielded neutrophils contaminated with variable amounts of PMPs (data not shown). We used specific antibodies to activate either the β2-integrin CD18 or the β3-integrin GPIIb/IIIa. We first studied NF-κB activation by Western blot experiments assaying the inhibitory IκBα-protein. Fig. 3A indicates that, in GM-CSF-treated suspension neutrophils that had not acquired GPIIb/IIIa receptors, neither an activating CD18 (KIM127) nor an activating GPIIb/IIIa antibody (LIBS-6) induced IκBα degradation. In contrast, GM-CSF-treated neutrophils that were loaded with PMPs showed NF-κB activation in response to an activating CD18 (KIM127) or an activating GPIIb/IIIa antibody (LIBS-6), respectively (Fig. 3B). NF-κB activation in PMP-loaded neutrophils was demonstrated by additional independent methods, namely a DNA binding assay (EMSA, Fig. 3C) and quantitative RT-PCR for NF-κB-dependent IκBα mRNA up-regulation (Fig. 3D). A blocking antibody to CD18 (7E4) prevented NF-κB activation by the activating GPIIb/IIIa and CD18 antibodies. Similarly, the blocking antibody to GPIIb/IIIa (P2) prevented NF-κB activation in response to the activating CD18 and GPIIb/IIIa antibodies (Fig. 3E). We observed similar findings when the assay was carried out on polyhemacrylate at a low cell density where we ensured by light microscopy that neutrophils had no contact to each other (data not shown). This culture condition was shown to securely exclude cell-plastic contact that may have provided a ligand for integrin receptors in the tube experiments and homotypic cell-cell contact (18). As an additional control experiment, we preincubated PMP-loaded neutrophils with a blocking antibody to another platelet receptor CD42b (glycoprotein Ibα). This blocking antibody did not prevent NF-κB activation in GM-CSF-treated cells in response to the activating CD18 antibody (Fig. 3F).

Thus far, our data indicate that acquisition of GPIIb/IIIa receptors via PMPs was a prerequisite for NF-κB activation in GM-CSF-treated neutrophils. We next tested whether or not PMP loading affected classical β2-integrin-mediated functions such as adherence to
fibronectin or spreading or other signaling pathways. Our data indicate that GM-CSF induced neutrophil adhesion to fibronectin and that this response was similar in neutrophils without and with PMP loading (Fig. 4, A and B). The results also document that the blocking CD18 antibody (7E4) completely abrogated adhesion and spreading, whereas the blocking GPIIb/IIIa antibody showed no effect (n = 4; *, p < 0.05). PI3K/Akt activation was done by Western blot experiments using a phospho-specific Akt antibody. The PI3K inhibitor LY294002 (10 μM) was used in parallel as a positive control. Equal loading was confirmed using an antibody to α-actin.

FIGURE 4. The acquisition of GPIIb/IIIa receptors via PMPs is not a prerequisite for β2-integrin-mediated functions such as adherence to fibronectin (panel A), spreading (panel B), or PI3K/Akt activation (panel C). PMP-loaded neutrophils and not PMP-loaded control neutrophils were incubated with blocking antibodies against CD18 (bCD18), GPIIb/IIIa (bCD41/61), and an isotype control (Iso), respectively. After 30 min, cells were stimulated with GM-CSF (20 ng/ml) on fibronectin-coated (10 μg/cm²) wells. Based on time course studies, samples were incubated for 60 min (adherence and spreading) or 15 min (PI3K/Akt activation). A blocking CD18 antibody completely abrogated adhesion and spreading, whereas the blocking GPIIb/IIIa antibody showed no effect (n = 4; *, p < 0.05). PI3K/Akt activation was done by Western blot experiments using a phospho-specific Akt antibody. The PI3K inhibitor LY294002 (10 μM) was used in parallel as a positive control. Equal loading was confirmed using an antibody to total Akt (n = 3).

Acquired GPIIb/IIIa by Neutrophils and NF-κB

β2-Integrins and GPIIb/IIIa Receptors Cooperate in NF-κB Activation of GM-CSF-stimulated Neutrophils That Interact with Fibronectin—During migration, neutrophils interact with extracellular matrix proteins such as fibronectin (19). We next studied whether or not signals transduced by acquired GPIIb/IIIa receptors were involved in NF-κB activation. We incubated PMP-loaded or not PMP-loaded control neutrophils on fibronectin. Although in non-loaded neutrophils, no NF-κB activation was detected for up to 3 h, and NF-κB activation was observed in PMP-loaded neutrophils at 30 and 60 min (panel A, n = 3). Blocking antibodies to either CD18 (bCD18, 20 μg/ml) or CD41/61 (bCD41/61, 20 μg/ml) prevented NF-κB activation in PMP-loaded GM-CSF-stimulated neutrophils on fibronectin (panel B, n = 4). Equal loading was confirmed using an antibody to α-actin.
Acquired GPIIb/IIIa by Neutrophils and NF-κB

**DISCUSSION**

We provide novel data indicating that human neutrophils may acquire GPIIb/IIIa receptors via PMPs. These acquired receptors are functional, allowing for NF-κB activation in GM-CSF-stimulated neutrophils that interact with fibronectin. Our experiments suggest that the underlying mechanisms include GPIIb/IIIa-β2 integrin receptor interactions. The therapeutic GPIIb/IIIa inhibitory compounds abciximab, eptifibatide, and tirofiban all abrogate this NF-κB activation, implicating that these anti-thrombotic drugs also exercise anti-inflammatory effects.

Neutrophil functions are not only important for host defense responses but also mediate undesired collateral injury. Several neutrophil functions are controlled by blood-circulating mediators, temperature, pH, oxygen tension, as well as by cell-cell and cell-matrix interactions. Recently, our understanding of circulating mediators was extended by the observation that such mediators may also include cell-derived microparticles that may contain cargo from several host cell types. Microparticles, also termed "cell dust," are generated from a variety of cell types, including platelets. In fact, in addition to the large body of evidence already documenting the importance of neutrophil interactions with the whole platelet, the functional significance of PMPs for neutrophils is increasingly recognized.
Acquired GPIIb/IIIa by Neutrophils and NF-κB

Neutrophil-platelet interactions contribute to the pathophysiology of unstable angina, myocardial infarction, cardiopulmonary bypass sequelae, thrombosis, and sepsis (23). Leukocyte-platelet adhesion increases in parallel with the extent of platelet activation (24), as demonstrated in in vitro studies and in patients with coronary artery disease (25, 26). However, platelets that are activated by various agonists, such as epinephrine and ADP (25, 27), also generate PMPs that are released into the circulation. PMPs are increased in the blood of patients with stroke (28), acute coronary syndromes (29), peripheral vascular disease (30), acute vasculitis, and hemodialysis (4). PMPs contain several proteins and lipids similar to those found on membranes of the platelet from whence they originated. Conceivably, PMPs transfer functional receptors to other cells, including neutrophils. These platelet-derived receptors, including the most abundant GPIIb/IIIa receptor (31), may then participate in neutrophil-induced inflammation. Characterization of such events may identify novel anti-inflammatory targets.

Our study is the first to demonstrate that PMPs transfer GPIIb/IIIa receptors to neutrophils. Because we wanted to study PMPs and not platelet-neutrophil interactions, we very carefully assured ourselves that whole platelets were removed from the preparation by centrifugation and filtration. We also felt that it was important to document GPIIb/IIIa receptor acquisition in whole blood experiments to exclude the possibility that our results merely reflect an in vitro phenomenon.

Flow cytometry and confocal microscopy clearly showed that PMPs, as opposed to whole platelets, transferred GPIIb/IIIa receptors to the neutrophil. However, an important subsequent question was to show whether or not acquired GPIIb/IIIa receptors had functional consequences for the neutrophil. Recently, Lo et al. (32) described findings indicating that PMPs mediate neutrophil activation via platelet glycoprotein Ib. Blocking antibodies against GPIIbα (CD42b) from the PMPs and against CD18 from the neutrophils abrogated respiratory burst activity and adherence, suggesting that the GPIIb-β2-integrin interaction was functionally important. However, GPIIbα is not the PMP-derived receptor mediating NF-κB activation in our experiments. Blocking antibodies did not abrogate NF-κB activation.

FIGURE 7: NF-κB activation depends on non-receptor tyrosine kinases, an intact actin cytoskeleton, and an active GPIIb/IIIa receptor. Src and Syk non-receptor tyrosine kinases as well as the actin cytoskeleton control NF-κB activation (panel A). Preincubation of PMP-loaded neutrophils with the Src inhibitor PP2 (10 μg/ml), the Syk inhibitor piceatannol (Pice, 10 μg/ml), and the actin polymerization inhibitor cytochalasin B (Cyto B, 5 μg/ml) prevented degradation of the inhibitory IκBα-protein, whereas the inhibitor of PI3K (LY294002) (LY) 10 μg/ml had no effect (n = 3). The therapeutic GPIIb/IIIa inhibitors abciximab (Ax, 100 ng/ml), eptifibatide (Ep, 1500 ng/ml), and tirofiban (Ti, 40 ng/ml) abrogated NF-κB activation by GM-CSF in neutrophils on fibronectin (panels B–E). Western blot experiments using an antibody to IκBα (n = 5) showed that all three drugs block IκBα degradation but not PI3K/Akt activation in cells subjected to GM-CSF (20 ng/ml) (n = 5) (panel B). Blockade of NF-κB activation was also shown with EMSA (n = 3) (panel C) and quantitative RT-PCR for TNF-α (n = 3) (panel D). Additionally, we proved that the inhibitory substances did not influence the percentage of neutrophils with acquired GPIIb/IIIa receptors (n = 4) (panel E).
Acquired GPIIb/IIIa by Neutrophils and NF-κB

the data imply that not all neutrophil responses are modified by GPIIb/IIIa receptor acquisition. However, in a GM-CSF-rich milieu, the resulting NF-κB activation and subsequent generation of NF-κB-mediated pro-inflammatory molecules would have significant consequences for the inflammatory process. For example, NF-κB-dependent TNF-α generation would affect activation and survival of both neutrophils and neighboring cells.

When we explored mechanisms of the GPIIb/IIIa receptor-mediated NF-κB activation, we found that acquired GPIIb/IIIa receptors and CD18 integrins cluster in close proximity on the neutrophil surface. On the functional level, we found that both receptors cooperate in NF-κB activation. We observed that activating either GPIIb/IIIa or CD18 was sufficient to induce NF-κB. No such effect was seen without prior GPIIb/IIIa receptor acquisition. We excluded cross-reactivity since the blocking antibody against CD18 abrogated adhesion and spreading, whereas the blocking GPIIb/IIIa antibody showed no effect. Interestingly, blocking platelet-derived GPIIb/IIIa abrogated CD18-induced NF-κB activation and vice versa. With GPIIb/CD11b/18 and the intercellular adhesion molecule ICAM-2 to leukocyte β2-integrin, additional examples for integrin-integrin cross-talk were described (26, 33–35). We do not yet know exactly how acquired GPIIb/IIIa receptors link to the neutrophil signaling machinery. However, similar to GPIIb/IIIa receptor signaling in platelets, Src and Syk kinases, as well as the actin cytoskeleton, seem to be involved. The exact mechanism as to how the GPIIb/IIIa receptor and the CD18 integrins cooperate to activate NF-κB needs clarification in further studies.

Regardless of the exact nature of the receptor interaction, we obtained novel data using the therapeutic GPIIb/IIIa inhibitors abciximab, epifibatide, and tirofiban. All three compounds completely prevented NF-κB activation in GM-CSF-treated neutrophils on fibronectin that had acquired GPIIb/IIIa receptors. We believe that the NF-κB inhibition observed in our study was due to GPIIb/IIIa inhibition rather than cross-reactivity to the CD18 β2-integrin. Cross-reaction with the activated form of CD11b/CD18 (αMβ2) (36) and the β3 integrin CD51/61 (vitronecin receptor) (37) was reported for abciximab (38) but not shown for epifibatide and tirofiban (39). Our assumption is further supported by the fact that the therapeutic compounds, similar to the specific GPIIb/IIIa blocking antibody P2, but unlike the blocking CD18 antibodies, showed no inhibitory effect on pure β2-dependent neutrophil adhesion and spreading. Thus, our data support the contention that abciximab, epifibatide, and tirofiban provide anti-inflammatory effects that involve the neutrophil. Acute coronary syndromes are increasingly appreciated as inflammatory diseases. It may well be that GPIIb/IIIa receptor blocker-mediated anti-inflammatory effects participate in the improved outcome of this disease.

In summary, we believe we have shown that GPIIb/IIIa receptors are acquired by neutrophils via PMPs. These newly acquired receptors cooperate with β2-integrins to activate NF-κB signaling. This effect is at work when GM-CSF-treated neutrophils interact with fibronectin. Therapeutic GPIIb/IIIa inhibitory compounds, such as abciximab, epifibatide, and tirofiban, prevent NF-κB activation under these conditions and may have novel implications in anti-inflammatory treatment protocols.

Acknowledgments—The confocal and fluorescence microscopy experiments were performed in the laboratory of Dr. Dieter Blottnig, Neuromuscular Group, Center for Space Medicine Berlin (ZWMB) with help from Dr. Michele Salanova.

REFERENCES

1. Martinez, M. C., Tesse, A., Zobairi, F., and Andriantsitohaina, R. (2005) Am. J. Physiol. 288, H1004–H1009
2. Mack, M., Kleinschmidt, A., Bruhl, H., Klier, C., Nelson, P. J., Cihak, J., Plachy, J., Stangassinger, M., Erfe, V., and Schondorf, D. (2000) Nat. Med. 6, 769–775
3. Mallat, Z., Benamer, H., Bugel, B., Benessiano, J., Steg, P. G., Freynssen, M. J., and Tedgui, A. (2000) Circulation 101, 841–843
4. Daniel, L., Fakhouri, F., Joly, D., Moutheon, L., Nusbaum, P., Grunfeld, J. P., Schifferli, J., Guillemin, L., Lesavre, P., and Hallwachs-Mecarelli, L. (2006) Kidney Int. 69, 1416–1423
5. Forlow, S. B., McEver, R. P., and Nollert, M. U. (2000) Blood 95, 1317–1323
6. Buffon, A., Biasucci, L. M., Liuzzo, G., D’Onofrio, G., Crea, F., and Maseri, A. (2002) N. Engl. J. Med. 347, 5–12
7. Choi, M., Rolle, S., Wellner, M., Cardoso, M. C., Scheideriet, C., Luft, F. C., and Kettritz, R. (2003) Blood 102, 2259–2267
8. Stephens, P., Romer, J. T., Spitali, M., Shock, A., Ortlepp, S., Fidgor, C. G., and Robinson, M. K. (1995) Cell Adhes Commun. 3, 375–384
9. Frelinger, A. L., III, Du, X. P., Plow, E. F., and Ginsberg, M. H. (1991) J. Biol. Chem. 266, 17106–17111
10. Coller, B. S. (1995) Circulation 92, 2373–2380
11. Coller, B. S. (1985) J. Clin. Investig. 76, 101–108
12. Gilchrist, I. C., O’Shea, J. C., Kosoglou, T., Jennings, L. K., Lorenz, T. J., Kint, M. M., Kleinman, N. S., Talley, D., Aguirre, F., Davidson, C., Runyon, J., and Tcheng, J. E. (2001) Circulation 104, 406–411
13. Konstantopoulos, K., Kamat, S. G., Saha, F. A., Ian, E. L., Jordan, S., Kleinman, N. S., and Hellums, J. D. (1995) Circulation 91, 1427–1431
14. Barrett, J. S., Murphy, G., Peerlinck, K., De Lepelle, L., Gould, R. J., Pan, Chian, D., Hand, E., Deckhyn, H., Vermylen, J., and Arnowt, J. (1994) Clin. Pharmacol. Ther. 56, 377–388
15. Merten, M., Pakala, R., Thiagajaran, P., and Benedict, C. R. (1999) Circulation 99, 2577–2582
16. Gemmell, C. H., Sefton, M. V., and Yeo, E. L. (1993) J. Biol. Chem. 268, 14586–14589
17. Ketritz, R., Choi, M., Rolle, S., Wellner, M., and Luft, F. C. (2004) J. Biol. Chem. 279, 2657–2665
18. Folkman, J., and Moscona, A. (1978) Nature 273, 345–349
19. Faull, R. J., and Ginsberg, M. H. (1996) J. Am. Soc. Nephrol. 7, 1091–1097
20. Obergell, A., Eto, K., Mcosai, A., Buescasco, C., Moorees, S. L., Brugge, J. S., Lowell, C. A., and Shattil, S. J. (2004) J. Cell Biol. 167, 265–275
21. Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 13298–13302
22. Gao, J., Zoller, K. E., Ginsberg, M. H., Brugge, J. S., and Shattil, S. J. (1997) EMBO J. 16, 6414–6425
23. Rinder, C. S., Bonan, J. L., Rinder, H. M., Mathew, J., Hines, R., and Smith, B. R. (1992) Blood 79, 1201–1205
24. Wagner, D. D. (2005) Arterioscler. Thromb. Vasc. Biol. 25, 1321–1324
25. Rinder, H. M., Bonan, J. L., Rinder, C. S., Ault, K. A., and Smith, B. R. (1991) Blood 78, 1730–1737
26. Konstantopoulos, K., Neelemangham, S., Burns, A. R., Hentzen, E., Kassas, G. S., Snapp, K. R., Berg, E. L., Hellums, J. D., Smith, C. W., McIntire, L. V., and Simon, S. I. (1998) Circulation 98, 873–882
27. Shattil, S. J., Cunningham, M., and Hoxie, J. A. (1987) Blood 70, 307–315
28. Lee, Y. J., Iw, W., Horstman, L. L., Janania, J., Reyes, Y., Kelley, R. E., and Ahn, Y. S. (1993) Thromb. Res. 72, 295–304
29. Nomura, S., Uehata, S., Saito, S., Osumi, K., Ozeki, Y., and Kimura, Y. (2003) Thromb. Haemostasis 89, 506–512
30. Tan, K. T., Tayebjee, M. H., Lynd, C., Blann, A. D., and Lip, G. Y. (2005) *Ann. Med.* **37**, 61–66
31. Shattil, S. J., and Newman, P. J. (2004) *Blood* **104**, 1606–1615
32. Lo, S. C., Hung, C. Y., Lin, D. T., Peng, H. C., and Huang, T. F. (2006) *J. Biomed. Sci.* **13**, 787–796
33. Simon, D. I., Chen, Z., Xu, H., Li, C. Q., Dong, J., McIntire, L. V., Ballantyne, C. M., Zhang, L., Furman, M. I., Berndt, M. C., and Lopez, J. A. (2000) *J. Exp. Med.* **192**, 193–204
34. Diacovo, T. G., deFougerolles, A. R., Bainton, D. F., and Springer, T. A. (1994) *J. Clin. Investig.* **94**, 1243–1251
35. Kuijper, P. H., Gallardo Tores, H. I., Lammers, J. W., Sixma, J. J., Koen-derman, L., and Zwaginga, J. J. (1998) *Thromb. Haemostasis* **80**, 443–448
36. Altieri, D. C., and Edgington, T. S. (1988) *J. Immunol.* **141**, 2656–2660
37. Charo, I. F., Fitzgerald, L. A., Steiner, B., Rall, S. C., Jr., Bekeart, L. S., and Phillips, D. R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 8351–8355
38. Coller, B. S. (1999) *Am. Heart J.* **138**, (Suppl.) S1–S5
39. Seshiah, P. N., Kereiakes, D. J., Vasudevan, S. S., Lopes, N., Su, B. Y., Flavahan, N. A., and Goldschmidt-Clermont, P. J. (2002) *Circulation* **105**, 174–180