Coexpression of GMP-140 and PAF by Endothelium Stimulated by Histamine or Thrombin: A Juxtacrine System for Adhesion and Activation of Neutrophils

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Abstract. The adhesion of polymorphonuclear leukocytes (PMNs) to vascular endothelial cells (EC) is an early and fundamental event in acute inflammation. This process requires the regulated expression of molecules on both the EC and PMN. EC stimulated with histamine or thrombin coexpress two proadhesive molecules within minutes: granule membrane protein 140 (GMP-140), a member of the selectin family, and platelet-activating factor (PAF), a biologically active phospholipid. Coexpression of GMP-140 and PAF is required for maximal PMN adhesion and the two molecules act in a cooperative fashion. The component of adhesion mediated by EC-associated PAF requires activation of CD11/CD18 integrins on the PMN and binding of these heterodimers to counterreceptors on the EC. GMP-140 also binds to a receptor on the PMN; however, it tethers the PMN to the EC without requiring activation of CD11/CD18 integrins. This component of the adhesive interaction is blocked by antibodies to GMP-140 or by GMP-140 in the fluid phase. Experiments with purified GMP-140 indicate that binding to its receptor on the PMN does not directly induce PMN adhesiveness but that it potentiates the CD11/CD18-dependent adhesive response to PAF by a mechanism that involves events distal to the PAF receptor. Tethering of the PMN to the EC by GMP-140 may also be required for efficient interaction of PAF with its receptor on the PMN. These observations define a complex cell recognition system in which tethering of PMNs by a selectin, GMP-140, facilitates juxtacrine activation of the leukocytes by a signaling molecule, PAF. The latter event recruits the third component of the adhesive interaction, the CD11/CD18 integrins.

ADHESION of polymorphonuclear leukocytes (PMN)[1] to endothelium is an early, requisite event in the acute inflammatory response (Movat, 1985; Osborn, 1990). Binding of PMNs to the endothelium can be rapid and transient, occurring within minutes under some conditions (Clark and Clark, 1935; Marchesi, 1985; Movat, 1985), or it can develop over hours depending on the factors that incite inflammation or tissue damage. This variable time course indicates that molecular mechanisms of adhesion are differently induced or regulated.

PMNs can rapidly alter adhesive molecules on their surfaces in response to specific stimuli. The CD11/CD18 glycoproteins (β_2 or leukocyte integrins) are constitutively expressed in the plasma membranes and in subcellular granules of PMNs (Springer, 1990). When the PMN is activated, there is a rapid qualitative alteration in CD11/CD18 heterodimers present on the PMN surface ("functional upregulation" or "activation") that makes them competent to bind to counterreceptors on the endothelial cells (EC) (Kishimoto et al., 1990). This mechanism is clearly involved in PMN adhesion and emigration in vivo (Anderson and Springer, 1987; Kishimoto et al., 1990). A second surface molecule that can be rapidly altered on the plasma membrane, LAM-1 or LEC-CAM-1, may play a role in targeting PMNs to inflammatory sites (Kishimoto et al., 1989; Watson et al., 1991). Other proadhesive molecules with altered expression on activated PMNs have been identified (Singer et al., 1989; Bohnsack et al., 1990), but their roles in PMN binding in acute inflammation have not been defined.

EC are also activated in inflammatory states and express molecules on their surfaces that mediate adhesion of PMNs (Zimmerman et al., 1990*a*,*c*; McEver, 1991). We previously

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1. *Abbreviations used in this paper*: EC, endothelial cells; GMP-140, granule membrane protein 140; PAF, platelet activating factor; PMN, polymorphonuclear leukocytes.

showed that cultured human EC stimulated with thrombin or certain other agonists become proadhesive for PMNs within minutes (Zimmerman et al., 1985; McIntyre et al., 1986; Lewis et al., 1988; Zimmerman and McIntyre, 1988; Zimmerman et al., 1990*a*). Similar events may occur in the endothelium of postcapillary venules in vivo (Clark and Clark, 1935; Grant, 1973). Rapid EC-dependent PMN adhesion involves the CD11/CD18 integrins; the binding is not completely blocked by anti-CD18 antibodies, suggesting a CD11/CD18-independent component of the adhesive interaction (Zimmerman and McIntyre, 1988). Some of the PMNs that bind to thrombin-stimulated EC are activated as evidenced by polarization (Zimmerman et al., 1985; McIntyre et al., 1986) and other criteria (our unpublished observations). Thus, molecules expressed by the activated EC mediate functional alterations in the PMNs as well as adhesion to the EC plasma membrane. In rapidly activated EC, platelet-activating factor (PAF), a biologically active phospholipid, is transiently synthesized and transported to the plasma membranes where it mediates PMN adhesion by ligating a receptor on the PMN (Zimmerman et al., 1990*a*). Since PAF is not released into the fluid phase under these conditions (Prescott et al., 1984; McIntyre et al., 1985), these experiments demonstrated an unusual mechanism of intercellular interaction involving the regulated surface expression of a phospholipid rather than a protein. However, our observations also suggested that adhesion is influenced by a second molecule that is expressed by EC with kinetics similar to PAF accumulation (Zimmerman et al., 1985). Granule membrane protein-140 (GMP-140) is the second proadhesive molecule.

GMP-140 (PADGEM, CD62) is a membrane glycoprotein constitutively present in secretory granules of platelets and EC. It is translocated within minutes to the plasma membranes following cellular activation (Stenberg et al., 1985; Berman et al., 1986; Hattori et al., 1989; Bonfanti et al., 1989; McEver et al., 1989). After surface expression on histamine-activated EC, GMP-140 is rapidly reinternalized (Hattori et al., 1989). GMP-140 has a predicted structure (Johnston et al., 1989) that is similar to that of ELAM-1 (Bevilacqua et al., 1989) and LAM-1 (Camerini et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Tedder et al., 1989) both of which are involved in EC-leukocyte interactions under other circumstances. These three related proteins have been termed selectins. Human neutrophils and other myeloid cells bind to purified, immobilized GMP-140, to cells transfected with GMP-140 cDNA, and to activated platelets and EC that express GMP-140 on their surfaces (Larsen et al., 1989; Geng et al., 1990; Hamburger and McEver, 1990).

The relative roles of PAF and GMP-140 in rapid EC–PMN interactions are unclear. It is also unknown if GMP-140 modifies the CD11/CD18 adhesive mechanism or other activation responses of neutrophils when expressed by activated EC. This issue is particularly important because others have reported that GMP-140 inhibits CD11/CD18-mediated adhesiveness of PMNs under some conditions (Gamble et al., 1990). Here we show that GMP-140 and PAF are coexpressed on rapidly activated EC and define the contributions of each to the binding and activation of PMNs. Unexpectedly, we found that they act cooperatively rather than in an additive fashion, and evidence that GMP-140, while not directly activating CD11/CD18 integrins on the PMN, facilitates the recruitment of this component of the adhesion mechanism by PAF.

Materials and Methods

Reagents

Histamine was from Sigma Chemical Co. (St. Louis, MO). PAF was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Purified human thrombin was a gift from John Fenton (Albany, NY). Recombinant human TNFα was provided by Genentech, Inc. (South San Francisco, CA) and recombinant human IL1α was provided by Peter Lomedico (Dept. of Molecular Genetics, Hoffman-La Roche Inc., Nutley, NJ). ^{111}In oxine was purchased from the Radiopharmacy Service at the University of Utah, and human serum albumin (25% USP) from Miles Inc. (Elkhart, IN). Medium 199 and HBSS were from Whittaker Bioproducts (Walkersville, MD).

Endothelial Cells

Monolayers of human umbilical vein EC were cultured in multiwell plates (16- or 24-mm wells; Costar, Inc. Cambridge, MD) as described (Zimmerman et al., 1990*b*). Tightly confluent, primary monolayers were used for these experiments.

Measurement of Neutrophil Binding to EC and to Protein Matrices

Human blood was obtained from volunteers after informed consent. Neutrophils were isolated and labeled with ^{111}Indium as described (Zimmerman et al., 1985). The binding assays have been reported previously (Zimmerman et al., 1985; Zimmerman and McIntyre, 1988). Briefly, EC monolayers were washed with HBSS and incubated with control buffer or an agonist in HBSS with 0.5% human serum albumin (HBSS/A) for variable time periods. In some experiments antibodies were added to the incubation mixtures as described in detail below. The incubation mixture was then removed, the monolayers were rapidly washed with HBSS, and ^{111}In-labeled PMNs were added and incubated for 5 min. In certain experiments, including those with EC treated with antibodies against GMP-140, the wash step was omitted (see below). In additional experiments, PMNs were pretreated with antibodies or with PAF receptor antagonists as described in detail below. After the incubation period nonadherent PMNs were removed, the monolayers were washed with HBSS, the adherent radiolabeled PMNs and the EC were solubilized, and the fraction of adherent PMNs (percent of total radiolabeled PMNs added) was quantitated. In incubations with TNFα or IL-1, the cytokine or control buffer was added to EC in complete culture medium for 3.5 or 4 h. The medium was then removed, the EC were washed, and ^{111}In-labeled PMN adhesion was determined after a 5-min incubation. In some experiments EC were washed, ^{111}In-labeled PMNs were added followed by addition of control buffer or an agonist (PAF or fMLP), and PMN binding was measured after 5 min.

^{111}In-labeled PMN binding to protein matrices, made by treating culture wells with gelatin (2,000 μg/ml; type A; Fisher Scientific Co., Fair Lawn, NJ) for 1 h at 37°C, was measured as previously described (Lewis et al., 1988; Bohnsack et al., 1990). Briefly, the matrices were washed once with HBSS (37°C), ^{111}In-labeled PMNs were layered over them, PAF or another agonist was added, and adhesion was measured after a 5- or 15-min incubation period.

Experiments with Antibodies to GMP-140

The preparation of rabbit polyclonal antisera (Stenberg et al., 1985) and monoclonal IgG's (Geng et al., 1990) against GMP-140 has been described previously. A polyclonal anti-GMP-140 was prepared by immunizing a goat with 1 mg of purified human platelet GMP-140 in complete Freund's adjuvant. The animal was boosted 1 mo later with 200 μg of GMP-140 in incomplete Freund's adjuvant. IgG was purified from serum by sequential 30 and 50% ammonium sulfate precipitations and gel filtration on a G200 column equilibrated in 0.1 M NaCl, 1 mM MOPS, and 0.02% sodium azide. The pooled IgG was monospecific for GMP-140 as assayed by Western blotting of human platelet lysates, reacting only with a band that co-migrated with purified human GMP-140. The goat IgG and mAbs were diluted to a concentration of 1 mg/ml in HBSS/A and frozen in aliquots until they were used.

In experiments to determine the effect of antibodies on PMN adhesion to activated EC, the EC monolayers were washed, HBSS/A was added, antibody against GMP-140 or control antibody was added, an agonist (histamine, thrombin) or control buffer was added, and the monolayers were incubated for the specified period of time in an atmosphere of 5% CO_2, 95% air at 37°C. The incubation solutions were removed and ^{111}In-labeled PMNs were added and incubated for 5 min at 37°C, 5% CO_2 without agitation. There was no wash step between incubation of the EC and addition of the PMNs, as there was in certain other experiments. In most experiments the PMN solution contained anti-GMP-140 or control antibody in the same concentration as did the solutions incubated with EC because maximal inhibition required that the antibody was present during incubation of the PMNs with the EC (an unpublished observation). Antibody was added to the PMN solutions and they were gently mixed at room temperature immediately before addition to the EC. Control antibodies were included in each experiment. Monoclonal IgG's S12, T10, and Tab were studied in experiments with monoclonal anti-GMP-140 antibodies. mAb S12 does not inhibit PMN binding to purified GMP-140 or to histamine-activated EC (Geng et al., 1990), even though it identifies an epitope on GMP-140 (McEver and Martin, 1984). In this series of experiments S12 also did not block PMN adhesion to EC activated with thrombin (not shown). mAb T10, directed against the platelet IIb/IIa complex (McEver et al., 1983), Tab, directed against platelet glycoprotein IIb (McEver et al., 1983), and nonimmune murine IgG did not block adhesion to histamine- or thrombin-stimulated EC. In experiments with polyclonal rabbit antisera or monospecific goat IgG against GMP-140, nonimmune sera and polyclonal antisera or purified IgG against von Willebrand factor, vitronectin, fibronectin, complement factors (C3, C3a), or platelet glycoproteins IIb or IIIa were studied in parallel; these reagents reduced PMN adhesion by 20% or less. The preparation of rabbit antisera against glycoprotein IIb/IIIa was previously described (Bray et al., 1986). Charles J. Parker generously provided rabbit antisera against vitronectin (Parker et al., 1988). Nonimmune rabbit serum and rabbit polyclonal anti-vWF were from Behring Diagnostics (La Jolla, CA) or Dako Corp. (Santa Barbara, CA), rabbit antiserum against C3a was from Behring Diagnostics. Mouse and goat nonimmune IgG, goat polyclonal anti-human fibronectin IgG, and goat polyclonal anti-human C3 were from Cappel (Cooper Biomedical Corp., Westchester, PA).

Experiments with Fluid-phase GMP-140

GMP-140 was purified from human platelets as described (Johnston et al., 1989*b*; Moore et al., 1990). It was stored as a stock solution in 0.1 M NaCl, 20 mM Tris and 0.01% (vol/vol) Lubrol® under sterile conditions at 4°C. In experiments to determine its effect on PMN adhesion to activated EC, the GMP-140 or control buffer was added to PMN solutions in various concentrations followed by incubation at room temperature with gentle mixing for 20 min. The PMN suspensions were then added to EC monolayers and adhesion was determined. Fluid-phase GMP-140 was continuously present during incubation of PMNs with the EC. Fibrinogen (Sigma Chemical Co.) and/or equivalent concentrations of Lubrol (vol/vol), the detergent used to solubilize the GMP-140, were used as controls in each experiment.

In experiments to determine the effect of GMP-140 on unstimulated PMN adhesion to resting unactivated EC (Fig. 6) or the response of GMP-140-treated PMNs to exogenous PAF or other agonists (Figs. 7–10), PMNs were treated with GMP-140, fibrinogen, or Lubrol as described above and then added to EC monolayers. In some experiments (Figs. 6–9) the PMNs were centrifuged at 450 *g* for 5 min at room temperature and resuspended in fresh HBSS/A before adding them to the EC. Adhesion was determined after a 5-min incubation period. In experiments in which the PMN were stimulated with PAF, fMLP, or PMA, the agonist was added in the fluid phase at the beginning of the 5-min incubation period with the EC.

Assays of GMP-140 Expression on the Plasma Membranes of Activated EC

Measurement of the binding of mAb S12 (McEver and Martin, 1984) to the surface of histamine-activated EC was done by a modification of the method of Hattori et al. (1989). EC were incubated with histamine (10^{-4} M) in HBSS/A for various times. The incubation solution was removed and the monolayers were fixed with 1% formaldehyde as described (Hattori et al., 1989). They were washed three times with buffer and incubated with buffer containing mAb S12, mAb T10, or nonimmune murine IgG for 45 min at room temperature. Each IgG was present in a final concentration of 2 μg/ml. The antibody solutions were removed and the EC monolayers were washed three times. A radiolabeled sheep anti-mouse IgG (Amersham, Arlington Heights, IL) (1:100 dilution) was then added and incubated for 20 min at room temperature. The monolayers were washed three times with buffer, solubilized with 1 N NH_4OH, and the radioactivity was counted in a gamma counter. In parallel incubations EC were treated with control buffer in place of histamine and binding of S12, T10, or IgG was measured.

Experiments with Competitive PAF Receptor Antagonists

PMNs were pretreated with control buffer or L659,989 (100 μM) for 5 min at room temperature before addition to EC monolayers. The specificity and efficacy of the competitive antagonist were assessed in the following way: In four experiments pretreatment of PMNs with L659,989 inhibited PMN binding to histamine-activated EC by 52% (range 32–77%). L659,989 inhibited PAF-stimulated (10^{-7} M) binding to quiescent EC (i.e., no histamine stimulation) by 76% (75–77% inhibition) but had no effect on fMLP-stimulated (10^{-7} M) adhesion in parallel incubations (0% inhibition). These results are similar to those using L659,989 and its analogs to block PMN adhesion to thrombin-activated EC (Zimmerman et al., 1990*a*). The conditions required for preparation of solutions of the antagonist and the appropriate control buffer, and additional experiments documenting its efficacy and specificity, were described previously (Zimmerman et al., 1990*a*). L659,989 was generously provided by John Chabala (Merck Research Laboratories, Rahway, NJ).

Experiments with CD11/CD18-deficient PMNs and Antibodies against CD11/CD18

Neutrophils from a subject with complete absence of surface expression of CD11/CD18 glycoproteins in both the resting and stimulated state (leukocyte adhesion deficiency) (Bohnsack et al., 1990), were isolated and radiolabeled using the same method as with neutrophils from control subjects. Binding assays were done as with control neutrophils, which were studied in parallel. The anti-CD18 mAbs IB4 (IgG 2a, Wright et al., 1983; a gift from Samuel Wright, Rockefeller University, New York) and 60.3 (IgG 2a; Beatty et al., 1983; a gift from Patrick Beatty, University of Utah) were diluted to a concentration of 1 mg/ml in PBS or HBSS and added to PMN suspensions in a final concentration of 10 μg/ml for each antibody. The PMNs were preincubated with the antibodies, or with control antibodies, for 10 min at room temperature before addition to EC monolayers. The control antibodies were mAb 60.5 (Gamble et al., 1985) or W6/32 (American Type Culture Collection, Rockville, MD) which are both IgG 2a immunoglobulins directed against HLA class I molecules. mAb 60.1 (supplied by Patrick Beatty), an anti-CD11b IgG1 (Beatty et al., 1983; Harlan et al., 1985), was stored in HBSS/A in a concentration of 4 mg/ml and was added to PMN suspensions in a final concentration of 40 μg/ml. Preincubations were done as with mAbs 60.3 and 1B4; nonimmune polyclonal murine IgG in the same concentration was the control.

Inhibition of PMN Activation Responses

PMNs were isolated and labeled with ^{111}Indium. They were then centrifuged at 1,350 RPM for 5 min at room temperature. The buffer was decanted and the pellet was resuspended in 10 ml of ice-cold 2.5% glutaraldehyde and incubated at 4°C for 5 min. The PMNs were centrifuged (1,350 RPM, 5 min at room temperature), resuspended in HBSS with 0.5% albumin (room temperature), and used in the adhesion assay. Control PMNs were treated in the same way but were incubated in buffer rather than glutaraldehyde. Fixed or control PMNs were added to EC monolayers that had been preincubated with buffer, thrombin, or histamine as described above. Adhesion of the PMNs was determined after a 5-min incubation. In additional experiments PMNs were fixed with paraformaldehyde as described (Bevilacqua et al., 1985), chilled to 4°C as described in the legend to Fig. 2, or treated with the metabolic inhibitors 2-deoxyglycose and sodium azide as described (Marlin and Springer, 1987). Control neutrophils were trea ed with appropriate solvents, etc., in parallel.

Results

GMP-140 and PAF Act Cooperatively and Both Are Required for Maximal PMN Adhesion to Thrombin- or Histamine-activated EC

We have shown that thrombin (Zimmerman et al., 1985) and histamine (Geng et al., 1990) stimulate EC to become adhe-

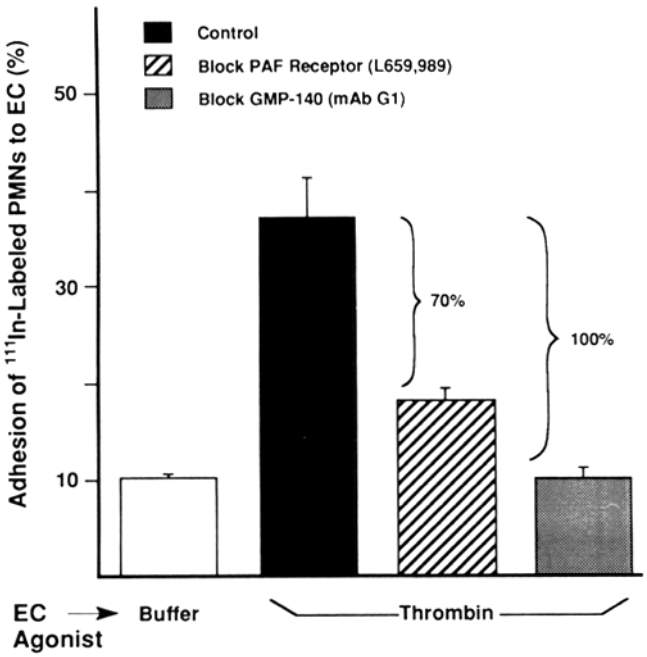

Figure 1. PAF and GMP-140 are required for maximal PMN binding to thrombin-activated EC. EC monolayers were washed with buffer and incubated with control buffer or thrombin (2 U/ml) in HBSS/A for 10 min. In some wells mAb G1 (10 μg/ml) was added immediately before the agonist. After the 10-min incubation, the solutions were removed and ^{111}In PMNs that had been pretreated with control buffer, or the competitive PAF receptor antagonist L659,989 (100 μM), were added. In monolayers treated with mAb G1, the antibody (10 μg/ml) was also present in the PMN suspensions. After a 5-min incubation PMN adhesion was determined. The percent inhibition resulting from blocking the PAF receptor on the PMNs or GMP-140 on the EC is shown in brackets. Both reagents (L659, 989, and mAb G1) added together in the same incubation resulted in 100% inhibition (not shown).

sive for neutrophils, and that under these conditions EC express two molecules, PAF and GMP-140, that could explain the increased adhesiveness. These responses occur within minutes, so we first determined the kinetics of the three events to see whether they were coincident or could be separated. We found that histamine stimulated the adhesion of neutrophils, expression of GMP-140 on the EC surface, and accumulation of PAF all with a peak between 5 and 10 min. Thus, the time of appearance of GMP-140 on the EC surface and the synthesis of PAF were compatible with their participation in the adhesion event. Polyclonal and monoclonal antibodies that block the adhesive properties of GMP-140 in purified systems also inhibited PMN adhesion to histamine-activated EC at the peak of the adhesive interaction, whereas control antibodies did not (Geng et al., 1990; and data not shown). The mean inhibition was 65–85% depending on the antibody. Anti-GMP-140 antibodies also inhibited PMN adhesion to EC activated with thrombin for 5–10 min (not shown). The inhibition was equally potent and specific as with histamine-activated EC. However, blocking the PAF receptor on the PMN potently inhibits PMN adhesion at this time point (Zimmerman et al., 1990*a*), indicating that PAF also mediates adhesion.

To examine the relative contributions of GMP-140 and PAF in the adhesive interaction, we blocked the PAF receptor on PMNs, GMP-140 on activated EC, or both in parallel incubations using a competitive antagonist of the PAF receptor and a blocking mAb to GMP-140. Under the conditions of these experiments, the mAb against GMP-140, G1, does not inhibit PAF-stimulated PMN adhesiveness, and the PAF receptor antagonist, L659,989, does not inhibit PMN adhesion to EC expressing GMP-140 but not PAF (Patel et al., 1991; and data not shown). In an experiment with thrombin-activated EC, each reagent, used alone, inhibited PMN adhesion by >50%, demonstrating that the components of adhesion mediated by GMP-140 and PAF are cooperative and not simply additive (Fig. 1). The pattern of inhibition also indicated a cooperative mechanism in histamine-activated EC (Table I). When the PAF receptor antagonist and mAb G1 were added together (Table I), PMN binding was blocked completely. Thus, PAF and GMP-140 appear to completely account for the EC-dependent PMN adhesion under these conditions.

The Effect of PAF Is to Upregulate CD11/CD18 Integrins on the PMNs; GMP-140 Tethers the Leukocyte Independently of CD11/CD18

The requirement for both PAF and GMP-140 for maximal adhesion and the apparent cooperative nature of the adhesion suggested that the molecular events involved are complex. Therefore, we examined the mechanism(s) by which each of these molecules contributes to the binding of PMNs to activated EC. The PAF receptor is linked to effector systems that require activation of the leukocyte (Prescott et al., 1990; Zimmerman et al., 1990*c*). We asked whether the adhesion of PMN to EC stimulated with histamine or thrombin required that the PMN be metabolically intact. PMNs were fixed with paraformaldehyde or glutaraldehyde, treated with metabolic inhibitors, or cooled to 4°C. In control experiments each of these manipulations blocked PMN adhesion in response to exogenous PAF in solution (not shown). Each of them also dramatically reduced, or completely blocked, the adhesion of PMN to thrombin- or histamine-activated EC (Fig. 2). In contrast, neither fixing nor cooling inhibits the binding of PMNs to GMP-140 (Geng et al., 1990; Moore et al., 1991).

The adhesive response of PMNs to PAF in solution depends on functional upregulation of CD11/CD18. In multiple experiments in which PMN adhesion was induced by exogenous PAF in solution, PAF incorporated into liposomes, or PAF associated with the plasma membranes of unactivated EC, neutrophil adhesion was blocked by antagonists of the PAF receptor or antibodies to CD18 (data not shown). In ad-

Table I. Coexpression of PAF and GMP-140 Mediates PMN Adhesion to Histamine-stimulated EC

EC agonist	Inhibitor	Mean percent inhibition
Histamine	L659,989	58% (range 32–77%)
	G1	80% (range 68–87%)
	L659,989 + G1	98% (range 95–100%)

PMN adhesion was measured in "side-by-side" incubations that were done as those described in the legend to Fig. 1. The mean results and ranges from three experiments are shown. The data are presented as the percent inhibition of PMN adhesion compared to controls containing no inhibitor.

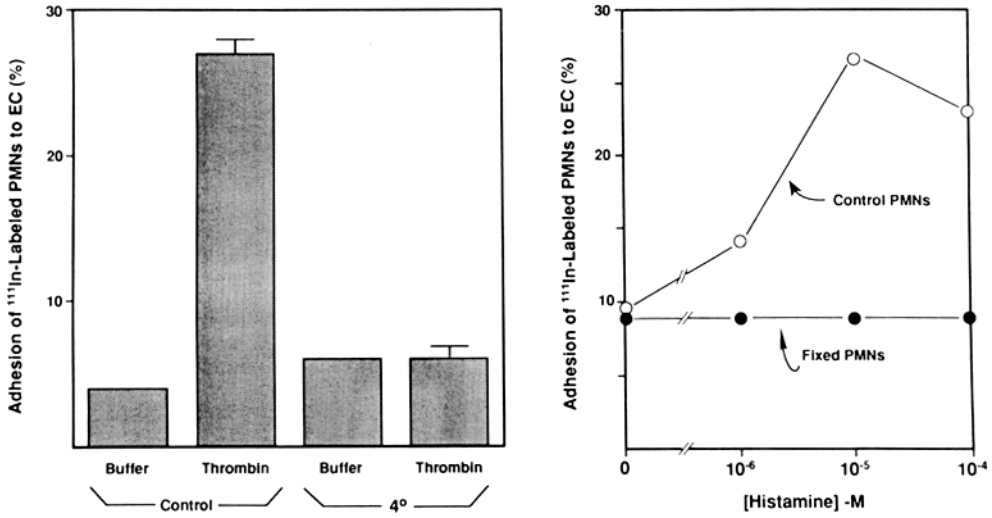

Figure 2. Neutrophil activation is required for maximal adhesion to thrombin- or histamine-activated EC. (*Left*) Chilling PMNs to 4°C inhibits adhesion. EC were pretreated with thrombin or buffer at 37° C for 10 min. Radiolabeled PMNs were equilibrated at 24° or 4°C for 10 min and then incubated with EC at the same temperature for 5 min. The error brackets indicate the range of duplicate measurements; bars without brackets indicate single measurements. In two additional experiments (not shown) cooling PMNs to 4°C inhibited their enhanced adhesion to thrombin-activated EC (enhanced adhesion reduced by 80% or greater compared to PMNs equilibrated at 37°C). (*Right*) Fixation of PMNs reduces their adhesion to rapidly activated EC. PMNs fixed with glutaraldehyde or control PMNs were added to EC that had been preincubated with buffer or histamine for 10 min. Adhesion was determined after a 5-min incubation. In additional experiments PMNs were fixed with paraformaldehyde, which also inhibited their adhesion to histamine- or thrombin-activated EC (not shown). Fixed PMN did not adhere to resting EC in response to PAF or fMLP but adhered to EC induced to express ELAM-1 by treatment with IL-1 or TNFα for 4 h (not shown).

dition, PMNs from a subject with CD11/CD18 deficiency did not adhere in response to PAF. These integrins also are upregulated by the PAF synthesized by EC in response to histamine. In three experiments PMNs were pretreated with a mAb (1B4) to CD18 or control murine IgG and their adhesion to histamine-activated EC was measured. The mAb to CD18 decreased binding by a mean of 52%. Anti-CD18 mAb also partially inhibit PMN adhesion to thrombin-stimulated EC (Zimmerman and McIntyre, 1988). Thus the adhesion of PMN to endothelium in response to PAF, whether the PAF is synthesized and expressed by EC or is pretreated in other ways, requires activation of CD11/CD18 integrins.

The lack of complete inhibition of PMN adhesion to thrombin- or histamine-activated EC by antibodies to CD18 indicated that there is a CD11/CD18-independent component. To confirm this, we studied PMNs from a CD11/CD18-deficient subject. The adhesion of these PMNs to histamine- or thrombin-activated EC was increased by two- to threefold over that to unstimulated EC (Fig. 3), although the increment in adhesion was significantly less than that observed with normal PMNs. These experiments document that there is an alternative binding mechanism. To test whether this component is due to GMP-140 we incubated CD11/CD18-deficient PMNs with rapidly activated EC and examined the ability of mAb G1 to block the binding. This antibody reduced the adhesion to background levels (Fig. 3, *right*). We conclude that GMP-140 can tether the PMN to the EC by a mechanism that does not require activation of CD11/CD18

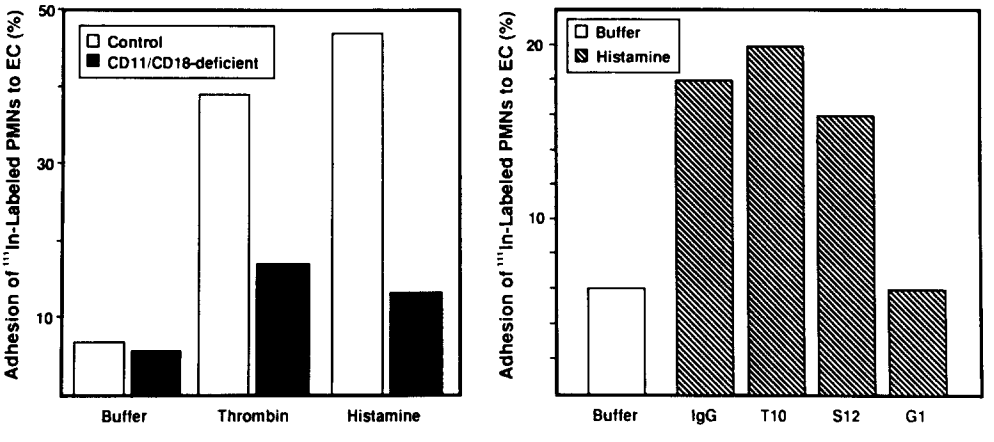

Figure 3. (*Left*) CD11/CD18-deficient PMNs adhere to rapidly activated EC. PMNs from a control subject or a patient with CD11/CD18 deficiency were added to EC that had been treated with control buffer, thrombin (2 U/ml), or histamine (10^{-4} M) for 5 min, and adhesion was determined after a 5-min incubation. The bars indicate single or the mean of duplicate points in one experiment. In three additional incubations the adhesion of CD11/CD18-deficient PMNs to histamine-stimulated EC was 2.5-fold greater than adhesion to control EC, and adhesion to thrombin-stimulated EC was twofold greater than control. (*Right*) GMP-140 expressed by activated EC mediates CD11/CD18-independent PMN binding. EC were washed and incubated with control buffer or histamine (10^{-4} M) for 5 min. In some wells, mAb's G1, T10, S12, or nonimmune murine IgG (10 μg/ml for each) were added immediately before the addition of the agonist. The incubation mixtures were removed and ^{111}In-labeled PMNs from the CD11/CD18-deficient subject were added and incubated for 5 min followed by determination of the fraction of adherent PMNs. In points treated with immunoglobulins, the mAb or control IgG was included in the PMN suspension.

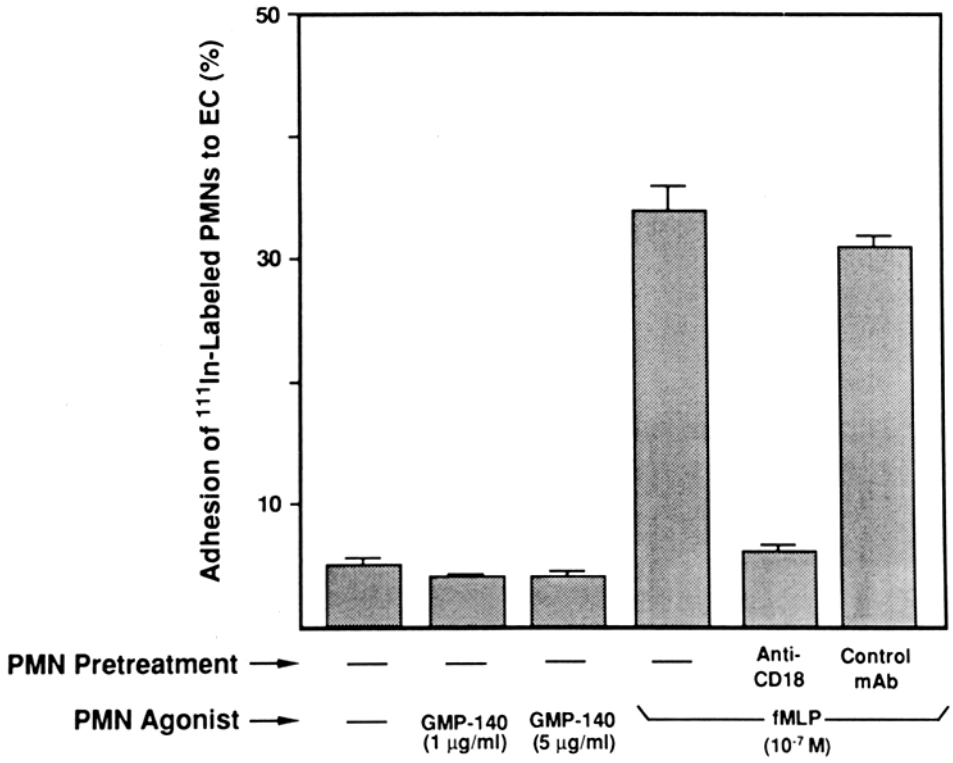

Figure 4. Fluid phase GMP-140 does not stimulate CD11/CD18-dependent adhesion to resting EC. PMNs were added to washed EC monolayers; buffer, GMP-140, or fMLP was added and adhesion was determined after a 5-min incubation period. Some of the PMNs were pretreated with the anti-CD18 mAb IB4 (10 μg/ml) or with the control mAb W6/32 (10 μg/ml) for 10 min at room temperature before addition to the EC. A 5-min incubation of PMNs with EC was used because of the transient nature of CD11/CD18-dependent binding of PMNs to EC (Zimmerman et al., 1985; Lo et al., 1989; Carveth et al., 1989). The bars indicate the mean and range of duplicate points in one of two experiments with similar results.

integrins. CD11/CD18-deficient PMNs specifically adhered to purified, immobilized GMP-140 at both 4° and 37°C (not shown), consistent with this interpretation.

The binding of GMP-140 to its receptor is Ca^{2+} dependent (Geng et al., 1990), and the CD11/CD18 adhesive mechanism is dependent on Mg^{2+} (Kishimoto et al., 1990). We found that maximal adhesion to thrombin-activated EC required both Ca^{2+} and Mg^{2+} (not shown). Thus, the divalent cation requirement for PMN adhesion to rapidly activated EC is consistent with a contribution from both GMP-140 and PAF.

Binding of GMP-140 to PMNs Inhibits Their Adhesion to Activated EC but not to Resting EC

GMP-140 binds to a sialic acid–containing glycoprotein on PMNs (Moore et al., 1991). It is unknown if this receptor is linked to signal transducing systems and if occupancy of the receptor activates the leukocyte. Therefore it is possible that, while GMP-140 does not require the CD11/CD18-dependent mechanism (Fig. 3, *right*), it activates CD11/CD18 integrins (Fig. 3, *left*). To examine this issue, we added purified GMP-140 in solution to PMNs and looked for CD11/CD18-dependent adhesion to resting, unactivated EC. In concentrations sufficient to saturate its binding sites on PMNs, 1–5 μg/ml (Moore et al., 1991), GMP-140 did not directly induce adhesiveness of PMNs (Fig. 4). In each experiment control incubations demonstrated that the leukocytes adhered via the CD11/CD18 mechanism when stimulated by fMLP or PAF.

In contrast to its lack of effect on PMN adhesion to resting EC, fluid phase GMP-140 inhibited PMN binding to histamine- or thrombin-treated EC. The inhibition was concentration-dependent, with maximal effect at 1–5 μg/ml GMP-140 (7–35 nM). The mean inhibition was 74% in four experiments with histamine-treated EC and was the same in five experiments with thrombin-activated EC. Neither a control protein, fibrinogen, nor the Lubrol present in the purified GMP-140 reproduced the inhibition of PMN adhesion (Fig. 5). The effect of fluid phase GMP-140 was specific in these experiments; it did not inhibit PMN adhesion to EC induced to express ELAM-1 by cytokine treatment (not shown).

GMP-140 Potentiates Neutrophil Adhesiveness Induced by PAF and Other Agonists

The data in Fig. 5 are consistent with the conclusion that fluid phase GMP-140 inhibited the adhesion of PMNs to histamine-activated EC by competing for its binding site on the PMNs. However, other workers have suggested that soluble GMP-140 inhibits CD11/CD18-dependent adhesiveness of stimulated PMNs (Gamble et al., 1990). Therefore we examined the effect of GMP-140 on PMN adhesion known to depend on CD11/CD18. We found that GMP-140 in the fluid phase enhanced the adhesion of PMNs to unactivated endothelium when they were stimulated with exogenous PAF (Fig. 6). The qualitative effect was consistent in multiple experiments done under a variety of conditions although the magnitude of the enhancement varied from experiment to experiment (see Figs. 6–9). The potentiation of adhesion was maximal at 1–5 μg/ml GMP-140 and was measurable when the PMNs were treated with GMP-140 for 2, 5, or 20 min (not shown). The enhancement occurred when the GMP-140 was continuously present (not shown) or when the PMNs were centrifuged and resuspended in fresh buffer after pretreatment with GMP-140 (see Figs. 6–9). Polymyxin B did not inhibit the potentiation, excluding the possibility that the effect was because of contaminating endotoxin (not shown). A control protein, fibrinogen, in the same concentrations and in the same solvent, did not reproduce the enhancement (see Figs. 6 and 9).

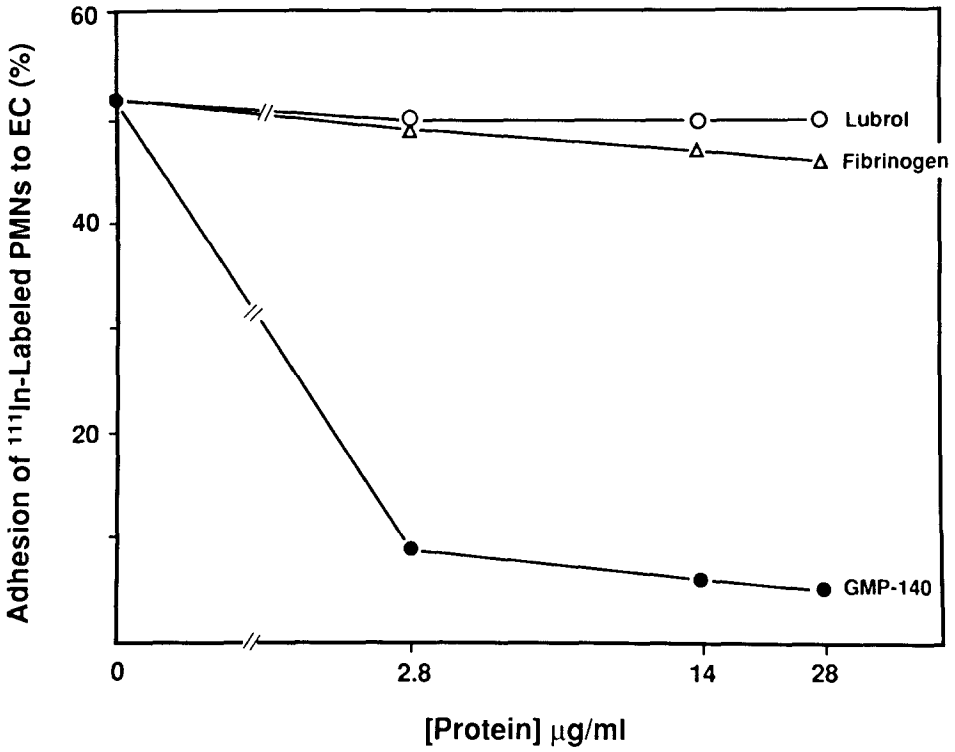

Figure 5. Fluid phase GMP-140 inhibits PMN adhesion to histamine-activated EC. PMNs were pretreated with GMP-140 solubilized from platelet membranes, with fibrinogen, or with Lubrol in the concentrations indicated for 20 min at room temperature with gentle mixing. They were then added to EC that had been pretreated with histamine (10^{-4} M) for 5 min. The concentrations of Lubrol (vol/vol) were equal to those in the PMN suspensions containing GMP-140. The figure indicates single points from one experiment.

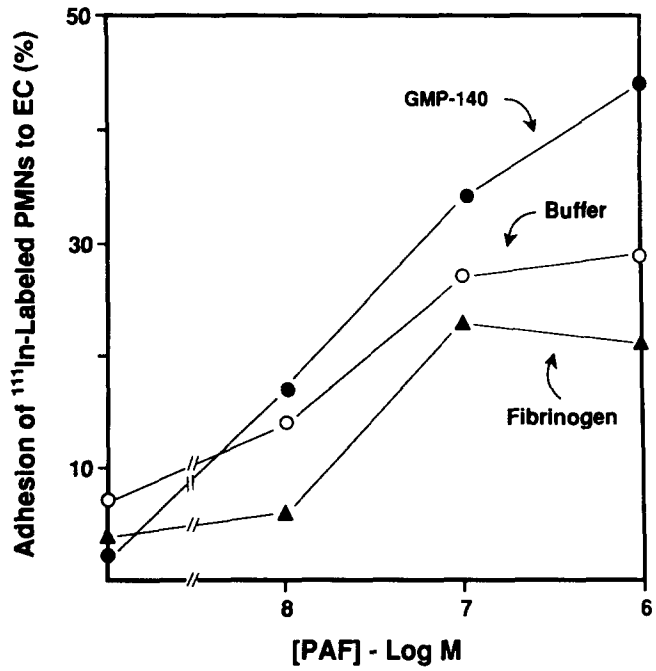

Figure 6. Fluid phase GMP-140 enhances PAF-stimulated adhesion to EC. PMNs were pretreated with GMP-140 (2 μg/ml), fibrinogen (2 μg/ml), or control buffer for 20 min at room temperature. The fibrinogen- and buffer-treated PMN suspensions also contained the same concentration of detergent (Lubrol; see Materials and Methods) as the GMP-140-treated PMNs. After the pretreatment period the PMNs were sedimented at 450 g for 5 min at room temperature, resuspended in fresh HBSS/A, and added to EC monolayers. Control buffer or PAF in the concentrations shown was added and adhesion to the monolayer was determined after a 5-min incubation.

The potentiating effect was blocked by mAb G1, indicating that the effect is specific, and that binding of GMP-140 to the neutrophil is necessary (Fig. 7). The adhesive response of GMP-140–treated PMNs to PAF was completely inhibited by a PAF receptor antagonist (Fig. 8), or by a mAb to CD18 (Fig. 8). These experiments indicate that ligation of PAF to its receptor on the PMNs and activation of CD11/CD18 integrins is required, and that the potentiating effect of GMP-140 is not due to recruitment of an alternative adhesive mechanism. The results shown in Figs. 7 and 8 demonstrate that mAb G1 blocks only the component of enhanced adhesion mediated by GMP-140, whereas blocking the PAF receptor or the CD11/CD18 integrins reduced the adhesion of PAF-stimulated PMN to, or below, that of PMNs treated with control buffer.

Certain lectins enhance the response of platelets to PAF by an apparently selective effect on the PAF receptor (Hwang and Wang, 1991). Since GMP-140 is a lectin, we asked whether its enhancement of neutrophil adhesion in response to PAF was specific or whether it also occurred with other agonists. Fluid phase GMP-140 enhanced PMN adhesion in response to fMLP, which acts through a different receptor, and to PMA, which directly activates protein kinase C (Fig. 9). In two additional experiments we examined the response of PMN to opsonized zymosan, which uses CD11b/CD18 as a receptor (Kishimoto et al., 1990). PMN were incubated in tissue culture wells that had been coated with GMP-140, albumin, or fibrinogen and then were stimulated with opsonized zymosan. PMN adherent to GMP-140 generated more superoxide anion than PMN incubated on the control surfaces (not shown). Thus, the increased PMN responsiveness in the presence of GMP-140 was not specific for PAF, but was observed with agonists that act via different receptors.

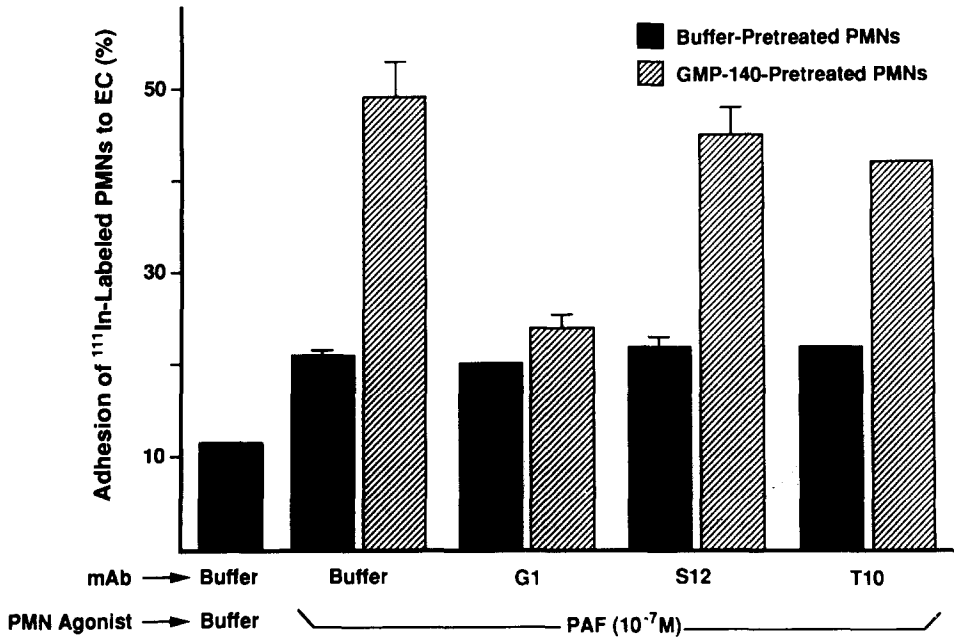

Figure 7. An anti-GMP-140 mAb, G1, inhibits GMP-140 enhancement of PAF-stimulated PMN adhesiveness. Control buffer (HBSS/A) or GMP-140 (2 μg/ml) was incubated with buffer or mAb G1, S12, or T10 for 60 min at 4°C as described (Moore et al., 1991). The solutions were then warmed to room temperature, PMNs were added, and the suspensions incubated for another 20 min at room temperature. The PMNs were then pelleted, resuspended in HBSS/A, and added to EC. Buffer or PAF was added and adhesion was determined after a 5-min incubation. In parallel incubations, PMN were pretreated with fibrinogen (2 μg/ml) that had been treated with buffer or the same mAb as GMP-140. Fibrinogen did not enhance PMN adhesion in response to PAF nor did any of the mAb alter the PAF-stimulated adhesion of fibrinogen-pretreated PMNs. Therefore the data are not shown to simplify the figure.

Discussion

The adhesion of PMNs to EC activated with histamine or thrombin requires three pairs of molecular interactions: binding of GMP-140 to its receptor on the PMN; binding of PAF to its receptor on the leukocyte; and binding of CD11/CD18 glycoproteins to counterreceptors on the EC (Fig. 10). The adhesive mechanism involves a novel example of juxtacrine cell-cell interaction (Massagué, 1990) in which cell-associated PAF acts as a signal that activates the PMN, recruiting participation of adhesion effectors, the CD11/CD18 integrins. Their binding amplifies the avidity of the tethering effect of GMP-140. The GMP-140 component, while relatively weak by itself under these conditions, is essential for effective operation of the PAF component and may facilitate PAF-stimulated CD11/CD18 activation.

The intercellular interaction begins with unactivated EC and unactivated PMNs. Histamine or thrombin, acting through EC surface receptors, induce translocation of GMP-140 from secretory granules to the plasma membrane where

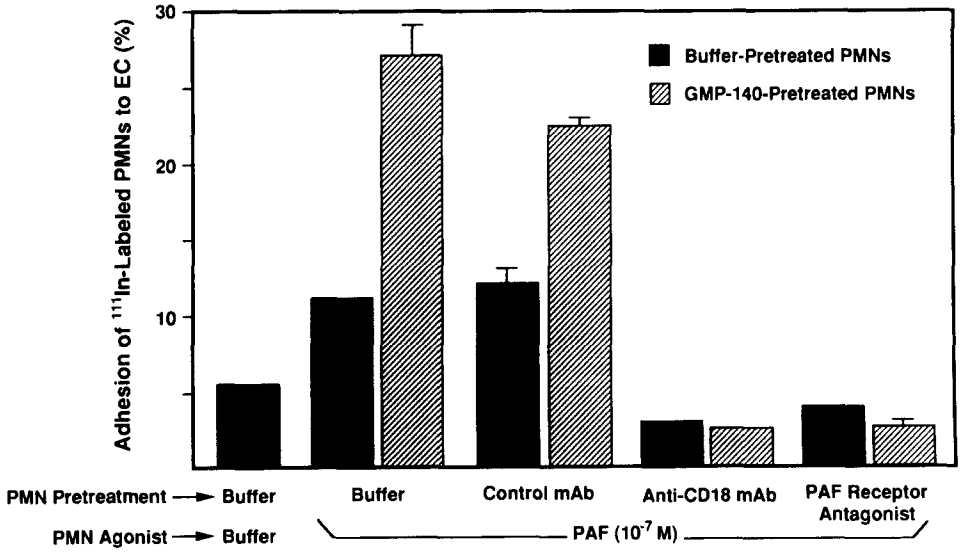

Figure 8. Enhanced adhesion of GMP-140–pretreated PMN in response to PAF requires the PAF receptor and CD11/CD18 integrins. PMN were pretreated with control buffer or GMP-140 (2 μg/ml) for 10 min as described in Fig. 6. They were then pelleted, resuspended in fresh HBSS/A, and incubated with a PAF receptor antagonist (L659,989, 100 μg/ml), an anti-CD18, mAb, (IB4, 10 μg/ml), a control mAb (W6/32, 10 μg/ml) or control buffer for an additional 10 min. Following this, they were added to EC monolayers, buffer or PAF was added, and adhesion was determined after a 5-min incubation. In a parallel set of points, PMN were pretreated with fibrinogen (2 μg/ml) and the PAF receptor antagonist, antibodies, or control solutions. Fibrinogen did not enhance PAF-stimulated adhesion, and the adhesion of PMNs pretreated with fibrinogen and stimulated with PAF was blocked by L659,989 and by IB4 but not by W6/32. The data are not shown to simplify the figure.

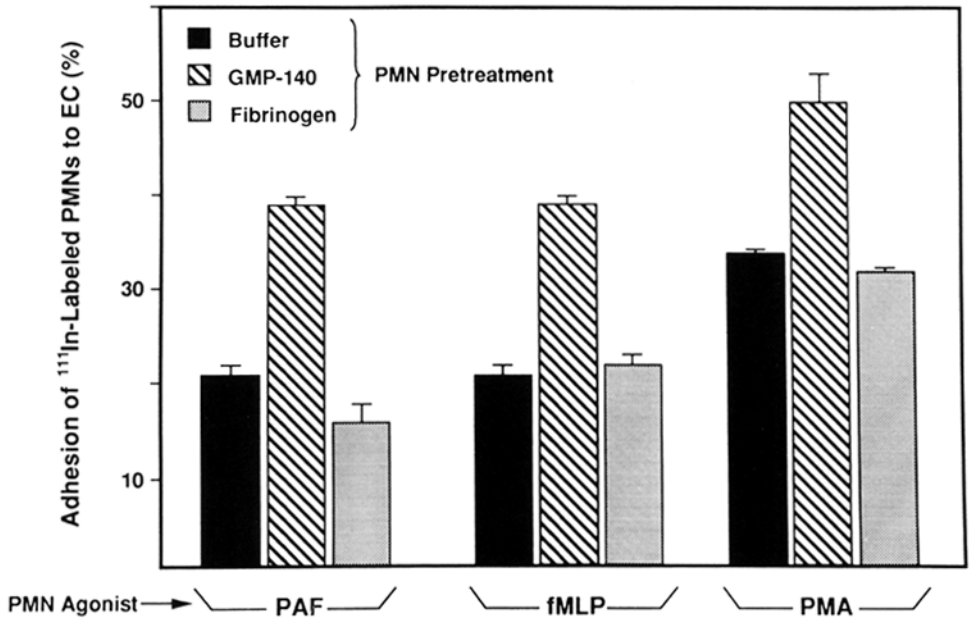

Figure 9. Fluid phase GMP-140 enhances FMLP- or PMA-stimulated PMN adhesion to EC. PMNs were pretreated with buffer, GMP-140 (2 μg/ml), or fibrinogen (2 μg/ml) as described in Fig. 6, pelleted, resuspended in fresh buffer, and added to EC monolayers. PAF (10^{-7} M), FMLP (10^{-7} M), or phorbol myristate acetate (PMA; 10^{-7} M) was added and adhesion was measured after 5 min. The data from three experiments are shown.

it ligates a glycoprotein on the PMN surface by a lectinlike interaction (Moore et al., 1991). The receptor appears to be different from that for ELAM-1, since fluid-phase GMP-140 inhibits PMN adhesion to thrombin- or histamine-activated EC but not to cytokine-activated EC under conditions in which the adhesion is highly dependent on ELAM-1. Because purified, immobilized GMP-140 supports adhesion of both fixed and native PMNs (Geng et al., 1990) it is likely that binding of GMP-140 to its receptor on the PMN is a bridging or capturing event that tethers the PMN to the EC. This does not require activation of the CD11/CD18 adhesive mechanism (Fig. 3, *right*). However, one or more activation responses of the PMN (Fig. 2), including functional upregulation of CD11/CD18 integrins (Fig. 3, *left*), is required for maximal PMN adhesion to rapidly activated EC. This contrasts with a lack of requirement for these events when PMNs bind to purified, immobilized GMP-140 (Geng et al., 1990), or to EC that express GMP-140 for prolonged periods (Patel et al., 1991) rather than transiently. Purified GMP-140 does not appear to directly activate the PMN adhesive mechanism (Fig. 4), consistent with the finding that it does not induce quantitative upregulation of CD11/CD18 integrins or shedding of LAM-1 (Moore et al., 1991) and does not directly induce other activation responses of PMNs (Lorant et al., manuscript in preparation). This indicates that a second proadhesive molecule is involved in the EC–PMN interaction.

PAF that is rapidly synthesized and translocated to the plasma membrane in histamine- or thrombin-activated EC also ligates a receptor on the PMN and mediates juxtacrine activation, a term originally used to describe activation of target cells by membrane-anchored growth factors (Anklesaria et al., 1990). This interaction is blocked by specific competitive receptor antagonists (Fig. 1 and Table I) or by desensitizing the PAF receptor (Zimmerman et al., 1985; McIntyre et al., 1986; Lewis et al., 1988). The neutrophil PAF receptor is present on the unactivated PMNs (Hwang, 1988) and is linked to GTPase and calcium regulatory signal transduction mechanisms (Hwang, 1988; Prescott et al.,

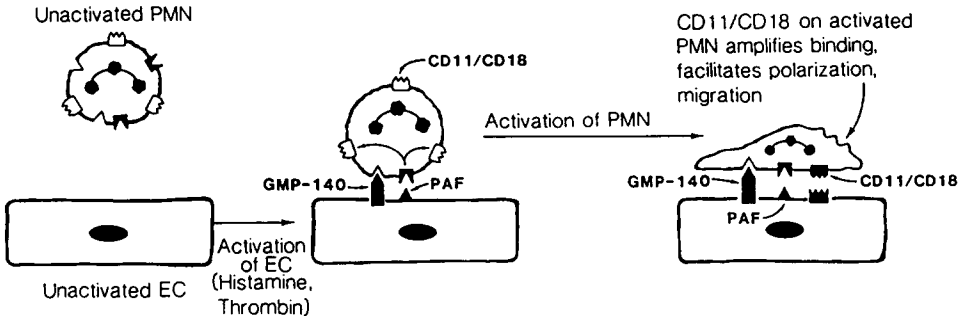

Figure 10. PMN adhesion to histamine- or thrombin-activated EC requires coordinate expression of GMP-140 and PAF. In histamine- or thrombin-activated EC GMP-140 is rapidly expressed on the EC surface where it binds to a receptor on the neutrophil, tethering it to the EC. This tethering event does not require CD11/CD18 heterodimers on the neutrophil. PAF is rapidly synthesized and is coexpressed with GMP-140 on the EC. PAF ligates a receptor on the PMNs, serving as a signal for activation-dependent alteration in CD11/CD18 glycoproteins (CD11a/CD18 and CD11b/CD18) on the PMN plasma membrane that makes them competent to bind to counterreceptors on the EC. The tethering component mediated by GMP-140 facilitates PAF interaction with its receptor on the PMN and enhances the PAF-stimulated adhesiveness. Binding sites for activated CD11/CD18 heterodimers on the PAF-stimulated PMNs are present on both unactivated (not shown) and activated EC. Antibodies to ICAM-1, an EC counterreceptor for CD11a/CD18 and CD11b/CD18, do not inhibit PAF-stimulated PMN adhesion to EC (our unpublished observation); therefore the identity of the EC binding site(s) is unknown. See text for details.

1990). A receptor for PAF was recently characterized by molecular cloning from guinea pig lung (Honda et al., 1991). Its structure predicts linkage to guanyl nucleotide-binding regulatory proteins ("G-proteins"), consistent with these characteristics and with our observation that PAF-stimulated adhesiveness of PMNs requires an activation response by metabolically competent cells. Activation of PMNs by PAF or other receptor-mediated agonists results in increased quantitative or functional expression of several surface molecules that may be involved in adhesion (Singer et al., 1989; Bohnsack, 1990; Stocks et al., 1990). However, PAF-stimulated PMN binding to EC did not occur when anti-CD18 mAb were present or when CD11/CD18-deficient PMNs were studied. Therefore the component of adhesion signaled by EC-associated PAF appears to exclusively require CD11/CD18 glycoproteins and not other PMN adhesion molecules. Both CD11a/CD18 and CD11b/CD18 mediate adhesion of neutrophils stimulated by PAF (our unpublished observation). PAF-induced adhesion mediated by these integrins is rapidly reversible (Zimmerman et al., 1985), consistent with the transient adhesion of PMNs to thrombin- or histamine-activated EC. The system of a signaling molecule that binds to a receptor and recruits a separate adhesion effector on the target cell is different from other examples of juxtacrine activation, where interaction of a cell-associated growth factor with a receptor on the second cell mediates both adhesion and activation (Anklesaria et al., 1990).

The fundamentally different mechanisms of adhesion provided by GMP-140 and PAF (tethering versus activation of PMNs and recruitment of CD11/CD18 integrins) predicted that their contributions to the adhesive interaction would be additive. However, blocking either the PAF or GMP-140 component in parallel incubations in the same experiment reduced adhesion by more than 50% in each case (Fig. 1 and Table I), indicating a cooperative mechanism of adhesion. In addition, in some experiments blocking GMP-140 completely inhibited adhesion, even though a significant PAF component could be demonstrated (Fig. 1). This suggests that the tethering effect of GMP-140 is required for efficient interaction of PAF in the EC plasma membrane with its receptor on the PMN. The physical orientation of PAF in the surface membranes of activated EC is unknown, but it may be in the outer leaflet of the lipid bilayer and thus relatively inaccessible because of surface proteins in adjacent areas (Zimmerman et al., 1990*a*). Transient surface expression of GMP-140 and tethering of PMNs by this mechanism may bring local domains of the neutrophil plasma membrane into close proximity with EC-associated PAF, facilitating receptor-ligand interaction. In other systems, membrane–membrane contact of cells mediated by one molecule is required to initiate specific interactions between others (Rutishauser et al., 1988).

In addition to physical considerations, binding of GMP-140 in the EC plasma membrane to its receptor on neutrophils may potentiate the PAF-stimulated component of adhesion. This is suggested by experiments in which PMNs were treated with fluid phase GMP-140 and then stimulated with exogenous PAF (Fig. 6–9). The mechanism by which this occurs is unknown, but the experiments in Fig. 9 suggest that ligation of GMP-140 to its receptor on the PMN potentiates CD11/CD18 adhesive responses stimulated by other agonists as well, and that protein kinase C–transduced events may be involved. Further definition of the mechanism will require identification of the GMP-140 receptor (Moore et al., 1991; Springer and Lasky, 1991) and characterization of the functional changes in the PMN that result from ligation of the receptor.

Our experiments indicating that fluid phase GMP-140 enhances PAF- or fMLP-stimulated PMN adhesion are at variance with observations that solubilized GMP-140 inhibits the ability of certain agonists to induce the CD11/CD18 adhesive mechanism (Gamble et al., 1990) and superoxide anion generation (Wong et al., 1991). Gamble et al. (1990) suggested that the putative soluble form of GMP-140 (Johnston et al., 1989*a*) may inhibit these responses as well. The negative effects might also be expected to be operative when GMP-140 is expressed in membranes, since the soluble form lacks only the membrane-spanning domain and does not have deletions in the amino terminal lectin domain that interacts with the PMN receptor (Johnston et al., 1989*a*). GMP-140 on the surface of activated EC might then inhibit CD11/CD18-dependent adhesiveness induced by EC-associated PAF. However, we found that antibodies to GMP-140 reduced adhesion (Fig. 1 and Table I) rather than enhancing it. Taken together, our experiments indicate that GMP-140 in EC membranes supports, rather than inhibits, CD11/CD18-dependent adhesiveness. The experiments in Figs. 6–9 exclude the possibility that the decrease in adhesion of PMNs treated with fluid phase GMP-140 to rapidly activated EC (Fig. 5) is due to inhibition of the CD11/CD18-dependent component of adhesion, and suggest that GMP-140 may potentiate the CD11/CD18 adhesive mechanism. The reason for the difference between our results and those of Gamble et al. (1990) is unknown.

If regulated coexpression of GMP-140 and PAF occurs in response to EC agonists in vivo, the juxtacrine system that we describe may confer additional biologic advantages besides tightness of adhesion. First, the tethering effect of GMP-140 provides specificity to the adhesive interaction since receptors for GMP-140 are present on PMNs and monocytes but not platelets (Moore et al., 1991). In contrast, platelets have PAF receptors and could potentially adhere to activated endothelium in the absence of a requirement for tethering by GMP-140. In other types of cell–cell interactions, combinations of recognition molecules provide additional degrees of specificity over that of one molecular system alone (Bixby et al., 1987; Rutishauser et al., 1988). Second, the GMP-140 and CD11/CD8-dependent mechanisms of adhesion may be variably operative under different conditions of flow and stasis. The GMP-140–mediated, CD11/ CD18-independent component may be required for the PAF-stimulated, CD11/CD18-dependent component to operate in vessels subjected to high shear stress early in the inflammatory response (Lawrence et al., 1990). Third, the transient coexpression of GMP-140 and PAF by EC may influence subsequent events critical to the acute inflammatory response. Adhesion of PMNs to endothelium, activation of the CD11/CD18 integrins, and polarization are required for effective migration of the leukocytes to extravascular sites (Movat, 1985; Anderson and Springer, 1987; Smith et al., 1989; Kishimoto et al., 1990; Springer, 1990). The signaling component provided by EC-associated PAF may facilitate this sequence of events. Coexpression of tethering and signaling molecules by activated endothelium

provides a mechanism for both adhesion and regulated functional alteration of the leukocyte at the first committed step in the acute inflammatory response. This paradigm, which we have defined in rapidly activated EC, may be generally applicable to other leukocyte-endothelial cell interactions.

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Note Added in Proof. Recently it was reported that GMP-140 and CD11/CD18 integrins act cooperatively to enhance the avidity of PMN adhesion in a model membrane system (Lawrence, M. B., and T. A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell*, 65:1–20), similar to the juxtacrine system in endothelium described here. That study also supports the observation that GMP-140 neither directly induces nor inhibits CD11/CD18-mediated adhesiveness of PMNs.

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