Endothelial Cell-associated Platelet-activating Factor: A Novel Mechanism for Signaling Intercellular Adhesion

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Abstract. The binding of neutrophils (polymorphonuclear leukocytes [PMNs]) to endothelial cells (ECs) presents special requirements in the regulation of intercellular adhesion. ECs that are stimulated by certain agonists, including thrombin and cytokines (tumor necrosis factor α, interleukin-1), generate molecular signals that induce the adhesion of PMNs (endothelial cell–dependent neutrophil adhesion). Our experiments demonstrate that the mechanism of binding induced by thrombin is distinct from that induced by the cytokines based on the time courses, the requirement for protein synthesis, and differential binding of HL60 promyelocytic leukemia cells to ECs activated by the two classes of agonists. The rapid EC-dependent PMN adhesion (initiated in minutes) that occurs when the ECs are stimulated by thrombin is temporally coupled with the accumulation of platelet-activating factor, a biologically active phosphoglyceride that remains associated with ECs and that activates PMNs by binding to a cell surface receptor. A portion of the newly synthesized platelet-activating factor (PAF) is on the EC surface, as demonstrated by experiments in which the rate of hydrolysis of PAF synthesized by activated ECs was accelerated by extracellular PAF acetylhydrolase. When ECs were treated with exogenous PAF they became adhesive for PMNs; the PMN binding was prevented by incubating the ECs with PAF acetylhydrolase or by treating the PMNs with competitive PAF receptor antagonists. Thus PAF associated with the EC plasma membrane induces PMN binding, an observation supported by experiments in which PAF in model membranes (liposomes) stimulated rapid PMN adhesion to ECs and to cell-free surfaces. In addition, competitive antagonists of the PAF receptor inhibited the binding of PMNs to ECs activated by thrombin and other rapidly acting agonists, but not to ECs activated by tumor necrosis factor α, indicating that PAF that is endogenously synthesized by ECs can mediate neutrophil adhesion. These experiments demonstrate a novel mechanism by which a cell-associated phospholipid, PAF, can serve as a signal for an intercellular adhesive event.

The interaction of circulating blood cells with the endothelial cells (ECs)[1] that line vessels presents special requirements in the signaling and regulation of intercellular adhesion, a fundamental biologic process. Under usual physiologic conditions blood cells remain separate from ECs or loosely associated with them, as in the case of the "marginal pool" of polymorphonuclear leukocytes (Athens et al., 1961). However, under other conditions individual types of blood cells adhere to localized areas of the vessel wall, in response to specific stimuli, to perform homeostatic functions. Examples include the accumulation of platelets at injured areas of vessels, which is required for hemostasis, and the adhesion of leukocytes to ECs as the first step in their migration to sites of extravascular infection or injury, a process that is required for host defense. Under physiologic conditions avid binding of myeloid leukocytes to ECs, which occurs in segments of the vasculature adjacent to sites harboring inflammatory stimuli, is reversible (allowing leukocytes to migrate to extravascular sites), and involves differential signaling for different kinds of leukocytes. For example, polymorphonuclear leukocytes (PMNs; neutrophils) accumulate rapidly at sites of acute inflammation, whereas the accumulation of mononuclear leukocytes occurs later and is more prolonged (van Furth et al., 1979).

Two general mechanisms for adhesion of PMNs to EC have been identified. The first involves activation of the PMNs by chemotactic factors (n-formylmethionyl-leucyl-phenylalanine [fMLP], leukotriene B_4, C5a) and agents such as PMA, resulting in altered expression of surface adhesive proteins (CD_{11}/CD_{18} heterodimers) (Anderson et al., 1986) that are members of the integrin superfamily (Kishimoto et al., 1987). At least one of these, CD_{11b}/CD_{18}, interacts with molecular targets on ECs (Wallis et al., 1986; Zimmerman

1. *Abbreviations used in this paper*: AH, acetylhydrolase; EC, endothelial cell; ELAM, endothelial cell leukocyte adhesion molecule; fMLP, n-formylmethionyl-leucyl-phenylalanine; HBSS/A, HBSS with 0.5% human albumin; IL, interleukin I; LDL, low density lipoprotein; PAF, platelet-activating factor; PMN, polymorphonuclear leukocyte; TNF, tumor necrosis factor.

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and McIntyre, 1988; Tonnesen et al., 1989). Intercellular adhesion molecule 1 (ICAM-1) on ECs appears to be a ligand for CD_{11}/CD_{18} molecules on stimulated PMNs (Smith et al., 1988). The CD_{11}/CD_{18}-dependent mechanism of adhesion is nonspecific, since it mediates aggregation of PMNs and their adhesion to matrix proteins and to a variety of other cells in addition to endothelial cells (Anderson et al., 1986; Wallis et al., 1986; Simon et al., 1986; Zimmerman and McIntyre, 1988; Wright et al., 1988).

In the second general mechanism ECs, rather than PMNs, are first activated, causing them to express molecules that promote PMN binding (EC-dependent PMN adhesion) (Zimmerman et al., 1985, 1986). EC-dependent PMN adhesion may be the relevant mechanism for the physiological interaction of neutrophils with ECs since it would target PMNs to specific areas of the intimal surface. There are at least two forms of EC-dependent adhesion of neutrophils. One occurs within minutes after exposure to thrombin and certain other agonists (Zimmerman et al., 1985; McIntyre et al., 1986; Lewis et al., 1988). The other appears over hours in response to the cytokines interleukin-1 (IL-1), tumor necrosis factor (TNFα), and lymphotoxin (TNFβ), or to endotoxin (Bevilacqua et al., 1985; Gamble et al., 1985; Schleimer and Rutledge, 1986; Broudy et al., 1987). The cytokine-stimulated response is temporally associated with the expression of a newly synthesized glycoprotein, termed endothelial cell leukocyte adhesion molecule-1 (ELAM-1), that mediates PMN adhesion and also promotes the binding of other myeloid cells (Bevilacqua et al., 1985, 1987, 1989). In contrast, EC-dependent PMN adhesion induced by thrombin and other rapidly acting agonists is temporally coupled with the synthesis of platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Prescott et al., 1984; Zimmerman et al., 1985; McIntyre et al., 1986; Lewis et al., 1988), a unique glycerophospholipid that activates PMNs, as well as platelets and other cells, by interacting with cell surface receptors (Hanahan, 1986; Hwang, 1988).

Earlier, we proposed that the PAF that is synthesized by activated EC is a signal for PMNs to bind (Prescott et al., 1984; Zimmerman et al., 1985). This conclusion was based on several lines of evidence, of which the most compelling was the demonstration that selective desensitization of the PAF receptor on PMNs blocked their adhesion to thrombin- or LTC_4-stimulated ECs (Zimmerman et al., 1985; McIntyre et al., 1986). However, the precise role of PAF was uncertain since we found that newly synthesized PAF remained associated with ECs, rather than being released into the fluid phase (Prescott et al., 1984; McIntyre et al., 1985), and it was unknown if it could function as a mediator of intercellular interaction when presented in association with a membrane surface (Zimmerman et al., 1985). Furthermore, the desensitization studies could have been flawed if there were effects other than "homologous desensitization" of the PAF receptor that were not manifest in our controls for selectivity (Sibley et al., 1987). In this report we present evidence demonstrating that EC-derived PAF mediates PMN binding to thrombin-stimulated endothelium. We also further differentiate the mechanism of binding stimulated by rapidly acting agonists and that stimulated by cytokines. These experiments demonstrate a novel mechanism for cell–cell interaction, in which a cell-associated lipid mediator, PAF, acts to induce rapid and reversible intercellular adhesion, a process usually ascribed to glycoproteins or lectins (Ekblom et al., 1986; Edelman, 1986; McClay and Ettensohn, 1987).

Materials and Methods

Materials

PAF was purchased from Avanti Polar Lipids, Inc. (Birmingham, Alabama), and [*sn*-2-^{3}H-acetyl]PAF, [1-O-^{3}H-alkyl]PAF, and [^{3}H]acetate were from New England Nuclear (Boston, MA). Purified human thrombin was a gift from John Fenton (Albany, NY). Recombinant human TNFα (5×10^7 U/mg; lot 3056–63) was provided by John Kaumeyer of Genentech, Inc. (San Francisco, CA) and recombinant human IL-1α (2.1×10^7 U/mg; lot SM-47) was provided by Peter Lomedico (Department of Molecular Genetics, Hoffman-La Roche Inc., Nutley, NJ). Leukotrienes B_4 and C_4 were gifts from Joshua Rokach, Merck-Frosst Laboratories (Montreal, Canada). Phospholipase A_2 from bee venom was from Sigma Chemical Co., (St. Louis, MO). ^{111}Indium oxine was purchased from the Radiopharmacy Service at the University of Utah, and human serum albumin (25% USP) from Miles Laboratories Inc. (Elkhart, IN). Medium 199, RPMI 1640, and Hanks' balanced salt solution (HBSS) were from Whittaker M. A. Bioproducts (Walkersville, MD), and FBS was from HyClone Laboratories (Logan, UT).

Endothelial Cells

Monolayers of human umbilical vein ECs were cultured in multiwell plates (16-mm wells; Costar Data Packaging Corp., Cambridge, MA) or individual 35-mm dishes as described (Zimmerman et al., 1985, 1989). Only tightly confluent, primary EC monolayers were used for these experiments.

Measurement of Neutrophil Binding to ECs and to Protein Matrices

Human neutrophils were isolated from heparinized or EDTA-treated blood and were labeled with ^{111}Indium as described (Zimmerman et al., 1985). The binding assays have been reported previously (Zimmerman et al., 1985). Briefly, EC monolayers were washed with HBSS and incubated with control buffer or an agonist in HBSS with 0.5% human albumin (HBSS/A) for variable time periods. 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. Nonadherent PMNs were removed, the monolayers were washed with HBSS, the adherent radiolabeled PMNs and the ECs were solubilized, and the fraction of adherent PMNs (percentage of total radiolabeled PMNs added) was quantitated. In incubations with TNF-α or IL-1, the cytokine or control buffer was added to complete culture medium for various time periods (usually 4 h). The medium was then removed, the ECs were washed, and ^{111}In-labeled PMN adhesion was determined after a 5-min incubation. In some experiments ECs were washed, ^{111}In-labeled PMNs were added followed by addition of control buffer or an agonist, and PMN binding was measured after a variable incubation time, usually 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 described (Zimmerman and McIntyre, 1988; Lewis et al., 1988). 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.

In experiments with fixed EC monolayers, the ECs were incubated with control buffer or an agonist, the solutions were removed, and then a fixative solution was added. These included glutaraldehyde (2.5%), paraformaldehyde (2%), or TCA (5%). The monolayers were incubated a minimum of 10 min at room temperature, washed three times with buffer, and incubated with ^{111}In-labeled PMNs for 5 min. The fraction of adherent PMNs was determined as with unfixed ECs.

Stimulation of PMN Binding with Liposomal PAF

Preparation of control and PAF-loaded liposomes was done using a modification of the method described by Lumb et al., (1983). Briefly, 20

mg of L-α-lecithin (Avanti Polar Lipids, Inc.) in 40 ml of chloroform was evaporated to dryness under nitrogen in a clean glass tube. 2 ml of HBSS was added, the lecithin was resuspended using a vortex device, and the resultant suspension was sonicated at 100 W for 30 min in a sonicator (1510; B. Braun Instruments, Burlingame, CA) and was then centrifuged for 30 min at 21,500 g (13,500 rpm) at 4°C in a centrifuge with a J21 rotor (Beckman Instruments, Inc., Palo Alto, CA). The liposome-rich supernatant was collected and a portion was used for loading with PAF. PAF in various amounts in chloroform was evaporated to dryness under nitrogen. 1 ml of liposome suspension was added and the suspension was vigorously mixed by vortexing. In parallel, radiolabeled PAF mixed with unlabeled PAF was dried and mixed with liposomes. The liposomal suspensions were loaded onto Sepharose 6B columns and eluted at 15 drops per tube with HBSS. The fractions containing liposomes, monitored by elution of liposomes loaded with radiolabeled PAF, were collected. The concentration of PAF in the liposomes was calculated based on the recovered labeled PAF and its specific activity, and the suspensions were diluted to the desired concentrations with HBSS. Control or PAF-loaded liposomes were added to ^{111}In-labeled PMN suspensions and the adhesion of the PMNs to ECs or protein matrices was measured as described previously.

Measurement of HL60 Cell Binding to ECs

HL60 promyelocytic leukemia cells were the gift of Jerry Kaplan, University of Utah, or were obtained from the American Type Culture Collection (Rockville, MD), and were maintained in tissue culture flasks in medium RPMI 1640 supplemented with 20% heat-inactivated FBS. The cells were passed (usually every 3 d) when they reached a concentration of 10^6/ml by diluting them to a concentration of 2×10^5 cells/ml. Their adhesion to ECs or to protein matrices was usually studied after 15–20 passages because basal adhesion was variable at earlier stages. For adhesion studies, HL60 cells were counted, centrifuged at 1350 rpm for 5 min at 20°C, and washed by resuspending them in 10 ml HBSS/A. They were again centrifuged as described, resuspended in 5 ml HBSS/A, and incubated with ^{111}Indium oxine (0.5 μCi/10^6) cells at 37°C for 20 min. The ^{111}In-labeled cells were centrifuged (1350 rpm, 5 min, 20°C), washed in 10 ml HBSS/A, and again pelleted using the same centrifugation conditions. The cells were then resuspended in an appropriate volume of HBSS/A to yield a final concentration of 5.5×10^6 cells/ml. The binding assay was done as described for ^{111}In-labeled PMNs. The adhesion of HL60 cells used in these experiments to ECs or gelatin matrices was not increased over control when the cells were incubated with PAF (10^{-9}–10^{-6} M), fMLP (10^{-9}–10^{-6} M), or PMA (10 or 100 ng/ml) for 5 or 30 min, indicating that they did not become adhesive in response to these agents.

Measurement of [^{3}H-Acetyl]PAF Accumulation in Stimulated ECs

PAF accumulation was measured by the incorporation of [^{3}H]acetate into [^{3}H-acetyl]PAF as described in detail elsewhere (McIntyre et al., 1985; Whatley et al., 1988).

Degradation of [^{3}H-Acetyl]PAF Associated with ECs by Exogenous Phospholipases

Medium was removed from EC monolayers, they were washed with HBSS, and incubated with [^{3}H]acetate and thrombin or another agonist as described in (McIntyre et al., 1985) and Fig. 3. After an incubation period that was chosen to induce maximal accumulation of [^{3}H-acetyl]PAF based on previous studies (Prescott et al., 1984; McIntyre et al., 1986) (when thrombin was the agonist, this was 10 min), the monolayers were rapidly washed and incubated for various times at room temperature or 37°C with control buffer or PAF acetylhydrolase (AH) preparations. The [^{3}H-acetyl]PAF that remained after incubation of the ECs under these conditions was extracted and quantitated as described (McIntyre et al., 1985). In some experiments ECs were stimulated to synthesize [^{3}H-acetyl]PAF, washed, fixed with glutaraldehyde or TCA and incubated with PAF AH or phospholipase A_2. PAF AH in low density lipoprotein (LDL) particles was prepared as described (Stafforini et al., 1987*a*,*b*). In some experiments the AH was inactivated by treatment with diisopropylfluoro-phosphate (DFP) as described; DFP was removed by dialysis before use of the LDL–AH preparations (Stafforini et al., 1989). Activity of the AH preparations was confirmed using our previously described assay (Stafforini et al., 1987*a*,*b*).

Experiments with Competitive PAF Receptor Antagonists

L652,731, L659,989, and kadsurenone were generously supplied by John C. Chabala, Ph. D of Merck, Sharp, and Dohme Research Laboratories (Rahway, NJ). The powdered reagent was not directly soluble in aqueous buffer and therefore 100-mM stock solutions were made in 100% DMSO and used immediately or frozen at −20°C until use. The inhibitor solution was then subdiluted to a concentration of 1 mM by slowly adding 25 μl of the 100-mM stock solution to 2.475 ml of HBSS that had been prewarmed to 37°C. It was essential to prewarm the buffer and to keep the subdiluted solution at 37°C during the experiment to prevent precipitation of the L652,731. In several experiments precipitates of L652,731 formed in the subdiluted solution even when kept at 37°C, requiring preparation of a new solution. 1 vol of the 1-mM solution was added to 9 vol of ^{111}In-labeled PMNs (room temperature) to yield a final concentration of 100 μM L652,731 and 0.1% DMSO, and the PMNs were incubated for 5 min at room temperature before addition to ECs or protein matrices. Aliquots of the 1-mM inhibitor solution were further diluted with prewarmed HBSS before adding to PMNs when lower concentrations of inhibitor were used. Control neutrophil suspensions were treated in side-by-side fashion with DMSO that was diluted in an identical way to the inhibitor solutions, yielding a final concentration of DMSO that was the same in both control and L652,731-treated PMN suspensions. L652,989 and kadsurenone solutions were prepared in the same way as with L652,731 and DMSO control solutions were used in parallel with these inhibitors as well. In each experiment, we documented inhibition of PMN binding induced by exogenous PAF by the competitive receptor antagonists as a positive control.

Results

EC-dependent PMN Adhesion Induced by Thrombin and by Cytokines Involve Different Molecular Mechanisms

Thrombin induces a 2–10-fold increase in the binding of PMNs to ECs. The enhanced adhesion occurs when thrombin is added to PMNs suspended in buffer (Fig. 1 *A*) or serum (not shown) overlying EC monolayers, as well as when ECs are pretreated with thrombin before addition of the PMN suspension (Fig. 1 *B*). The response has a similar time dependency under both conditions (Zimmerman et al., 1985, 1986). We found in direct side-by-side experiments that the time courses of EC-dependent PMN adhesion induced by thrombin and by TNFα are dramatically different. The thrombin-stimulated response is measurable within minutes, peaks at 5–10 min, and decays over the next 30–50 min (Fig. 1), whereas TNF-induced EC-dependent adhesion of PMN requires 30–60 min before enhanced PMN binding occurs, is maximal at ∼240 min, and is sustained over several hours (Fig. 1; Gamble et al., 1985). The rapid onset of neutrophil binding induced by thrombin is similar to that induced by leukotrienes C_4 and D_4 and hydrogen peroxide (McIntyre et al., 1986; Lewis et al., 1988), and by histamine (Zimmerman, G. A., manuscript in preparation). Thus, the kinetics of binding, together with the transient duration, differentiate the effect of thrombin and other rapidly acting agonists from the TNFα effect. The TNFα time dependency is similar to that induced by IL-1 and by lipopolysaccharide (Bevilacqua et al., 1985; Schleimer and Rutledge, 1986). A second difference is the requirement for protein synthesis for the EC to become proadhesive. EC-dependent leukocyte binding stimulated by TNFα and other cytokines requires protein synthesis and is inhibited by cycloheximide or actinomycin D (Gamble et al., 1985; Bevilacqua et al., 1985). We found (two experiments) that the thrombin-induced effect was not blocked

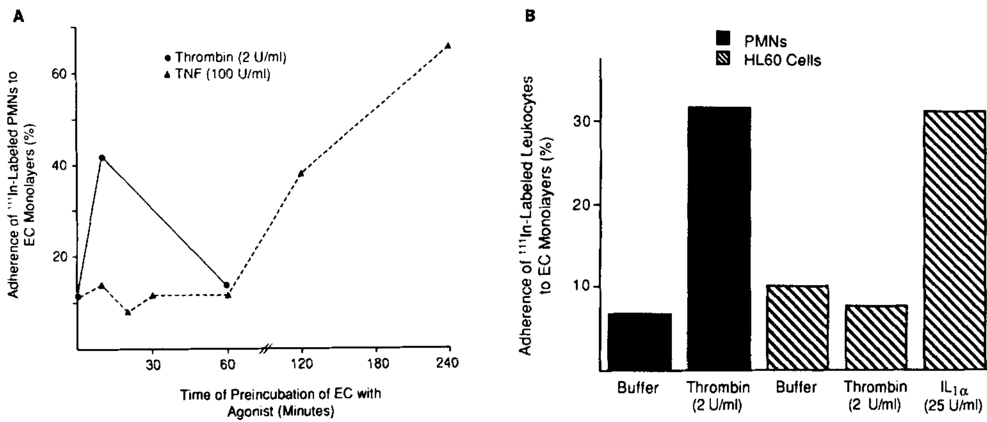

Figure 1. (*A*) Rapid and slow endothelial cell-dependent PMN adhesion induced by thrombin and TNFα. EC monolayers were pretreated with recombinant TNFα for the indicated times, washed, and ^{111}In-labeled PMNs were added; adhesion of labeled PMNs was determined after a 5-min period. In parallel incubations ^{111}In-labeled PMNs were added to washed EC monolayers, thrombin (2 U/ml, 18.5 nM) was added, and adhesion of labeled PMNs was determined at the times indicated. The latter protocol was chosen to demonstrate the rapidity with which thrombin induces EC-dependent PMN adhesion. We previously showed that pretreatment of EC monolayers with thrombin for various times, followed by washing and incubation with ^{111}In-labeled PMNs, caused EC-dependent adhesion with the same time course as that illustrated (Zimmerman et al., 1985) (see also Fig. 1 *B*). (*B*) Thrombin induces EC-dependent binding of PMNs but not HL60 promyelocytic leukemia cells. EC monolayers were pretreated with buffer or thrombin (2 U/ml) in HBSS/A for 10 min and washed, ^{111}In-labeled PMNs or HL60 cells were added, and the adherence of the leukocytes was determined after a 5-min incubation. Additional monolayers were treated with IL-1α for 4 h in complete medium and washed, and the adhesion of ^{111}In-labeled HL60 cells was determined after a 5-min incubation. Each bar represents the mean results from three experiments except for IL-1α (two determinations). The standard deviations for each bar were, for PMNs, 2% (buffer), and 13% (thrombin); for HL60 cells, 6% (buffer), 4% (thrombin), and 8% (IL-1α).

by pretreatment of the ECs with cycloheximide (10 μg/ml for 4 h) before thrombin stimulation. In contrast, the same concentration of cycloheximide inhibited EC-dependent binding in response to various concentrations of TNFα (4 h) by 75–85%. A third feature differentiates the rapid and slow EC responses. HL60 promyelocytic leukemia cells, as well as PMNs, adhere to cytokine-treated EC (Bevilacqua et al., 1985, 1987, 1989). We compared the binding of these cells and PMNs to EC activated with TNF or thrombin. There was no increase in binding of HL60 cells to thrombin-treated ECs compared with control EC monolayers (Fig. 1 *B*), whereas there was a 3–10 fold increase in adherence of HL60 cells to ECs treated with TNFα or IL-1 (Table I). Thus, the rapid, thrombin-induced PMN binding occurs by a mechanism that is fundamentally different from that induced by cytokines. The experiments with HL60 cells exclude the possibility that the thrombin-dependent adhesion involves rapid expression of preformed ELAM-1, consistent with the observation by Bevilacqua et al. (1987, 1989) that neither ELAM-1 nor messenger RNA for the protein is present in unstimulated ECs. The inability of HL60 cells to bind to thrombin-activated ECs is consistent with their lack of a functional receptor for PAF (Valone, 1988) (see below).

The Proadhesive Activity on Thrombin-stimulated ECs Has Characteristics of a Phospholipid

Experiments to define the chemical nature of the proadhesive activity were hampered by the transient nature of the response in thrombin-treated ECs. However, we found that the adhesive surface of the ECs was preserved if the monolayers were stimulated with thrombin and then fixed before incubation with radiolabeled PMNs (Fig. 2). In contrast, there was no enhanced PMN binding if the EC monolayers were fixed before treatment with thrombin, demonstrating that a response of the ECs was required. Washing of thrombin-treated, fixed EC monolayers with albumin-containing buffer did not remove the adhesive activity (Fig. 2). However, treatment of the monolayers with methanol caused loss of the activity (Fig. 2), which suggested that it was a lipid. We next treated thrombin-stimulated, fixed monolayers with phospholipase A_2 and found that it abolished the adhesive activity, indicating the involvement of a phospholipid (Fig. 2). In contrast, the adhesive activity remained after treatment of the monolayers with a variety of proteases (papain, proteinase K, thermolysin; not shown). These results were compatible with our hypothesis that PAF is the EC-derived signal for PMNs

Table I. Binding of HL60 Promyelocytic Leukemia Cells to Human Endothelial Cells

ECs pretreated with	HL60 binding	*n*
Buffer, 10 min	7 ± 4%	9
Thrombin (2 U/ml), 10 min	6 ± 3%	9
(5 U/ml), 10 min	6 ± 2%	3
PAF (10^{-6} M), 10 min	10 ± 5%	5
Buffer, 240 min	10 ± 6%	7
IL-1α (25 U/ml), 240 min	37 ± 13%	7
TNFα (100 U/ml), 240 min	34 ± 11%	4

Primary monolayers of confluent human ECs were washed and preincubated with buffer, thrombin, or PAF for 10 min (5% CO_2, 37°C). The incubation buffer was removed, the monolayers were washed with HBSS/A, ^{111}In-labeled HL60 cells were added and incubated for 5 min, and the percentage of leukocytes that adhered to the monolayer was then determined (Materials and Methods). In one experiment there was no difference in the binding of HL60 cells to buffer- or thrombin-treated ECs when the leukocytes were incubated with the EC monolayers for 30 min rather than 5 min.

In additional incubations buffer, recombinant IL-1α, or recombinant TNFα was added to ECs in complete culture medium and the monolayers were incubated for 4 h. They were then washed, ^{111}In-HL60 cells were added, and binding was determined after a 5-min incubation period.

In several experiments, ^{111}In-labeled PMN binding was measured in parallel with HL60 adhesion; treatment of the monolayers with thrombin (10 min), PAF (5 or 10 min), TNFα (4 h), or IL-1 (4 h) induced PMN binding as expected (see text and Fig. 1 B).

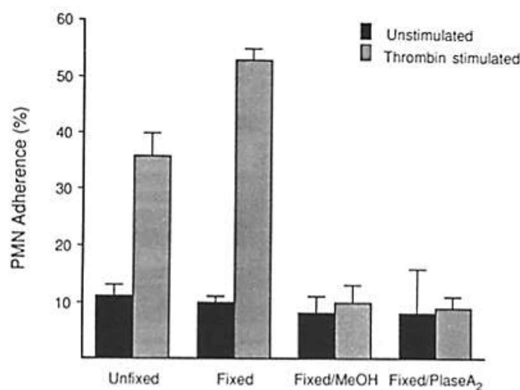

Figure 2. The proadhesive activity associated with thrombin-activated EC is degraded by phospholipase A_2. EC monolayers were incubated with control buffer or thrombin (2 U/ml) for 10 min and then fixed by treatment with 1.5% glutaraldehyde for 90 min at room temperature. Replicate monolayers were treated identically but were not fixed. Fixed and unfixed monolayers were rapidly washed three times with albumin-containing buffer, ^{111}In-labeled PMNs were added, and PMN binding was measured after a 5-min incubation. Additional control or thrombin-stimulated monolayers were fixed and incubated with methanol or with phospholipase A_2 (16 U/ml) for 15 min at room temperature, followed by washing with buffer and measurement of radiolabeled PMN binding after a 5-min incubation. Phase-contrast microscopy demonstrated that the monolayers remained intact after fixation and after treatment with methanol or phospholipase A_2. In a second experiment, phospholipase A_2 treatment of TNFα-stimulated, fixed ECs did not reduce PMN binding.

to bind to thrombin-stimulated ECs. An additional feature suggesting this is that pretreatment of ECs with nonspecific inhibitors of phospholipase A_2 such as *p*-bromophenacyl bromide (Lewis et al., 1988), mepacrine, or trifluoperizine (our unpublished observations) inhibited thrombin-stimulated PAF synthesis and also inhibited thrombin-induced, EC-dependent PMN adhesion (Lewis et al., 1988). Thus inhibition of the first step of the deacylation–reacetylation mechanism for PAF synthesis (remodeling pathway) (Snyder, 1987), PLA_2-catalyzed hydrolysis of 1-0-alkyl-2-acyl-*sn*-glycero-3-phosphocholine yielding 1-0-alkyl-*sn*-glycero-3-phosphocholine ("lyso-PAF"), the immediate precursor of PAF, also inhibited rapid EC-dependent PMN adhesion. However, we showed previously that the newly synthesized PAF remains associated with stimulated ECs and is not released into the fluid phase (Prescott et al., 1984; McIntyre et al., 1985). Thus, for endogenously synthesized PAF to interact with its receptor on the PMNs, some or all of it would have to be on the surface of the EC.

Newly Synthesized PAF is on the Surface of ECs

To determine if newly synthesized PAF was on the cell surface, we induced the synthesis of [^{3}H-acetyl]PAF by incubating the ECs with [^{3}H]acetate and thrombin or another agonist (pulse). After an incubation time chosen to induce maximal [^{3}H-acetyl]PAF synthesis (Prescott et al., 1984; McIntyre et al., 1986), we changed to buffer without [^{3}H]acetate or thrombin and which contained an enzyme that degrades PAF (chase). At subsequent times the loss of [^{3}H-acetyl]PAF was measured. The enzyme used in these experiments was the specific PAF AH from human plasma (EC 3.1.1.47) (Stafforini et al., 1987*a*,*b*, 1989). This strategy was the same as that used by others to determine the "sidedness" of phospholipids in a membrane: the phospholipase is not internalized, and, therefore, degradation of a given phospholipid indicates that it is on the outside of the membrane (for review see Op den Kamp, 1979). In our approach, this assay was even more specific since the PAF AH is highly selective for short acyl chains at the *sn*-2 position (Stafforini et al., 1987*b*). Thus, since it will not degrade the usual membrane phospholipids with long acyl chains at *sn*-2, its actions are unlikely to be due to permeabilization or disruption of the plasma membrane.

Fig. 3 demonstrates that exogenous AH accelerates the degradation of EC-associated PAF. In contrast, AH in which the catalytic activity had been abolished with DFP (Stafforini et al., 1989) did not accelerate the rate of loss. In this experiment we used isolated LDL particles as a source of partially purified AH (LDL–AH) (Stafforini et al., 1987*a*,*b*), because the lipoprotein environment of the AH affects its catalytic activity and the most favorable environment is in the LDL particle (Stafforini et al., 1989). However, we also found that purified AH (43,500 mol wt) accelerated the degradation (not shown). The possibility that the LDL–AH was internalized by the ECs, and acted on PAF located at intracellular

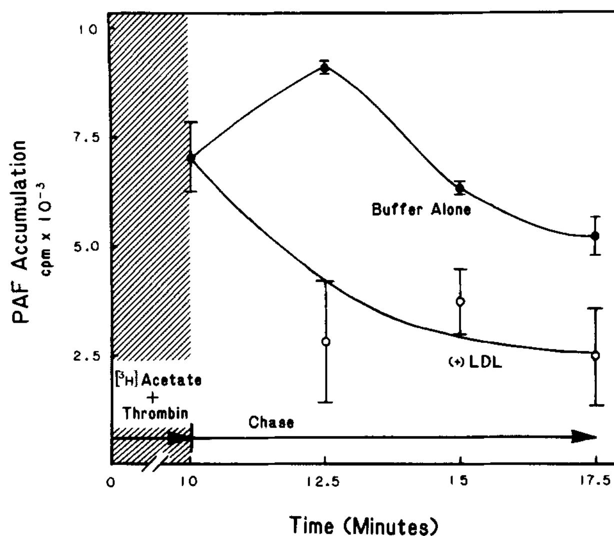

Figure 3. EC-associated PAF is accessible to extracellular PAF acetylhydrolase. ECs were stimulated with thrombin (2 U/ml) in the presence of [^{3}H]acetate for 10 min at room temperature (pulse) and then washed with buffer. A chase was then performed by incubating the ECs at room temperature with buffer alone, buffer containing LDL as a source of plasma AH (*+LDL*), or LDL that had been treated with diisopropylfluorophosphate (DFP) to inactivate the AH activity. Isolation of LDL–AH and its inactivation with DFP were done as described (Stafforini et al., 1989). The concentration of LDL was 0.5 U/ml, which is equivalent to 20% of the mean amount in normal plasma (Stafforini et al., 1989). At the times shown the reaction was stopped by treating the monolayers with acidified methanol and the content of [^{3}H-acetyl]PAF was measured (McIntyre et al., 1985) as an indicator of the rate of degradation. Each point indicates the mean and standard deviation of triplicate determinations. 7,750 ± 1,625 cpm and 9,063 ± 313 cpm of [^{3}H-acetyl]PAF were associated with monolayers incubated with DFP-treated LDL at 15 and 17.5 min, respectively.

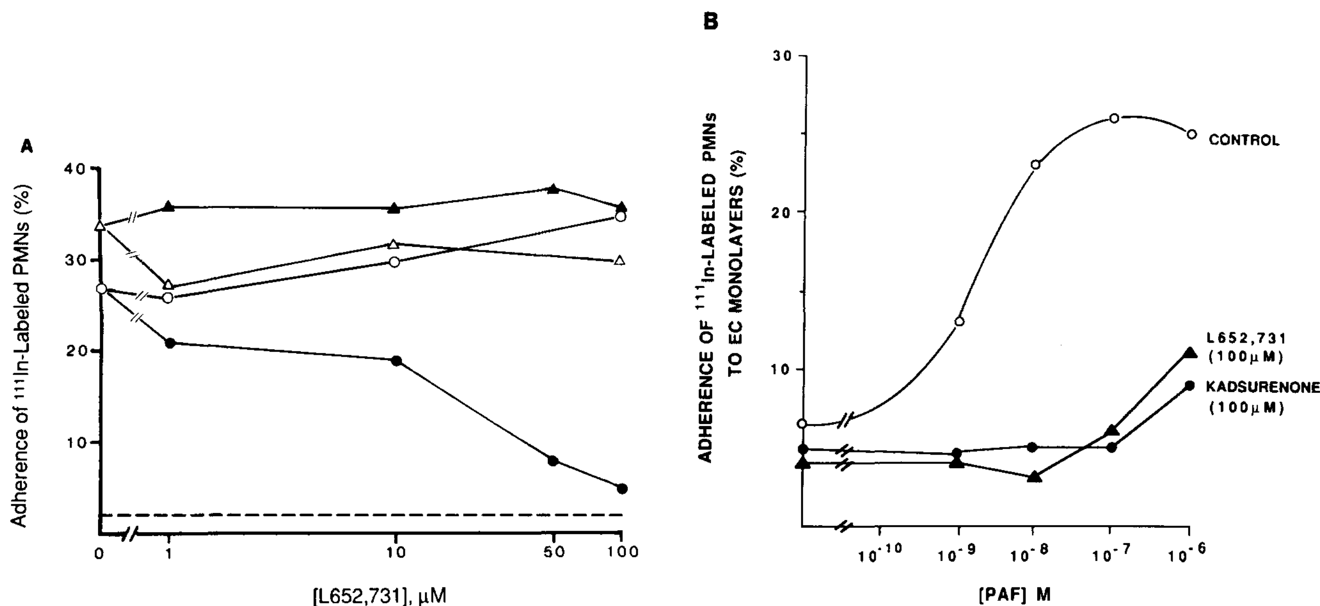

Figure 4. Competitive PAF receptor antagonists inhibit PAF-stimulated neutrophil binding to protein matrices and to EC. (*A*) PMNs were pretreated with various concentrations of L652,731 or control buffer containing equal concentrations of DMSO for 5 min (see Materials and Methods) and were then layered over gelatin matrices. Buffer, PAF (10^{-7} M), or fMLP (10^{-7} M) was added, and PMN binding was assayed after a 15-min incubation. The dashed line indicates the binding of unstimulated PMNs. (○) DMSO-treated PMNs, PAF; (●) L652,731-treated PMNs, PAF; (△) DMSO-treated PMNs, fMLP; (▲) L652,731-treated PMNs, fMLP. (*B*) ^{111}In-labeled PMNs were pretreated with control buffer, L652,731, or kadsurenone, added to EC monolayers, and their adhesion was determined after a 5-min incubation in the presence of various concentrations of PAF. In parallel incubations the adhesion of control PMNs and L652,731-treated PMNs in response to fMLP (10^{-7} M) was 30 and 25%, respectively.

sites, is unlikely since the effect of the LDL-AH was rapid and LDL particles are slowly internalized by confluent human ECs (our unpublished observation; Cotzee et al., 1979). Furthermore, in six experiments AH in LDL particles degraded [^{3}H-acetyl]PAF associated with fixed ECs, excluding the possibility that internalization of the AH was required for hydrolysis of the metabolically labeled PAF. In these experiments ECs were stimulated to synthesize [^{3}H-acetyl]PAF with thrombin or calcium Ionophore A23187 as in Fig. 3, fixed, and incubated with LDL-AH, DFP-treated LDL-AH, or control buffer. In each experiment the LDL-AH caused degradation of [^{3}H-acetyl]PAF (82–95% depending on the time, the concentrations of LDL, and the fixation conditions), whereas the control solutions did not cause hydrolysis. In one of these, the binding of ^{111}In-labeled PMN was measured in parallel. The binding of PMNs to thrombin-stimulated, fixed ECs that had been treated with buffer, LDL-AH, or DFP-treated LDL-AH was 31, 8.5, and 25%, respectively, compared with 9.5% adhesion to unstimulated, fixed EC treated with LDL-AH. There was 94% hydrolysis of [^{3}H-acetyl]PAF by LDL-AH in the parallel incubations (5,771 cpm in thrombin-activated ECs treated with buffer vs. 321 cpm in thrombin-stimulated ECs treated with LDL-AH).

From these experiments we conclude that a substantial portion of the PAF synthesized by ECs is on the surface of the cell, as indicated by its accessibility to exogenous AH. Thus the location of PAF is compatible with the hypothesis that it serves as a signal for PMNs to adhere.

Competitive Antagonists of the PAF Receptor Inhibit EC-dependent Neutrophil Adhesion Induced by Thrombin

As an additional test of this hypothesis, we blocked the responses of PMNs to PAF with competitive antagonists of the PAF receptor. Kadsurenone, L652,731, and L659,989 specifically compete with PAF for binding to cellular receptors and block PAF-induced activation of platelets and leukocytes (Shen et al., 1985; Hwang et al., 1985; Hwang et al., 1986; Ponpipom et al., 1988). We rigorously characterized the effects of these antagonists on PAF-induced adhesive responses of neutrophils. In the first series of experiments we examined their inhibition of PAF-stimulated adhesiveness in the absence of ECs. L652,731 inhibited PMN aggregation and PMN adhesion to gelatin matrices in a concentration-dependent fashion with a maximal effect at 50–100 μM (Fig. 4). The IC_{50} was ∼4 μM when 10^{-8} M PAF was used to stimulate PMN binding to gelatin matrices, and 15 μM when 10^{-7} M PAF was used. The inhibition was specific for PAF because L652,731, in the same concentrations, had little or no effect (30% inhibition or less) on PMN adhesion induced by fMLP (Fig. 4), leukotriene B_4, or PMA (not shown). Kadsurenone caused a similar concentration-dependent and -specific inhibition; its potency was equal to, or less than, that of L652,731 at equivalent concentrations, as previously reported in other systems (Hwang et al., 1988; Ponpipom et al., 1988). The IC_{50} for each compound varied from experiment to experiment, which may have been due to variable states of the neu-

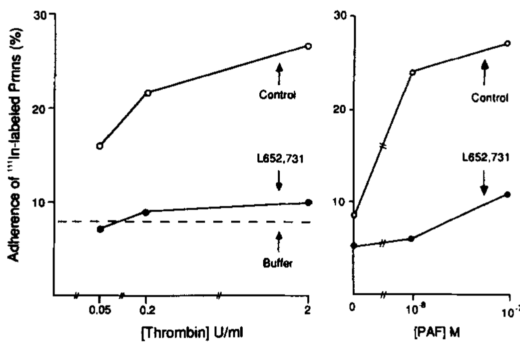

Figure 5. L652,731, a competitive PAF receptor antagonist, inhibits PMN binding to EC activated by thrombin. ECs were preincubated with control buffer or buffer containing thrombin in the indicated concentrations for 5 min at 37°C, the solutions were removed, control PMNs or PMNs treated with L652,731 (100 μM) were added, and their adherence was determined after a 5-min incubation (*left*). In parallel incubations, the same concentration of L652,731 inhibited PMN binding to ECs induced by the addition of exogenous PAF (*right*), but had a trivial effect (0–12% inhibition) on PMN binding induced by the addition of 10 or 100 ng/ml PMA (not shown).

trophil PAF receptor(s) (Hwang, 1988). We then showed that the inhibitors blocked PMN adhesion to ECs in response to exogenous PAF in solution (Fig. 4), with a similar concentration dependence and specificity to that for adhesion to gelatin. L652,731 did not inhibit PAF synthesis by ECs.

Once the conditions for competitive, specific inhibition of neutrophil responses to PAF were established, we asked if the receptor antagonists blocked PMN adhesion to thrombin-treated ECs. We pretreated PMNs with L652,731 and measured their binding to ECs that had been activated with thrombin (Fig. 5). In three experiments in which ECs were activated with various concentrations of thrombin and then incubated with PMNs, L652,731 inhibited PMN adhesion to thrombin-stimulated ECs by 40–100%. The magnitude of inhibition was dependent on the concentration of thrombin; that is, it was less when higher concentrations of thrombin were used (mean inhibition 57 ± 28% at 2 U/ml thrombin), consistent with its action as a competitive inhibitor of the PAF receptor and higher concentrations of EC-associated PAF at higher concentrations of thrombin (Prescott et al., 1984). Since the affinity of L652,731 for the PAF receptor is two to three orders of magnitude lower than PAF (Hwang, 1988; Ponpipom et al., 1988), the ratio of inhibitor to ligand may have been unfavorable for complete inhibition of PMN binding to ECs stimulated with maximal concentrations of thrombin. A second possibility is that another molecular species participates in signaling neutrophils to bind to thrombin-activated ECs (Zimmerman et al., 1985; Zimmerman and McIntyre, 1988). However, in the experiment shown in Fig. 5, L652,731 caused essentially complete inhibition of adhesion at all concentrations of thrombin tested, demonstrating that PAF can be the sole mediator. In three experiments kadsurenone, L652,731, and a third competitive receptor antagonist, L659,989 (Ponpipom et al., 1988), were compared. Their relative binding affinities to PAF receptors is L659,989 > L652,731 ⩾ kadsurenone (Hwang, 1988; Ponpipom et al., 1988), and we found that their relative potencies as inhibitors of PMN adhesion to thrombin-stimulated ECs had the same rank order.

In addition to thrombin, we previously showed that LTC_4 and H_2O_2 induce rapid PAF synthesis and EC-dependent PMN adhesion (McIntyre et al., 1986; Zimmerman and McIntyre, 1988; Lewis et al., 1988). Since our results with thrombin supported a direct role for PAF in signaling PMNs to adhere, we asked whether this was a common mechanism for other rapidly acting agonists. In two of three experiments L652,731 inhibited PMN adhesion to ECs activated with LTC_4 (1 μM for 30 min) by 46 and 65%. In three additional experiments, L652,731 inhibited PMN adhesion to ECs activated with H_2O_2 (2.5–10 μM for 20 min) by 80–95%, and in a fourth experiment kadsurenone inhibited PMN binding to H_2O_2-activated ECs by 25–60%, depending on the concentration of H_2O_2. Thus, competitive antagonists of the PAF receptor inhibit PMN binding to ECs activated by three agonists (thrombin, LTC_4, H_2O_2) that each induce rapid EC-dependent neutrophil adhesion that temporally parallels the accumulation of PAF. These data indicate that PAF that is synthesized by activated ECs mediates PMN binding, and are consistent with our previous observations that desensitization of PMNs to PAF reduced their binding to ECs activated with these agonists (Zimmerman et al., 1985; McIntyre et al., 1986; Lewis et al., 1988).

In contrast to the result with ECs stimulated by rapidly acting agonists, L652,731 did not inhibit PMN adhesion to ECs activated with TNFα (adhesion of control PMNs to ECs treated with 10 U/ml TNFα for 4 h, 65 ± 11%; adhesion

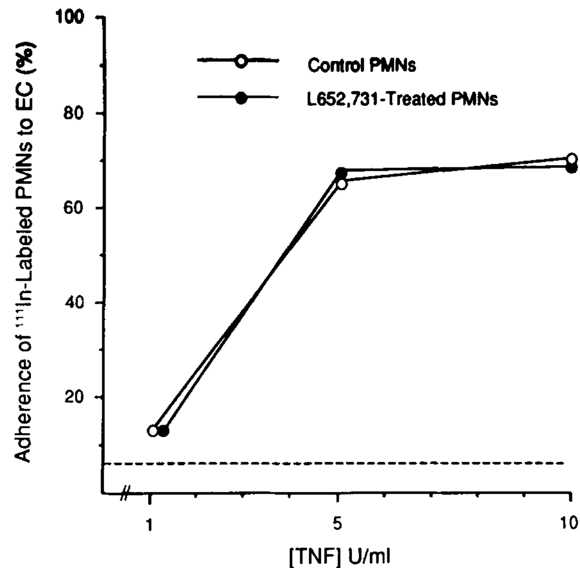

Figure 6. L652,731 does not inhibit PMN binding to EC activated with TNFα. Control buffer or TNFα was added to EC monolayers in complete culture medium. After a 4-h incubation, the medium was removed, the monolayers were washed once with HBSS, ^{111}In-labeled PMNs that were pretreated with L652,731 (100 μM) or control solution were added, and binding was determined after a 5-min incubation. The dashed line indicates the binding of PMNs to EC treated with control buffer. In parallel incubations, L652,731 completely inhibited PMN binding induced by the addition of exogenous PAF (10^{-8} or 10^{-7} M) (not shown).

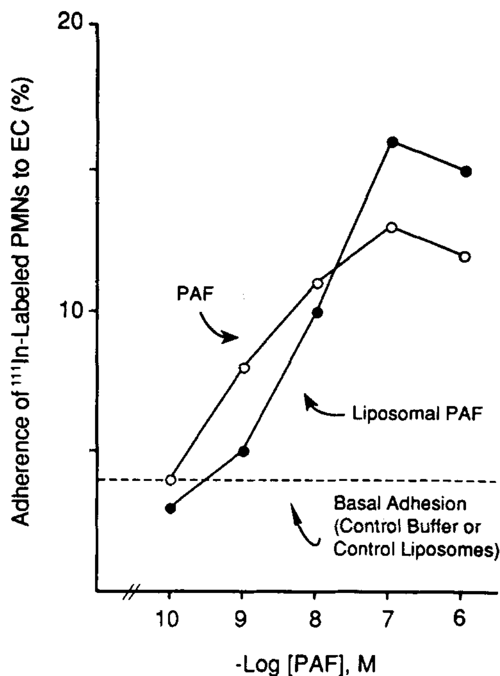

Figure 7. PAF in liposomes induces PMN binding to EC monolayers. PAF in various concentrations was sonicated with phosphatidylcholine to form PAF-loaded liposomes (see Materials and Methods), or was diluted in HBSS/A. PAF in HBSS/A or liposomal PAF was added to ^{111}In-labeled PMNs overlying EC monolayers and adhesion of PMNs to the ECs was determined after a 5-min incubation. The binding of PMNs in response to control buffer or control liposomes was 4% in each case. In parallel incubations, control PMNs or PMNs treated with L652,731 (100 μM) were incubated with 10^{-8} M PAF or liposomal PAF, and their adhesion to gelatin matrices was measured after a 5-min incubation. The results were 33% for PAF + control PMNs; 4% for PAF + L652,731-treated PMNs; 28% for liposomal PAF + control PMNs, and 2% for liposomal PAF + L652,731-treated PMNs.

of L652,731-treated PMNs, 65 $\pm$ 8%; n = 4), regardless of the concentration of the cytokine (Fig. 6). These observations demonstrate that the PAF receptor antagonist does not have toxic or nonspecific effects on EC-dependent neutrophil adhesion, and indicate that, although treatment of ECs with TNFα has been reported to induce the synthesis of PAF (Bussolino et al., 1988), the major mechanism of PMN binding to cytokine-activated ECs involves molecules other than PAF (Bevilacqua et al., 1987, 1989; Smith et al., 1988). Recently, Brevario et al. (1988) concluded that PAF synthesized by IL-1-activated ECs does not contribute significantly to PMN adhesion to them.

PAF in Liposomes and Associated with the EC Surface Induces Neutrophil Binding

An unexplained feature of these results is how a small molecule such as PAF can remain associated with the surface of one cell and still interact with its receptor on the target cell. We examined this issue with two experimental strategies. In the first, we incorporated PAF into liposomes, added these, or control liposomes, to PMN suspensions, and measured adhesive responses of the neutrophils. In parallel incubations we found that [^{3}H-acetyl]PAF remained associated with liposomes under the conditions of these experiments and was not released into buffer alone, or buffer containing 0.5 mg/ml human albumin. PAF-loaded liposomes induced adherence of PMNs to protein matrices with the same potency as monomeric PAF, while control liposomes caused adhesion that was similar to buffer alone (buffer, 9 $\pm$ 0% adherence; control liposomes, 11.5 $\pm$ 3.5%; monomeric PAF at 10^{-6} M, 71 $\pm$ 8%; liposomes loaded with 10^{-6} M PAF, 72 $\pm$ 8%; n = 2). The enhanced adhesion induced by liposomal PAF was completely blocked by pretreatment of the PMNs with the receptor antagonist L652,731 (Fig. 7). Furthermore, liposomal PAF also induced the rapid adhesion of PMNs to ECs (Fig. 7). Thus, PAF presented in model membranes can induce PMN binding to surfaces by interacting with receptors on the neutrophils, suggesting that PAF associated with EC plasma membranes may do the same. We also found that PAF-loaded liposomes stimulate the aggregation of human platelets (unpublished observation) and it has previously been reported that liposomal PAF can activate macrophages (Hayashi et al., 1985), although in the latter experiments the response required hours and may have involved internalization of the lipid vesicles.

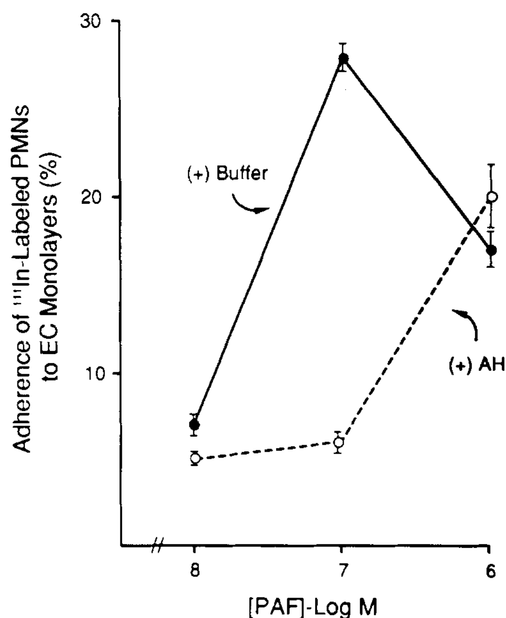

Figure 8. Exogenous PAF associated with EC plasma membranes induces PMN binding. EC monolayers were pretreated with HBSS/A or HBSS/A containing various concentrations of PAF for 5 min at 37°C; the solutions were removed, the monolayers were washed once with HBSS/A, and then were incubated with control buffer (●) or purified AH (○) for 15 min at 37°C. The buffer or AH was removed, ^{111}In-labeled PMNs were added, and their binding was determined after a 5-min incubation. The figure indicates the mean of duplicate determinations at each point. The adhesion of ^{111}In-labeled PMNs to EC treated with control buffer (no PAF) was 5%.

In several additional experiments in which PAF was "presented" to PMNs associated with the EC surface (as in this figure) or in the fluid phase (as in Fig. 4), we saw a descending limb in the concentration–response relationship, similar to that shown here, at concentrations of PAF greater than 10^{-7} M. A biphasic pattern including decreased response at increasing concentrations of agonist has been reported for several PMN activities and with a variety of agonists, including PAF (Fernandez et al., 1978; Donabedian and Gallin, 1981; O'Flaherty et al., 1981; Shaw et al., 1981).

In the second experimental approach, we asked whether PAF added exogenously to the EC surface could directly induce PMN binding. We treated EC monolayers with PAF or buffer for various times, washed the ECs with albumin-containing buffer, and then measured the adhesion of PMNs. Pretreatment of ECs with PAF (5 min) resulted in enhanced adhesion (buffer-pretreated ECs, 7.5 ± 3%; 10^{-7} M PAF, 24 ± 11%, $n = 4$) (Fig. 8). In contrast, pretreatment of ECs under identical conditions with the biologically inactive compound, lyso-PAF, did not induce neutrophil binding. Pretreatment of ECs with PAF (10^{-7} or 10^{-6} M) did not induce enhanced binding of HL60 cells (Table I).

We incubated ECs with PAF radiolabeled in the alkyl chain at *sn*-1 ([^{3}H-alkyl]PAF) and demonstrated a low affinity binding site for PAF. This binding was not blocked by a 1,000-fold excess of the receptor antagonist L652,731, indicating that it was not a typical receptor for PAF. The nature of the association of exogenous PAF with the EC plasma membrane is unknown, but it may insert into the phospholipid bilayer as it does in model membranes (Lumb et al., 1983). There was no degradation of the exogenous [^{3}H-alkyl]PAF that had bound to ECs during a 30-min incubation (two experiments), indicating that the exogenous PAF was not internalized. Furthermore, incubation of ECs that had exogenous [^{3}H-alkyl]PAF bound to their surface with the plasma PAF acetylhydrolase for 5–30 min resulted in hydrolysis of the labeled phospholipid (80–89%, depending on the time of incubation and the concentration of [^{3}H-alkyl]PAF), with an accompanying increase in [^{3}H-alkyl] lyso-PAF. These experiments demonstrated that PAF remained at the EC surface under conditions that resulted in PMN binding (Fig. 8).

The increased binding of PMNs induced by treatment of EC with PAF could have been due to interaction of PAF localized on the EC surface with the neutrophils, or to activation of the ECs by PAF (Bussolino et al., 1987*b*; Hirafuji et al., 1988), causing them to express a second adhesive molecule. To differentiate between these two possibilities, we pretreated ECs with PAF and then incubated the monolayers with AH, or with control buffer, before adding PMNs and measuring their adhesion (Fig. 8). Incubation of EC monolayers with AH completely prevented PMN binding to ECs that had been pretreated with 10^{-8} M or 10^{-7} M PAF, but did not significantly alter binding to ECs pretreated with 10^{-6} M PAF. The latter result is explained by our finding that, when 10^{-6} M PAF was used to pretreat the ECs, only 89% of it was hydrolyzed in the subsequent incubation with AH, based on degradation of [^{3}H-alkyl]PAF measured in parallel. Thus, the residual PAF ($\approx 10^{-7}$ M) was sufficient to account for the PMN binding. To further exclude the possibility that PAF induced a functional alteration in the ECs by interacting with cell surface receptors, we incubated ECs with PAF (10^{-7} M) in the presence or absence of a 1,000-fold higher concentration of L652,731. Inclusion of the receptor antagonist in the incubation, followed by washing and addition of ^{111}In-labeled PMNs, had no effect on the enhanced PMN adhesion to PAF-treated monolayers. In contrast, inclusion of L652,731 in the buffer with ^{111}In-labeled PMNs completely blocked their binding to EC pretreated with PAF (10^{-8}–10^{-6} M), demonstrating that the mechanism of binding involved ligation of the neutrophil PAF receptor by PAF on the EC surface. These experiments demonstrate that PAF on the surface of ECs can induce PMN adhesion via its receptor on neutrophils.

Discussion

The experiments in this report demonstrate a novel mechanism for cell–cell interaction: a phospholipid, PAF, synthesized by ECs in response to an agonist, is rapidly and transiently expressed on the cell surface and signals neutrophils to bind. Although it has been observed recently that glycolipids, like glycoproteins, can mediate cell–cell and cell–matrix interaction (Cheresh et al., 1986; Sariola et al., 1988), and that membrane phospholipid content and distribution may influence erythrocyte intercellular interactions (Franck et al., 1985; Schwartz et al., 1985; Mikkelsen et al., 1988), neutrophil binding in response to EC-associated PAF represents the first instance in which a phospholipid that is synthesized in a regulated fashion and that is retained by its parent cell induces intercellular adhesion. These results support previous observations that suggested this hypothesis (Zimmerman et al., 1985; McIntyre et al., 1986; Zimmerman and McIntyre, 1988; Lewis et al., 1988): (*a*) agonists that induce rapid PAF synthesis by ECs (thrombin, LTC_4, H_2O_2), also induce rapid EC-dependent PMN adhesion; (*b*) the concentration–response relationships and specificities of this spectrum of agonists are the same for the two responses; (*c*) there is tight temporal coupling between the accumulation and degradation of PAF and the development and reversal of EC-dependent adhesion; and, (*d*) "desensitization" of the PAF receptor on PMNs specifically inhibits their binding to ECs activated with these agonists. In addition to these characteristics, preliminary experiments indicate that PAF synthesis and rapid EC-dependent PMN adhesion share similar signal transduction mechanisms: they have the same requirement for extracellular Ca^{++} and, in the case of receptor-mediated agonists, may share a common guanyl nucleotide-binding regulatory protein (our unpublished results).

Two pathways for PAF synthesis have been identified in mammalian cells (Snyder, 1987). Human ECs do not appear to use the DTT-insensitive cholinephosphotransferase pathway (Blank et al., 1986; Bussolino, 1987*a*), but instead synthesize PAF in a two-step reaction in which a phospholipid precursor, 1-0-alkyl-2-acyl-*sn*-glycero-3-phosphocholine, is hydrolyzed by a phospholipase A_2 yielding 1-0-alkyl-*sn*-glycero-3-phosphocholine, which is then acetylated at the *sn*-2 position by an acetyltransferase to yield PAF (Prescott et al., 1984; Whatley et al., 1989). Although we cannot exclude the possibility that PAF is synthesized in the plasma membrane, we assume that the major site of PAF synthesis in ECs is the endoplasmic reticulum based on the location of phospholipid synthesis in mammalian cells (Bishop and Bell, 1988), and the localization of PAF acetyltransferase in microsomes from several cells and tissues (Ribbes et al., 1985; Snyder, 1987; Mollinedo et al., 1988). In human PMNs, both PLA_2 and acetyltransferase activities are found in intracellular organelles; the acetyltransferase is localized in microsomal and granular fractions (Mollinedo et al., 1988). Observations in our laboratory indicate that there is a similar localization of acetyltransferase activity in intracellular organelles of human ECs (our unpublished results). The mechanism of its intracellular transport and the specific orientation of the fraction found in the plasma membrane of

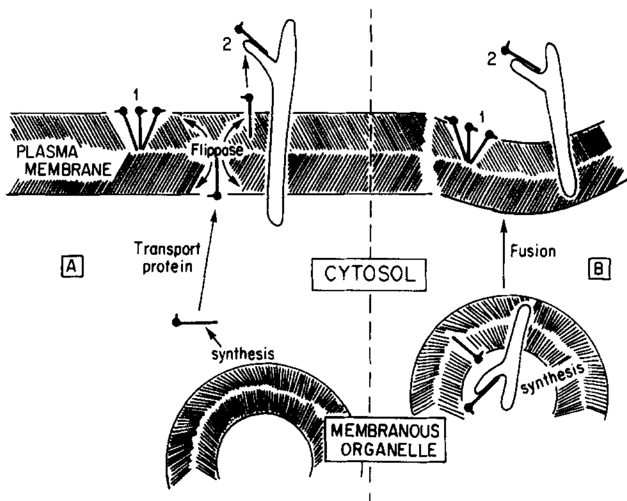

Figure 9. Potential mechanisms for PAF localization in the EC plasma membrane. PAF is synthesized in an intracellular membranous organelle, presumably the ER. A portion of the newly synthesized PAF is then rapidly transported to the inner leaflet of the plasma membrane by a phospholipid transport protein (Bishop and Bell, 1988), shown in *A*; putative PAF transport proteins have been identified in other cell types (Banks et al., 1988). A "flippase" (Bishop and Bell, 1988) catalyzes the rapid transmembrane transfer of PAF to the outer leaflet of the plasma membrane where PAF is localized in the phospholipid milieu (position 1) or by noncovalent binding to one or more surface proteins (position 2). The flippase may also transfer the PAF back to the inner leaflet, where it is accessible to degradation by the intracellular PAF AH. Alternatively, PAF may be translocated from the site of synthesis to the outer leaflet of the plasma membrane by vesicular flow and fusion (*B*); association with a "presenting protein" may occur in the intracellular organelle(s) rather than at the EC surface.

ECs remain to be defined. Several possibilities are shown in the model illustrated in Fig. 9. PAF may reach the surface by a lipid transport protein (*A* in Fig. 9) or by vesicular fusion (*B* in Fig. 9) (Bishop and Bell, 1988). In the plasma membrane, PAF may be in the outer leaflet of the bilayer (location *1* in Fig. 9). Our experiments with PAF-loaded liposomes indicate that PAF in a lipid membrane can interact with its receptors on neutrophils. A second possibility is that PAF is noncovalently linked to one or more surface proteins of ECs (location *2* in Fig. 9). This mechanism of presentation is an attractive possibility since it would place PAF in a more prominent and accessible location than if it were inserted in the phospholipid bilayer. However, our experiments with proteases did not support such a model, unless the putative binding protein is resistant to cleavage. Also, if our experiments with liposomes reflect what occurs at the surface of a biologic membrane, a "presenting protein" is not required. How PAF in a bilayer interacts with a receptor on another cell is not clear. One potential mechanism, which is not shown in the figure, is that PAF transfers from the EC membrane to its receptor on the PMN when the two cells are in close proximity. Against this possibility is our previous observation that endogenously synthesized PAF is not extracted from the ECs by high concentrations of albumin (McIntyre et al., 1985), which binds PAF. However, it is possible that the affinity of the neutrophil receptor for PAF is higher enough than that of albumin to alter the partitioning of the phospholipid. It is also possible that PAF can remain in the bilayer and cause localized changes that allow it to be recognized by the receptor. For example, PAF causes disordering in model membranes (Bratton et al., 1988) and, particularly if it is clustered in domains in the EC plasma membrane (location *1* in Fig. 9), might cause such an effect (Karnovsky et al., 1982; Spector and Yorek, 1985).

The EC-dependent binding of PMNs induced by PAF appears to require a response from the PMN that then results in the adhesive interaction. This is suggested by preliminary experiments in which we found that PMN binding to thrombin-activated ECs was inhibited by fixation of the neutrophils or by cooling them to 4°C (our unpublished observation). One such mechanism could be upregulation of CD_{11}/CD_{18} glycoproteins in the PMN plasma membrane in response to EC-associated PAF. However, monoclonal antibodies against CD_{11}/CD_{18} only partially inhibited PMN adhesion to thrombin-stimulated ECs (Zimmerman and McIntyre, 1988). This result has several potential interpretations, including the possibilities that CD_{11}/CD_{18} glycoproteins are expressed in localized domains (Poo et al., 1988; Wright et al., 1988) that were not accessible to the antibodies, that the affinity of binding of CD_{11}/CD_{18} heterodimers to their "ligands" on activated ECs is different from the affinity of binding to ligands on resting ECs, or that additional proadhesive molecules are expressed by thrombin-activated ECs. PMN binding to rapidly activated ECs may be similar to lymphocyte adhesion to vascular endothelium, which potentially involves at least three distinct molecular interactions (Dustin and Springer, 1988).

PAF synthesis occurs in ECs that are stimulated in situ (Whatley et al., 1988), as well as in cultured ECs, and when human ECs are stimulated with thrombin in serum rather than buffer (our unpublished observation). Furthermore, other agonists that induce its rapid synthesis in endothelium (histamine, leukotrienes C_4 and D_4, H_2O_2) are important inflammatory mediators. Thus, rapidly induced PAF synthesis by ECs may be a mechanism that influences cell–cell interaction in physiologic or pathologic inflammation. The mechanism has characteristics that may be particularly advantageous for regulating the binding of PMNs to endothelium in vivo. The accumulation of PAF is maximal within minutes (Prescott et al., 1984; McIntyre et al., 1986; Lewis et al., 1988), a response time that is consistent with the rapid targeting of PMNs to localized areas that has been observed under some conditions in vivo (Van Furth et al., 1979; Harlan, 1985). Also, the PAF that is synthesized by activated EC is degraded at a variable rate (Prescott et al., 1984; McIntyre et al., 1985; McIntyre et al., 1986), providing a means to modulate the duration of the signal. Consistent with this, EC-dependent PMN adhesion induced by thrombin and other rapidly acting agonists is reversible with an "off-rate" that parallels the degradation of newly synthesized PAF (Fig. 1) (Zimmerman et al., 1985, 1986; McIntyre et al., 1986). A crucial point is that PAF is retained, rather than being released, by ECs (McIntyre et al., 1985; Whatley et al., 1988). This has the potential to localize the signal, and thereby prevent indiscriminant activation of PMNs in the fluid phase. Thus, synthesis of PAF by ECs provides a mechanism for rapid induction of EC-dependent PMN adhesion of variable, transient duration whereas the synthesis of ELAM-1 is a slower, more sustained mechanism for such ad-

herence (Fig. 1). Since the time course of rapidly induced PAF accumulation overlaps that of ELAM-1 under some conditions (McIntyre et al., 1986), the two mechanisms have the potential to act in concert to cause both immediate and prolonged EC-dependent recruitment of neutrophils at sites of inflammation.

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