Roles of prostacyclin, EDRF and active oxygens in leukocyte-dependent platelet adhesion to endothelial cells induced by platelet-activating factor *in vitro*

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1 The mechanism of polymorphonuclear leukocyte (PMN)-dependent platelet adhesion to cultured endothelial cells induced by platelet-activating factor (PAF) was investigated to determine whether PMNs release or generate any factor(s) capable of inducing platelet adhesion, and the roles of prostacyclin and endothelium-derived relaxing factor (EDRF).

2 Cell-free supernatants, sonicates or rapid filtrates of PAF-stimulated PMN suspensions did not induce platelet adhesion to endothelial cells, but the PMN sonicates induced platelet adhesion when endothelial cells were pretreated with both aspirin and N^G-nitro-L-arginine (L-NOARG). Its microphotograph showed that mainly platelet aggregates adhered to the endothelial cell surface.

3 Platelet adhesion induced by the PMN sonicates to aspirin- and L-NOARG-pretreated endothelial cells was dose-dependently prevented by OP-41483 (1–100 nM), and slightly by L-arginine (1 mM). The inhibition of platelet adhesion by OP-41483 and L-arginine was potentiated by their combination.

4 WEB 2170 (3 µM), a PAF antagonist, inhibited platelet adhesion induced by the PMN sonicates. However, PAF alone did not induce significant platelet adhesion to aspirin- and L-NOARG-treated endothelial cells.

5 Platelet adhesion induced by the PMN sonicates was not suppressed by AA-861 and indomethacin. However, both superoxide dismutase and catalase significantly inhibited platelet adhesion, and, in combination, their inhibitory effect was synergistically potentiated. Mannitol had no effect. It was also significantly inhibited by α₁-antitrypsin, whereas chymostatin and elastatinal had no effect.

6 PAF-induced platelet adhesion to endothelial cells in the presence of intact PMNs was not suppressed by indomethacin and AA-861, or by protease inhibitors. SOD alone, and in combination with catalase, caused a slight but significant inhibition, while catalase and mannitol by themselves had no effect.

7 PMN-induced platelet adhesion was slightly inhibited by OP-41483 (100 nM). L-Arginine (1 mM) alone had no effect, but slightly potentiated the effect of OP-41483. This platelet adhesion was not accompanied by suppression of prostacyclin synthesis.

8 The results with the PMN sonicates show that prostacyclin, EDRF and active oxygens are important modulators of intercellular interactions between platelets and endothelial cells. These results further suggest that the mechanism of intact PMN-dependent platelet adhesion is primarily through platelet-endothelial cell interactions in which leukocyte-derived active oxygens play a role, but does not involve platelet-platelet interactions inhibitable by prostacyclin.

Keywords: Active oxygens; prostacyclin; EDRF; platelet adhesion; endothelial cells; leukocytes; platelet-activating factor (PAF)

Introduction

Interaction of platelets and polymorphonuclear leukocytes (PMNs) with the blood vessel walls at sites of vascular injury is considered to be an essential early event in the pathogenesis of inflammation, thrombosis and atherosclerosis. In a previous report, we demonstrated that platelet-activating factor (PAF) induced platelet adhesion to endothelial cells in the presence of PMNs *in vitro*, and had no effect in the absence of PMNs (Hirafuji & Shinoda, 1991a). Another potent PMN activator, N-formyl-methionyl-leucyl-phenylalanine also induced platelet adhesion to endothelial cells when PMNs were present, while leukotriene B₄ had no effect, suggesting that the induction of platelet adhesion is not a general property of PMN activators (Hirafuji & Shinoda, 1991b). Although there are several possible mechanisms for the platelet adhesion, microscopic examination has suggested the possibility that some factor(s) released from PAF-stimulated PMNs mediate(s) platelet adhesion by acting on platelets or/and endothelial cells. Activated PMNs are known to generate and release various bioactive substances, including arachidonate

metabolites, proteases and active oxygens, which can modulate not only platelets but also endothelial cell functions (Harlan, 1987).

On the other hand, endothelial cells normally have highly anti-thrombogenic properties which can be attributed to their capacity to synthesize and release a variety of substances, including prostacyclin and endothelium-derived relaxing factor (EDRF) which have been shown to inhibit platelet aggregation and adhesion (Moncada *et al.*, 1976; Azuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1987a,b; Sneddon & Vane, 1988). Therefore, PMN-dependent platelet adhesion induced by PAF would also occur due to the loss of these anti-thrombogenic properties. In the present study, to clarify the mechanism of PMN-dependent platelet adhesion, we investigated whether PAF-stimulated PMNs release or generate any factor(s) capable of inducing platelet adhesion to endothelial cells. We also examined the role of prostacyclin and EDRF released from endothelial cells in this mechanism.

Methods

Endothelial cells, platelets and PMNs

Endothelial cells, platelets and PMNs were prepared as described previously (Hirafuji & Shinoda, 1991a). In brief, endothelial cells were isolated from human umbilical cord veins by collagenase digestion. Primary endothelial cells were seeded into 24-well culture plates (Primaria, Falcon), and grown until confluent (4–6 days) in Ham's F-12 medium containing 15% foetal calf serum, $25 \mu\text{g ml}^{-1}$ endothelial cell mitogen, $90 \mu\text{g ml}^{-1}$ heparin, 50 u ml^{-1} penicillin and $50 \mu\text{g ml}^{-1}$ streptomycin. Just prior to experiments, endothelial cells were washed 3 times with Hanks' balanced salt solution containing 10 mM HEPES (pH 7.4) and 0.25% bovine serum albumin (BSA; HBSS).

Washed platelets were prepared from freshly collected rabbit blood with EDTA (5 mM) as anticoagulant. Platelets were finally resuspended in HBSS at 5×10^8 platelets ml^{-1} . To radio-label platelets, platelets in platelet-rich plasma were incubated with [^3H]-adenine, $0.5 \mu\text{Ci ml}^{-1}$ for 30 min at 37°C , according to the method of Curwen *et al.* (1982). After washing out free radioactivity, cold adenine ($100 \mu\text{M}$) was added to the final platelet suspension.

Methods for rabbit PMN preparation included dextran sedimentation of erythrocytes, hypotonic shock to eliminate erythrocyte contamination, and purification by centrifugation on Lymphoprep solution. PMNs were finally suspended in calcium- and magnesium-free HBSS at 1×10^7 cells ml^{-1} . Calcium (1.3 mM) and magnesium (1.0 mM) were added just prior to the experiments.

Platelet adhesion assay

Platelet adhesion to endothelial cells was determined in the presence of either intact PMNs or their cell-free preparations. When intact PMNs were used, radiolabelled platelets (7.5×10^7 per well) and/or PMNs (7.5×10^5 per well) were added to endothelial cell monolayers, and incubated for 20 min at 37°C with gentle shaking in a total volume of $400 \mu\text{l}$. Platelets and endothelial cells were also incubated with various cell-free preparations derived from PMN suspensions. In some experiments, platelets and PMN preparations were incubated with endothelial cells that had been pretreated with aspirin ($500 \mu\text{M}$) and/or N^G -nitro-L-arginine (L-NOARG; $300 \mu\text{M}$) for 15 min. Following incubation, the wells were gently washed 3 times with HBSS to remove non-adherent cells. Distilled water containing 1% Triton X-100 was added to each well to lyse the remaining cells, and the radioactivity in the wells was counted.

Cell-free preparations of PMNs

To prepare cell-free supernatants, PMN suspensions ($1 \times 10^7 \text{ ml}^{-1}$) were incubated for 10 min at 37°C with or without 26.7 nM PAF, and then centrifuged at $230 g$ for 10 min at 4°C . To obtain PMN sonicates, PMN suspensions were sonicated for 20 s (Tomy UR-20P, Japan) at 0°C after PAF stimulation. To obtain rapid filtrates, PMNs were stimulated for 10 min with PAF in a disposable syringe. Following incubation, PMN suspensions were rapidly filtered through a $0.22 \mu\text{m}$ membrane filter (Millipore, Japan) attached to the syringe, and the filtrates were immediately (within 1 s) added to endothelial cell monolayer and platelets.

Determination of prostacyclin

Following incubation of endothelial cells, platelets or PMNs for 20 min at 37°C as indicated in the text, the medium in the well was transferred to microvials containing indomethacin ($10 \mu\text{M}$, final concentration), chilled on ice and then centrifuged at $8,000 g$ for 2 min. The supernatants were stored at -20°C until use. Prostacyclin was determined as 6-keto-

prostaglandin $\text{F}_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) by a specific radioimmunoassay (Hirafuji & Shinoda, 1992).

Materials

PAF (1-O-hexadecyl -2-acetyl-sn-glycero-3-phosphocholine), bovine serum albumin, adenine, indomethacin, N^G -nitro-L-arginine, α_1 -antitrypsin, superoxide dismutase, and catalase were purchased from Sigma; Ham's F-12 culture medium, foetal calf serum, penicillin and streptomycin from Gibco; endothelial cell mitogen from Biomedical Technologies Inc.; Lymphoprep (9.6% sodium metrizoate and 5.6% Ficoll) from Nycomed AS; collagenase (*Clostridium histolyticum*), mannitol, L-arginine and HEPES from Wako Chemicals; chymostatin and elastatinal from Peptide Institute Inc.; Hanks' balanced salt solution from Nissui Pharmaceut.; aspirin (DL-lysine salt) from Midorijuhji Co.; [^3H]-adenine (24 Ci mmol^{-1}) from Amersham, Buckinghamshire; [^3H]-6-keto-PGF $_{1\alpha}$ from NEN, U.S.A. AA-861 (2-(12-hydroxydeca-5, 10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone) and WEB 2170 (6-(2-chloro-phenyl)-8,9-dihydro-1-methyl-8-(4-morpholinyl-carbonyl)-4H, 7H-cyclopenta[4,5]thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]diazepine) were gifts from Takeda Chemical Indust., Japan, and Dr H. Heuer, Boehringer-Ingelheim KG, Germany, respectively. OP-41483- α -cyclodextrin (5(E)-6,9 α -methylene-15-cyclopentyl-16, 17, 18, 19, 20-pentanor-prostaglandin I_2 - α -cyclodextrin clathrate), 6-keto-PGF $_{1\alpha}$ and its antiserum were also kindly provided by Ono Pharmaceuticals Indust., Ltd., Japan.

Statistical analysis

Platelet adhesion was expressed as a percentage of the total radioactivity in cell suspension added to the wells. Results are expressed as mean $\pm$ s.e.mean of n experiments performed in triplicate which agreed within 10% or of replicate determinations. Statistical analysis was made by Student's t test, and P values less than 0.05 were considered as significant.

Results

Effects of cell-free preparations of PMNs

Platelets and endothelial cells were incubated with various cell-free preparations from PMNs stimulated without or with PAF (26.7 nM) for 10 min to determine if PMNs generate or release any factor(s) capable of inducing platelet adhesion. However, as shown in Table 1, neither the cell-free supernatants, the sonicates, nor the rapid filtrates, of PMN suspensions stimulated with or without PAF caused significant platelet adhesion to endothelial cells.

Effects of pretreatment of endothelial cells with aspirin and L-NOARG

Endothelial cells were pretreated for 15 min with aspirin ($500 \mu\text{M}$) and/or L-NOARG ($300 \mu\text{M}$), and incubated with platelets in the presence of the sonicates of PAF-stimulated PMNs. As shown in Figure 1, when endothelial cells were pretreated with either aspirin or L-NOARG, the PMN sonicates did not induce significant platelet adhesion to the endothelial cells. However, when endothelial cells were pretreated with both aspirin and L-NOARG, the PAF-stimulated sonicates significantly ($P < 0.01$) induced platelet adhesion (from 2.8 ± 0.3 to $8.4 \pm 0.6\%$ platelet adhesion, mean $\pm$ s.e.mean, $n = 4$ experiments performed in triplicate). As demonstrated in Figure 2, microscopic examination showed that some single platelets and mainly aggregates of platelets adhered to endothelial cell surfaces without any gaps or retraction of endothelial cell monolayers. The sonicates of unstimulated PMNs did not induce platelet adhesion to the aspirin- and/or L-NOARG-pretreated endo-

Table 1 Effects of various cell-free preparations from PMNs on platelet adhesion to endothelial cells

Additions	Platelet adhesion (%)
Basal	2.6 ± 0.2
Cell-free supernatants ^a	
Unstimulated PMNs	2.3 ± 0.1
PAF-stimulated PMNs	2.2 ± 0.3
Sonicates ^b	
Unstimulated PMNs	2.4 ± 0.2
PAF-stimulated PMNs	2.3 ± 0.1
Rapid filtrates ^c	
Unstimulated PMNs	3.0 ± 0.2
PAF-stimulated PMNs	2.9 ± 0.4

[³H]-adenine-labelled platelets and cell-free preparations of PMN suspension, either unstimulated or stimulated with 26.7 nM PAF for 10 min, were added to endothelial cell monolayers and incubated for 20 min. Mean ± s.e.mean of 3 experiments performed in triplicate.

^aAfter PAF stimulation, PMN suspensions (1×10^7 cells ml⁻¹) were centrifuged at 230 g for 10 min at 4°C.

^bAfter PAF stimulation, PMN suspensions were sonicated for 5 × 4 s at 0°C.

^cAfter PAF stimulation, PMN suspensions were rapidly filtered through a 0.22 µm membrane filter, and the filtrates were immediately (within 1 s) added to platelets and the endothelial cell monolayer.

Figure 1 Effects of aspirin- and N^G-nitro-L-arginine (L-NOARG)-pretreatment of endothelial cells on platelet adhesion in the presence of PMN sonicates. Endothelial cells were pretreated with 500 µM aspirin and/or 300 µM L-NOARG for 15 min at 37°C. After aspirating the incubation medium, radiolabelled platelets and sonicates of PMNs stimulated (stippled columns) or unstimulated (open columns) with PAF were added to endothelial cells and incubated for 20 min at 37°C. Mean with s.e.mean of 4 experiments performed in triplicate. ***P* < 0.01.

thelial cells. The cell-free supernatants of the PAF-stimulated and unstimulated PMNs also did not induce platelet adhesion to the thrombogenic endothelial cells (data not shown).

Effects of L-arginine and OP-41483 on platelet adhesion

Figure 3 demonstrates the effects of L-arginine and OP-41483 on platelet adhesion to aspirin- and L-NOARG-pretreated endothelial cells induced by sonicates of PAF-stimulated PMNs. When endothelial cells were pretreated with aspirin and L-NOARG in the presence of L-arginine (1 mM), platelet adhesion induced by PMN sonicates was slightly prevented ($20.5 \pm 5.1\%$ inhibition, $n = 4$ in triplicate, *P* < 0.05). Platelet adhesion induced by the PMN sonicates was dose-dependently

Figure 2 Phase-contrast photomicrograph (original magnification; ×200) of platelet adhesion to endothelial cells. Platelets and sonicates of PAF-stimulated PMNs were incubated for 20 min with aspirin- and N^G-nitro-L-arginine (L-NOARG)-pretreated endothelial cells. Details are the same as described in the legend of Figure 1. Following three washings, the well was fixed with 2% paraformaldehyde-2% glutaraldehyde. Scale bar = 20 µm.

Figure 3 Effects of L-arginine and OP-41483 on platelet adhesion to aspirin- and N^G-nitro-L-arginine (L-NOARG)-pretreated endothelial cells induced by sonicates of PAF-stimulated PMNs. Endothelial cells were pretreated with aspirin and L-NOARG in the presence (stippled columns) or the absence (open columns) of L-arginine (1 mM). Following the pretreatment, endothelial cells were incubated with platelets and the sonicates in the presence or the absence of OP-41483 at the indicated concentrations. Results are expressed as a percentage of the control platelet adhesion induced without L-arginine and OP-41483. Mean with s.e.mean of 4 experiments in triplicate. All treatments were statistically significant (*P* < 0.05).

prevented by OP-41483 with complete prevention at 100 nM. The effect of OP-41483 was potentiated when combined with L-arginine, which was remarkable at lower concentration. OP-41483 alone at 1 nM caused $12.2 \pm 3.6\%$ inhibition ($n = 4$, *P* < 0.05) of platelet adhesion, while it caused $46.6 \pm 4.0\%$ inhibition ($n = 4$, *P* < 0.01) in combination with L-arginine.

Effects of PAF and PAF antagonist on platelet adhesion

As illustrated in Figure 4, a potent PAF antagonist, WEB 2170 (3 µM) completely inhibited platelet adhesion to aspirin-

Figure 4 Effects of WEB 2170 on platelet adhesion to aspirin- and N^G -nitro-L-arginine (L-NOARG)-pretreated endothelial cells induced by sonicates of PAF-stimulated PMNs. Radiolabelled platelets without (open columns) or with (stippled columns) sonicates of PAF-stimulated PMNs were added to the aspirin- and L-NOARG-pretreated endothelial cells in the presence of a PAF antagonist, WEB 2170 (3 μ M), and incubated for 20 min. Mean with s.e.mean of 3 experiments in triplicate. ** $P < 0.01$.

and L-NOARG-pretreated endothelial cells induced by sonicates of PAF-stimulated PMNs, but had no effect on the basal adhesion.

On the other hand, PAF (10 nM) alone without the sonicates did not induce platelet adhesion to aspirin- and/or L-NOARG-pretreated endothelial cells (data not shown).

Effects of various inhibitors on platelet adhesion induced by PMN sonicates

Table 2 shows the effects of inhibitors of arachidonate metabolism and proteases, and of scavengers of active oxygens on the sonicates-induced platelet adhesion to thrombogenic endothelial cells. Platelet adhesion induced by the sonicates of PAF-stimulated PMNs was not suppressed when PMNs were stimulated in the presence of AA-861 (3 μ M), a 5-lipoxygenase inhibitor (Ashida *et al.*, 1983), or indomethacin (3 μ M). However, both superoxide dismutase (SOD; 300 u ml⁻¹) and catalase (1000 u ml⁻¹) significantly ($P <$

0.05) inhibited platelet adhesion, and their inhibitory effect was potentiated by their combination. Mannitol (1 mM) had no effect. Platelet adhesion was also significantly ($P < 0.05$) inhibited by α_1 -antitrypsin (1 mg ml⁻¹), whereas chymostatin (10 μ g ml⁻¹) and elastatinal (10 μ g ml⁻¹) had no effect.

Effects of various inhibitors on platelet adhesion induced by intact PMNs

PAF induces platelet adhesion to endothelial cells when intact PMNs were coincubated (Hirafuji & Shinoda, 1991a). The effects of various inhibitors and scavengers of active oxygens were also evaluated on PAF-induced platelet adhesion to endothelial cells in the presence of intact PMNs (Table 2). Indomethacin and AA-861 again had no effect on platelet adhesion. Furthermore, α_1 -antitrypsin, chymostatin and elastatinal had no significant effect. Among scavengers of active oxygens, SOD alone, and in combination with catalase, caused a slight but significant ($P < 0.05$) inhibition of platelet adhesion. However, catalase and mannitol by themselves were without effect.

Effects of L-arginine and OP-41483 on intact PMN-induced platelet adhesion

PAF (10 nM) caused a significant ($P < 0.01$) platelet adhesion to endothelial cells when intact PMNs were present (3.9 ± 0.5 in basal and 9.9 ± 0.6 in PAF-stimulated group, $n = 6$ in triplicate). As illustrated in Figure 5, OP-41483 at 100 nM slightly inhibited platelet adhesion induced by PAF in the presence of PMNs ($92.5 \pm 1.5\%$ of control, $n = 4$, $P < 0.05$), whereas the lower concentrations had no effect. L-Arginine at 1 mM alone had no effect ($94.7 \pm 5.7\%$ of control, $n = 4$), but slightly potentiated the effect of OP-41483 ($77.6 \pm 3.9\%$ of control, $n = 4$, $P < 0.05$).

Prostacyclin production during intact PMN-induced platelet adhesion

Table 3 demonstrates a representative result of the effect of PAF on prostacyclin production during platelet adhesion. Incubation of endothelial cells with platelets but not PMNs remarkably increased the basal prostacyclin production. Addition of PAF (10 nM) caused 2, 3.2 and 3.5 fold increase in prostacyclin production by endothelial cells alone, with platelets and with PMNs, respectively, when compared with each basal production. PAF also caused a 3.6 fold increase in

Table 2 Effects of scavengers of active oxygens and various inhibitors on platelet adhesion to endothelial cells induced by sonicates of PAF-stimulated PMNs and by intact PMNs

Additions	Platelet adhesion induced by	
	PMN sonicates ^a	Intact PMNs ^b
	% of control (n)	
Indomethacin (3 μ M)	97.6 $\pm$ 3.4 (3)	104.6 $\pm$ 5.0 (3)
AA-861 (3 μ M)	98.9 $\pm$ 2.9 (3)	90.1 $\pm$ 15.8 (3)
α_1 -Antitrypsin (1 mg ml ⁻¹)	34.7 $\pm$ 5.1 (3)*	89.7 $\pm$ 8.4 (3)
Chymostatin (10 μ g ml ⁻¹)	95.6 $\pm$ 3.2 (3)	92.4 $\pm$ 5.5 (3)
Elastatinal (10 μ g ml ⁻¹)	93.0 $\pm$ 4.7 (3)	95.7 $\pm$ 4.0 (3)
SOD (300 u ml ⁻¹)	78.7 $\pm$ 7.5 (4)*	87.9 $\pm$ 2.8 (4)*
Catalase (1000 u ml ⁻¹)	59.9 $\pm$ 9.8 (4)*	96.6 $\pm$ 1.4 (4)
SOD + catalase	19.8 $\pm$ 4.8 (4)**	81.0 $\pm$ 4.5 (4)*
Mannitol (1 mM)	92.7 $\pm$ 5.6 (3)	93.5 $\pm$ 5.5 (3)

For abbreviations, see text.

^aEndothelial cells were pretreated with 500 μ M aspirin and 300 μ M N^G -nitro-L-arginine for 15 min. After aspirating the incubation medium, [³H]-adenine-labelled platelets and sonicates of PAF-stimulated PMNs were incubated for 20 min with endothelial cells in the presence of the indicated agent, except that indomethacin and AA-861 were added to PMN suspensions during the PAF stimulation.

^bRadio-labelled platelets and intact PMNs were incubated for 20 min with untreated endothelial cells in the presence of PAF (10 nM) and the indicated agent.

Mean $\pm$ s.e.mean of n experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

Figure 5 Effects of L-arginine and OP-41483 on platelet adhesion induced by PAF in the presence of intact PMNs. Radiolabelled platelets, endothelial cells and intact PMNs were stimulated by PAF (10 nM) for 20 min without or with OP-41483 (100 nM) in the absence (open columns) or presence (stippled columns) of L-arginine (1 mM). Results are expressed as a percentage of the control platelet adhesion induced by PAF. Mean with s.e.mean of 4 experiments in triplicate. * $P < 0.05$.

Table 3 Effect of PAF on prostacyclin production by intercellular interactions among endothelial cells (ECs), platelets and PMNs

Conditions	Prostacyclin (ng per well)	
	Basal	PAF (10 nM)
ECs	0.06 ± 0.005	0.12 ± 0.003
ECs + platelets	3.62 ± 0.26	11.68 ± 0.62
ECs + PMNs	0.06 ± 0.003	0.21 ± 0.07
ECs + platelets + PMNs	3.60 ± 0.30	13.07 ± 0.51

Endothelial cells without or with platelets and/or PMNs were incubated in the absence (Basal) or the presence of PAF (10 nM) for 20 min at 37°C. At the end of incubation, the medium was added with indomethacin (10 µM) and centrifuged at 8,000 *g* for 2 min. Prostacyclin in the supernatants was determined by radioimmunoassay as 6-keto-PGF_{1α}. Mean ± s.e.mean of a representative of 3 experiments performed in triplicate.

prostacyclin production by endothelial cells with platelets and PMNs, a condition under which platelet adhesion to endothelial cells occurred. The experiment was repeated three times with essentially similar results, although the basal levels and response to PAF of prostacyclin productions in each condition were very variable depending on the experiment, possibly due to the variation in batches of endothelial cells.

Discussion

In a previous paper, we demonstrated that PAF induced platelet adhesion to endothelial cell surfaces only in the presence of PMNs *in vitro* (Hirafuji & Shinoda, 1991a). In the present study, to determine whether PMNs release or generate any factor(s) capable of inducing platelet adhesion, the effects of various cell-free preparations from PMNs on platelet adhesion were first examined. However, neither the cell-free supernatants containing released factors, the sonicates containing various factors and their generating systems, nor the rapid filtrates containing stable and unstable released factors, of PMN suspensions stimulated with or without PAF, caused platelet adhesion to endothelial cells. This indicates that PMN-dependent platelet adhesion cannot be

simply explained by factor(s) released or generated by activated PMNs. As platelet adhesion occurs only in the presence of intact PMNs, it may require the concomitant intercellular interactions among these cells.

However, when endothelial cells were depleted of prostacyclin and EDRF by pretreatment with both aspirin, a cyclo-oxygenase inhibitor, and L-NOARG, a potent inhibitor of EDRF production (Ishii *et al.*, 1990; Moore *et al.*, 1990), the sonicates of PAF-stimulated PMNs induced platelet adhesion. Pretreatment with either aspirin or L-NOARG alone had no effect. Furthermore, L-arginine partially prevented platelet adhesion induced by the PMN sonicates, possibly antagonizing the effect of L-NOARG, and the inhibitory effect was potentiated in combination with OP-41483, a stable prostacyclin analogue (Fujitani & Wakitani, 1990). However, these results may not necessarily imply that prostacyclin and EDRF prevent platelet adhesion to endothelial cells by acting in concert as in platelet aggregation (Radomski *et al.*, 1987b; MacDonald *et al.*, 1988). Platelet adhesion seems to be regulated by elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) but not adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Radomski *et al.*, 1987a; Sneddon & Vane, 1988; Venturini *et al.*, 1992), while OP-41483 elevates cyclic AMP level in platelets like prostacyclin (Fujitani & Wakitani, 1990), and this compound alone effectively inhibited platelet adhesion induced by the PMN sonicates. Furthermore, since photomicrographs of sonicate-induced platelet adhesion showed that mainly platelet aggregates adhered to the endothelial cell surface, platelet adhesion cannot be readily separated from platelet aggregation in this system. Therefore, the inhibition of platelet adhesion by OP-41483 may be due to dissociation of adherent platelet aggregates which have formed in the presence of PAF-stimulated PMN sonicates. The synergism between endogenous prostacyclin and EDRF in preventing platelet adhesion may be also due to inhibition of platelet aggregate formation rather than platelet-endothelial cell interactions.

Platelet adhesion to aspirin- and L-NOARG-pretreated endothelial cells induced by sonicates of PAF-stimulated PMNs was completely inhibited by a PAF antagonist, WEB 2170 (Heuer *et al.*, 1990). This result indicates that the sonicates contain PAF which was not inactivated by acetylhydrolase (Lee *et al.*, 1982), and that platelet adhesion to thrombogenic endothelial cells requires, at a minimum, the activation of both, or either, the platelets and/or endothelial cells by PAF. Since PAF is a potent agonist in rabbit platelets and a weak agonist in human endothelial cells (Hirafuji & Shinoda, 1992), platelets may be the target cells of PAF. However, PAF alone at a concentration (10 nM) that induces full aggregation did not induce platelet adhesion even to the thrombogenic endothelial cells without the sonicates, suggesting that some factor(s) in the sonicates are prerequisite to the ultimate platelet-endothelial cell interactions. Since the platelet adhesion was significantly inhibited by SOD, catalase and mostly by their combination, active oxygens in the PMN sonicates may be those factors. Likewise, Salvemini *et al.* (1989) have found that SOD inhibits thrombin-stimulated platelet aggregation and adhesion to gelatin-coated plastic, although catalase has no effect, suggesting that superoxide anions enhance platelet aggregation and adhesion. Therefore, in our system, active oxygens may potentiate both aggregation and adhesion, resulting in the deposition of platelet aggregates on the endothelial cells. Certain proteases also seem to play minor roles, because the platelet adhesion was also significantly inhibited by a non-specific protease inhibitor, α₁-antitrypsin.

Thus, an experimental system using the PMN sonicates suggested that prostacyclin, EDRF and active oxygens were important modulators of intercellular interactions between platelets and endothelial cells. Therefore, we also evaluated the involvements of these factors in platelet adhesion to endothelial cells induced by intact PMNs. The results suggest that alteration of prostacyclin synthesis, at least, is not

involved in this system for the following reasons. First, OP-41483 at 100 nM caused a minimal inhibition of platelet adhesion induced by intact PMNs, whereas this concentration of OP-41483 completely inhibited the sonicate-induced platelet adhesion. Second, when prostacyclin production during the intercellular interactions was determined, PMN-dependent platelet adhesion induced by PAF was not accompanied by a suppression in prostacyclin synthesis. Furthermore, single platelets are the predominant adhering cells in intact PMN-induced platelet adhesion (Hirafuji & Shinoda, 1991a), while aggregates of platelets are mainly adhering in the sonicate-induced platelet adhesion (Figure 2). Hence, the mechanism by which intact PMNs induce platelet adhesion is probably different from that of the sonicate-induced platelet adhesion. It may be primarily platelet-endothelial cell interactions mediated by activated PMNs, but does not involve platelet-platelet interactions inhibitable by prostacyclin.

Since the platelet adhesion induced by intact PMNs was significantly inhibited by scavengers of active oxygens, active oxygens derived from activated PMNs may play a role in the platelet-endothelial cell interactions. However, these scavengers had less of an effect in this system than in the system using the PMN sonicates. This minor effect could be explained by recent electron microscopic-histochemical studies (Visser *et al.*, 1985; Hirai *et al.*, 1991). These studies have demonstrated that PMNs, activated by specific agonists, release active oxygens only at the area of contact between

PMNs and nonphagocytosable surfaces or phagocytosable particles, which is hence inaccessible to macromolecules such as scavenger proteins. Since pharmacological studies seem to possess such a limitation, further studies with other approaches may be required to clarify the involvement of active oxygens.

It is also not clear from the present study whether EDRF is involved in the PMN-dependent platelet adhesion. EDRF, in contrast to prostacyclin, strongly inhibits platelet adhesion by stimulating guanylate cyclase (Radomski *et al.*, 1987a; Sneddon & Vane, 1988; Venturini *et al.*, 1992). Furthermore, a possible mechanism by which active oxygens increase platelet adhesion may be through inhibition of guanylate cyclase (Salvemini *et al.*, 1989). Therefore, it is conceivable that PMN-derived active oxygens decrease the cyclic GMP level in platelets by inhibiting guanylate cyclase and by destroying EDRF released from endothelial cells (Gryglewski *et al.*, 1986) ultimately cause platelet adhesion. It would be of interest to determine levels of cyclic GMP or EDRF during the intercellular interactions to clarify these possibilities.

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