Br. J. Pharmacol. (1991), **103**, 1333–1338

Platelet-leukocyte interaction in adhesion to endothelial cells induced by platelet-activating factor *in vitro*

[1]Masahiko Hirafuji & Hisashi Shinoda

Department of Pharmacology, Tohoku University School of Dentistry, 4-1 Seiryo-machi, Sendai 980, Japan

1 Platelet-activating factor (PAF, 10 nM) did not induce platelet adhesion to endothelial cells cultured in monolayer but it induced their adhesion to protein-coated plastic. However, PAF induced a marked platelet adhesion to endothelial cells when polymorphonuclear leukocytes (PMNs) were present. Lyso-PAF had no effect.

2 Phase-contrast microscopic examination showed that single platelets rather than their aggregates adhered to the endothelial cell surface around aggregating and adhering PMNs.

3 Significant platelet adhesion was induced by PAF at concentrations higher than 0.01 nM with the maximal response at 10 nM. Platelet adhesion occurred within minutes after PAF addition, reaching a maximum approximately after 30 min. Platelet adhesion also occurred significantly at a PMN : platelet ratio of 1 : 800, and linearly up to 1 : 50.

4 The PAF-induced platelet adhesion was suppressed by three structurally unrelated PAF antagonists, WEB 2086, ONO 6240 and BN 52021, in a concentration-dependent manner.

5 PAF also increased PMN adhesion to endothelial cell monolayers, which was further augmented by the presence of platelets.

6 The present study demonstrates that PAF induces platelet adhesion to endothelial cells *in vitro* when PMNs are present and that there is a close interaction between platelets and PMNs in their adhesion to endothelial cells. The present study further suggests that PMNs could play a central role in platelet adhesion to vascular endothlium in certain pathological conditions.

Keywords: Platelet-activating factor (PAF); platelets; leukocytes; endothelial cells; platelet adhesion; vascular injury

Introduction

Cell-cell interactions may play a critical role in the pathogenesis of inflammation, thrombosis, and atherosclerosis. In particular, interaction of platelets and polymorphonuclear leukocytes (PMNs) with the blood vessel wall at sites of vascular injury is considered to be an early event in the pathogenesis of these diseases. Morphological studies of platelet plugs and thrombi in vascular lesions frequently demonstrate that they are also rich in PMNs (Henry, 1965; Bourgain *et al.*, 1984), suggesting a close interaction between platelets and PMNs, and possibly among these cells along with the vascular endothelial cells. However, the molecular mechanisms that mediate the cell-cell interactions are still unclear.

PAF (platelet-activating factor) is a lipid mediator that is produced by various cells types including platelets, PMNs or endothelial cells in response to a variety of immunological or non-immunological stimuli (Snyder, 1985). PAF is also a potent activator of these cells, and induces various cellular responses that may play a critical role in the process of vascular diseases (Vargaftig *et al.*, 1989). Several *in vitro* studies have demonstrated or suggested that newly formed PAF is an important mediator of direct interactions between platelets and PMNs (Benveniste *et al.*, 1972; Shaw & Henson, 1980; Oda *et al.*, 1986; Coëffier *et al.*, 1987), or PMNs and endothelial cells (Zimmerman *et al.*, 1985; Breviario *et al.*, 1988). Cooperation between platelets and PMNs in PAF formation has also been described (Coëffier *et al.*, 1990). However, the role of PAF in the cell-cell interaction between PMNs, platelets and endothelial cells in the complex processes leading ultimately to platelet and PMN adhesion to the endothlium remains to be clarified. Therefore, in the present study, we investigated the effect of exogenous PAF on the adhesion of platelet and PMN mixtures to endothelial cells *in vitro*, to determine if PAF has any influence on platelet and PMN adhesion to endothelial cells, especially if the presence of one cell type might modulate the adhesion of another cell type. Here we demonstrate that PAF induces platelet adhesion to endothelial cells when PMNs are present, and that there is a close interaction between platelets and PMNs in the adhesion process to endothelial cells. Some of these results have been presented at the 63rd Annual Meeting of the Japanese Pharmacological Society (Hirafuji & Shinoda, 1990).

[1] Author for correspondence.

Methods

Endothelial cells

Endothelial cells were isolated from human umbilical cord veins by collagenase digestion as described previously (Hirafuji *et al.*, 1987). Primary endothelial cells were seeded into 24-well culture plates (Primaria, Falcon), and grown until forming a complete monolayer (3–5 days) in Ham's F-12 medium containing 15% foetal calf serum, 50 u ml^{-1} penicillin and 50 $\mu g\,ml^{-1}$ streptomycin. When protein-coated culture plates were used, wells of the plate were incubated overnight at 37°C with the culture medium. Just prior to experiments, endothelial cells and the wells were washed 3 times with Hanks' balanced salt solution containing 10 mM HEPES and 0.25% bovine serum albumin (BSA; pH 7.4; HBSS).

Platelets and polymorphonuclear leukocytes

Blood was collected from the central ear artery of unanaesthetized adult male rabbits and immediately mixed with EDTA (5 mM, final concentration). Platelet-rich plasma (PRP) obtained by centrifugation of the whole blood at 500 *g* for 20 min was then centrifuged at 1,500 *g* for 15 min at room temperature. Platelets were resuspended in calcium-free Tyrode buffer (composition, mM: NaCl 137, KCl 2.6, NaH_2PO_4 0.4, $MgCl_2$ 1.0 and gluccose 5.6, pH 6.5) containing 10 mM HEPES, 0.25% BSA and 0.2 mM EGTA, washed twice with the same buffer, and counted in a Celltac Counter (MEK-

4150, Nihon Kohden, Japan). The platelet pellet from the final wash was resuspended in a small volume of the same buffer, and finally diluted to a concentration of 5×10^8 platelets ml^{-1} with HBSS, and kept at room temperature until use. Contamination by other blood cells in the platelet suspension was negligible, as determined with a haemocytometer.

The cells obtained after removing PRP were mixed with the same volume of 3% dextran (average Mr 2.5×10^5) in physiological saline, and allowed to stand at room temperature for approximately 30 min. During this time the red blood cells sedimented. The supernantant was aspirated, and cells were collected by centrifugation at 230 *g* for 10 min at 4°C. The resultant pellet was resuspended in 1 ml of cold physiological saline. The suspension was added to 10 ml of ice-cold distilled water to lyse contaminating erythrocytes. After 40 s 10 ml of a cold 1.8% NaCl solution was added to restore isotonicity. After centrifugation (at 230 *g* for 10 min at 4°C), the cell pellet was resuspended in 2 ml physiological saline containing 20% autologous platelet-poor plasma, underlayerd with 6 ml Lymphoprep solution and subjected to centrifugation at 500 *g* for 20 min at 20°C. The pellet (PMNs) was washed twice by centrifugation in calcium-, and magnesium-free HBSS, finally resuspended in this buffer, and kept at 0°C until use. The cell-number was adjusted to 1×10^7 cells ml^{-1} after counting in a haemocytometer. Cell purity and viability, as assessed by microscopic examination and exlusion of trypan blue dye, respectively, exceeded 95%. Calcium (1.3 mM) and magnesium (1.0 mM) were added just prior to the experiments.

Labelling of platelets and polymorphonuclear leukocytes

Platelets or PMNs were radiolabelled with [^{3}H]-adenine according to the methods of Curwen *et al.* (1982) and Gimbrone & Buchanan (1982). Platelets in PRP or PMNs in calcium- and magnesium-free HBSS were incubated with 0.5 μCi ml^{-1} [^{3}H]-adenine for 30 min or 2.0 μCi ml^{-1} for 15 min at 37°C, respectively. Platelets and PMNs incorporated 80–90% and 20–30% of the total radioactivities, respectively. After washing out free radioactivity, cold adenine (100 μM) was added to the final suspension of radiolabelled cells.

Adhesion assay

In standard experiments, platelets (7.5×10^7 per well) and/or PMNs (1.5×10^6 per well) were added to culture plates that had been coated with proteins or where endothelial cells had reached confluence and incubated for 20 min at 37°C with gentle shaking (0.5 Hz) in the presence of test compounds in a total volume of 400 μl. At end of the incubation time, the wells were gently washed 3 times with 500 μl HBSS to remove non-adherent cells. In some experiments, the incubation period and the number of PMNs added to the well were varied as indicated in the text. Distilled water (500 μl) containing 1% Triton X-100 was added to each well to lyse the remaining cells. After 30 min incubation at 37°C, the radioactivity in the wells was counted in a scintillation counter. For inverted phase-contrast photomicrographs, cells remaining in the well after washing procedure were fixed overnight with 2% paraformaldehye–2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C.

Materials

PAF (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), lyso-PAF (1-O-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine), BSA (fatty acid-free) and adenine were obtained from Sigma, U.S.A.; HEPES from Dojin, Japan; collagenase (*Clostridium histolyticum*) and dextran from Wako, Japan; Hanks' balanced salt solution, calcium- and magnesium-free Hanks' balanced salt solution, Ham's F-12 culture medium, foetal bovine serum, penicillin and streptomycin from Gibco, U.S.A.; Lymphoprep (9.6% sodium metrizoate and 5.6% Ficoll, Nycomed AS, Norway); [^{3}H]-adenine (24 Ci $mmol^{-1}$; 1 μCi = 37 kBq) from Amersham, Buckinghamshire. BN 52021 (3-tert-butyl-hexahydro-4,7b,11-trihydroxy-8-methyl-9H-1,7a-epoxy-methano-1H, 6aH-cyclopenta[c]furo[2,3b]furo[3',2' : 3,4] cyclopenta[1,2-d]furan-5,9,12(4H)trione) was a kind gift from Dr J.M. Mencia-Huerta, Institut Henri Beaufour, France; WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) from Dr H. Heuer, Boehringer-Ingelheim KG, FRG; ONO 6240 (1-O-hexadecyl-2**RS**-O-ethyl-3-O-(7-thiazolinoheptyl)-glycerol methanesulphonate) from Ono Pharmaceutical Indust. Ltd., Japan.

PAF and lyso-PAF were dissolved in physiological saline containing 0.25% BSA at a concentration of 10 mM and stored at −20°C. WEB 2086 and ONO 6240 were dissolved in physiological saline. BN 52021 was dissolved in dimethylsulphoxide at a concentration of 20 mM. These stock solutions were diluted to the desired concentrations with the incubation medium before use.

Statistical analysis

Platelet or PMN adhesion was expressed as a percentage of the total radioactivity in cell suspension added to the wells. Results are expressed as mean ± s.e.mean of (n) experiments performed in replicate which agrees within 10% coefficient of variation or of replicate determinations. Statistical analysis was made by ANOVA followed by Student's unpaired t test or Cochran-Cox test, and P values less than 0.05 were considered as significant.

Results

Release of [^{3}H]-adenine from radiolabelled cells

Centrifugation at 2,000 *g* for 3 min at room temperature of the final suspensions of radiolabelled platelets and PMNs showed that 3.2 ± 0.8% (mean ± s.e.mean, $n = 5$) and 3.6 ± 0.1% ($n = 3$), respectively, of the radioactivity was spontaneously released from the cells 1 h after the preparation and less than 10% within the time frame of experiments. Incubation for 20 min at 37°C of radiolabelled PMNs and endothelial cells without or with 10 nM PAF demonstrated that PAF did not increase the radioactivity release (16.2 ± 0.4%, $n = 3$, and 17.2 ± 0.9%, $n = 3$, of the total radioactivity without or with PAF, respectively). When radiolabelled platelets were incubated for 20 min at 37°C with an endothelial cell monolayer, 6.5 ± 1.4 ($n = 3$) and 21.9 ± 2.1 ($n = 3$)% of the radioactivity was released in the absence and the presence of 10 nM PAF, respectively. The presence of PMNs did not affect the release.

Uptake of [^{3}H]-adenine by unlabelled cells

Incubation for 20 min at 37°C of either an endothelial cell monolayer or protein-coated plastic with [^{3}H]-adenine (1×10^5 d.p.m. per well; approximately equivalent to the activity in a typical labelled platelet or PMN suspension) in the presence of excess cold adenine (37.5 μM) demonstrated that less than 1% (0.8 ± 0.08 and 0.5 ± 0.04%, respectively, $n = 3$) of the radioactivity remained associated with the well after the standard washing procedure. Also, incubation for 20 min at 37°C of PMNs (3.75×10^6 ml^{-1}; equivalent to the number used for the adhesion assay) or platelets (1.9×10^8 ml^{-1}) with [^{3}H]-adenine in the presence of excess cold adenine caused negligible incorporation of the radioactivity. Thus, the radioactivity remaining in the well after the washing procedure was a reflection of the number of adherent cells.

Platelet adhesion to protein-coated plastic

Figure 1a illustrates the effects of PAF (10 nM) on platelet adhesion to protein-coated plastic in the presence or the

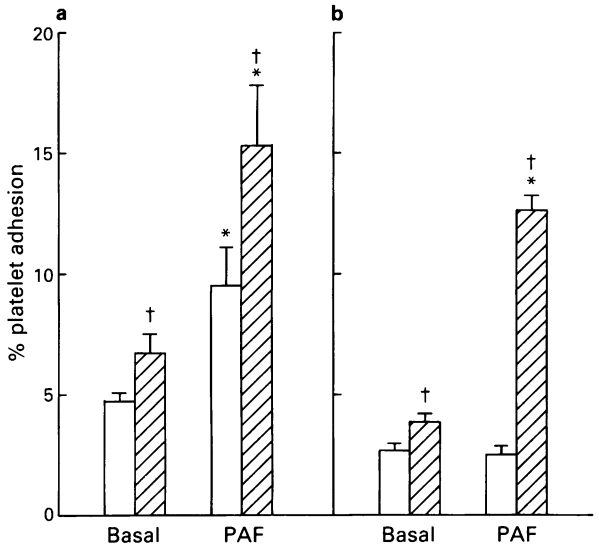

Figure 1 Effect of PAF on platelet adhesion to protein-coated plastic and endothelial cells. Radiolabelled platelets without (open columns) or with polymorphonuclear leukocytes (PMNs, hatched columns) were added to protein-coated culture plate (a) or endothelial cell monolayers (b), and incubated at 37°C for 20 min in the presence or the absence of 10 nM PAF. At the end of incubation time, the wells were washed 3 times with HBSS, and the remaining radioactivity was counted. Results are expressed as percentage of added total radioactivity. Each column represents mean of 4 separate experiments performed in triplicate; vertical bars show s.e.mean; * and † indicate significant differences from each basal and value without PMNs, respectively.

absence of PMNs. Without endothelial cells, PAF significantly increased the platelet adhesion to the protein-coated plastic in the absence of PMNs (4.8 ± 0.2 and 9.5 ± 1.6% platelet adhesion for the control and PAF-treated groups, respectively, $n = 4$ experiments tested in triplicate). When PMNs were coincubated, the basal platelet adhesion slightly but significantly increased (6.8 ± 1.1% platelet adhesion). The platelet adhesion in the presence of PMNs was increased further when PAF was also added to the incubation medium (15.3 ± 2.5% platelet adhesion). Inverted phase-contrast microscopic examination at the end of the incubation period showed that, as well as PMNs, single and mainly aggregated platelets adhered to the plastic surface. This concentration of PAF (10 nM) induced a maximal platelet aggregation when assayed in an aggregometer (83.5 ± 2.9% of light transmittance, $n = 3$).

Platelet adhesion to endothelial cells

In contrast, as illustrated in Figure 1b, PAF had no effect on platelet adhesion to endothelial cell monolayers in the absence of PMNs (2.7 ± 0.2 and 2.5 ± 0.3% platelet adhesion for the control and PAF-treated groups, respectively, $n = 4$ experiments tested in triplicate), a fact that was also confirmed by phase-contrast microscopy. When PMNs were added together with platelets, the basal platelet adhesion was slightly but significantly increased (3.9 ± 0.2% platelet adhesion). However, PAF caused a marked increase in the platelet adhesion when PMNs were also present (12.6 ± 0.5% platelet adhesion). As demonstrated in Figure 2, microscopic examination clearly showed that single platelets rather than small aggregates adhered to endothlial cell surface around aggregating and adhering PMNs. There was no visible endothelial cell lysis or gap formation between the cells during the incubation time. Although it was not easy to determine accurately at this level of resolution, platelets also seemed to adhere to single and possibly aggregated PMNs.

As presented in Figure 3, platelet adhesion was dependent on the PAF concentration, and was significantly induced by 0.01 nM PAF with a maximal response at 10 nM, whereas lyso-PAF, the deacetylated metabolite of PAF, at a concentration of 10 nM, had no effect. Figure 4 presents the time course of PAF-induced platelet adhesion in the presence of PMNs. The platelet adhesion was a rapid response, which was induced

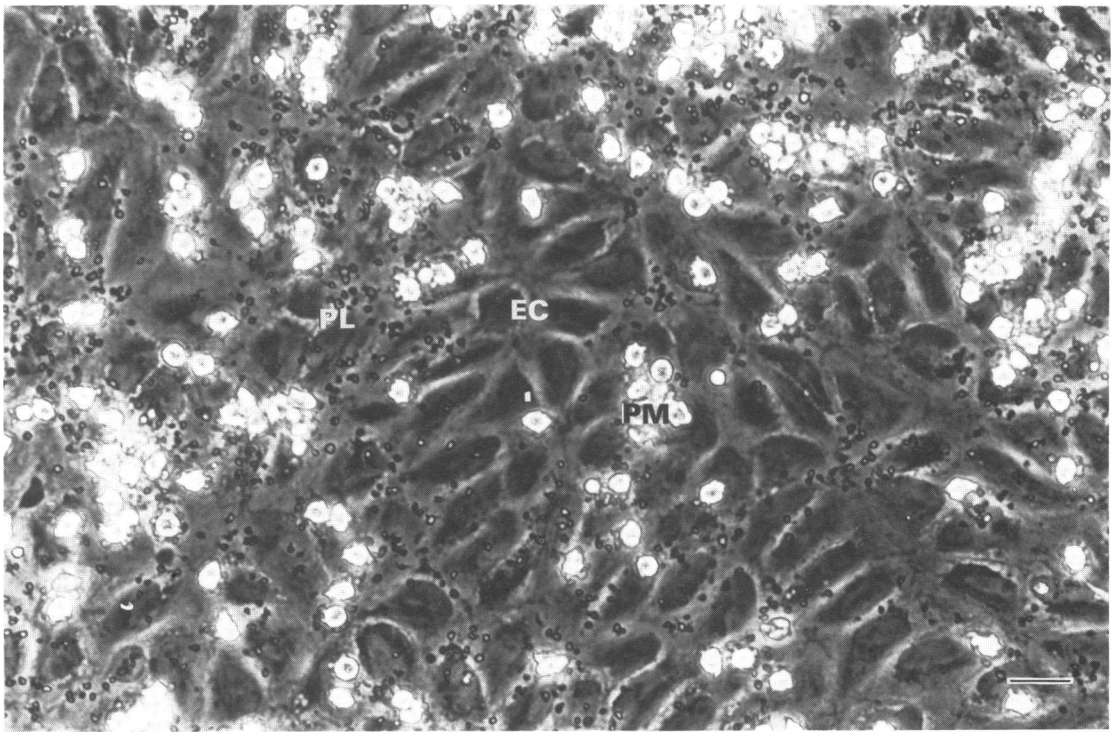

Figure 2 Phase-contrast photomicrograph (original magnification; ×200) of the adhesion of platelets and polymorphonuclear leukocytes (PMNs) to endothelial cells. Endothelial cell monolayer (EC) was incubated for 20 min with platelets (PL), PMNs (PM) and PAF (10 nM). At the end of adhesion assay, the well was washed and fixed with 2% paraformaldehye–2% glutaraldehyde. Scale bar = 20 μm.

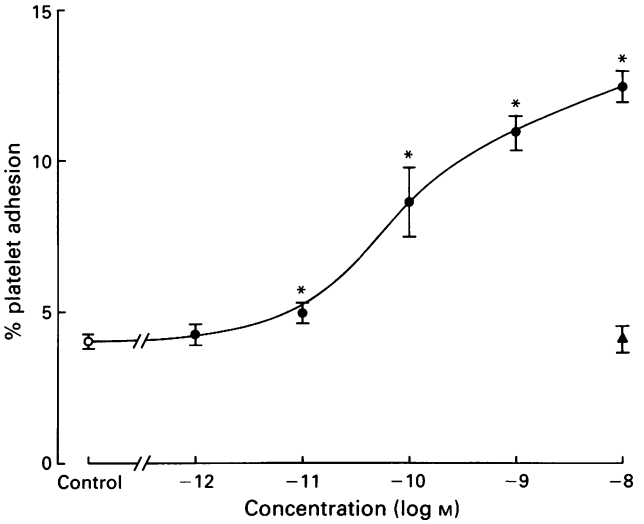

Figure 3 Effects of PAF and lyso-PAF on platelet adhesion to endothelial cells. Radiolabelled platelets were added to endothelial cell monolayers with polymorphonuclear leukocytes (PMNs) and incubated for 20 min without (control, ○) or with indicated concentrations of PAF (●) or lyso-PAF (▲). Mean of 4 and 2 experiments performed in triplicate for PAF and lyso-PAF, respectively; vertical bars show s.e.mean.

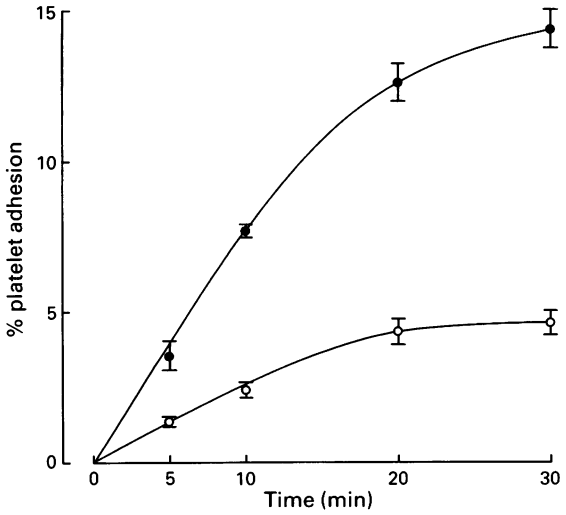

Figure 4 Time course of PAF-induced platelet adhesion to endothelial cells. Radiolabelled platelets were added to endothelial cell monolayers with polymorphonuclear leukocytes (PMNs), and incubated in the absence (○) or the presence (●) of 10 nM PAF. At the indicated time, the wells were washed, and the remaining radioactivity was counted. Mean of 3 experiments in triplicate; vertical bars show s.e.mean.

Table 1 Effect of PAF and platelets on polymorphonuclear leukocyte (PMN) adhesion to endothelial cells

	% PMN adhesion	
	− Platelets	*+ Platelets*
Control	14.6 ± 1.2	20.6 ± 0.2†
PAF 10 nM	22.1 ± 1.4*	36.2 ± 3.2*†

PMNs labelled with [^{3}H]-adenine were added to endothelial cell monolayers with or without platelets and incubated at 37°C for 20 min in the absence (control) or the presence of 10 nM PAF. Results are expressed as percentage of total radioactivity added. Mean ± s.e.mean of a representative experiment performed in quadruplicate. * and † indicate statistical significance compared with control values and the values in the absence of platelets, respectively.

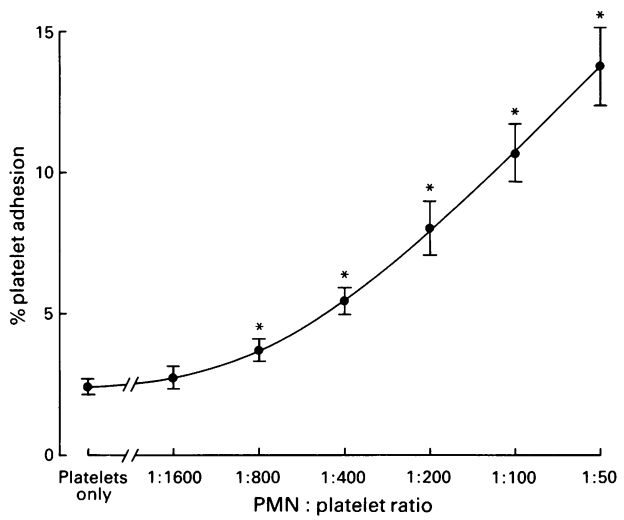

Figure 5 Effect of polymorphonuclear leukocyte (PMN): platelet ratio on PAF-induced platelet adhesion to endothelial cells. Fixed number of radiolabelled platelets (7.5×10^7 per well) with different numbers of PMNs at the indicated PMN : platelet ratio was added to endothelial cell monolayers and incubated for 20 min in the presence of 10 nM PAF. Mean of 3 experiments in duplicate; vertical bars show s.e.mean.* Indicates significant differences from the value with platelets only.

within minutes after PAF addition and reached its maximum by approximately 30 min. Figure 5 demonstrates the effect of PMN number on PAF-induced platelet adhesion to endothelial cells. A significant increase of PMN-dependent platelet adhesion was observed at a PMN : platelet ratio of 1 : 800 when compared to platelets only. At this ratio, platelet adhesion to the endothelial cell surface could be observed microscopically. The platelet adhesion was increased linearly up to a PMN : platelet ratio of 1 : 50.

As shown in Figure 6, the platelet adhesion induced by 10 nM PAF was suppressed in a concentration-dependent manner by three structurally unrelated PAF antagonists, WEB 2086, ONO 6240 and BN 52021.

Polymorphonuclear leukocyte adhesion to endothelial cells

Table 1 presents the effect of PAF (10 nM) on PMN adhesion to endothelial cell monolayers in the absence or in the presence of platelets. PAF induced a 1.5 fold increase in PMN adhesion to endothelial cells in the absence of platelets. When platelets were present, basal PMN adhesion was increased,

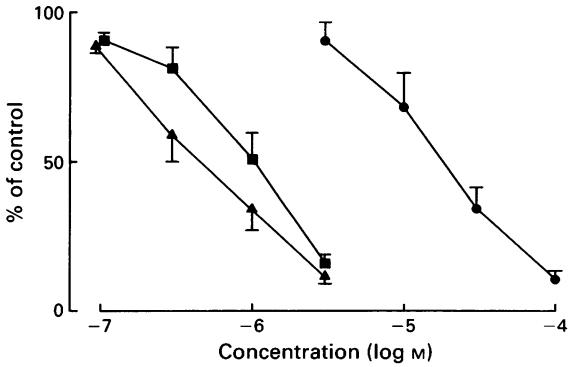

Figure 6 Effect of PAF antagonists on PAF-induced platelet adhesion to endothelial cells. Radiolabelled platelets were added to endothelial cell monolayers with polymorphonuclear leukocytes, preincubated for 1 min with different concentrations of WEB 2086 (▲), ONO 6240 (■) or BN 52021 (●), and then stimulated with 10 nM PAF for 20 min. Results are expressed as percentage of the control platelet adhesion induced by PAF. Each point represents mean of 3 experiments in triplicate; vertical bars show s.e.mean.

and PAF induced a further 1.8 fold increase in PMN adhesion to endothelial cells. As shown in Figure 2, microscopic examination at the end of the incubation period showed that single PMNs and numerous aggregated PMNs were adherent to the endothelial cell surface. In contrast, when PMNs were stimulated by PAF in the absence of platelets, mainly single PMNs adhered to the endothelial cells.

Discussion

Circulating blood platelets normally do not adhere to the intact vascular endothelium. This property of endothelium could be attributed to the ability to produce or release several factors such as prostacyclin (Higgs *et al.*, 1978), 13-hydroxyoctadecadienoic acid (Buchanan *et al.*, 1985), or endothelium-derived relaxing factor (Radomski *et al.*, 1987; Sneddon & Vane, 1988), although the relative contributions of these factors have to be clarified. This anti-platelet property of endothelial cells seems to function efficiently even when PAF, the most potent platelet activating substance, is present. Indeed, this lipid mediator did not induce platelet adhesion to cultured endothelial cells even at a concentration that induced the maximal aggregation and stimulated the platelet adhesion to protein-coated plastic.

However, in the present study, we observed that PAF induced platelet adhesion to endothelial cells when PMNs were present. By phase-contrast microscopy, it was confirmed that single platelets rather than aggregates adhered to the endothelial cell surface around aggregating and adhering PMNs. Platelets are known to be adhesive to PMNs *in vitro* (McGregor *et al.*, 1980). Although it was not clear at this level of resolution, platelets also seemed to adhere to single and possibly agregated PMNs, which can be confirmed only by electron microscopy. Therefore, when platelet adhesion to endothelial cell monolayers was studied in the presence of PMNs, the radioactivity remaining in the wells after the washing procedure should be interpreted as being a total reflection of the number of platelets associated with adhering PMNs as well as those adhered to endothelial cell surface. Since the methodology adopted in the present study cannot discriminate between the relative contribution of these two types of platelet adhesion, we have referred to them as 'platelet adhesion to endothelial cells' for convenience. Furthermore, a critical point of our adhesion assay using platelets labelled with [^{3}H]-adenine might be the slight release of radioactivity in response to PAF, although our technique has some advantages compared with those using ^{51}Cr or ^{111}In-oxine (Curwen *et al.*, 1982; Heaton *et al.*, 1989). Although Curwen *et al.* (1982) found that no detectable release of radioactivity was observed after incubation of [^{3}H]-adenine-labelled PRP with aggregating stimuli, in our hands, the radioactivity released from labelled platelets incubated with an endothelial monolayer was 15.4% higher in the presence of 10 nM PAF (the highest concentration used in the present study) than in control. This means that PAF-dependent platelet adhesion is underestimated by at most 15.4% in our experimental conditions. However, even taking into account this release, it was clear from our study with the parallel microscopic observation that PAF induced platelet adhesion to endothelial cells when PMNs were present.

The platelet adhesion to endothelial cells in the presence of PMNs occurred very rapidly, i.e. within minutes after PAF stimulation, and was dependent on the PAF concentrations and the PMN number coincubated with platelets and endothelial cells. Lyso-PAF, the biologically inactive metabolite of PAF, had no effect. Three structurally unrelated PAF antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987), ONO 6240 (Miyamoto *et al.*, 1985) and BN 52021 (Braquet *et al.*, 1985), dose-dependently suppressed the platelet adhesion. PAF also activated PMNs, resulting in the stimulation of PMN adhesion to endothelial cell monolayer. These results suggest that the platelet adhesion is mediated by activation of PAF specific membrane receptors, most probably on PMNs. However, PAF is an activator not only of platelets but also of endothelial cells (Bussolino *et al.*, 1987; Hirafuji *et al.*, 1988) so a possibility remains that the simultaneous PAF activation of platelets and/or endothelial cells as well as PMNs is a prerequisite to the platelet adhesion.

Although several mechanisms for the PMN-dependent platelet adhesion to endothelial cells induced by PAF can be postulated, microscopic examination suggests that some factor(s) released from PAF-stimulated PMNs mediates the adhesion by acting on platelets or/and endothelial cells. During direct interaction of platelets and PMNs, activated PMNs are known to produce and release a number of soluble factors that modulate platelet activity; for example, oxygen-derived free radicals (Levine *et al.*, 1976; Clark & Klebanoff, 1980), proteases (Chignard *et al.*, 1986; Del Maschio *et al.*, 1990), arachidonic acid metabolites (Coëffier *et al.*, 1987), or PAF (Benveniste *et al.*, 1972; Coëffier *et al.*, 1987). Most of these factors are also modulators of endothelial cell function (Harlan, 1987). One of the most likely factors would be superoxide anions derived from PMNs, since Salvemini *et al.* (1989) have shown that oxygen radicals enhance platelet adhesion to gelatin-coated plastic and aggregation induced by thrombin, in a process that is inhibited by superoxide dismutase (SOD). However, preliminary experiments have shown that SOD at a high concentration only slightly inhibited the platelet adhesion to endothelial cells (Hirafuji & Shinoda, 1990), suggesting the involvement of other mechanisms. Further experiments are now being undertaken to clarify the mechanism(s) underlying the PMN-dependent platelet adhesion observed in the present study.

The present study also indicated that PAF increased PMN adhesion to endothelial cells, which was augmented *vice versa* by the presence of platelets. This result is in agreement with that of Boogaerts *et al.* (1982), who have shown that platelets release products which enhance granulocyte-endothelial cell adherence. Such an augmentation by direct interaction of PMNs with platelets has also been reported to occur in a nylon-fibre assay of PMN adherence (Rasp *et al.*, 1981).

Thus, the present study demonstrates that PAF induces platelet adhesion to endothelial cells *in vitro* when PMNs are present. As well, a close interaction between platelets and PMNs in the adhesion process to endothelial cells is demonstrated. Our results further support the central role of PMNs in platelet adhesion to the vascular endothelium under certain pathological conditions. In this respect it is noteworthy that the intravenous injection of inflammatory stimuli such as FMLP to the rabbit, triggers leukocyte and platelet deposition at the inflammatory site, which is prevented by leukocyte depletion (Issekutz *et al.*, 1983). The interaction of platelets and PMNs demonstrated in the present study may have a significant relevance to the process of vascular diseases, since the adhesion of these cells to the endothelium is considered to be one of the initial steps in the development of thrombosis, atherosclerosis, or inflammation.

The authors are grateful to Dr T. Adachi and the staff at the Adachi Maternity Hospital for kindly providing the umbilical cords, and to Dr J.M. Mencia-Huerta, Institut Henri Beaufour, France; Dr H. Heuer, Boehringer-Ingelheim KG, FRG; Ono Pharmaceutical Indust., Ltd., Osaka, Japan, for the generous gift of PAF antagonists. This work was supported input by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

BENVENISTE, J., HENSON, P.M. & COCHRANE, C.G. (1972). Leukocyte-dependent histamine release from rabbit platelets: the role of IgE, basophils and a platelet-activating factor. *J. Exp. Med.*, **136**, 1356–1377.

BOOGAERTS, M.A., YAMADA, O., JACOB, H.S. & MOLDOW, C.F. (1982). Enhancement of granulocyte-endothelial cell adherence and granulocyte-induced cytotoxicity by platelet release products. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 7019–7023.

BOURGAIN, R.H., VERMARIËN, H., ANDRIES, R., VEREECKE, F., JACQUELOOT, J., RENNIES, J., BLOCKEEL, E. & SIX, F. (1984). A standardized *in vitro* method for the study of experimental arterial thombosis. Description of a method. *Adv. Exp. Med. Biol.*, **180**, 635–649.

BRAQUET, P., SPINNEWYN, B., BRAQUET, M., BOURGAIN, R.H., TAYLOR, J.E., ETIENNE, A. & DRIEU, K. (1985). BN 52021 and related compounds: a new series of highly specific PAF-acether receptor antagonists isolated from *Ginkgo biloba*. *Blood Vessels*, **16**, 559–572.

BREVIARIO, F., BERTOCCHI, F., DEJANA, E. & BUSSOLINO, F. (1988). IL-1-induced adhesion of polymorphonuclear leukocytes to cultured human endothelial cells. Role of platelet-activating factor. *J. Immunol.*, **141**, 3391–3397.

BUCHANAN, M.R., HAAS, T.A., LAGARDE, M. & GUICHARDANT, M. (1985). 13-Hydroxyoctadecadienoic acid is the vessel wall chemorepellant factor, LOX. *J. Biol. Chem.*, **260**, 16056–16059.

BUSSOLINO, F., CAMUSSI, G., AGLIETTA, M., BRAQUET, P., BOSIA, A., PESCARMONA, G., SANAVIO, F., D'URSO, N. & MARCHISIO, P.C. (1987). Human endothelial cells are target for platelet-activating factor. I. Platelet-activating factor induces changes in cytoskeleton structures. *J. Immunol.*, **139**, 2439–2446.

CASALS-STENZEL, J., MUACEVIC, G. & WEBER, K.-H. (1987). Pharmacological actions of WEB 2086, a new specific antagonist of platelet activating factor. *J. Pharmacol. Exp. Ther.*, **241**, 974–981.

CHIGNARD, M., SELAK, M.A. & SMITH, J.B. (1986). Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 8609–8613.

CLARK, R.A. & KLEBANOFF, S.J. (1980). Neutrophil-platelet interaction mediated by myeloperoxidase and hydrogen peroxide. *J. Immunol.*, **124**, 399–405.

COËFFIER, E., JOSEPH, D., PRÉVOST, M.-C. & VARGAFTIG, B.B. (1987). Platelet-leukocyte interaction: activation of rabbit platelets by FMLP-stimulated neutrophils. *Br. J. Pharmacol.*, **92**, 393–406.

COËFFIER, E., DELAUTIER, D., LE COUEDIC, J.-P., CHIGNARD, M., DENIZOT, Y. & BENVENISTE, J. (1990). Cooperation between platelets and neutrophils for paf-acether (platelet-activating factor) formation. *J. Leukocyte Biol.*, **47**, 234–243.

CURWEN, K.D., KIM, H.-Y., VAZQUEZ, M., HANDIN, R.I. & GIMBRONE, M.A. JR. (1982). Platelet adhesion to cultured vascular endothelial cells. A quantitative monolayer adhesion assay. *J. Lab. Clin. Med.*, **100**, 425–436.

DEL MASCHIO, A., EVANGELISTA, V., RAJTAR, G., CHEN, Z.M., CERLETTI, C. & DE GAETANO, G. (1990). Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am. J. Physiol.*, **258**, H870–H879.

GIMBRONE, M.A. JR. & BUCHANAN, M.R. (1982). Interactions of platelets and leukocytes with vascular endothelium: *in vitro* studies. *Ann. N. Y. Acad. Sci.*, **401**, 171–183.

HARLAN, J.M. (1987). Consequence of leukocyte-vessel wall interactions in inflammatory and immune reactions. *Semin. Thromb. Hemost.*, **13**, 434–444.

HEATON, W.A., HEYNS, A. DU P. & JOIST, J.H. (1989). Measurement of *in vivo* platelet turnover and organ distribution using ^{111}In-labeled platelets. In *Methods in Enzymology*, Vol. 169. ed. Hawiger, J. pp. 172–185. San Diego: Academic Press.

HENRY, R.L. (1965). Leukocytes and thrombosis. *Thromb. Diath. Haemorrh.*, **13**, 35–46.

HIGGS, E.A., MONCADA, S., VANE, J.R., CAEN, J.P., MICHEL, H. & TOBELEM, G. (1978). Effect of prostacyclin (PGI_2) on platelet adhesion to rabbit arterial endothelium. *Prostaglandins*, **16**, 17–22.

HIRAFUJI, M., MAEYAMA, K., WATANABE, T. & OGURA, Y. (1988). Transient increase of cytosolic free calcium in cultured human vascular endothelial cells by platelet-activating factor. *Biochem. Biophys. Res. Commun.*, **154**, 910–917.

HIRAFUJI, M., MENCIA-HUERTA, J.M. & BENVENISTE, J. (1987). Regulation of PAF-acether (platelet-activating factor) biosynthesis in cultured human vascular endothelial cells stimulated with thrombin. *Biochim. Biophys. Acta*, **930**, 359–369.

HIRAFUJI, M. & SHINODA, H. (1990). Effects of platelet-activating factor on platelet and neutrophil adhesion to vascular endothelial cells *in vitro*. *Jpn. J. Pharmacol.*, **52**(Suppl.), 204P.

ISSEKUTZ, A.C., RIPLEY, M. & JACKSON, J.R. (1983). Role of neutrophils in the deposition of platelets during acute inflammation. *Lab. Invest.*, **49**, 716–724.

LEVINE, P.H., WEINGER, R.S., SIMON, J., SCOON, K.L. & KRINSKY, N.I. (1976). Leukocyte-platelet interaction. Release of hydrogen peroxide by granulocytes as a modulator of platelet reactions. *J. Clin. Invest.*, **57**, 955–963.

McGREGOR, D.H., DAVIS, J.W., LIU, P.I., GATES, E. & POINDEXTER, A.R. (1980). Platelet satellitism. Experimental studies. *Lab. Invest.*, **42**, 343–355.

MIYAMOTO, T., OHNO, H., YANO, T., OKADA, T., HAMANAKA, N. & KAWASAKI, A. (1985). ONO-6240: a new potent antagonist of platelet-activating factor. In *Advances in Prostaglandin, Thromboxane, and Leukotriene Research*, Vol. 15. ed. Hayaishi, O. & Yamamoto, S. pp. 719–720. New York: Raven Press.

ODA, M., SATOUCHI, K., YASUNAGA, K. & SAITO, K. (1986). Polymorphonuclear leukocyte-platelet interactions: acetylglyceryl ether phosphocholine-induced platelet activation under stimulation with chemotactic peptide. *J. Biochem.*, **100**, 1117–1123.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet*, **ii**, 1057–1058.

RASP, F.L., CLAWSON, C.C. & REOINE, J.E. (1981). Platelets increase neutrophil adherence in vitro to nylon fiber. *J. Lab. Clin. Med.*, **97**, 812–819.

SALVEMINI, D., DE NUCCI, G., SNEDDON, J.M. & VANE, J. R. (1989). Superoxide anions enhance platelet adhesion and aggregation. *Br. J. Pharmacol.*, **97**, 1145–1150.

SHAW, J.O. & HENSON, P.M. (1980). The binding of rabbit basophil-derived platelet activating factor to rabbit platelets. *Am. J. Pathol.*, **98**, 791–810.

SNEDDON, J.M. & VANE, J.R. (1988). Endothelium-derived relaxing factor reduces platelet adhesion to bovine endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2800–2804.

SNYDER, F. (1985). Chemical and biochemical aspects of platelet activating factor: a novel class of acetylated ether-linked chlolinephospholipids. *Med. Res. Rev.*, **5**, 107–140.

VARGAFTIG, B.B., PRETOLANI, M., COËFFIER, E. & CHIGNARD, M. (1989). Platelet activating factor: biology, receptors and antagonists. In *Mediators of the Inflammatory Process*, Handbook of Inflammation, Vol. 6. ed. Henson, P.M. & Murphy, R.C. pp. 113–146. Amsterdam: Elsevier.

ZIMMERMAN, G.A., McINTYRE, T.M. & PRESCOTT, S.M. (1985). Thrombin stimulates the adherence of neutrophils to human endothelial cells in vitro. *J. Clin. Invest.*, **76**, 2235–2246.

(Received August 3, 1990
Revised December 28, 1990
Accepted January 23, 1991)