BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 101–105 (1997)
ARTICLE NO. RC977435

The Release Mechanism of Platelet-Activating Factor during Shear-Stress Induced Platelet Aggregation

Shin-ichi Iwamoto, Tomio Kawasaki,[1] Jun-ichi Kambayashi,* Hideo Ariyoshi, Nobutoshi Shinoki, Masato Sakon, Yasuo Ikeda,† and Morito Monden

*Department of Surgery II, Osaka University Medical School, Osaka, Japan; *Otsuka America Pharmaceutical Inc., Maryland Office of Clinical Research, Rockville, Maryland; and †Department of Internal Medicine, Keio University, Tokyo, Japan*

Received August 25, 1997

We previously reported that 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet-activating factor, PAF), released from activated platelets stimulated with thrombin plus collagen, is associated with platelet microparticles. In the present study, we found that PAF is concentrated and associated with microparticles released from high shear stress-induced activated platelets. The total amount of PAF released from 3×10^8 platelets under high shear stress (108 dyne/cm^2) was $3.2\pm0.6\times10^{-15}$mol (n=5, mean± S.D.). Eighty percent of the PAF released from the platelets was recovered in the microparticle fraction after ultracentrifugation in the presence of albumin. Under high shear stress, PAF was not released from platelets within 3 minutes, although microparticles were released. In conclusion, microparticles released from activated platelets in the rheological condition of high shear stress are major carriers of PAF.

Shear-induced platelet aggregation (SIPA) is an important mechanism in thrombogenesis(1). The mechanism of SIPA has been studied by several investigators(2-5). Under low shear stress (12 dyne/cm^2), platelet aggregation can be induced by the binding of fibrinogen and glycoprotein (GP) IIb/IIIa complex. In contrast, aggregation induced under high shear stress (108 dyne/cm^2) is mediated by von Willebrand factor (vWF), by interacting with its platelet-binding sites of GP Ib followed by the exposure of fibrinogen binding site of GP IIb/IIIa(2-5). The latter mechanism is particularly important because it may occur in the stenosed coronary artery. Several agonists such as calcium ionophore (A23187) or thrombin plus collagen induce platelet aggregations and increase the synthesis of platelet-activating factor (PAF)(6,7), which is a potent phospholipid mediator possessing numerous biological activities(8). We recently reported the mechanism of the release of PAF from platelets and clarified that PAF is released from activated platelets associated with microparticles(MPs)(9), which also possess clot-promoting activity(2). In the present study, we investigated the mechanism of the release of PAF following the shear stress-dependent activation of platelets. The results may be clinically relevant because PAF associated with MPs released from platelets may play an important role in the pathogenesis of thrombosis in the rheological condition of high shear stress, which may occur in partially occluded arteries or arterioles.

[1] To whom correspondence should be addressed at Department of Surgery II, Osaka University Medical School, 2-2, Yamadaoka, Suita-city, Osaka,565, Japan. Fax: (81)6-879-3259.

METHODS

Materials were obtained from the following suppliers: Prostaglandin I_2 was a gift from Ono Pharmaceutical Co., Osaka, Japan. Fluorescen 5-isothiocyanate (FITC)-labeled goat anti-mouse IgG was obtained from Organon Teknika Co., Durham, NC. Murine monoclonal antibody NNKY1-32, specific for the glycoprotein IIb/IIIa complex on both resting and activated platelets, was kindly provided by Dr. Shousaku Nomura of Kansai Medical University, Osaka, Japan.

Von Willebrand factor (vWF), fibrinogen and bovine serum albumin(BSA) were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of platelets. Fresh human blood was collected into a plastic syringe containing a one-tenth volume of 3.8% trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the citrated blood at 130×g for 15 min. All the preparations were done at room temperature. Prostaglandin I_2 was added to the PRP at the final concentration of 5ng/ml for the purpose of cytoprotection, and PRP was centrifuged at 650×g for 13min. The platelet pellet was resuspended in a HEPES buffer (142mM NaCl, 6.2mM KCl, 2.4mM $MgSO_4$, 6.5mM HEPES, 5mM glucose, pH7.4). The same washing procedure was repeated once more and resuspended in the HEPES buffer without prostaglandin I_2. The final platelet count was adjusted to be 3×10^8/ml. The suspension thus obtained was found to be free of any contaminants by a microscopic examination.

Measurement of shear stress-induced platelet aggregation. The method used to measure SIPA with a cone-plate viscometer was

0006-291X/97 $25.00

described previously(10). Briefly, the cone is rotated with a rotor motor with a diameter of 4 centimeters regulated by a computer. With this instrument, the cone could be rotated at a maximum rotation speed of 2,000 rpm to generate a constant shear stress without turbulent flow. Helium-neon laser light at 633nm was passed through the streaming samples, and the transmitted light intensity was continuously recorded. The percent platelet aggregation was calculated according to Lambert-Beer's equation. To measure the SIPA, 400 μl of washed human platelets was applied to the plate with fibrinogen (final concentration (fc), 100 μg/ml), vWF(fc. 1 μug/ml) and $CaCl_2$ (fc. 1mM) and exposed to shear stress at 25°C. The rotation rate of the cone can be set at low (200rpm=12dyne/cm^2), middle (1000rpm= 60 dyne/cm2) and high speeds (1800rpm=108 dyne/cm^2).

Preparation of MPs. The samples were exposed to shear stress for 3 or 6 minutes. The reaction was terminated by the addition of EGTA(fc. 5mM) and cooling on an ice bath. The preparation of MPs was done according to the modified method previously reported by us(9). Briefly, the mixture was centrifuged at 14,000×g for 1 min in order to precipitate platelets, and the supernatant was further centrifuged at 100,000×g for 150 min to sediment the MPs. At second centrifugation, the samples were washed with 0.1% BSA to remove any nonspecific binding of PAF to MPs.

Flow cytometry. In order to distinguish the MPs and platelets from the background noise, the MPs and platelets were immunocytochemically stained according to the method reported recently(9,11). Aliquots of platelets and/or MPs(100 μl) were fixed with NNKY-32 (anti-glycoprotein IIb/IIIa complex antibody) for 30 min at room temperature and then with a saturated concentration of FITC-labeled goat anti-mouse antibody for 30 min at room temperature. They were then diluted with 1ml of phosphate-buffered saline (pH7.5), and the fluorescence levels of 10,000 platelets were measured with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). The FACScan flow cytometer was used with its standard band pass filter for FITC fluorescence at 530/30 nm. Data were obtained with FACScan Research software. The samples were treated with FITC fluorescence signals to exclude background scatter. The population solely positive for glycoprotein IIb/IIIa complex was then gated to distinguish the MPs and platelets from electric noise. Before being analyzed by the FACScan, a known amount of fluorescent beads were added to the diluted samples. The absolute amount of MPs were determined by comparing with known amount of fluorescent-control beads. The accuracy of this assay method was assessed by Yano et al(11). Each of the MP/platelet fractions was gated for flowcytometric analysis and the relative volume ratio of MPs/ platelets was recalculated from the mean size obtained from a forward side scatter(FSC) analysis.

Lipid extraction and bioassay. Phospholipids were extracted according to the method of Bligh and Dyer's, and the samples were kept at −20°C until use. The preparation of washed rabbit platelets collected from a New Zealand white rabbit was performed by the same procedure used for the preparation of washed human platelets. The final platelet count was adjusted to 3×10^5/ul in HEPES-buffered saline. The platelet aggregating activity was detected by its ability to aggregate washed rabbit platelets in an aggregometer. Fibrinogen and epinephrine were added to the washed rabbit platelet suspension in order to potentiate the platelet aggregation by PAF(12). The dried residue of the sample was reconstituted in a 0.1% bovine serum albumin-saline solution. The measurement of PAF was determined by comparing the activity of each sample to that of known amounts of standard synthetic 1-O-hexadecyl-2-acetyl-sn-3-phosphocholine. The following criteria were used for the identification of the platelet aggregation activity as PAF: the ability to induce platelet aggregation in the presence of aspirin (1mM)(an inhibitor of cyclooxygenase), and the inhibition of the aggregation by a selective concentration of E5880(50nM), a specific PAF receptor antagonist donated from Eisai Pharmaceutical CO., Tokyo, Japan.

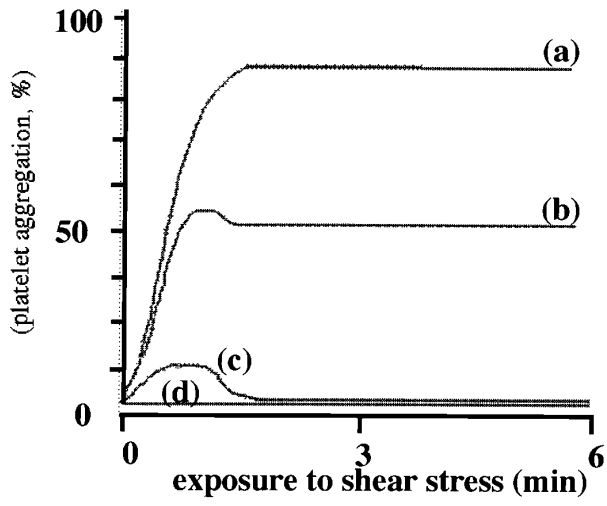

FIG. 1. Measurement of platelet aggregation. Tracing of platelet aggregation patterns obtained at various shear stresses. (a) high shear stress [=108 dyne/cm^2, maximum percentage of aggregation of 87+/−5.3 (mean+/−S.D, n=5)]. (b) Middle shear stress [=60 dyne/cm^2, maximum percentage of aggregation of 47+/−8.3 (mean+/−S.D, n=5)]. (c) Low shear stress [=12 dyne/cm^2, maximum percentage of aggregation of 9+/−2.3 (mean+/−S.D, n=5)]. (d) High shear stress in the absence of fibrinogen and vWF (=108 dyne/cm^2, n=5).

RESULTS

Platelet aggregation induced by shear-force. The trace of shear-induced platelet aggregation is shown in Fig. 1. In the presence of vWF and fibrinogen, washed platelets were aggregated within 1 minute at the designated shear stress in a shear force-dependent manner. However, no significant platelet aggregation was observed even at a high shear value without vWF and fibrinogen. A small aggregation was observed under low shear stress and was disaggregated within 3 minutes.

Characterization of MPs. Figure 2 shows the scattering patterns of platelets and MPs detected with the FITC-labeled anti-glycoprotein IIb/IIIa complex antibody (NNKY 1-32) and a known concentration of FITC-labeled control beads. Only the populations that were positive for glycoprotein IIb/IIIa complex were gated to distinguish the beads and MPs from electric noise. Only a small percentage of MPs was detected in the washed human platelets at rest (Fig. 2-a). MPs were released from platelets in a time- and shear force-dependent manner. After platelets were exposed to shear stress for either 3 or 6 minutes and centrifuged at 14,000×g for 1 minute, the supernatant was analyzed in a FACScan(Fig. 2-b-e). The pellet obtained by centrifugation of the reaction mixture at 100,000×g for 150 minutes, yielded an almost identical distribution of MPs as that obtained in the flowcytometric analysis, and the supernatant was found to not contain any MPs (data not shown).

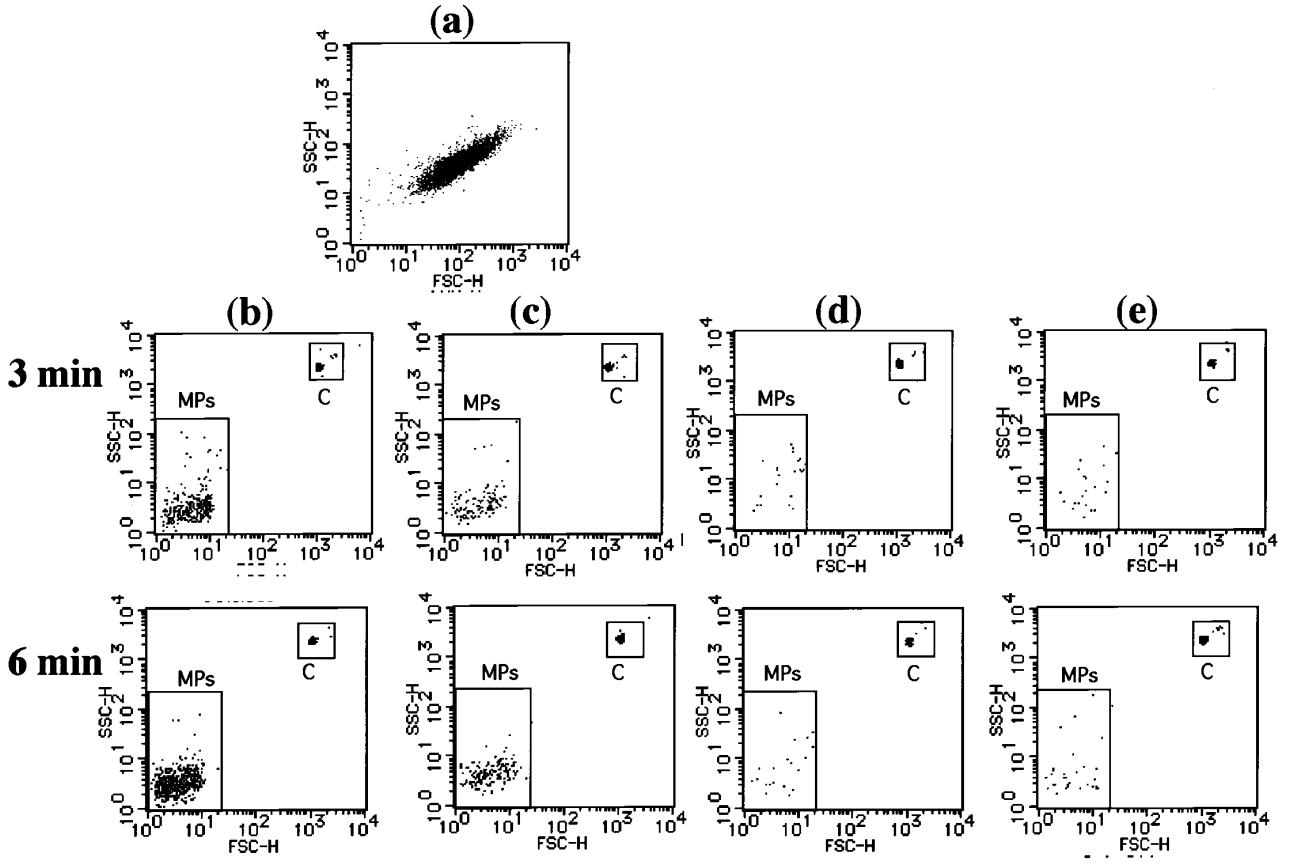

FIG. 2. Characterization and quantitative determination of platelet MPs. Scattering pattern of platelets and MPs detected by flow cytometry with FITC-labeled anti-glycoprotein IIb/IIIa complex antibody (NNKY1-32) and FITC-labeled control beads. "MPs" indicates a gate for MPs and "C" indicates a gate for control beads. (a) Washed human platelet at rest. (b) MP fraction obtained from platelets exposed to high shear stress (108 dyne/cm^2). (c) MP fraction obtained from platelets exposed to middle shear stress (60 dyne/cm^2). (d) MP fraction obtained from platelets exposed to low shear stress (12 dyne/cm^2). (e) MP fraction obtained from platelets exposed to high shear stress in the absence of fibrinogen and vWF.

Identification and distribution of PAF. In order to ascertain the chemical identity of the platelet aggregating activity of the lipid extracted from platelets and MPs, the activity was compared with a synthetic PAF of 1-O-hexadecyl-2-acetyl-3-phosphocholine. The lipid extracted from platelets and MPs aggregated rabbit platelets in the presence of 1mM aspirin (an inhibitor of cyclooxygenase), and did not aggregate platelets in the presence of 50nM E5880 (a specific PAF receptor antagonist). As shown in Table 1(A), the total amount of PAF produced by 3.0×10^8 platelets in 6 minutes of high shear stress was $3.7\pm0.5\times10^{-15}$mol. (n=5, mean± S.D.) About 0.5×10^{-15}mol of PAF was released into the medium, and $0.4\pm 0.3\times10^{-15}$mol of PAF was co-precipitated with MPs after ultracentrifugation. Only $0.1\pm0.05\times10^{-15}$mol of PAF was identified in the suspension medium. Most of the PAF activity associated with the MPs was not recovered in the suspension after ultracentrifugation in the presence of 0.1% BSA-saline solution. In 3 minutes with high shear stress, PAF was identified only in the platelet fraction(Table 1(B)). The six-minutes exposure at low shear stress, and high shear stress without fibrinogen and vWF failed to synthesize PAF in any platelet fraction. With 6 minutes of middle shear stress, 1.8+/−0.3 mol of PAF was detected in the platelet fraction, and PAF was not identified in the MP fraction.

Quantitative identification of MPs and PAF released from platelets. The absolute levels of MPs and PAF released from platelets are shown in Fig. 3. The platelet MP levels are expressed by the MP/ control beads ratio. The MPs produced under high shear stress increased in a time- dependent manner and displayed linearity(0.85± 0.28 MPs/control beads in 3 minutes, 1.68±0.15 MPs/control beads in 6 minutes). Six minutes of high shear stress without fibrinogen and vWF showed no increase of MPs (0.06± 0.02 MPs/control beads in 3 minutes, 0.08±0.03 MPs/control beads in 6 minutes). An exposure to low shear stress resulted in no increase of MPs (0.04±0.02 MPs/control beads in 3 minutes, 0.05±0.01 MPs/control beads in 6 minutes),

TABLE 1

Identification and Distribution of PAF

Fractions	108 dyne/cm^2	60 dyne/cm^2	12 dyne/cm^2
	(A) 3-minute exposure to shear stress		
Platelets	$2.4 \pm 0.3 \times 10^{-15}$ [a]	ND	ND
MPs	ND	ND	ND
Medium	ND	ND	ND
Total	$2.4 \pm 0.3 \times 10^{-15}$	ND	ND
	(B) 6-minute exposure to shear stress		
Platelets	$3.2 \pm 0.6 \times 10^{-15}$ [a]	$1.8 \pm 0.3 \times 10^{-15}$ [a]	ND
MPs	$0.4 \pm 0.3 \times 10^{-15}$ [a]	ND	ND
Medium	$0.1 \pm 0.05 \times 10^{-15}$ [a]	ND	ND
Total	$3.7 \pm 0.5 \times 10^{-15}$	$1.8 \pm 0.3 \times 10^{-15}$	ND

Note. Release of PAF (mol) from human platelets (3×10^8/ml) after 3 and 6 minutes exposure to shear stress. PAF was produced under a 6-minute exposure to high shear stress, and eighty-percent was retained in the platelets. ND, not detected.

[a] n = 5, mean ± S.D.

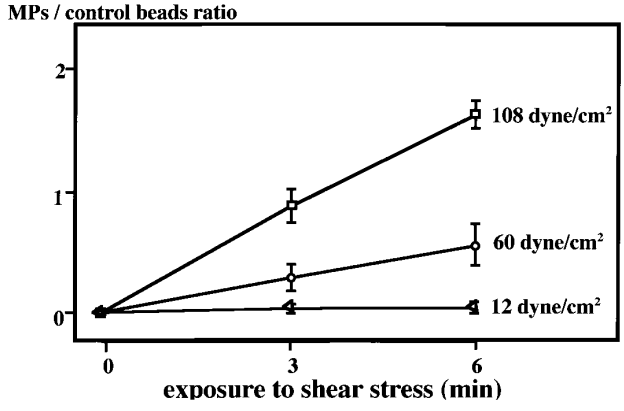

FIG. 3. Quantitative identification of MPs and PAF released from platelets. The amount of platelet MPs is expressed by the microparticles (MPs)/control beads ratio. (□) MPs released after exposure to high shear stress (108 dyne/cm^2). (○) MPs released after exposure to middle shear stress (60 dyne/cm^2). (△) MPs released after exposure to low shear stress (12 dyne/cm^2). Results are expressed as means±S.D.(n=5) ■ indicates PAF associated with MPs after exposure to high shear stress. The 6-minutes exposure of platelets to high shear stress resulted in the release of PAF from platelets on the surface of MPs. However, PAF was not released within 3 minutes under the high shear stress, although about half of the volume of MPs was released and intercellular PAF was synthesized.

and a small amount of MPs is released under middle shear stress (0.34± 0.1 MPs/control beads in 3 minutes, 0.55±0.18 MPs/control beads in 6 minutes). Although MPs were released after the 3-minute exposure to high shear stress, PAF was not detected in MPs from platelets or in the medium fraction, but was detected in platelets.

Relative volume ratio of MP/platelet. To investigate the relative volume ratio of MP and platelet, each fraction of MPs and platelets was gated and the average sizes of the MPs and platelets were calculated using FACScan Research software (Fig. 4). The FSC analysis, revealed the mean sizes of MPs/platelets were 5.03/119.96(=0.0419). The relative platelet/MP volume ratio is thus calculated to be 116.47.

DISCUSSION

PAF is a lipid mediator released from activated cells including platelets when stimulated by various kinds of agonists(13-15), and about 80% of produced PAF is known to be retained in cells(16,17). We previously reported that PAF is released from activated platelets by thrombin plus collagen in the firmly bound form to MPs, and that was susceptible to plasma acetylhydrolase(9). Compared to the agonists such as thrombin or collagen, shear stress is relatively slow and more physiological stimuli. The system described in the present study will be a better model to investigate the release mechanism of PAF from platelets. In the present experiment, the six-minute exposure to high shear stress resulted in the release of PAF in the binding form to MPs from which PAF could not be removed by the washing procedure in the presence of albumin. The high shear stress used may be attained in stenosed or partially occluded arterial vessels.

Bratton et al.(18) reported that the release of PAF from the cell in which it is synthesized would depend first on the localization to the plasma membrane followed by the transbilayer movement accompanying the

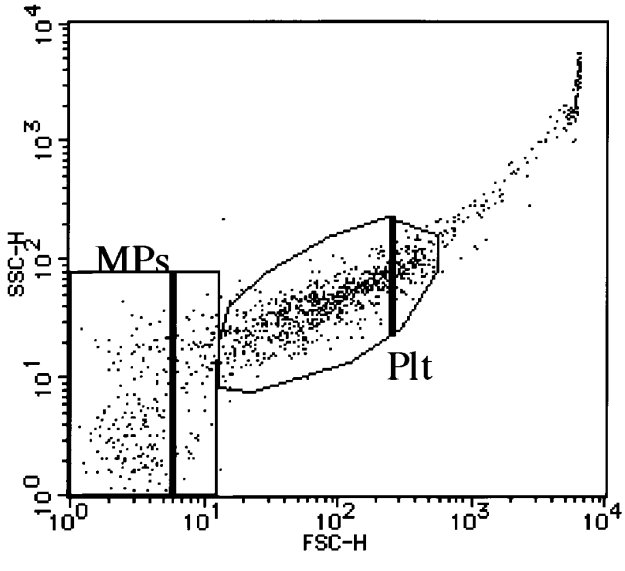

FIG. 4. Calculation of average size of MP/platelet fraction. Each fraction of MP/platelet was gated, and the mean sizes of MPs and platelets were calculated using FACScan Research software. In the FSC analysis, the mean size of MPs/platelets was 5.03/119.96 (=0.042). Bold line (—) indicates the average sizes of MPs/platelets.

loss of membrane asymmetry. They suggested the existence of "acceptor molecules" which remove PAF from the plasma membrane. We demonstrated that the net release of PAF from activated platelets mainly bound to MPs which acted as if they were an "acceptor molecules". In our data, the production of MPs from platelets after the 6-minute exposure to high shear stress was 0.4±0.1 MP/platelet(data not shown). The volume ratio of a MP to a platelet was approximately 1/116.47, calculated from the FSC analysis. Since the volume ratio of MPs and platelets was too small considering the PAF distribution produced between the platelets and MPs (9:1), PAF may be 30-fold concentrated on MPs upon activation.

Interestingly, PAF was not released under the 3-minute exposure to high shear stress, although about half of the volume of MPs was released and intercellular PAF was produced. We hypothesize that the intracellular PAF was produced in the 3-minute period of high shear stress, but did not reach the membrane releasing as PAF, and in the subsequent 3 minutes, the intracellular PAF was released associated with MPs. To corroborate the validity of this mechanism, the three-fold amount of MPs which was released in the 3-minute of high shear stress was provided to the PAF bioassay, only to fail to detect PAF. Furthermore, an additional 6-minute exposure to high shear stress of the separated microparticles released within a 3-minute exposure of platelets to high shear stress did not produce PAF(data not shown). This indicates that PAF is not produced in the released MPs.

Platelet MPs, composed of plasma membrane, are known to be rich in membrane glycoproteins Ib, IIb and IIIa(19,20) and to have procoagulant activity on their surface(21). An increased number of circulating microparticles has been found in various pathological conditions such as cardio-pulmonary bypass, acute respiratory distress syndrome and idiopathic thrombocytopenic purpura(22-24).

In conclusion, we observed the release of PAF under high shear stress, and suggest that the mechanism underlying this release contributes to the delivery system of PAF in the rheological condition of high shear stress comparable to occluded arteries or arterioles.

ACKNOWLEDGMENTS

This study was supported by grants from the Grant-in-Aid for Scientific Research 09671228, The Ministry of Education, Science, and Culture, Japan, and Heparin conference.

REFERENCES

1. Turitto, V. T. (1982) *Prog. Hemost. Thromb.* **6,** 139–177.
2. Ikeda, Y., Handa M., Kawano, K., Kamata T., Murata, M., Araki, Y., Anbo, H., Kawai, Y., Watanabe, K., Itagaki, I., Sakai, K., and Ruggeri, Z. M. (1991) *J. Clin. Invest.* **87,** 1234–1240.
3. Peterson, D. M., Stathopoulos, N. A., Giorgio, T. D., Hellums, J. D., and Moake, J. L. (1987) *Blood* **69,** 625–628.
4. Chow, T. W., Hellums, J. D., Moake, J. L., and Kroll, M. H. (1992) *Blood* **80,** 113–120.
5. Ikeda, Y., Handa, M., Kamata, T., Kawano, K., Kawai, Y., Watanabe, K., Kawakami, K., Sakai, K., Fukuyama, M., Itagaki, I., Yoshioka, A., and Ruggeri, Z. M. (1993) *Thromb. Haemost.* **69,** 496–502.
6. Alam, I., Smith, J. B., and Silver, M. J. (1983) *Thromb. Res.* **30,** 71–79.
7. Coeffier, E., Ninio, E., Le Couedic, J. P., and Chignard, M. (1986) *Br. J. Haematol.* **62,** 641–644.
8. Snyder, F. (1990) *Am. J. Physiol.* **259,** C697–C708.
9. Iwamoto, S., Kawasaki, T., Kambayashi, J., Ariyoshi, H., and Monden, M. (1996) *Biochem. Biophys. Res. Commun.* **218,** 940–944.
10. Fukuyama, M., Sakai, K., Itagaki, I., Kawano, K., Murata, M., Kawai, Y., Watanabe, K., Handa, M., and Ikeda, Y. (1989) *Thromb. Res.* **54,** 253–260.
11. Yano, Y., Kambayashi, J., Kawasaki, T., and Sakon, M. (1994) *Int. J. Cardiol.* **47,** s13–s19.
12. Shinozaki, K., Kawasaki, T., Kambayashi, J., Sakon, M., Shiba, E., Uemura, Y., Ou, M., Iwamoto, N., and Mori, T. (1994) *Life Sci.* **54,** 429–437.
13. Lynch, J. M., and Henson, P. M. (1986) *J. Biol. Chem* **137,** 2653–2661.
14. Ludwig, J. C., McManus, P. O., Clark, D. J., Hanahan, D. J., and Pinckard, R. N. (1984) *Arch. Biochem. Biophys.* **232,** 102–110.
15. Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3534–3538.
16. Lynch, J. M., and Henson, P. M. (1986) *J. Immunol.* **137,** 2653–2661.
17. Oda, M., Satouchi, K., Yasunaga, K., and Saito, K. (1985) *J. Immunol.* **134,** 1090–1093.
18. Bratton, D. L., Kailey, J. M., Clay, K. L., and Henson, P. M. (1991) *Biochem. Biophys. Acta.* **1062,** 024–34.
19. Sims, P. J., Faioni, E. M., Wiedmer, T., and Shattil, S. J. (1988) *J. Biol. Chem.* **263,** 18205–18212.
20. Fox, J. E., Austin, C. D., Reynolds, C. C., and Steffen, P. K. (1991) *J. Biol. Chem.* **266,** 13289–13295.
21. Wolf, P. (1967) *Br. J. Haematol.* **13,** 269–288.
22. George, J. N., Pickett, E. B., and Sauceman, S. (1986) *J. Clin. Invest.* **78,** 340–348.
23. Abrams, C. C., Ellison, M., Budzynski, A. Z., and Shattil, S. J. (1990) *Blood* **76,** 1–16.
24. Wenche, J. Y., Horstman, L. L., Arce, M., and Ahn, Y. S. (1992) *J. Lab. Clin. Med.* **119,** 334–345.