

# High Shear Stress Can Initiate Both Platelet Aggregation and Shedding of Procoagulant Containing Microparticles

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Previous studies have demonstrated that a high level of shear stress can produce platelet aggregation without the addition of any agonist. We investigated whether high shear stress could cause both platelet aggregation and shedding of microparticles from the platelet plasma membrane. A cone-plate viscometer was used to apply shear stress and microparticle formation was measured by flow cytometry. It was found that microparticle formation increased as the duration of shear stress increased. Both microparticles and the remnant platelets showed the exposure of procoagulant activity on their surfaces. Investigation of the mechanisms involved in shear-dependent microparticle generation showed that binding of von Willebrand factor (vWF) to platelet glycopro-

tein Ib, influx of extracellular calcium, and activation of platelet calpain were required to generate microparticles under high shear stress conditions. Activation of protein kinase C (PKC) promoted shear-dependent microparticle formation. Epinephrine did not influence microparticle formation, although it enhanced platelet aggregation by high shear stress. These findings suggest the possibility that local generation of microparticles in atherosclerotic arteries, the site that pathologically high shear stress could occur, may contribute to arterial thrombosis by providing and expanding a catalytic surface for the coagulation cascade.

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ONE OF THE responses of activated platelets to certain stimuli is the shedding of microparticles. Many studies have been attempted to characterize the role of microparticles under various clinical situations<sup>1-5</sup> or experimental conditions.<sup>6-15</sup> It has been established that there is a close relationship between platelet procoagulant activity, which involves the exposure of amino phospholipids on the platelet plasma membrane, and the shedding of procoagulant-containing microparticles by platelets. Therefore, it seems possible that generation of microparticles from platelets at sites of vascular injury may play an important role in the normal coagulation process. On the other hand, it is also possible that local generation of microparticles in atherosclerotic small arteries or arterioles may promote acute arterial occlusion by providing and expanding a catalytic surface for the coagulation cascade.

Pathological levels of fluid shear stress may occur in diseased small arteries and arterioles partially obstructed by atherosclerosis or vasospasm and such shear stress may induce the activation and aggregation of circulating platelets.<sup>16-21</sup> This type of platelet aggregation appears to make a crucial contribution to thrombogenesis in various pathological states.<sup>16,21,22</sup> Using a cone-plate viscometer to precisely apply shear forces, previous studies have partially unraveled

the process of high shear stress-induced platelet aggregation (SIPA).<sup>16-20</sup> The interaction of multimeric von Willebrand factor (vWF) with glycoprotein (GP) Ib, followed by the opening of calcium channels and the transmembrane influx of extracellular calcium ions is a prerequisite for subsequent binding of vWF to GP IIb/IIIa, which may be necessary to support shear-induced platelet aggregation. The shear-induced diacylglycerol-independent pathway of protein kinase C (PKC) activation may also make some contribution to platelet aggregation.<sup>23</sup> Furthermore, protein tyrosine phosphorylation is induced by high shear stress, but whether it participates in the process of shear-induced activation is not clear.<sup>24,25</sup>

These previous findings suggested that high shear stress in atherosclerotic arteries might induce the formation of procoagulant-containing platelet microparticles and thus contribute to the pathogenesis of arterial thrombosis. Accordingly, we investigated the shedding of microparticles from platelets in response to a constant high shear stress generated using a cone-plate viscometer, as well as the process of microparticle formation under such conditions.

## MATERIALS AND METHODS

**Materials.** Calpeptin<sup>26</sup> was the generous gift of Dr Jun-ichi Kamabayashi of Osaka University (Osaka, Japan). Purified annexin V was a kind gift from Dr Masahiro Maki of the Akita Red Cross Blood Center (Akita, Japan). 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), ethanol, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), staurosporine, fluorescein 5-isothiocyanate (FITC)-labeled goat antimouse IgG, 3,3'-diaminobenzidine tetrahydrochloride (DAB), and horseradish peroxidase conjugated goat antimouse IgG were purchased from Wako Chemicals (Tokyo, Japan). Thrombin, calcium ionophore A-23187, and apyrase were obtained from Sigma Chemical Co (St Louis, MO). EGTA was obtained from Dojindo Lab (Kumamoto, Japan), collagen from Horme (Munich, Germany), and the Arg-Gly-Asp-Ser tetrapeptide (RGDS) from the Peptide Institute (Osaka, Japan). Human vWF was purified as described previously.<sup>27,28</sup> A monoclonal antibody (MoAb) for actin-binding protein (ABP), PM6/317,<sup>29</sup> was purchased from Serotec Co (Oxford, England), while a murine MoAb directed against GP IX (KMP-9) was produced by immunizing BALB/c mice.<sup>30</sup> In a preliminary study, it was confirmed that KMP-9 did not influence agonist-induced platelet aggregation, that its binding to platelets was not im-

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paired by the prior binding of specific anti-GP Ib MoAbs, and that it showed competition for platelet binding with a specific MoAb to platelet GP IX (FMC-25; Serotec Co) (unpublished observations). FITC-labeled KMP-9 was prepared and used to detect platelets and platelet-derived microparticles. The following MoAbs were produced in our laboratory and have been reported previously. NMC-4<sup>30</sup> is an anti-vWF MoAb that inhibits ristocetin- or botrocetin-induced binding of vWF to platelet GP Ib by combining with the GP Ib-binding domain(s) of vWF. NNKY1-32<sup>14,31,32</sup> is a specific anti-GP IIb/IIIa MoAb that recognizes this complex on either resting or activated platelets and totally inhibits adenosine diphosphate (ADP)-induced platelet aggregation. NNKY5-5<sup>31,33,34</sup> is an anti-GP Ib MoAb that completely inhibits ristocetin-induced platelet aggregation.

**Preparation of platelets.** Blood was collected from healthy volunteers, who had not taken any drugs for 14 days, by venipuncture into tubes containing a 1:10 volume of 3.8% (wt/vol) trisodium citrate and gently mixed. Isolation of platelets was performed at room temperature. Platelet-rich plasma was prepared by centrifugation of whole blood at 200g for 10 minutes and was acidified to pH 6.5 with acid citrate dextrose (ACD). Platelets were separated from the platelet-rich plasma by centrifugation at 800g for 10 minutes in the presence of 1 U/mL apyrase and 1  $\mu$ mol/L PGE<sub>1</sub> and were washed twice in a platelet-washing buffer (113 mmol/L NaCl, 4.3 mmol/L K<sub>2</sub>HPO<sub>4</sub>, 4.2 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 24.4 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, and 5.5 mmol/L glucose, pH 6.5) that also contained 1 U/mL apyrase and 1  $\mu$ mol/L PGE<sub>1</sub>. The platelet pellets were resuspended in HEPES-Tyrode's buffer (138 mmol/L NaCl, 2.8 mmol/L KCl, 2 mmol/L CaCl<sub>2</sub>, 0.5 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 12 mmol/L NaHCO<sub>3</sub>, 10 mmol/L glucose, and 10 mmol/L HEPES, pH 7.4) at a concentration of  $3 \times 10^8$  mL and used within 1 hour. Before applying shear stress, vWF was added to the platelet suspensions at a final concentration of 10  $\mu$ g/mL. In some experiments, washed platelets were incubated for 5 minutes with MoAbs, calpeptin, staurosporine, H-7, PGE<sub>1</sub>, apyrase, or RGDS before applying shear stress. Epinephrine was added just before the application of shear stress. Staurosporine and calpeptin were prepared as stock solutions in DMSO, with the solvent concentration never exceeding 0.1% (vol/vol). PGE<sub>1</sub> was maintained as a stock solution in ethanol and diluted in HEPES-Tyrode's buffer shortly before use so that the final ethanol concentration was under 0.01% (vol/vol).

**Measurement of SIPA.** A modified cone-and-plate type viscometer was used for the measurement of SIPA as described in detail previously.<sup>16,17,35</sup> In brief, washed platelets (400  $\mu$ L) were added to the viscometer and exposed to shear stress at room temperature in the presence of exogenous calcium (2 mmol/L) and vWF (10  $\mu$ g/mL). The rotation rate of the cone was 100 rpm for the first 15 seconds and was then increased to either 1,800 rpm (high shear stress: 108 dynes/cm<sup>2</sup>) or 200 rpm (low shear stress: 12 dynes/cm<sup>2</sup>) over the next 10 seconds, after which a constant shear stress was applied for 5 minutes (Fig 1).

**Assessment of microparticle formation.** Microparticle formation was determined as the number of microparticles per 10,000 intact platelets using flow cytometry, as described below. Ten microliters of stimulated or unstimulated platelet suspension ( $3 \times 10^8$  mL) was added to 100  $\mu$ L of HEPES-Tyrode's buffer containing 5 mmol/L EGTA, and both intact and aggregated platelets were removed by centrifugation at 1,000g for 15 minutes to yield a supernatant containing microparticles only. Next 10  $\mu$ L of washed intact platelets ( $3 \times 10^8$  mL) was added to the supernatant and incubation with KMP-9 (the FITC-labeled antiplatelet GP IX MoAb) was performed for 30 minutes in the dark at room temperature. After incubation, samples were diluted 1:10 with HEPES-Tyrode's buffer containing 5 mmol/L EGTA and analyzed using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA).<sup>14,31,33</sup> The FACScan was

used in the standard configuration with a 15 mW, 488 nm air-cooled argon laser, and the standard band-pass filter for FITC fluorescence (530/30 nm). Data were obtained with FACScan Research software. Washed platelets incubated with FITC-labeled goat antimouse IgG were run first to set the threshold of FITC fluorescence so as to exclude background scatter. Only the cells and particles positive for GP IX were gated to distinguish platelets and microparticles from electronic noise. To differentiate between platelets and microparticles, the lower limit of the platelet gate was set at the left-hand border of the forward scatter profile of resting platelets. Ten thousand FITC-positive platelets were counted first, and the FITC-positive particles in the microparticle gate were then counted to determine the number of microparticles released per 10,000 platelets.

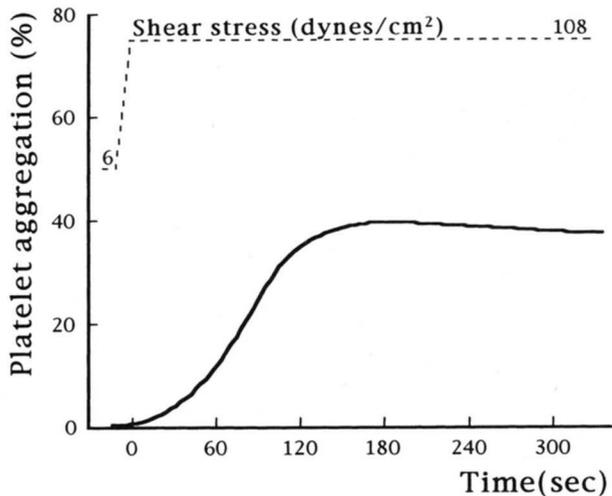
**Assessment of microparticle formation in whole blood.** To study the release of microparticles from platelets in whole blood, part of the whole blood collected for preparation of washed platelets was directly added to the viscometer and exposed to high shear stress as described in "Measurement of SIPA." Immediately after exposure to high shear stress for 5 minutes, 10  $\mu$ L of stimulated whole blood was added to 100  $\mu$ L of HEPES-Tyrode's buffer containing 5 mmol/L EGTA, and cells other than microparticles were removed by centrifugation at 1,000g for 15 minutes. Then 10  $\mu$ L of washed intact platelets (the platelet count was adjusted to that of unstimulated whole blood) was added to the supernatant and microparticle formation in whole blood was determined as described in "Assessment of microparticle formation." Platelet aggregation in whole blood was determined by comparing the platelet count before and after shear stress.

**Assessment of annexin V binding to platelets and microparticles.** Purified annexin V was labeled with FITC as described previously.<sup>36</sup> Suspensions of stimulated or unstimulated platelets (25  $\mu$ L) were incubated with FITC-labeled annexin V (150 nmol/L) for 10 minutes at room temperature, diluted with 500  $\mu$ L of HEPES-Tyrode's buffer, and analyzed using a FACScan as described above. The saturating concentration of FITC-labeled annexin V was predetermined on the basis of platelet activation by A23187, which promotes maximal microparticle formation.<sup>7,36</sup> No binding of annexin V to platelets or microparticles was detected in the presence of 5 mmol/L EGTA.

**Investigation of ABP degradation.** The proteolysis of ABP was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Platelet stimulation was terminated by the addition of an equal volume of 2 times concentrated Laemmli's SDS sample buffer<sup>37</sup> with reducing reagent and EGTA. After boiling, SDS-PAGE was performed on 7.5% homogeneous gels. The separated proteins were then transferred to a nitrocellulose membrane, which was incubated with 3  $\mu$ g/mL of the anti-ABP antibody<sup>29</sup> at 37°C for 30 minutes and developed with peroxidase-conjugated goat antimouse IgG and DAB. To assess the degradation of microparticle ABP, microparticles were isolated by differential centrifugation.<sup>10,11</sup> In brief, EGTA at a final concentration of 5 mmol/L was added to suspensions of stimulated platelets, which were then centrifuged for 15 minutes at 1,000g to remove intact and aggregated platelets. The resulting microparticle-containing supernatant was then recentrifuged at 12,000g for 1 hour to concentrate the microparticles. After lysis of the microparticle pellets in SDS sample buffer, these particles were analyzed by SDS-PAGE and immunoblotting.

## RESULTS

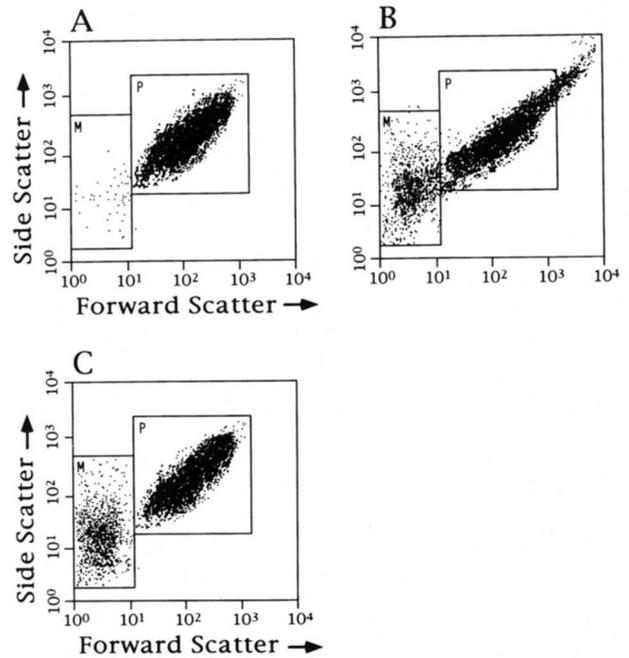
**Microparticle formation during SIPA.** Exposure of washed platelets to high shear stress in the presence of vWF and extracellular calcium resulted in platelet aggregation without the addition of fibrinogen or any exogenous agonist



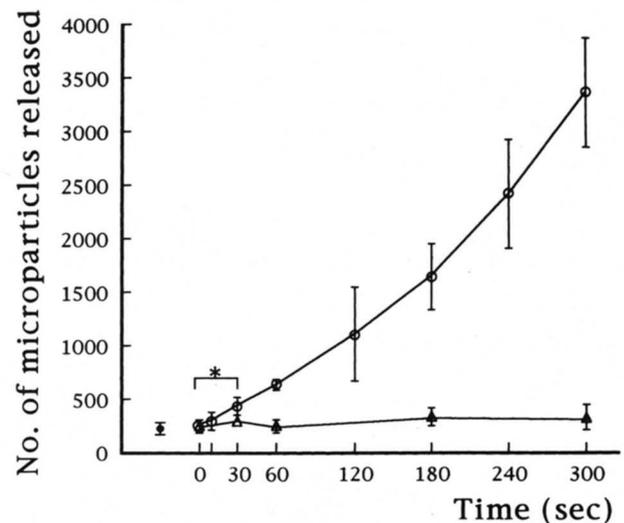
**Fig 1.** Typical pattern of platelet aggregation under constant high shear stress. Washed platelet suspensions with added calcium (2 mmol/L) and vWF (10  $\mu$ g/mL) were placed in a cone-and-plate viscometer. After an initial 15 seconds at 6 dynes/cm<sup>2</sup>, the shear stress was increased to 108 dynes/cm<sup>2</sup> over a 10-second period and then maintained at that level for 5 minutes at room temperature. The shear force applied to the platelets is shown by the broken line.

(Fig 1), as has been observed previously.<sup>16,19,23</sup> We then examined whether high shear stress caused the release of microparticles from the platelet plasma membrane. Platelets and platelet-derived particles were labeled with an FITC-conjugated antiplatelet GP IX MoAb (KMP-9) and analyzed by flow cytometry. The scatter pattern obtained indicated that exposure of platelets to high shear stress caused the release of microparticles (Fig 2). We next examined the time course of microparticle formation during SIPA. As shown in Fig 3, significant release of microparticles occurred within 30 seconds of the application of shear stress and the number of microparticles continued to increase as the shear time was prolonged. In contrast, exposure of washed platelet suspensions containing 2 mmol/L calcium, 10  $\mu$ g/mL vWF, and 100  $\mu$ g/mL fibrinogen to low shear stress did not cause either platelet aggregation (data not shown) or microparticle formation (Fig 3). We also investigated the release of microparticles from platelets in whole blood (Table 1). Both the extent of platelet aggregation and the number of microparticles released showed no significant differences between whole blood and washed platelet.

**Generation of microparticles by agonists and shear stress.** Platelet agonists, such as thrombin and collagen, are known to cause the release of microparticles from platelets.<sup>6,11</sup> As shown in Table 2, we found that the release of microparticles in the presence of high shear stress was greater after both 1 minute and 5 minutes than the release caused by thrombin (0.5 U/mL) or collagen (20  $\mu$ g/mL). In addition, microparticle generation by high shear stress was more rapid than generation by the combination of thrombin and collagen, although the extent of microparticle release after 5 minutes was comparable (Table 2). Addition of fibrinogen (100  $\mu$ g/mL) did not influence microparticle release by high shear



**Fig 2.** Flow cytometric light scatter patterns of platelets and microparticles. Platelets and microparticles were detected by an anti-GP IX MoAb (KMP-9). P and M indicate the gates for platelets and microparticles, respectively. (A) unstimulated platelets. (B) Platelets exposed to high shear stress for 5 minutes. (C) Intact and aggregated platelets were removed from a stimulated sample by centrifugation at 1,000g for 15 minutes, and unstimulated platelets were added to the resultant microparticle suspension. Data are shown for a representative single experiment of 5 different experiments.



**Fig 3.** Time course of microparticle formation under shear stress. The number of microparticles generated per 10,000 platelets was determined at each time point. (○), High shear stress (108 dynes/cm<sup>2</sup>). (△), Low shear stress (12 dynes/cm<sup>2</sup>). (●), Untreated platelets. Results are shown as the mean  $\pm$  SD of 3 to 5 experiments. \**P* < .03 by Student's *t*-test.

**Table 1. Platelet Microparticle Formation by High Shear Stress in Whole Blood and Washed Platelet Suspension**

Sample	Platelet Aggregation (%)	Microparticles
Washed platelet suspension	44.8 ± 7.9	3,358 ± 506
Whole blood	40.3 ± 2.9	2,578 ± 458

Platelet aggregation is determined as described in Materials and Methods. Microparticle formation after 5 minutes is expressed as the number of microparticles per 10,000 intact platelets. Data are shown as the mean ± SD of 5 different experiments.

stress. Low shear stress (12 dynes/cm<sup>2</sup>), which does not cause platelet aggregation at normal blood calcium concentrations,<sup>16</sup> did not provoke statistically significant release of microparticles.

*Shear stress-induced exposure of amino phospholipids, platelets, and microparticles.* To examine whether high shear stress caused the expression of procoagulant activity, we used FITC-conjugated annexin V to detect exposed amino phospholipids by flow cytometric analysis.<sup>36</sup> Our preliminary study using collagen, epinephrine, and calcium ionophore A23187 as agonists gave results in agreement with those reported previously.<sup>38</sup> A23187 showed the strongest ability to trigger the exposure of amino phospholipids and microparticle formation, while collagen had a moderate ability to do so, and epinephrine could not trigger these reactions (data not shown). Figure 4A shows that flow cytometry detected the exposure of amino phospholipid on the surface of platelets subjected to shear stress. The microparticles shed from platelets under high shear stress conditions also had amino phospholipids exposed on their surfaces (Fig 4B).

*Effect of SIPA inhibitors on microparticle formation.* Since SIPA depends on the binding of vWF to both GP Ib and GP IIb/IIIa,<sup>16</sup> as well as the transmembrane influx of extracellular calcium<sup>17</sup> and the endogenous ADP level,<sup>20,25</sup>

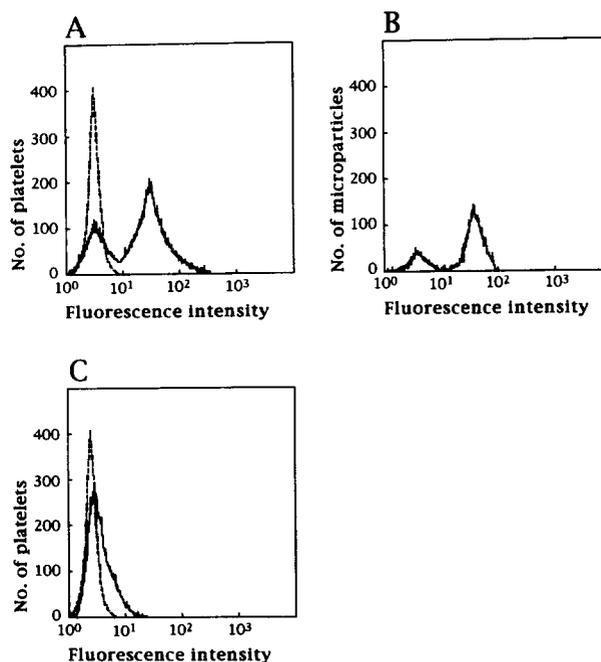
**Table 2. Effect of Various Stimuli on Platelet Microparticle Formation**

Stimulant	Number of Microparticles Released (per 10 <sup>4</sup> platelets)	
	1 min	5 min
None	ND	231 ± 55
Low shear stress (12 dynes/cm <sup>2</sup> )	252 ± 64	330 ± 122
High shear stress (108 dynes/cm <sup>2</sup> )	636 ± 47	3,358 ± 506
+ Fibrinogen (100 µg/mL)	662 ± 152	3,661 ± 658
Thrombin (0.5 U/mL)	387 ± 52	1,423 ± 331
Collagen (20 µg/mL)	420 ± 57	1,406 ± 364
Thrombin (0.5 U/mL) + Collagen (20 µg/mL)	525 ± 44*	2,864 ± 417

Microparticle formation after 1 minute and 5 minutes is expressed as the number of microparticles per 10,000 intact platelets. + Fibrinogen: high shear stress in the presence of fibrinogen, vWF (10 µg/mL) and calcium (2 mmol/L). Data are shown as the mean SD ± of 3 to 5 experiments. Thrombin, collagen, and thrombin + collagen were added with stirring.

Abbreviation: ND, not determined.

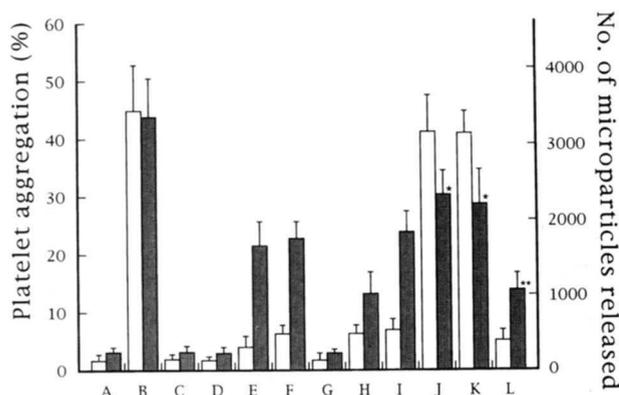
\* <.05 (Student's *t*-test) for thrombin + collagen versus high shear stress after 1 minute.



**Fig 4. Expression of amino phospholipids on the surface of platelets and microparticles.** Washed platelet suspensions were subjected to high shear stress (108 dynes/cm<sup>2</sup>) for 5 minutes in the absence (A and B) or presence (C) of calpeptin (30 µg/mL). Flow cytometric analysis of the binding of FITC-conjugated annexin V to platelets (A and C) and generated microparticles (B) was done with gating by light scatter as shown in Fig 2. The broken lines in (A and C) show the binding of FITC-conjugated annexin V to untreated platelets. Data are shown for a single representative experiment of 3 different experiments.

inhibition of various events can result in the suppression of shear-related aggregation. To investigate the process of high shear stress-induced microparticle formation, we analyzed the effect of SIPA inhibitors on microparticle release (Fig 5). Disturbance of the binding of vWF to GP Ib by anti-GP Ib or anti-vWF MoAbs and chelation of extracellular calcium by addition of EGTA caused complete inhibition of both SIPA and microparticle formation. Inhibition of vWF binding to GP IIb/IIIa by the anti-GP IIb/IIIa MoAb or RGDS also resulted in complete suppression of SIPA, but statistically significant release of microparticles was still observed. The effect of apyrase, an ADP scavenger, was similar to that of RGDS.

PKC has been shown to contribute to the maximal extent of SIPA.<sup>23</sup> On the other hand, platelet aggregation by high shear stress still occurs after the activation of PKC and other protein kinases has been inhibited.<sup>23,25</sup> We examined whether PKC activation was involved in the process of SIPA-associated microparticle formation (Fig 5). No impairment of SIPA was observed after the addition of a broad-spectrum protein kinase inhibitor, staurosporine. Interestingly, despite the absence of the potential as an inhibitor of SIPA, staurosporine partially inhibited SIPA-associated microparticle formation. Similar results were observed with H-7,<sup>39</sup> a specific PKC inhibitor. In addition, a statistically significant decrease of microparticle formation was observed in the presence of anti-



**Fig 5. Effect of SIPA inhibitors on microparticle generation.** The maximum SIPA (%) is shown by open bars and the number of microparticles released per 10,000 platelets is shown by shaded bars. A constant very low shear stress (6 dynes/cm<sup>2</sup>, bar A) or high shear stress (108 dynes/cm<sup>2</sup>, bars B ~ L) was applied to platelet suspensions for 15 seconds (A) or 5 minutes (B ~ L). Before applying shear stress, platelets were incubated for 5 minutes with the antibodies or reagents listed below: (A and B), HEPES-Tyrodé's buffer alone; (C), anti-GP Ib MoAb (NNKY5-5: 10  $\mu$ g/mL); (D), anti-vWF MoAb (NMC-4: 10  $\mu$ g/mL); (E), anti-GP IIb/IIIa MoAb (NNKY1-32: 60  $\mu$ g/mL); (F) RGDS (2 mmol/L); G, EGTA (5 mmol/L); (H), PGE<sub>1</sub> (2  $\mu$ mol/L); (I), apyrase (2 U/mL); (J), staurosporine (1  $\mu$ mol/L); (K), H-7 (100  $\mu$ mol/L); (L), NNKY1-32 (60  $\mu$ g/mL) + H-7 (100  $\mu$ mol/L). Results are shown as the mean  $\pm$  SD for 3 to 5 experiments. \* $P$  < .03 (Student's  $t$ -test) for addition of staurosporine or H-7 versus buffer alone. \*\* $P$  < .05 (Student's  $t$ -test) for addition of anti-GP IIb/IIIa MoAb + H-7 versus anti-GP IIb/IIIa MoAb alone.

GP IIb/IIIa MoAb plus H-7 when compared with the MoAb alone.

PGE<sub>1</sub> increases platelet cyclic AMP levels and inhibits SIPA without influencing the influx of extracellular calcium.<sup>17</sup> Although complete suppression of SIPA by addition of PGE<sub>1</sub> was reproducible, we found that a few microparticles were released in the presence of PGE<sub>1</sub>. Aspirin, which does not inhibit SIPA,<sup>20</sup> did not affect SIPA-associated microparticle formation (data not shown).

**Effect of epinephrine on microparticle formation during SIPA.** It has been shown that epinephrine augments SIPA, while ADP and collagen do not.<sup>40</sup> This effect was obvious when epinephrine was added at 10 ng/mL in our experimental system (Fig 6). However, addition of epinephrine at 1 to 30 ng/mL had no significant effect on microparticle formation induced by high shear stress (Fig 6).

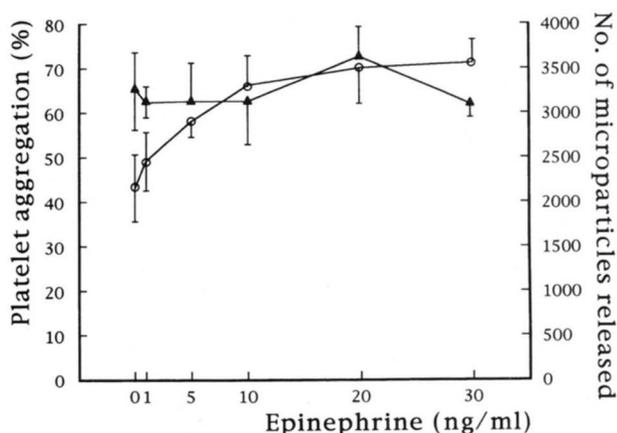
**Degradation of ABP during SIPA.** Proteolytic degradation of platelet ABP by activated calpain is a crucial event in both the expression of procoagulant activity and the shedding of microparticles induced by thrombin, collagen, and calcium ionophore A23187,<sup>6,7,41,42</sup> while complement-induced vesiculation of platelets does not depend on it.<sup>11</sup> We, therefore, examined whether cleavage of ABP occurred during SIPA and detected degradation of this protein (Fig 7). Although the extent of ABP proteolysis varied between experiments, a 190-kD fragment was generally observed after 30 seconds of shear stress and a 90-kD fragment always appeared after 5 minutes of shear stress. Microparticles con-

tained only the proteolytic products of ABP and not the intact form.

**Effects of calpeptin on SIPA, microparticle formation, and ABP degradation.** Finally, we examined the effects of calpeptin, a membrane-permeable calpain-specific inhibitor,<sup>26,41</sup> on SIPA, microparticle formation, and amino phospholipid exposure. As shown in Fig 8, calpeptin significantly ( $P$  < .01 by the Student's  $t$ -test) reduced the shedding of microparticles at 10  $\mu$ g/mL and almost completely suppressed microparticle formation at 30  $\mu$ g/mL (Fig 8). ABP degradation was obviously reduced by calpeptin at 10  $\mu$ g/mL and completely inhibited at 30  $\mu$ g/mL (Fig 7). Expression of amino phospholipids on the platelet surface was also inhibited by calpeptin, as determined by the change in the binding of annexin V (Fig 4C). However, the maximum extent of SIPA was not influenced by calpeptin, even at a concentration of 300  $\mu$ g/mL (Fig 8). Inhibition of both SIPA and microparticle formation, by blocking the vWF-GP Ib interaction or by chelation of extracellular calcium, also completely inhibited ABP degradation. In contrast, obvious degradation of ABP was observed when SIPA alone was inhibited by disturbance of vWF-GP IIb/IIIa binding or addition of PGE<sub>1</sub> (Fig 7).

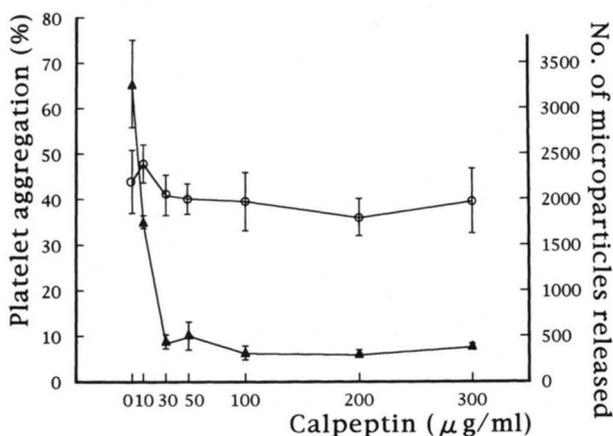
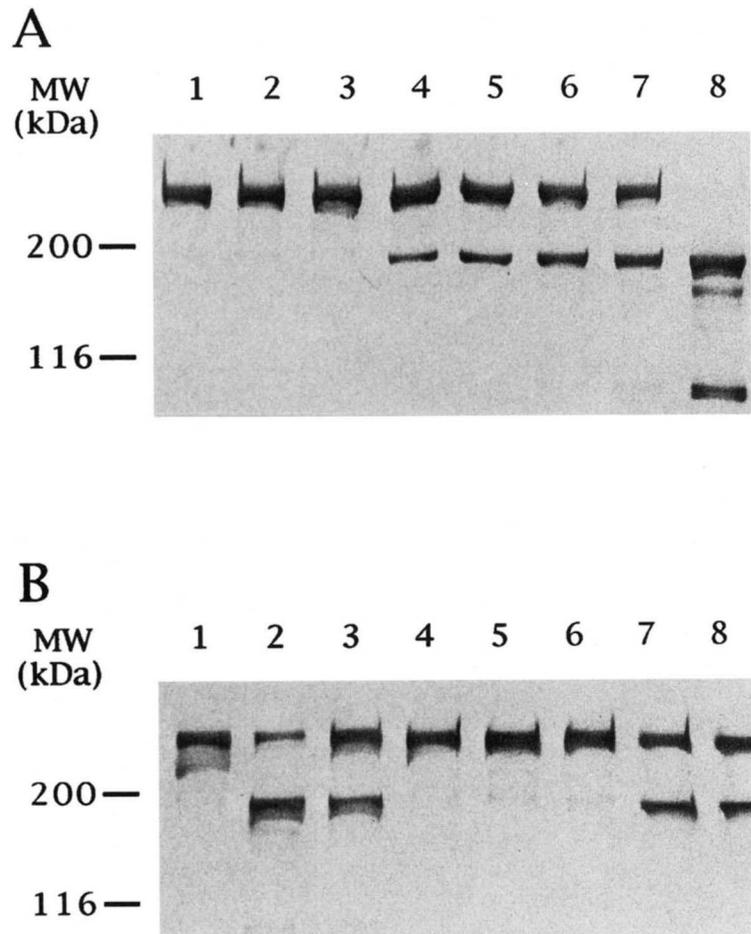
## DISCUSSION

Exposure of washed platelets to high shear stress causes vWF-dependent and fibrinogen-independent aggregation in the presence of extracellular calcium.<sup>16,17,19,20</sup> High shear stress can also induce the enhancement of platelet procoagulant activity.<sup>43</sup> In the present study, we first examined whether shear stress could initiate the shedding of microparticles from the platelet membrane. From among the various methods that have been employed to detect platelet microparticles,<sup>6,7,10,11,44-46</sup> we chose flow cytometry because of its rapidity and ability to quantify and characterize microparticles. On the other hand, it is important to note that our method is likely to underestimate the actual number of mi-



**Fig 6. Effect of epinephrine on SIPA and microparticle formation.** Epinephrine was added to platelet suspensions just before applying high shear stress and the maximum SIPA (○) and the number of microparticles released per 10,000 platelets (▲) within 5 minutes were determined. Each data point represents the mean  $\pm$  SD of 3 to 5 experiments.

**Fig 7.** Degradation of ABP during SIPA and its inhibition by reagents and MoAbs. (A) Platelet suspensions subjected to high shear stress were immediately lysed at the indicated time point, followed by SDS-PAGE and immunoblotting with an anti-ABP MoAb to detect ABP (270 kD) and its proteolytic fragments (190 kD and 90 kD). Lane 1, resting platelets; lanes 2 to 7: 0 seconds, 10 seconds, 30 seconds, 1 minute, 2 minutes, and 5 minutes after the onset of high shear stress (108 dynes/cm<sup>2</sup>). Lane 8, platelet-free microparticles isolated by differential centrifugation from a platelet suspension exposed to high shear stress for 5 minutes. Representative data from 1 of 4 different experiments are shown. (B) Platelet suspensions were preincubated with MoAbs or reagents, and exposed to high shear stress (108 dynes/cm<sup>2</sup>) for 5 minutes, followed by SDS-PAGE and immunoblotting. Lane 1, resting platelets; lane 2, HEPES-Tyrode's buffer alone; lane 3, calpeptin (10  $\mu$ g/mL); lane 4, calpeptin (30  $\mu$ g/mL); lane 5, anti-vWF MoAb (NMC-4: 10  $\mu$ g/mL); lane 6, EGTA (5 mmol/L); lane 7, RGDS (2 mmol/L); lane 8, PGE<sub>1</sub> (2  $\mu$ mol/L). Representative data from 1 of 3 different experiments are shown.



**Fig 8.** Effect of calpeptin on SIPA and microparticle formation. Calpeptin or solvent alone (DMSO) was added to platelet suspensions and incubation was done for 5 minutes before applying high shear stress. The maximum SIPA (○) and the number of microparticles released per 10,000 platelets (▲) within 5 minutes were determined. Each data point represents the mean  $\pm$  SD of 3 to 5 experiments.

croplastic due to loss during centrifugation and due to the limited forward scatter resolution of the FACScan. Evident release of microparticles was observed when washed platelet suspensions were subjected to high shear stress (108 dynes/cm<sup>2</sup>) for 5 minutes in the presence of exogenous vWF (10  $\mu$ g/mL) and extracellular calcium (2 mmol/L). The extent of microparticle formation was not influenced by the addition of fibrinogen (100  $\mu$ g/mL). Previous studies have shown that production of procoagulant-containing microparticles is dependent on either the binding of fibrinogen to GP IIb/IIIa, induced by thrombin, collagen,<sup>6,14</sup> or an anti-CD9 MoAb,<sup>15</sup> or the direct stimulation of platelets by complement proteins<sup>10,11</sup> and nonphysiologic agonists, such as calcium ionophore and local anesthetics.<sup>7</sup> Thus, it was an interesting finding that the binding of vWF to platelet GPs under high shear stress conditions led to microparticle release and the expression of procoagulant activity. In contrast, we confirmed that microparticles were not generated by ristocetin- or botrocetin-induced vWF-dependent platelet aggregation (unpublished observations). Our time course experiments (Fig 3) indicated that the shedding of microparticles commenced within 30 seconds after the onset of high shear stress and that microparticle formation was closely related with the progression of SIPA during the initial 2 minutes of shear

stress. Taking these findings together with the previous observation that an immediate increase of intracellular calcium occurs concurrently with SIPA due to the influx of extracellular calcium,<sup>17,18</sup> it seems likely that the processes of microparticle generation and transmembrane calcium influx are closely related in the early phase of SIPA. However, microparticle formation continued from 3 to 5 minutes in a time-dependent manner (Fig 3), although SIPA (Fig 1) and transmembrane calcium influx<sup>17</sup> had already reached a plateau. These observations suggest that some mechanism responsible for the amplification of microparticle production was especially active in the later phase of SIPA.

We confirmed that shedding of platelet microparticles occurred when whole blood was subjected to high shear stress. Interestingly, the number of microparticles released was somewhat lower in whole blood, although there was no significant difference compared with washed platelets. Although the cause of this slight reduction is not clear, it is possible that platelet microparticles may be trapped by other cells when generated in whole blood or that coagulation factors in the blood may modulate microparticle release.

The shedding of platelet microparticles is reported to be closely associated with exposure of anionic phosphatidylserine on the outer surfaces of both the microparticles and the remnant platelets,<sup>6-11</sup> and this exposure of anionic phospholipids accelerates the coagulation cascade by providing a catalytic surface for the binding and interaction of coagulation factors Va and Xa.<sup>8,10</sup> It was recently shown that the calcium-dependent aminophospholipid-binding protein annexin V can be used to detect the expression of procoagulant activity on platelets and microparticles.<sup>36,38</sup> In a preliminary study, we confirmed that the binding of annexin V to the platelet surface varied in proportion to that of an anti-factor Va MoAb, which recognized platelet  $\alpha$ -granule-derived factor Va bound to surface Va binding sites. Furthermore, annexin V completely inhibited the binding of exogenously added factor Xa to activated platelets<sup>38</sup> (and unpublished data). These preliminary results indicate that exposure of annexin V binding sites is directly related to the development of procoagulant activity. Accordingly, we used FITC-labeled annexin V to detect amino phospholipids exposed on the surface of platelets and microparticles in the present study. The flow cytometric study showed that high shear stress can increase platelet procoagulant activity by accelerating the translocation of phosphatidylserine from the inner leaflet to the outer surface of the platelet plasma membrane and can cause the shedding of phosphatidylserine-containing microparticles. These findings suggested that pathologically high shear stress in small arteries and arterioles narrowed by atherosclerosis or vasospasm<sup>16,19,21</sup> may induce platelet aggregation and the expression of procoagulant activity, both of which may contribute to vascular occlusion. The potential pathological effect of SIPA-associated microparticle formation is emphasized by our finding in the present study that the speed and extent of microparticle formation were greater with high shear stress than with any single physiological platelet agonist. Moreover, production of microparticles by high shear stress was still more rapid than by the combination of two strong platelet agonists,

thrombin and collagen, although the eventual number of microparticles released within 5 minutes was comparable.

Next, we investigated the mechanisms responsible for microparticle generation by high shear stress. Inhibition of the binding of vWF to either GP Ib or GP IIb/IIIa results in the complete inhibition of SIPA,<sup>16</sup> but the crucial difference between these two interventions is that blocking vWF binding to GP Ib inhibits the transmembrane influx of extracellular calcium, while blocking vWF binding to GP IIb/IIIa does not.<sup>17,18</sup> We found that inhibition of the vWF-GP Ib interaction by anti-GP Ib or anti-vWF MoAbs and the chelation of extracellular calcium by addition of EGTA resulted in the complete inhibition of both SIPA and microparticle generation. In contrast, inhibition of binding of vWF to GP IIb/IIIa by an anti-GP IIb/IIIa MoAb or by the tetrapeptide RGDS resulted in the complete inhibition of SIPA, but only partial inhibition of microparticle formation. In addition, a few microparticles were generated under high shear stress conditions in the presence of PGE<sub>1</sub>, which inhibited SIPA without affecting the influx of extracellular calcium.<sup>17</sup> These results suggest that the transmembrane influx of extracellular calcium ions, which depends on the shear-induced interaction of vWF with GP Ib,<sup>17,18</sup> is crucial to the initiation of microparticle formation, while promotion of SIPA by vWF binding to GP IIb/IIIa may subsequently potentiate the shedding of microparticles.

Although activation of PKC by shear stress (90 dynes/cm<sup>2</sup>) was demonstrated previously,<sup>23</sup> it was not clarified whether or not PKC had a role in SIPA.<sup>23,25</sup> We found that inhibition of PKC activation impaired microparticle formation during SIPA, but did not cause significant suppression of SIPA itself (Fig 5). These results suggest that the activation of PKC may contribute to the potentiation of microparticle formation, but not to initiation of this process. In addition, the finding that the combination of an anti-GP IIb/IIIa MoAb and H-7 suppressed microparticle shedding more effectively than the MoAb alone suggested that PKC was at least partially activated by signals from the interaction of vWF with GP Ib and the subsequent transmembrane influx of extracellular calcium, even in the absence of vWF binding to GP IIb/IIIa and platelet aggregation. This suggestion is supported by previous reports that influx of calcium ions across the plasma membrane can induce local activation of PKC,<sup>47</sup> that induction of vWF binding to GP Ib by ristocetin causes PKC activation,<sup>48</sup> and that vWF triggers the activation of platelet PKC under high shear stress conditions.<sup>23</sup> In contrast to the contribution of PKC,<sup>23</sup> the cyclooxygenase pathway is reported to be not necessarily involved in SIPA.<sup>16,20</sup> In fact, we found that inhibition of cyclooxygenase activity by aspirin had no effect on SIPA or high shear stress-dependent microparticle generation (Fig 5).

Since the addition of epinephrine produced reproducible augmentation of SIPA (Fig 6), we also expected to find the enhancement of microparticle formation, but epinephrine actually had no effect on microparticle formation during SIPA over the concentration range of 1 to 30 ng/mL (Fig 6). These results suggest that epinephrine may act via the  $\alpha_2$ -adrenergic receptor to increase the binding affinity of vWF receptors under high shear stress conditions without

affecting the process of microparticle generation. Indeed, it was previously reported<sup>49</sup> that epinephrine alone does not induce the activation of PKC, which appears to be one of the factors potentiating SIPA-associated microparticle formation.

Previous observations have indicated that degradation of ABP by calpain plays a key role in thrombin- or calcium ionophore-induced microparticle formation.<sup>6,7</sup> On the other hand, complement proteins C5b-9 can still induce the shedding of microparticles after calpain activity has been inhibited by leupeptin.<sup>11</sup> As shown in Figs 7 and 8, we found that ABP was cleaved during SIPA. This cleavage commenced between 10 and 30 seconds after the onset of shear stress, ie, concurrently with microparticle generation. Inhibition of calpain activity by calpeptin completely prevented ABP cleavage, microparticle formation, and amino phospholipid exposure on the platelet surface during SIPA. In addition, blocking of the vWF-GP Ib interaction or the chelation of extracellular calcium by EGTA, but not the prevention of vWF-GP IIB/IIIa binding, resulted in the complete inhibition of both SIPA-associated microparticle formation and ABP degradation. Accordingly, SIPA-associated microparticle formation and the expression of procoagulant activity under high shear stress conditions may require the cleavage of ABP by calpain, which is induced by signals arising from the interaction of vWF with GP Ib and subsequent transmembrane calcium influx. Because ligand binding to GP IIB/IIIa is required to cause activation of platelet calpain after stimulation by agonists,<sup>50</sup> it is interesting to note that activation of calpain can be initiated without the vWF-GP IIB/IIIa interaction under high shear stress conditions.

In conclusion, the present study demonstrated that a pathological level of shear stress has the potential to initiate not only platelet aggregation but also the release of procoagulant-containing microparticles. It appears that high shear stress-induced microparticle formation and the expression of procoagulant activity are both linked to some process that is completely dependent on the vWF-GP Ib interaction with a subsequent influx of calcium ions and calpain activation. This process is potentiated by PKC activation, is independent of the cyclooxygenase pathway, and is not potentiated by epinephrine. The finding that a pathological level of shear stress can cause not only platelet aggregation but also the expansion of procoagulant activity underlines the potential significance of shear stress in vascular occlusion.

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