Platelets play a pivotal role in the coagulation response by adhering and aggregating at the site of vessel injury. This sequestering of platelets provides substantial phospholipid surface for the assembly of membrane-dependent procoagulant enzyme complexes. The prothrombinase complex, consisting of the serine protease factor Xa, its cofactor protein factor Va, calcium ions, and a phospholipid bilayer, is one of the most thoroughly studied. This complex, which converts the zymogen prothrombin to the active enzyme thrombin, has been shown to assemble on the membranes of platelets activated in suspension and platelets adhered to von Willebrand factor (vWF).

The functional site of prothrombinase complex assembly is currently a controversial topic. In vitro studies of platelets in suspension suggest that as a consequence of platelet activation with the physiological agonists thrombin and collagen, a portion of the platelet plasma membrane is shed as microvesicles. A larger portion of the plasma membrane is released with agonists such as calcium ionophore A23187 or complement proteins C5b-9. It is proposed that the platelet-released microvesicles, not the platelet membrane itself, are the site of platelet procoagulant activity.

The mechanism of microvesicle release from platelets in suspension has not yet been clearly defined. Calpain, a calcium-dependent protease, is known to cleave several platelet cytoskeletal proteins and may play a role in cytoskeletal degradation and microvesicle release. However, microvesicles are also generated in the absence of calpain activation, suggesting that several mechanisms may exist for the formation and release of platelet microvesicles in vitro. Shear stress on the membranes of platelets activated in suspension does result in significantly more microvesicle release than platelets not subjected to shear.

The mechanism and function of microvesicle shedding from platelets in vivo also remains unclear. A low level of microvesicle shedding may be part of the natural aging process of the platelet. Studies in rabbits show that circulating platelets continually decrease in size and procoagulant activity as they age, possibly as a result of microvesicle release. In humans, increased microvesicle shedding is reported in some disease states.

**KEY WORDS** microvesicles platelets procoagulant activity prothrombinase complex

Received May 28, 1993; revision accepted August 16, 1993.

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states. High levels of circulating microvesicles have been detected in cases of thrombocytopenic purpura and in patients undergoing cardiopulmonary bypass surgery, and they may play a role in disseminated intravascular coagulation. Lack of microvesicle formation may also be representative of a disease condition.

To study the possibility of microvesicle release from adherent platelets, platelets were studied under conditions chosen to mimic those during the initial in vivo hemostatic response to vascular injury, in which platelets adhere to the exposed subendothelium to begin formation of the clot. This model system was established for total internal reflection fluorescence spectroscopy (TIRFS) binding studies. Platelets were allowed to adhere to a surface coating of vWF. These platelets mimic the deposition-phase platelets seen in the first stage of platelet adherence to vWF under flow conditions. Platelet adherence to exposed subendothelium via vWF is particularly important in areas of high shear, such as the microvasculature. Although morphological changes occur on platelet adherence to a vWF-coated surface in both flow systems and the static model, platelet adherence to vWF in the static model does not induce full platelet activation as seen under flow conditions. Microvesicle release from vWF-adherent platelets in the static system could thus be studied as a function of the adherence event and subsequently as a function of platelet stimulation with thrombin, a physiologically relevant agonist. The TIRFS study examined prothrombinase complex assembly on the membranes of adherent and thrombin-stimulated adherent platelets, but it did not address the possible release of microvesicles into the solution phase induced by thrombin addition. In this study, the solution phase was examined by flow cytometry to determine if platelet-released microvesicles were present in the buffer fractions above adherent platelets and fully stimulated adherent platelets. The microvesicles could be distinguished from detached platelets on the basis of size and relative fluorescence by using a fluorescein-conjugated antibody to platelet membrane glycoprotein (GP) IIb-IIIa. Electron microscopy was used to scan for the presence of adherent microvesicles. Microvesicles contain GPIb and GPIb-IIIa, both of which bind to vWF when expressed in the plasma membranes of platelets, suggesting that microvesicles may have the ability to adhere to the surface coating.

A prothrombinase activity assay was developed to determine several parameters. A comparison of the prothrombinase activities supported by adherent and thrombin-stimulated adherent platelets allowed determination of the requirement of platelet activation for expression of functional prothrombinase binding sites. In addition, the ability of the released microvesicles to support functional prothrombinase complex assembly was compared with the activity supported by the intact adherent platelet membranes to determine the primary site of procoagulant activity.

Methods

Materials

Calpeptin, a lipido-soluble calpain inhibitor, was the generous gift of Dr Toshima Tsujinaka, Osaka University School of Medicine, Osaka, Japan. Dansylarginine \( N\)-(3-ethyl-1,5-pentanediyl)amide (DAPA), an active-site-directed thrombin inhibitor, was provided by Haematologic Technologies, Inc, Essex Junction, Vt. Fluorescein-5-isothiocyanate (FITC) 10% adsorbed on Celite was obtained from Molecular Probes. Spectrozyme-TH (\( H-D\)-hexahydropyrosyl-\( L\)-alanyl-\( L\)-arginine-\( p\)-nitroanilide diacetate salt), a chromogenic substrate for thrombin, was purchased from American Diagnostica, Inc. Rat tail type I collagen was obtained from Sigma.

Protein Purification and Fluorescent Labeling

vWF was isolated from fresh frozen human plasma by cryoprecipitation and was further purified by chromatography on a Sepharose CL-4B (Pharmacia) column. The vWF-containing fractions were pooled and run on a 2% agarose gel to verify the presence of high-molecular-weight multimers. Protein concentrations of vWF pools were determined by protein assay. Factor V was isolated from bovine plasma and was activated with thrombin to generate the active cofactor, factor Va. Factor X and prothrombin were purified from fresh frozen human plasma. Factor Xa was prepared from factor X by using factor X activator from Russell's viper venom. A portion of the prothrombin pool was activated to generate thrombin. The thrombin proteolytic activity was determined by comparison with a standard of known activity. Purified murine monoclonal immunoglobulin (Ig) G antibody HP1-1D was provided by Dr William R. Church, University of Vermont, Burlington. HP1-1D recognizes platelet membrane GPIb-IIIa independent of platelet activation. Factor Va, factor Xa, prothrombin, thrombin, and IgG concentrations were determined by using the appropriate extinction coefficients: \( E_{155}^{1\%} \times 10^3 = 17.4, 11.6, 13.8, 18.3, \) and 14.0, respectively. HP1-1D was labeled with FITC 10% adsorbed on Celite. The fluorophore/protein ratio was 4:1.

Isolation of Human Platelets

Venous blood was drawn from consenting individuals who had not taken aspirin in the previous 10 days. The blood was anticoagulated with acid-citrate-dextrose (ACD) 6 mL ACD/30 mL blood. Heparin (50 U/mL) was also added to the ACD before blood collection. Platelets were isolated from 60 to 120 mL blood/ACD by using the technique of Mustard et al. The whole blood was centrifuged at 120g for 15 minutes at 26°C to obtain platelet-rich plasma. Platelets were isolated from platelet-rich plasma by a second centrifugation at 240g for 15 minutes at 26°C. The platelets were washed two to three times in 10 mL buffer containing 137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO\(_3\), 0.3 mmol/L NaH\(_2\)PO\(_4\), 2 mmol/L CaCl\(_2\), 1 mmol/L MgCl\(_2\), 5.5 mmol/L dextrose, and 0.35% bovine serum albumin, pH 7.35 (Tyrode's albumin). The first wash buffer also contained 50 U/mL heparin. Platelets were incubated in the wash buffers for 15 minutes at 37°C and were then sedimented at 240g for 5 minutes at 26°C. After the final wash, the platelet pellet was resuspended in 2 to 4 mL wash buffer containing 5 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES–Tyrode’s albumin).
Flow Cytometry

Individual wells in 24-well cell-culture plates were coated with 10 μg/mL vWF in 50 mmol/L sodium carbonate buffer, pH 9.0, for 15 hours at 4°C. The wells were washed three times with HEPES-Tyrode's albumin. Platelets (2 x 10^6) were added to each well and incubated for 15 minutes. The nonadherent platelets were washed from the well, and 200 μL fresh buffer was added; 2.8 ± 0.7 x 10^7 platelets adhered per well. Either 20 nmol/L (2 U/mL) human thrombin (5-minute incubation) or 1 μmol/L calcium ionophore A23187 (20-minute incubation) was added to stimulate the adherent platelets. Samples were also prepared in the presence of calpeptin to inhibit calpain proteolysis of platelet cytoskeletal proteins. Platelets (2 x 10^6) were incubated with 10 to 50 μg/mL calpeptin for 1 hour at 37°C. The platelets were then added to a vWF-coated well and stimulated with 20 nmol/L (2 U/mL) thrombin. A second incubation method was also used in which 10 to 50 μg/mL calpeptin was added to wells containing adherent platelets. The wells were incubated at 37°C for 1 hour before platelet stimulation with 20 nmol/L (2 U/mL) thrombin. To test the effects of a combination of agonists, wells were coated with 10 μg/mL type 1 collagen in 50 mmol/L sodium carbonate buffer for 15 hours at 4°C. The wells were washed, and 2 x 10^6 platelets were added with a 15-minute incubation. The nonadherent platelets were removed and fresh HEPES-Tyrode's albumin buffer was added. Half of the collagen-adherent platelet samples were stimulated with 20 nmol/L (2 U/mL) thrombin (5-minute incubation). Control wells with untreated adherent platelets and with vWF or collagen alone were prepared for background measurements. The vWF and collagen samples were treated with thrombin as were the corresponding adherent platelet samples. After incubation at 37°C for 15 minutes, the buffers from the wells were removed. The buffer fraction contained detached platelets and any released microvesicles. The nonadherent platelet wash and a sample of unstimulated platelets in suspension were diluted to 2 x 10^6 platelets/200 μL (1 x 10^6 platelets/mL). FITC-conjugated 1D (45 nmol/L) was added to each sample with a 10-minute incubation at 37°C. The samples were diluted with an additional 200 μL HEPES-Tyrode's albumin before analysis. All samples were analyzed for forward- and right-angle scatter and for green fluorescence with a Coulter Elite Fluorescence Activated Cell Sorter. Each sample was analyzed for 3 seconds. Two gates were chosen to distinguish particles positive for green fluorescence: one that included >95% of the platelet population (gate A) and one set for the smaller microvesicle population (gate B). Gate B was set to include all particles between the smaller-sized platelets and the background noise limit of the instrument. Controls were run to examine microvesicle release as a function of time, between 5 and 30 minutes. Within the given incubation times for the agonists, 5 minutes for thrombin and 20 minutes for A23187, microvesicle release from the platelets was complete. The mass percent microvesicle release was determined by the number and size range of particles counted as microvesicles in the gate B range and by the size range of platelet population determined by examination of platelets in suspension. Number of platelets was defined as the number of platelets remaining in a particular well subsequent to the wash steps (2.8 ± 0.7 x 10^7 platelets/well). The average size range of the microvesicle population was compared with the average size of an unstimulated platelet in suspension (as determined by flow cytometry). The mass percent of a microvesicle in terms of percent of total platelet membrane could then be determined. The average microvesicle represented approximately 5% of the average platelet membrane. Based on the number of microvesicles released (particles detected in gate B) and the total number of platelets in an individual well, the mass percent of microvesicles released from a particular sample could be determined. Between five and eight experiments were run for each set of conditions outlined, with a panel of five donors used for platelet isolation.

Scanning Electron Microscopy

Glass coverslips 12 mm in diameter were placed in 24-well cell-culture plates and coated with 10 μg/mL vWF in 50 mmol/L sodium carbonate buffer for 15 hours at 4°C. The wells were washed before the addition of 2 x 10^6 platelets, and samples were prepared as for flow cytometry. Half of the adherent platelet samples were stimulated with 20 nmol/L (2 U/mL) thrombin. The remaining platelet samples were not stimulated. The samples were fixed for scanning electron microscopy within 1 hour of preparation. One milliliter of 2.5% glutaraldehyde in Millonig's buffer (41.4 mL of 2.26% NaH2PO4 and 8.5 mL of 2.52% NaOH, pH 7.4) was added to the adherent platelets and incubated at 4°C for 1 to 2 hours. The platelets were washed four times with Millonig's buffer, and 1 mL of 1% osmium was applied for 30 minutes. The samples were dehydrated with a graded series of ethanol solutions (10% to 100%) and were critical-point dried in a CO2 system. The dried samples were then mounted on specimen stubs and were gold-coated in a sputtering device. The samples were viewed with a Joel 100CX electron microscope. Control samples with vWF alone were also viewed to determine if particulate matter was present in the buffer solution or inherent in the vWF coating.

Prothrombinase Activity Assay

vWF- and collagen-adherent platelet samples were prepared as for flow cytometry. vWF-adherent platelets were stimulated with 20 nmol/L (2 U/mL) thrombin (5-minute incubation) or 1 μmol/L calcium ionophore A23187 (20-minute incubation). Collagen-adherent platelets were stimulated with 20 nmol/L (2 U/mL) thrombin. Wells with unstimulated adherent platelets and vWF and collagen alone were also prepared. vWF- and collagen-coated wells were treated with thrombin or A23187 as were the corresponding wells containing adherent platelets. The platelet and control samples were incubated with or without agonists before the buffer fraction was removed. The buffer above the layer of adherent platelets, containing detached platelets and any released microvesicles, was placed in a separate uncoated well, and fresh buffer was added to the adherent platelets. The adherent platelets and buffer fractions were assayed separately. The total volume in each well was 500 μL. The plates were positioned on a gel shaker set at 800 rpm at 26°C. DAPA (3 μmol/L) was added to each sample to block thrombin feedback during the assay. Bovine factor Va (5 nmol/L) and 5 nmol/L human factor Xa were added with a 5-minute incubation. Human prothrombin (1.4 μmol/L) was
added to initiate the reaction. Aliquots (50 μL) were removed at five time points between 30 seconds and 15 minutes. The aliquots were immediately quenched in 150 μL of 0.02 mmol/L tris(hydroxymethyl)aminomethane, 0.15 mmol/L NaCl, 0.05 mol/L EDTA, and 0.1% polyethylene glycol 8000 at 4°C. Samples (100 μL) of the quenched aliquots were removed, placed in a 96-well assay plate, and assayed with 0.4 mmol/L Spectrozeme-TH to examine thrombin generation over time. The chromogenic assay was performed with a Molecular Devices Kinetic Microplate Reader. The samples were assayed for 5 minutes at 8-second intervals at 405 nm with continuous mixing of the well contents. A thrombin standard of known activity was used to calibrate the amount of thrombin generated over time under various sample conditions. Between five and eight experiments with five different donors were run for each set of conditions. The background activity (<10% of sample activity) in wells without vWF or collagen coating alone was subtracted from the measured activity for the appropriate adherent platelet samples. The buffer samples from the adherent platelet wells, containing detached platelets and any released microvesicles, were not centrifuged to pellet the detached platelets. Flow cytometry experiments indicated that the centrifugation step induced additional microvesicle formation.

Results

Flow cytometry was used to characterize the release of platelet-derived microvesicles from vWF- or collagen-adherent platelets. Monolayers of adherent platelets were stimulated with 20 nmol/L (2 U/mL) thrombin (5-minute incubation) or 1 μmol/L calcium ionophore A23187 (20-minute incubation) as a positive control for microvesicle release. After incubation with either agonist, the buffer above the adherent platelets was removed. Any detached platelets and released microvesicles present in the buffer were labeled with FITC-conjugated HP1-1D, allowing the use of fluorescence, as well as size, to distinguish between platelets, microvesicles, and the background noise of the instrument. HP1-1D recognizes the GPIIb-IIIa complex in both the unactivated and activated receptor states.41 The GPIIb-IIIa complex found in the microvesicle population could then be labeled regardless of the activation state of the complex. No particulate matter was detected in buffers from wells coated with vWF or collagen alone, with or without the addition of 20 nmol/L thrombin. A histogram of the forward- and right-angle scatter properties of washed platelets in suspension (Fig 1A) was used to set gates for data acquisition. Gate A was set to include >95% of the platelet signal. Gate B was set between the platelet signal and the smallest particle size detectable by the instrument to cover a range of microvesicle sizes. From 2% to 4% of the unstimulated platelets in suspension were detected in the smaller size range in gate B. The profile obtained from the buffer removed from a layer of unstimulated vWF-adherent platelets (Fig 1B) was nearly identical to that seen for unstimulated platelets in suspension. More than 95% of the signal was due to detached platelets. The number of detached platelets represented 15% to 20% of the total number of platelets in the well, indicating that >80% of the platelets remained bound to vWF throughout the experimental procedure. After thrombin stimulation of the vWF-adherent platelets, the histogram (Fig 1C) shows a slight shift in the platelet size range in gate A, indicating the presence of a smaller-sized population of platelets relative to the unstimulated samples. The amount of particulate matter identified in gate B also marginally increased. However, the number of detached platelets remained within the 15% to 20% detected for the unstimulated vWF-adherent platelets. An increase in the number of detached platelets was therefore not responsible for the change in the forward- and right-angle scatter profiles. The shift in platelet size detected in gate A and the increase in the number of particles counted in gate B was also seen after treatment of adherent platelets with calcium ionophore A23187 (Fig 1D).

For each flow cytometry experiment, 2×10⁸ platelets were added to the vWF- or collagen-coated wells. The nonadherent platelets, still in suspension, were collected after a 15-minute incubation period during which 10% to 14% (2.8±0.7×10⁸) of the added platelets adhered to the vWF surface. The nonadherent platelet sample from a vWF-coated well was analyzed to detect the presence of microvesicles released from the platelets as they adhered to the surface. Approximately 4% of the total platelet mass was detected in gate B in the nonadherent platelet sample. Analysis of unstimulated platelets in suspension and buffer from unstimulated vWF-adherent platelets showed that approximately 4% of the total platelet mass was counted in gate B for both of these samples as well (Fig 2A through 2C). This low percentage found in the size range covered by gate B probably represented a natural level of very small
proteolysis of platelet cytoskeletal proteins may induce microvesicle release. The mass percent of particles detected in unstimulated platelets in suspension, no increase in microvesicle release over thrombin-stimulated vWF-adherent platelets was seen (Fig 2F). Approximately 8% of the total platelet mass was released as microvesicles after thrombin stimulation of the collagen-adherent platelets. The positive control for microvesicle release, A23187-treated vWF-adherent platelets, showed approximately 21% of the total platelet mass being released as microvesicles (Fig 2G). There were significantly more microvesicles released after platelet treatment with A23187 than with the physiological agonists thrombin and collagen.

Microvesicles contain several platelet membrane glycoproteins. When expressed in the platelet plasma membrane, GPIb and the GPIIb-IIIa receptor complex both recognize vWF. The surface of a vWF-coated coverslip with adherent platelets was examined by scanning electron microscopy to detect the presence of adherent microvesicles. A series of photomicrographs was obtained at several magnifications to examine the overall appearance of the platelet and vWF surfaces. At a low magnification, the morphologies of unstimulated and thrombin-stimulated adherent platelets were remarkably different (Fig 3A and 3B). At higher magnification, at which individual platelets became visible, the unstimulated adherent platelets (Fig 3C) were fairly round with some pseudopod extension, whereas the thrombin-stimulated adherent platelets (Fig 3D) showed loss of individual platelet definition and greatly increased pseudopod formation. While adherence did induce morphological changes in the platelets, stimulation with an agonist was necessary to fully activate the adherent platelets. With increased magnification, a number of submicron-diameter particles attached to the exposed vWF coating between unstimulated adherent platelets could be detected (Fig 4A and 4C). Particles were also visible between thrombin-stimulated adherent platelets (Fig 4B and 4D). No particles were seen adhering to the platelets themselves. The particles were not inherent in either the buffer or the vWF coating, but were due to the presence of platelets. The size range of the particles as well as the dependence on the presence of platelets suggested that the particles were platelet-released microvesicles. The small number of particles visualized indicated that adherence is not a primary function of microvesicles. It was necessary to screen at least 50 fields such as those seen in Fig 4B and 4D to obtain evidence of any particles in the microvesicle size range. The mass percent of microvesicle release detected by flow cytometry is, there-
Before an accurate measure of total microvesicle release by adherent platelets.

The ability of the microvesicle-containing fraction and the adherent platelet membranes to support functional prothrombinase complex assembly was determined with a prothrombinase activity assay. After incubation of the platelet samples with or without an agonist, the buffer fraction containing detached platelets and any released microvesicles was removed. Fresh buffer was added to the adherent platelets. The adherent platelets and initial buffer fraction were then assayed as separate samples. Samples were incubated with 5 nmol/L factor Va and 5 nmol/L factor Xa in the presence of 3 μmol/L DAPA to inhibit thrombin feedback. After the addition of 1.4 μmol/L prothrombin to initiate the reaction, aliquots were removed at time points between 30 seconds and 15 minutes. The aliquots were assayed for the presence of thrombin generated over time and were compared with a thrombin standard of known activity. The prothrombinase activity of each sample was normalized as thrombin generated per minute per $2 \times 10^7$ platelets at each time point. After subtraction of the background activity in the wells with vWF, collagen, or buffer alone, >85% of the total prothrombinase activity was localized on the membranes of unstimulated vWF-adherent platelets. The remaining activity was associated with the buffer containing detached platelets and any released microvesicles (Fig 5A and 5B). (Approximately 15% of the total number of platelets counted in each well were either not removed in the wash step or were detached from the surface. These platelets were found in the buffer fraction.) Because the number of detached platelets was consistent between different samples, the activity associated with the detached platelets from the unstimulated vWF-adherent platelet sample could be used as a background level of platelet activity in the buffer fraction. Thrombin stimulation of the vWF-adherent platelets yielded the same results as seen for the unstimulated adherent platelets. More than 85% of the total activity remained with the membranes of the stimulated adherent platelets. There was no measurable increase in

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**Fig 3.** Scanning electron micrographs of von Willebrand factor (vWF)-adherent platelets. A, Unstimulated adherent platelets (original magnification ×700; bar=20 μm). B, Thrombin-stimulated (20 nmol/L) adherent platelets (original magnification ×700; bar=20 μm). C, Unstimulated adherent platelets (original magnification ×2800; bar=3 μm). D, Thrombin-stimulated adherent platelets (original magnification ×2800; bar=3 μm).
activity in the buffer fraction (Fig 5C and 5D). Whereas the number of detached platelets remained constant between the unstimulated and thrombin-stimulated samples, microvesicle release was associated with the stimulation event. Approximately 8% of the total platelet mass was released as microvesicles as a consequence of thrombin activation of the vWF-adherent platelets. However, there was no corresponding increase in activity due to microvesicle shedding in the buffer fraction. Calpeptin inhibition of calpain activity in the thrombin-stimulated vWF-adherent platelets did not shift the activity profile from that seen for thrombin-stimulated adherent platelets in the absence of calpeptin (Fig 5E and 5F). The slight increase in activity seen in the buffer fraction was most likely due to interference of calpeptin in the assay. (No change in the number of detached platelets or released microvesicles in the calpeptin-treated sample versus the untreated sample was detected by flow cytometry.) The combined effects of collagen and thrombin activation were also examined.

No increase in total activity was detected for the thrombin-stimulated collagen-adherent platelets; >85% of the total activity remained associated with the membranes of the adherent platelets. The activity of the buffer fraction showed no increase over the level of activity seen for thrombin-stimulated vWF-adherent platelets or unstimulated vWF-adherent platelets (Fig 5G and 5H). These results indicated that the activity found in the buffer fraction was due to detached platelets and showed no correlation with microvesicle release. Calcium ionophore A23187 treatment of vWF-adherent platelets yielded very different results from those of the naturally occurring agonists collagen and thrombin. The total prothrombinase activity was greater and equally divided between the membranes of the adherent platelets and the buffer fraction containing detached platelets and microvesicles. The mechanism of microvesicle release exhibited after A23187 treatment of vWF-adherent platelets may be substantially different than the mechanism of microvesicle shedding with...
The activity of each sample was normalized as nanomoles per liter thrombin generated per minute per $2 \times 10^7$ platelets. Five to eight experiments were run for each sample, from which the average normalized as nanomoles per liter thrombin generated per minute. The activity of each sample was normalized as nanomoles per liter thrombin generated per minute per $2 \times 10^7$ platelets. Five to eight experiments were run for each sample, from which the average normalized as nanomoles per liter thrombin generated per minute. The activity of each sample was normalized as nanomoles per liter thrombin generated per minute per $2 \times 10^7$ platelets. Five to eight experiments were run for each sample, from which the average normalized as nanomoles per liter thrombin generated per minute.

** FIG 5. Bar graph showing prothrombinase activity of adherent platelets and microvesicle-containing buffer fractions at 1 minute. The activity of each sample was normalized as nanomoles per liter thrombin generated per minute per $2 \times 10^7$ platelets. Five to eight experiments were run for each sample, from which the average normalized as nanomoles per liter thrombin generated per minute.**

Physiologically relevant agonists. The microvesicle “activity” profile versus the extent of microvesicle release showed that the microvesicles released (21% of the total platelet mass) from A23187-treated vWF-adherent platelets supported activity equal to the remaining 79% of the platelet membranes. Calcium ionophore A23187 may produce more “active” microvesicles as well as a greater number of microvesicles relative to the physiological agonists thrombin and collagen.

**Discussion**

The role of platelet membranes versus microvesicles in providing procoagulant membrane surface has been a subject of controversy. The debate has encompassed the release of microvesicles from platelets, their function in vivo, and the link between platelet activation and generation of procoagulant activity. In vivo studies outline conditions under which a high level of circulating microvesicles may be present. Functional studies outlining the possible role of microvesicle release have been done in vitro by using microvesicles released from platelets activated in suspension. However, in vivo, one of the primary actions of platelets in maintaining hemostasis is to adhere and aggregate at a site of vessel injury.

Microvesicle shedding from adherent platelets was examined under a variety of conditions. Flow cytometry demonstrated that significant levels of microvesicles were shed only from adherent platelets on stimulation with a potent physiological agonist such as thrombin or the nonphysiological agonist calcium ionophore A23187. The adherence event did not induce measurable microvesicle release from the platelet membranes even when collagen, a platelet agonist, was used as the surface coating. Subsequent to thrombin stimulation of the vWF- or collagen-adherent platelets, approximately 8% of the total platelet mass was released as microvesicles. The majority of the platelet membranes remained associated with the surface.

Because microvesicles released from platelets in suspension have been reported to contain significant amounts of the vWF-binding glycoproteins GPIb and GPIb-IIIa, it was possible that microvesicles were not only released from the platelet membranes, but also bound to the vWF coating as well. The platelet and vWF surfaces were examined by scanning electron microscopy to determine if microvesicles were indeed present. Microvesicles did not appear to attach to the adherent platelets, and few were bound to the exposed vWF multimers between the platelets. Adherence did not seem to be a primary function of microvesicles shed from thrombin-stimulated vWF-adherent platelets. Because the microvesicles were instead released from the platelet membrane and supporting surface, the mass percent of microvesicle release determined by flow cytometry was an accurate measure of microvesicle shedding by the adherent platelets.

The function of microvesicles released from the platelet surface, in vivo or in vitro, is currently unknown. Studies of microvesicles generated from platelets in suspension suggest that the microvesicles support high levels of procoagulant activity. Examination of the procoagulant capability of microvesicles released from thrombin-stimulated collagen- or vWF-adherent platelets showed that >85% of the total prothrombinase activity remained associated with the membranes of the adherent platelets. Analysis of unstimulated adherent platelets showed that the remaining activity, localized in the buffer fraction, could be attributed to detached platelets. Because there was no increase in prothrombinase activity in the buffer fraction from thrombin-stimulated adherent platelets (containing microvesicles and detached platelets) over the activity in the buffer fraction from unstimulated adherent platelets (containing detached platelets but no detectable levels of microvesicles), the microvesicles released from the stimulated adherent platelets did not appear to support significant procoagulant activity. (The number of detached platelets did not vary between samples of unstimulated adherent platelets and thrombin-stimulated adherent platelets.) Under these conditions, which mimic those during the initial hemostatic response of platelets to vascular injury, microvesicle release was not essential in the expression of platelet procoagulant activity.

In the case of adherent platelets, this raises questions as to the validity of reports suggesting a link between platelet activation, release of microvesicles, and expres-
sion of platelet procoagulant activity. TIRFS binding studies demonstrated that adherent platelets and thrombin-stimulated adherent platelets expressed equivalent numbers of prothrombinase binding sites. Activity measurements in this study showed that vWF-adherent platelets, which were not fully activated and released no microvesicles on adherence, supported prothrombinase activity equivalent to that supported by thrombin-stimulated vWF-adherent platelets. Although the vWF-adherent platelets would have become fully activated during the experiment as thrombin was converted to thrombin, the initial activity measurements would have been lower than those seen for thrombin-stimulated vWF-adherent platelets if activation and microvesicle release were vital in expression of functional prothrombinase binding sites on the platelet surface. The generation of thrombin by the unstimulated vWF-adherent platelets would have lagged behind the values measured for thrombin-stimulated vWF-adherent platelets if platelet activation was key in promoting procoagulant activity in adherent platelets. Under the conditions studied, no link between platelet activation, release of microvesicles, and expression of sites for functional prothrombinase complex assembly was demonstrated. Adherence to vWF, which does not promote full platelet activation, was sufficient to induce exposure of functional binding sites for the prothrombinase complex. Although microvesicles were released as a consequence of thrombin stimulation of adherent platelets, they did not appear to serve in a procoagulant capacity and were not associated with exposure of prothrombinase binding sites on the platelet membranes. These results answer the question raised in the TIRFS study of prothrombinase complex assembly on adherent platelets. Complex assembly was monitored via an energy transfer phenomenon between fluorescein-labeled factor V and rhodamine-labeled factor Xa. Prothrombinase assembly could be demonstrated on both unstimulated and thrombin-stimulated vWF-adherent platelets. The 7% decrease in the energy transfer signal subsequent to thrombin stimulation of the platelets was attributed to competition for labeled factor Xa between the labeled factor Va and unlabeled platelet-released factor Va. (No energy transfer signal would be detected for the binding of labeled factor Xa to unlabeled factor Va.) In this study, no microvesicle release was detected from platelets on adherence, and prothrombinase complex assembly on microvesicles was not detected by activity assay; hence, the decrease in the energy transfer signal is not likely to be due to microvesicle shedding on platelet stimulation. When platelets are allowed to adhere to a surface, the function of microvesicles shed from platelet membranes still remains unclear. They do not appear to serve in a procoagulant capacity, nor do they seem to be associated with exposure of binding sites for prothrombinase complex on the platelet membrane surface. Adherent and thrombin-stimulated adherent platelets support equivalent assembly of functional prothrombinase complexes, demonstrated by both TIRFS binding studies and activity assays. In this model system, full platelet activation is not required for procoagulant activity. It appears likely that platelets that adhere at a site of vascular damage may promote prothrombinase activity before the generation of thrombin. Activation of thrombin by the complexes would induce additional platelet adherence and aggregation during the repair process. Because platelet-released microvesicles do not appear to play a role in this process, the adherent platelet membranes would be the focal point of procoagulant enzyme complex activity. Surface-dependent coagulation would serve to localize clot formation through specific binding interactions among platelets, exposed subendothelial components, and plasma coagulation factors. In turn, activity would be amplified due to generation of high local concentrations of procoagulant enzymes. Shedding of procoagulant microvesicles would only lead to dissemination of clotting activity. This process may occur in some disease states, but it does not appear to occur under the in vitro conditions that more closely mimic the initial injury repair stage of normal hemostasis, ie, the deposition phase of platelet adherence to subendothelial components.

Acknowledgments
This work was supported by research grants PO1-HL46703 and RO1-H16973 and Hemostasis Training Grant T32-HL07594 from the National Heart, Lung, and Blood Institute. We thank Gregory Hendricks and Jamie Levis, who kindly prepared and analyzed the platelet samples for electron microscopy. We also thank Debra Allen for her help with the prothrombinase activity assay.

References
Intact platelet membranes, not platelet-released microvesicles, support the procoagulant activity of adherent platelets.

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doi: 10.1161/01.ATV.13.11.1613

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