Morphological analysis of microparticle generation in heparin-induced thrombocytopenia

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Heparin-induced thrombocytopenia (HIT) with thrombosis is a serious complication of heparin use. HIT sera can generate platelet-derived microparticles, which are produced in a heparin-dependent manner and are hypothesized to be important initial pathological participants because they promote vascular occlusion. To date, microparticles have been studied using flow cytometric techniques. However, it is uncertain whether the small-sized material seen in flow cytometric studies represents true platelet microparticles shed from activated platelets or whether they are platelets that have contracted after releasing their internal components. This report describes a morphological investigation of platelet-derived microparticles in HIT using, among other techniques, confocal, scanning electron, and transmission electron microscopy. Following incubation with HIT sera, the existence of small membrane-bound vesicles in the milieu of activated platelets was demonstrated. A population of microparticles, expressing platelet-specific glycoproteins, was separated from platelets by centrifugation over a sucrose layer. These microparticles had identical flow cytometric profiles, size heterogeneity, and GPIb$_{\alpha}$ and GPIIb/IIIa staining intensity as the microparticle population in unfractionated samples. When microparticles were generated in situ and fixed onto grids, they were demonstrated to be distinct membrane-bound vesicles that originated near the platelet body and terminal ends of pseudopods on activated platelets. These microparticles appeared to be generated by localized swelling, budding, and release. Collectively, these morphological studies document the existence of true microparticles in platelets activated by HIT sera. The microparticles may play an important role in the pathogenesis of HIT. (Blood. 2000;96:188-194)

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Introduction

Heparin-induced thrombocytopenia (HIT), one of the most common immune-mediated adverse drug reactions, occurs in 1%-3% of patients receiving therapeutic doses of heparin.[1,2] Unlike other immunological drug-induced reactions, many patients with HIT experience serious thrombotic morbidity including venous thrombosis, arterial thrombosis, and disseminated intravascular coagulation (DIC).[1-3] Studies of the pathogenesis of this prothrombotic condition demonstrated that circulating heparin-dependent antibodies bind to a complex of heparin and platelet factor 4 (PF4).[4-8] Several years ago, we demonstrated that the binding of heparin-dependent immunoglobulin G (IgG) to platelet FcγRII receptors leads to platelet activation[9,10] and the generation of platelet-derived microparticles.[11] These microparticles were produced in a heparin-dependent fashion and were shown to have procoagulant activity.[11,12] Although microparticles are thought to be initial participants in venoocclusive events, their mechanism of formation and confirmation of existence are still a matter of controversy.

Presently, flow cytometry is the most frequently reported technique used to study platelet-derived microparticles, and it has been used by our group to develop a diagnostic test for heparin-induced thrombocytopenia.[13] However, some investigators have raised questions about the analysis of flow cytometry experiments. Studies by Bode et al[14] showed that the light scatter distribution of platelets is broad and that it is difficult to identify clearly where the population of intact platelets ends and the population of smaller particles begins. This observation of a continuum of particle size rather than 2 distinct platelet and microparticle populations questioned the nature of microparticles and their degree of heterogeneity. Studies by Matzdorff et al[15] demonstrated that counting microparticles becomes unreliable when platelet counts drop to a low number. This was thought to be explained by the fact that saturating amounts of antibodies may become unspecifically adsorbed to other particles or may form antibody complexes. The observations that an antibody surplus leads to antibody complexes and that these complexes can interfere with platelet and microparticle counting have also been reported by other groups.[16,17] Finally, George et al[18] demonstrated that microparticle preparations derived from washed activated platelets contained a heterogeneous array of membrane fragments, vesicles, and granules. These observations suggested that morphological documentation of microparticles in heparin-induced thrombocytopenia was necessary for the confirmation of their existence.

In previous studies, immunoassays and flow cytometry studies demonstrated procoagulant properties of microparticles but failed to provide information on their structural origin or mechanism of generation. In this study we conducted a morphological analysis of platelet-derived microparticles to document the existence of microparticles in heparin-induced thrombocytopenia. Several different

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techniques were used including flow cytometry, confocal microscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) to provide a network of support for the existence of true microparticles in heparin-induced thrombocytopenia. Through a novel approach of activating platelets directly on EM grids with heparin-induced thrombocytopenia sera, we visually documented the structural origin of microparticles in situ. Finally, microparticles from platelet reactions were isolated by centrifugation over a sucrose layer. We were able to demonstrate that microparticles are physically distinct from platelets and have scatter plot displays, size heterogeneity, and staining intensity similar to that of microparticles in unfractionated platelet reaction mixtures.

Patients, materials, and methods

Patient samples

This study was approved by the University and Hospital Ethics Review Committee at McMaster University Medical Center, Hamilton, Ontario, Canada. Patient and control samples were collected according to the university-approved Ethics Review Committee recommendations, and all entrants provided signed consent.

Studies were performed using sera from patients with HIT who met the clinical criteria for diagnosis as described previously.[9] In all patients, the diagnosis of HIT was confirmed using the ^{14}C-serotonin platelet release assay.[9,19] Control sera were obtained from patients who tested negative for HIT using the ^{14}C-serotonin platelet release assay and from healthy aspirin-free volunteers. All samples were heat-inactivated for 30 minutes at 56°C, centrifuged at 8000*g* for 10 minutes, and frozen at −70°C until use.

Materials

All materials were purchased from Sigma Chemical Co (St Louis, MO) unless otherwise stated. Where applicable, brand names are noted in parentheses.

Antibodies

Flow cytometry studies were performed using the platelet-specific monoclonal antibody (mAb) anti-GPIb$_\alpha$ (TW-1).[20] This antibody was conjugated directly with fluorescein isothiocyanate (FITC). Confocal microscopy studies were performed using the platelet-specific mAbs anti-GPIb$_\alpha$ (TW-1) and anti-GPIIb/IIIa (Raj-1).[20] The secondary antibody used in these studies was a Texas Red–conjugated (TR-conjugated) goat antimouse IgG antibody (Jackson Immuno-Research Laboratories, West Grove, PA).

Platelet ^{14}C-serotonin release assay

Diagnostic testing for HIT was performed using the ^{14}C-serotonin release assay as previously described.[9,19] All tests were performed in triplicate.

Isolation of platelets

Whole blood was collected from healthy aspirin-free volunteers and mixed with acid citrate dextrose (ACD, 6:1, vol/vol [pH 4.5]) containing 1 mmol/L theophylline and 1 µg/mL prostaglandin E_1. Platelets were isolated by differential centrifugation at 160*g* for 20 minutes and washed once in calcium- and albumin-free Tyrode's solution (pH 6.2) containing apyrase. For microparticle generation studies, washed platelets were resuspended in albumin-free Tyrode's solution containing calcium and magnesium (pH 7.4).

Preparation of platelets

In 12 × 75-mm round-bottom polystyrene tubes (Becton Dickinson, Lincoln Park, NJ), 0.7 mL washed platelets (300 × 10^9 cells per L) were added to 0.2 mL test serum and 0.1 mL buffer, 0.1-0.3 U/mL final concentration of heparin (Leo Laboratories, Ajax, Ontario, Canada), 0.1-1.0 U/mL final concentration of thrombin,[3] or 10 µm calcium ionophore A23187.[4] Following a 1-hour incubation, without stirring or shaking, at room temperature, the reaction was stopped by adding an equal volume of 1% paraformaldehyde (PFA) (wt/vol) in phosphate-buffered saline (PBS)(pH 7.4) for 1 hour at room temperature.

For flow cytometry studies, samples were diluted 1:2 with filtered 0.22 µm PBS (pH 7.4) containing 0.2% glycine (wt/vol). A 100-µL sample of PFA-fixed platelets was incubated with 10 µL FITC-labeled anti-GPIb$_\alpha$ (20 µg/mL) for 1 hour at 37°C. Following incubation, samples were further diluted with 0.25-mL filtered PBS (pH 7.4) and kept in the dark until fluorescence activated cell sorter (FACS) analysis (FACScan; Becton Dickinson, San Jose, CA).

For confocal microscopy studies, PFA-fixed samples were washed with PBS/glycine buffer (pH 7.4), centrifuged at 2000*g* for 15 minutes, and resuspended in 3% bovine serum albumin (BSA)/PBS blocking buffer (pH 7.4) containing 50 µg/mL normal mouse IgG. A 0.5-mL sample of PFA-fixed platelets was incubated with anti-GPIb$_\alpha$ (1/100) or anti-GPIIb/IIIa (1/100) for 2 hours at 37°C. The platelets were washed once with blocking buffer and incubated with TR-conjugated goat antimouse IgG secondary antibody (1/100). Following a 2-hour incubation at 37°C, the platelets were washed, spread onto glass coverslips, and analyzed using confocal microscopy. For all confocal microscopy studies, samples were kept in the dark until analysis.

Flow cytometry

Platelet-derived microparticles were identified using a FACScan. FITC fluorescence was detected using a 530-nm bandpass filter. Data for forward light angle scatter (FCS), side angle scatter (SSC), and FITC fluorescence were obtained with gain settings in the logarithmic mode. For each sample, 5000 events were acquired. Microparticles were distinguished from platelets on the basis of their characteristic flow cytometric profile of FCS versus FITC fluorescence. Analysis of the fluorescence histograms (counts vs fluorescence) was used to quantitate platelet microparticles, which were defined as GPIb$_\alpha$-positive events that exhibited less fluorescence than 95%-99% of nonactivated FITC GPIb$_\alpha$-labeled platelets. This setting gave background microparticle levels of less than 6% in control samples.

Confocal microscopy

Platelets and platelet-derived microparticles were identified using the platelet-specific mAbs, anti-GPIb$_\alpha$ and anti-GPIIb/IIIa, and a TR-conjugated goat antimouse IgG secondary antibody. Confocal microscopy was performed with a Universal Confocal Laser Scan Research Microscopy System (Carl Zeiss, Oberkochen, Germany), objective magnification × 100, and individual excitation lasers and filters for the TR fluorochrome. Controls included platelets incubated with and without antibody, normal mouse IgG, or primary antibody with an irrelevant fluorescent secondary antibody. Acquired images were imported into Micrografx Picture Publisher (version 7, Micrografx Inc, Richardson, TX).

Electron microscopy

Using 12 × 75-mm round-bottom polystyrene tubes (Becton Dickinson), 0.7 mL washed platelets (resuspended in albumin-free Tyrode's solution containing calcium and magnesium; pH 7.4) at a concentration of 300 × 10^9 cells per L were added to 0.2 mL heat-treated test serum and 0.1-mL buffer or heparin at a final concentration of 0.1-0.3 U/mL. Following a 1-hour incubation, without stirring or shaking, at room temperature, the reaction was stopped by adding an equal volume of 1% glutaraldehyde (wt/vol) in 0.1 mol/L phosphate buffer (pH 7.2) for 1 hour at room temperature. Samples were pelleted at 2000*g* for 15 minutes, embedded in glycolmethacrylate, and processed for routine TEM. Thin platelet sections were cut on a Reichert-Jung Ultracut ultramicrotome (Leica AG, Vienna, Austria), contrast-stained with uranyl acetate and lead citrate, and analyzed under a JEOL 1200EX Transmission Electron Microscope (Tokyo, Japan).

The washed platelets were resuspended in albumin-free Tyrode's solution containing calcium and magnesium (pH 7.4) and incubated with a heat-treated test serum and buffer or heparin at a final concentration of 0.1-0.3 U/mL. After incubation for 30 minutes at room temperature, the samples were fixed with 2% glutaraldehyde (wt/vol) in phosphate buffer (pH 7.2). A drop of the platelet suspension was allowed to settle on a

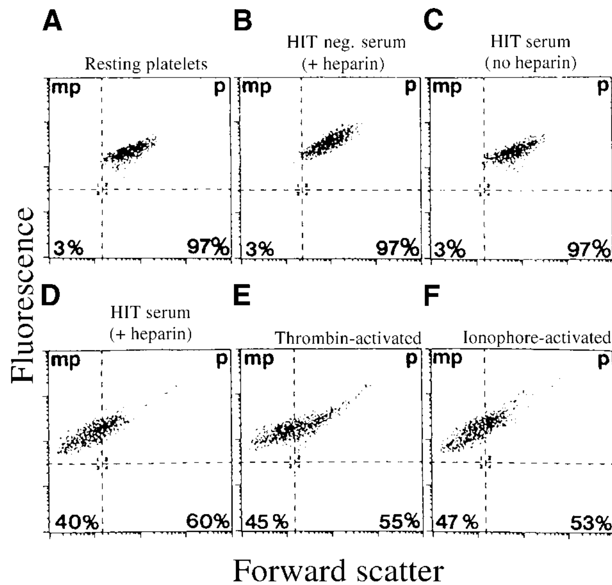

Figure 1. Flow cytometric analysis of platelets activated with HIT sera, thrombin, and calcium ionophore. Platelets (p) and microparticles (mp) were identified using fluorescence (FL1, FITC anti-GPIbα) (y-axis) and size (FSC) (x-axis) characteristics. Control platelets included; (A) platelets incubated in buffer alone; (B) platelets incubated with patient serum, which tested negative for HIT, in the presence of 0.1 U/ml heparin; and (C) platelets incubated with HIT serum with no heparin added. (D) Platelets were incubated with HIT serum in the presence of 0.1 U/ml heparin. (E) As a positive control, platelets were also incubated with 1 U/mL thrombin or (D) 10 µm calcium ionophore A23187. Microparticles (MP) were generated with heparin-induced thrombocytopenia serum, thrombin, and calcium ionophore and not with the control serum. The percent of microparticles (the percent of fluorescent events in the microparticle gate) generated by HIT serum was 40%; thrombin, 45%; and calcium ionophore, 47%. The results of a representative experiment are shown.

poly-L-lysine–coated glass coverslip. Samples were postfixed in 1% osmium tetroxide, dehydrated in graded alcohol, and dried by the critical-point method. The dried samples were sputter-coated with gold and observed under a JEOL 1200EX Scanning Electron Microscope.

A 5-µL sample of washed platelets was then resuspended in albumin-free Tyrode's solution containing calcium and magnesium (pH 7.4). Using a pipette, the platelets were gently put onto a BSA-precoated formvar grid and allowed to settle undisturbed for 2 minutes. A 3-µL sample of test serum and 2 µL buffer,[1] 0.1-0.3 U/mL final concentration of heparin,[2] 0.1-1.0 U/mL final concentration of thrombin,[3] or 10 µL calcium ionophore A23187[4] were then gently put onto the grid with a pipette. Following a 5- to 20-minute incubation at room temperature, the reaction was stopped by the addition of 2% glutaraldehyde (wt/vol) in phosphate buffer (pH 7.2). Samples were negatively stained with 2% phosphotungstic acid and analyzed under a JEOL 1200EX Transmission Electron Microscope.

Density centrifugation

Microparticles were isolated from activated platelets by sedimenting platelet reactions across a sucrose layer according to Pasquet et al.[21,22] This procedure is a modification of the procedure used in binding experiments to separate platelet-bound cells from unbound ligands.[23] Briefly, PFA-fixed platelet reactions were centrifuged at 1000*g* for 10 minutes to pellet the bulk of the platelets. The supernatant was layered onto 5 mL 27% sucrose (wt/vol), prepared in assay buffer, and centrifuged at 2000*g* for 10 minutes. The residual platelets sedimented through the sucrose, and the upper phase, containing the microparticles, was harvested for flow cytometry and confocal microscopy analysis.

For flow cytometry studies, samples were diluted 1:2 with filtered PBS (pH 7.4) containing 0.2% glycine (wt/vol). A 100-µL sample was incubated with 10-µL FITC-labeled anti-GPIb$_\alpha$ at a concentration of 20 µg/mL for 1 hour at 37°C. Following incubation, the samples were further diluted with 0.25 mL filtered PBS (pH 7.4) and analyzed by FACS analysis.

For confocal microscopy studies, PFA-fixed samples were washed with PBS/glycine buffer (pH 7.4), centrifuged at 15 000*g* for 15 minutes, and resuspended in 3% BSA/PBS blocking buffer (pH 7.4) containing normal mouse IgG at a concentration of 50 µg/mL. A 0.5-mL sample was incubated with anti-GPIIb/IIIa (1/100) or anti-GPIb$_\alpha$ (1/100) for 2 hours at 37°C. Samples were washed once with blocking buffer and incubated with a TR-conjugated goat antimouse IgG secondary antibody (1/100). Following a 2-hour incubation at 37°C, samples were washed and spread onto glass cover slips. The samples were kept in the dark until confocal microscopy analysis.

Results

Flow cytometry was used to detect platelet-derived microparticles following incubation of normal washed platelets with patient sera in the presence or absence of heparin. Microparticles were distinguished from platelets on the basis of size and relative fluorescence of an FITC-conjugated antibody to platelet membrane GPIb$_\alpha$ (Figure 1). Briefly, the FL1/FSC (fluorescence of FITC anti-GPIb$_\alpha$/

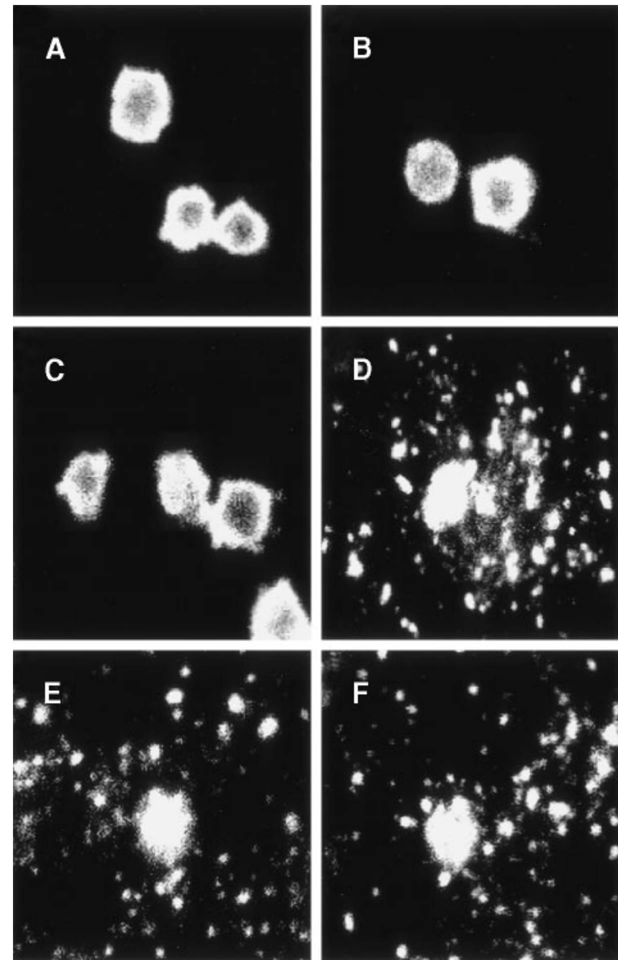

Figure 2. Confocal microscopy images of microparticles generated by HIT serum, thrombin, and calcium ionophore. Platelets and microparticles were identified using a platelet-specific primary antibody (mAb anti-GPIIb/IIIa) and a fluorescent secondary antibody (TR-conjugated goat antimouse). (A) Control platelets were incubated with patient serum, which tested negative for HIT in the presence of 0.1 U/mL heparin, (B) heparin alone, and (C) HIT serum in the absence of heparin. (D) Platelets were incubated with HIT serum in the presence of 0.1 U/mL heparin. As a positive control, (E) platelets were also incubated with 1 U/mL thrombin or (F) 10 µm calcium ionophore A23187. Platelet reactions with HIT serum in the presence of heparin demonstrated numerous brightly stained particles surrounding homogeneously stained platelets. This "starry sky pattern" was also observed in platelet reactions with thrombin and calcium ionophore. In comparison, very few stained particles were observed in platelet reactions with control serum or heparin alone. The results of a representative experiment are shown.

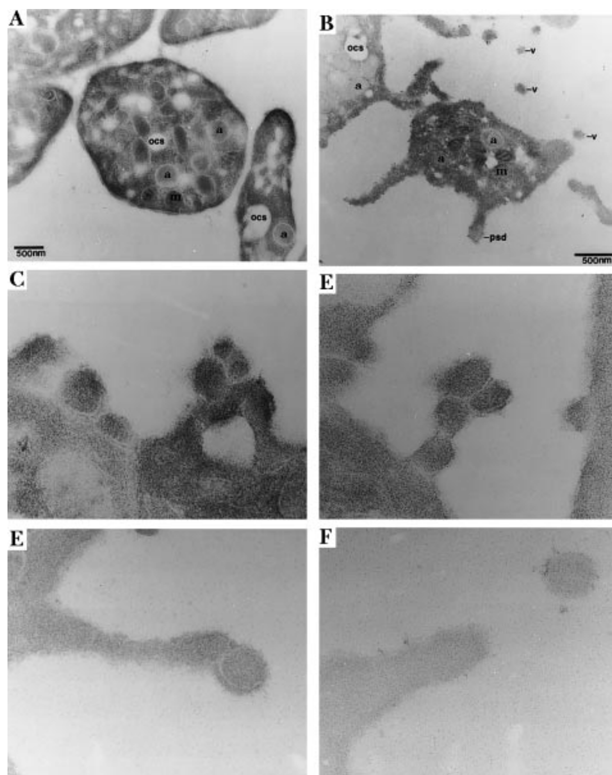

Figure 3. Representative transmission electron micrographs of resting platelets and platelets incubated with HIT serum. Resting platelets or platelets incubated with HIT serum in the presence of 0.1 U/mL heparin were fixed with 0.5% glutaraldehyde and embedded in glycolmethacrylate. (A) The morphology of a representative normal resting platelet. Alpha granules (a), mitochondria (m), and channels of the open-cannalicular system (ocs) can be seen throughout the platelet. The platelets appear round or discoid in shape, and there is an absence of pseudopodia. (B-F) The morphology of representative platelets incubated with HIT serum in the presence of heparin. Platelets are activated with a centralized clustering of alpha granules (a) and mitochondria (m). (B) Pseudopodia (psd) can be seen extending from the platelet body, and several distinct membrane-bound structures resembling small vesicles (v) are observed near the platelet. Numerous pseudopodia demonstrated a region of bulging (E) at the terminal end of the pseudopod or (C) along the body of the pseudopod. Frequently, several areas of bulging were observed on (D) the same pseudopod and (C) the platelet body itself. (F) In these platelets, microparticles appeared to be released by the budding of pseudopods. (Original magnification (A, B) × 15 000; (C, D) × 25 000; and (E, F) × 50 000.)

forward lightscatter) representation of platelets activated with HIT sera (n = 3) showed 2 populations: (1) a major population, corresponding to platelets, and (2) a smaller second population, corresponding to the microparticle fraction defined in previous studies.[13] This second population was absent in platelets incubated with control sera (n = 3) or heparin alone. Microparticle generation by thrombin and calcium ionophore produced a population of particles similar to that observed for HIT sera. Based on fluorescence histograms (data not shown), the percentage of microparticles generated from control sera was less than 5% in comparison with 40% for HIT sera, 45% for thrombin, and 47% for calcium ionophore.

Although flow cytometry demonstrated that significant levels of microparticles were generated from platelets incubated with HIT sera, it was not clear whether microparticles were a population of compressed smaller platelets or true microparticles. To resolve these issues, we used several additional techniques including confocal microscopy, TEM, and SEM to visually analyze the morphology of microparticles generated by HIT sera.

Confocal microscopy studies

In these studies, microparticles were distinguished from platelets on the basis of size. Platelet microparticles were defined as GPIIb/IIIa$^+$ particles, which were less than 0.1 to 1.0 μm in diameter. Platelet reactions with HIT sera in the presence of heparin demonstrated numerous brightly stained particles surrounding homogeneously stained platelets (Figure 2). This "starry sky pattern" was also observed in platelet reactions with thrombin and calcium ionophore. In comparison, very few stained particles were observed in platelet reactions with control sera. Confocal analysis of the microparticles generated by HIT sera, thrombin, or calcium ionophore did not demonstrate apparent differences in their size heterogeneity or the absolute numbers of microparticles generated. Furthermore, there was no apparent difference in staining intensity of their particles using antibodies for GPIb$_\alpha$ or GPIIb/IIIa. Although these studies suggested that platelet microparticles were generated by activating platelets with HIT sera, electron microscopy was used to further investigate the morphology of the microparticles and the mechanism of their generation.

Electron microscopy studies

TEM was used to study the ultrastructure of platelets incubated with HIT sera. Figure 3 shows representative TEM pictures of platelets incubated with control serum or platelets incubated with HIT serum in the presence of heparin. Platelets from control reactions, where there was no stimulus for platelet activation, were discoid in shape. This morphology is characteristic of resting platelets. In comparison, platelets activated by HIT sera displayed numerous pseudopodia and loss of their resting discoid shape. Separate from the pseudopods, there appeared to be some discrete

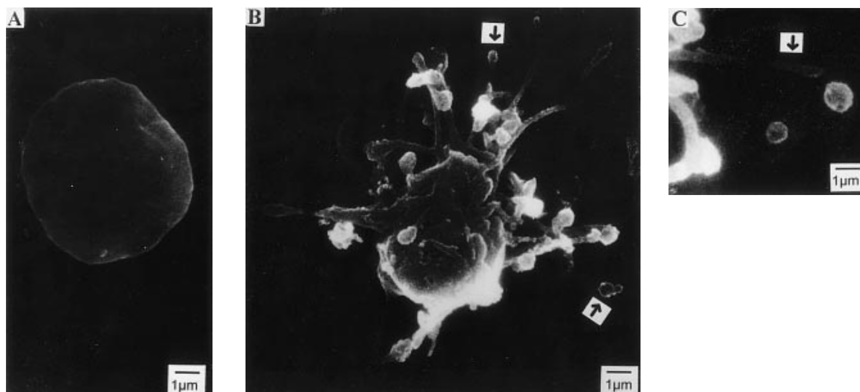

Figure 4. Representative scanning electron micrographs of resting platelets and platelets activated with HIT serum in the presence of heparin. Resting platelets or platelets incubated with HIT serum in the presence of 0.1 U/mL heparin were fixed with 2% glutaraldehyde and processed for SEM. (A) The morphology of a representative normal resting platelet. Resting platelets were observed to generally maintain a discoid form. (B) The morphology of a representative platelet incubated with HIT serum. These platelets demonstrated several morphological changes including absence of a discoid form,[1] presence of pseudopodia,[2] and presence of microparticles near the ends of pseudopodia[3] (indicated by arrows). (C) Microparticles that are clearly distinct and separate from the platelet body localize near the terminal end of a pseudopod (indicated by arrow).

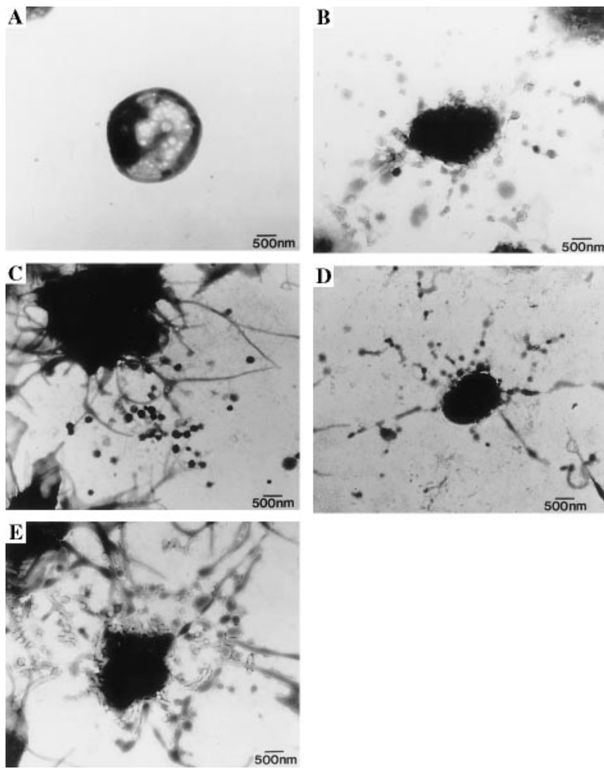

Figure 5. Electron micrographs of negatively stained platelets activated in situ with HIT sera. Platelets were allowed to settle on BSA-coated formvar grids and then incubated with patient serum or platelet agonists. (A) Control platelets were incubated with patient serum, which tested negative for HIT, or heparin alone (data not shown). (B) Platelets were inoculated with HIT serum in the presence of 0.1 µ/mL heparin. As a positive control, platelets were also incubated with (C) 1 U/mL thrombin or (D) 10 µm calcium ionophore A23187. Platelets were then fixed with 2% glutaraldehyde and negatively stained with 2% phosphotungstic acid. (A) Control platelets demonstrated a round or discoid shape, which is characteristic of resting platelets. (B) Platelets incubated with HIT serum demonstrated numerous microparticles surrounding the platelet body. Frequently, these platelets were observed with pseudopodia extending from the platelet body. Microparticles ranged in size from less than 0.1 to 1.0 µm in diameter and appeared as discrete membrane-bound particles. Platelets incubated with (C) thrombin and (D) calcium ionophore demonstrated a similar platelet morphology, with microparticles surrounding the platelet body. (Original magnification (A-E) × 13 000.) (E) These particles were observed to be near the terminal ends and body of the pseudopods, and numerous points of bulging or swelling along the body and terminal ends of the pseudopodia were observed.

small particles that were located adjacent to platelets and pseudopodia. Numerous pseudopodia demonstrated a region of bulging at their terminal end or along their body, suggesting these structures might be the origins of microparticles. Areas of bulging were observed on both pseudopods and the central platelet body. To address the issue that microparticles might correspond to transverse sections of pseudopods in these studies, using SEM, we further investigated the morphology of platelets activated with HIT sera. In these studies, the entire platelet was viewed, and cross-sectioning was not performed.

SEM studies (Figure 4) demonstrated that platelets incubated with control sera or heparin alone (not shown) maintained a discoid shape. These morphological observations were consistent with those of resting platelets observed in TEM studies. Platelets activated with HIT sera demonstrated morphological changes consistent with those observed in cross-sectioned platelets including absence of a discoid form; presence of pseudopodia; and presence of microparticles, in which no linkage to a platelet body or pseudopod could be discerned. These same morphological changes were also observed in platelets activated with thrombin and calcium ionophore (data not shown).

In all of the previously described methods, some washing steps were required as part of the platelet processing protocol. To address the issue that some microparticles may have been lost in these washing steps, we analyzed negatively stained platelets, which were activated and fixed directly onto electron microscopy grids. In this technique, washing steps were not used. Platelets incubated with control sera (Figure 5) or therapeutic concentrations of heparin alone (data not shown) appeared round or discoid in shape, which is consistent with a resting state. When platelets were activated directly on formvar-coated grids with HIT sera, formed microparticles were observed. These platelets demonstrated morphological changes that were consistent with those observed in TEM and SEM studies. Frequently, these platelets were observed with pseudopodia extending from the platelet body and with numerous microparticles surrounding the pseudopods and platelet body. These microparticles ranged in size from less than 0.1 to 1.0 µm in diameter and appeared as discrete membrane-bound particles. Microparticles were observed to be localized near the terminal ends and body of pseudopods, often near points of bulging or swelling.

Platelet reactions with thrombin and calcium ionophore demonstrated a similar phenomenon, with numerous microparticles surrounding the platelet body. The apparent morphology or size of

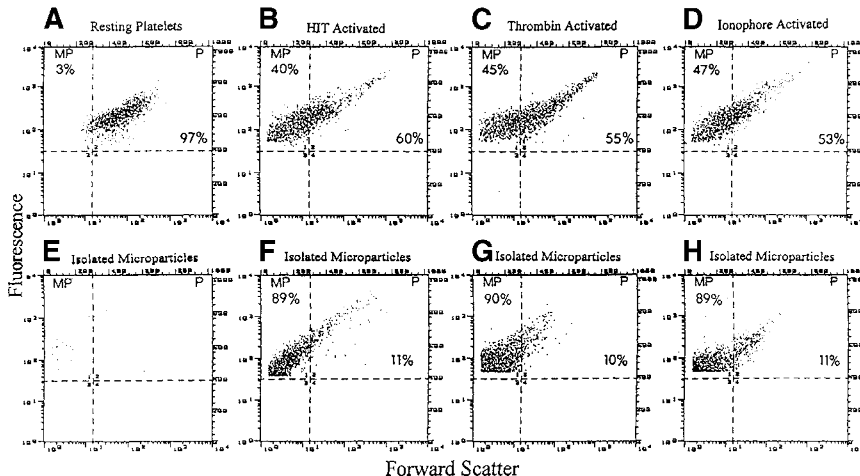

Figure 6. Flow cytometric analysis of isolated microparticles from platelets activated with HIT sera, thrombin, and calcium ionophore. Very few microparticles were observed in control reactions, in which platelets were incubated with patient serum that tested negative for (E) heparin-induced thrombocytopenia or heparin alone (data not shown). Microparticles (lower row) were identified using fluorescence (FL1, FITC anti-GPIbα) (y-axis) and size (FSC) (x-axis) characteristics. Microparticles isolated from platelets incubated with (F) heparin-induced thrombocytopenia serum, (G) thrombin, and (H) calcium ionophore demonstrated similar light scatter profiles as microparticles in unfractionated platelet reactions (upper row). The results of a representative experiment are shown.

microparticles did not appear to differ, whether microparticles were generated from HIT sera, thrombin, or calcium ionophore. Furthermore, some microparticles were observed to be clustered together at points, suggesting that microparticles may be integrated into aggregates upon release.

Microparticle isolation studies

We have described a simple procedure to isolate microparticles from platelets by sedimenting the latter across a sucrose layer. These studies were performed to further confirm that microparticles are a distinct population and not connected to activated platelets. When microparticle-rich fractions were isolated from platelet reactions incubated with HIT sera, FACS analysis confirmed that the supernatant contained very few contaminating platelets. Furthermore, isolated microparticles and microparticles in unfractionated reactions had identical physical properties: scatter plot displays (Figure 6), size heterogeneity (Figure 7), and GPIb$_\alpha$ and GPII/IIIa staining intensity (Figure 7). The observation that very few microparticles were isolated from platelets incubated with control serum or heparin alone demonstrated that the sucrose isolation procedure did not generate artefactual microparticle formation during the centrifugation step. These studies demonstrated that microparticles were physically distinct from platelets, as evidenced by their ability to be isolated from platelet reaction mixtures.

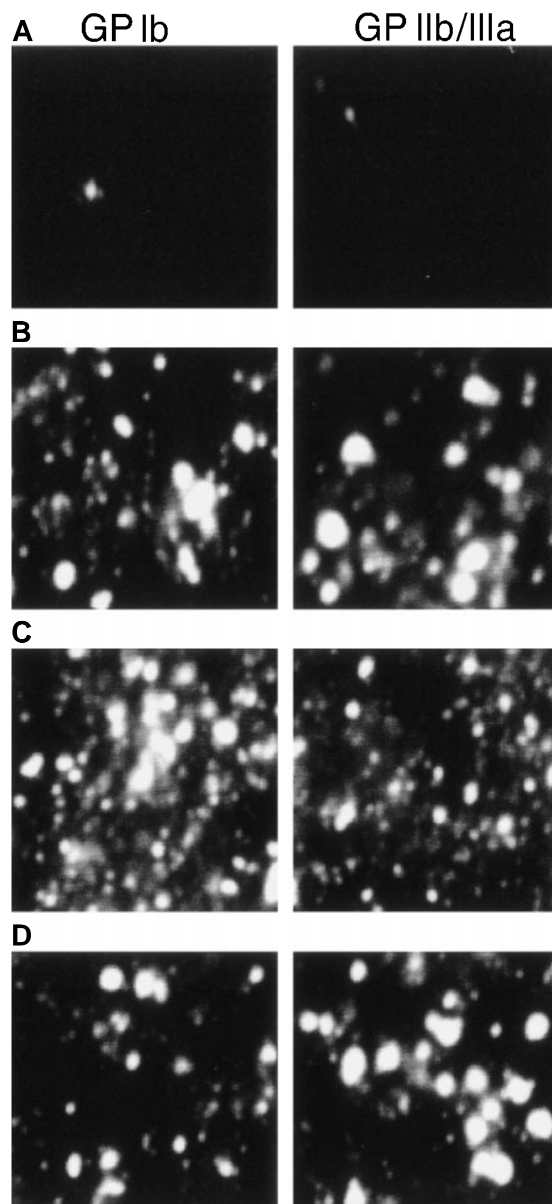

Figure 7. Confocal microscopy images of isolated microparticles from platelets activated with HIT sera, thrombin, and calcium ionophore. Microparticles were identified using platelet-specific primary antibody (mAb anti-GPIb and anti-GPIIb/IIIa) and a fluorescent secondary antibody (TR-conjugated goat antimouse). (A-D) Isolated microparticles following centrifugation of the platelet reaction over a sucrose layer. Very few microparticles were isolated from control reactions, in which platelets were incubated with patient serum that tested negative for (A) heparin-induced thrombocytopenia or heparin alone (data not shown). Microparticles isolated from platelets incubated with (B) heparin-induced thrombocytopenia serum, (C) thrombin, and (D) ionophore were less than 0.1 to 1.0 μm in diameter and demonstrated a similar degree of size heterogenosity and staining intensity as microparticles in unfractionated platelet reactions (not shown). The results of a representative experiment are shown.

Discussion

Several years ago, we demonstrated that the binding of heparin-dependent IgG to platelet FcγRII receptors lead to platelet activation[9,10] and the generation of platelet-derived microparticles.[11] These microparticles were produced in a heparin-dependent fashion and were shown to have procoagulant activity, as demonstrated by coagulation[12] and amidolytic assays.[11] Using flow cytometry, increased levels of circulating microparticles have also been observed in patients with acute HIT.[11] The observation of small numbers of microparticles in the plasma of normal human blood[14,24,25] and in increased amounts in HIT has led to their diagnostic measurement.[13] Although these microparticles are thought to be distinct from those of activated platelets, their true existence has not been morphologically confirmed. In this study, we document for the first time that true microparticles are generated from HIT sera and that this microparticle population is separate and distinct from that of activated platelets.

Several of the experimental conditions and techniques used in this study provide important morphological evidence that true microparticles are generated when platelets are activated by HIT sera. Using negative staining, confocal microscopy, TEM, and SEM, we documented consistent morphological observations. First, when platelets were activated with HIT sera, they lost their discoid shape, and pseudopodia were observed. Second, numerous sites of swelling were observed on both the platelet body and pseudopodia, and small discrete membrane-bound particles were frequently observed near these sites. Using SEM and TEM on unfractionated platelet reaction mixtures, we were able to confirm that a generation of microparticles are not attached to the platelet body or to pseudopodia. The microparticles generated by HIT sera ranged in size from less than 0.1 to 1.0 μm in diameter. These microparticles could be physically isolated from platelet reaction mixtures by centrifugation over a sucrose gradient, and they were indistinguishable from the population of microparticles in unfractionated platelet reaction mixtures in their size, membrane glycoprotein expression, and physical properties.

Based on the morphological observations associated with microparticle generation, we hypothesize that microparticle shedding in HIT involves (1) formation of localized points of swelling on the platelet body, pseudopod body, or terminal pseudopod tips; (2) further development of these points of swelling into well-defined buds; and (3) transformation of buds into microparticles and concomitant release.

The clinical significance of microparticles in HIT has not been

established. There is now evidence to suggest that the incidence of HIT depends on the clinical context of the patients receiving heparin therapy and the duration of heparin exposure.[26] This incidence appears to be greater in older patients with underlying hemostatic activation such as inflammation, orthopedic trauma, or cardiopulmonary bypass surgery.[26] In this context, the presence of activated platelets appears to favor the development of HIT.[26] In our study we noted that microparticles generated from HIT sera, thrombin, or calcium ionophore were quite similar, irrespective of the agonist. These results suggest that the process of microparticle formation may involve a similar mechanism irregardless of the agonist. Recent studies suggest that agonist-induced microparticle formation is closely related to the elevation of intracellular calcium, which affects the reorganization of the platelet cytoskeletal architecture.[27-29] It is unclear whether microparticles in HIT are primarily markers of platelet activation or whether they serve a more significant physiologic role.

It is unclear whether HIT-induced microparticles or microparticles observed in other thrombotic conditions, such as transient ischemic attacks,[30] pulmonary embolism,[31] thrombotic thrombocytopenic purpura,[32] or DIC,[33] have similar functional properties. We observed that microparticles generated by HIT sera, thrombin, or calcium ionophore express similar amounts of $GPIb_{\alpha}$ and GP IIb/IIIa. It is possible that the microparticle membrane characteristics may not vary with respect to specific inducers of microparticle formation.

Our study describes the ultrastructure of microparticle production in platelets incubated with HIT sera. Using electron microscopy, these microparticles appear to bud from pseudopodia and/or regions of the platelet membrane following platelet activation, and they are distinct and separate from activated platelets. The observations that microparticles are no longer bound to activated platelets, carry intense procoagulant activity, and have a smaller circulating size suggest that they may be important promoters of thrombotic events in HIT. Our study provides important morphologic clues as to the nature of microparticle production in platelets. These results may contribute to the understanding of thrombotic complications that characterize heparin-induced thrombocytopenia.

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