Platelet Adhesion to Collagen in Individuals Lacking Glycoprotein IV

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The Nak" isoantigen is expressed on glycoprotein (GP) IV (CD36), a platelet membrane GP that has been identified as having a role in platelet interactions with collagen and thrombospondin and in binding Plasmodium falciparum-infected erythrocytes to endothelial cells and melanoma cells. We have studied normal platelets and Nak" platelets from two Japanese donors that have 1% of GPIV by concentration-dependent antibody binding and flow cytometry. We found that thrombin-stimulated Nak" platelets expressed the same amount of thrombospondin as did normal platelets. From our studies with Nak" platelets, we cannot identify a definitive role for GPIV in platelet aggregation, in adhesion to types I, III, and IV collagen, or in endogenous thrombospondin binding to platelets.

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MATERIALS AND METHODS

Collagen preparation. Type I collagen was isolated from commercial crude preparations of calf skin acid-soluble type I collagen (Sigma, St Louis, MO) by extraction in 0.5 mol/L acetic acid (HAc) at 4°C. The extracted material was precipitated by 4% NaCl and was redissolved in 0.5 mol/L HAc at 4°C. It was dialyzed for 24 hours against 0.02 mol/L sodium phosphate, pH 9.0, until the pH of the retained material was >7.0. The precipitate was then collected by centrifugation at 4°C for 30 minutes at 7,500g, redissolved in 0.1 mol/L HAc, and dialyzed against 0.1 mol/L HAc at 4°C with 6 changes. The collagen solution was lyophilized and frozen at -70°C until used. Before use, 10 to 15 mg of the lyophilized collagen was dissolved in 10 mL 5.0 mmol/L HAc by gentle mixing overnight at 4°C. It was centrifuged at 100,000g for 1 hour at 4°C, and the collagen was stored at 4°C. The final preparation consisted solely of monomeric type I collagen. No noncollagenous protein was present as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). From the Hematology Service, National Institutes of Health, Bethesda, MD.

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Platelet adhesion to collagen

Fig 1. Platelet aggregation to 100 μg/mL of type I collagen is shown. Nak⁺⁺ platelets (A) showed shorter lag times and more total aggregation than normal platelets (B; n = 3).

Fig 2. Time course of normal (*) and Nak⁺⁺ platelet (■) adhesion to type I collagen in the presence of 2 mmol/L Mg⁺⁺ (A) and in the presence of 100 μmol/L EDTA (B) is shown. Washed,¹¹¹In labeled platelets were incubated from 4 to 60 minutes at room temperature on collagen coated wells. There was no significant difference between normal (n = 4) and Nak⁺⁺ (n = 6) platelets at any time point (P > .05). Normal and Nak⁺⁺ platelet adhesion in the presence of 100 μmol/L EDTA was decreased at all time points compared with adhesion in the presence of Mg⁺⁺. Values shown are the means ± 1 SD.

Platelet adhesion. Platelet aggregation studies were performed in a Chronolog Dual Channel Lumi-Aggregometer (Chronolog, Havertown, PA). Blood was drawn using a 2-syringe technique and was anticoagulated with 0.1 mL 3.8% sodium citrate per 10 mL whole blood (final concentration, 10.9 mmol/L). PRP was prepared by centrifugation at 750g for 3 minutes at room temperature. The platelet count was adjusted to 200,000/μL by the addition of platelet poor plasma (PPP). PPP was prepared by centrifugation at 2,000g for 10 minutes at room temperature. Adenosine triphosphate (ATP) release was measured using luciferase-luciferin reagent (Sigma, St Louis, MO).

Flow cytometry. Flow cytometric analysis was performed on PRP and on Lexx- (Consulting Associates, Inc. Tacoma, WA) purified platelets using an EPICS V flow cytometer (Coulter Electronics,わない).
Fig 3. Anti-β3 integrin (176D7) inhibition of platelet adhesion to type I collagen in the presence of 2 mmol/L Mg++ is shown. (●) Nak" or (○) normal platelets were incubated at room temperature for 20 minutes with 176D7 before they were added to collagen-coated wells for 60 minutes. Values shown are the mean ± 1 SD, for Nak" platelets (n = 8) and for normal platelets (n = 12; P = .1).

MoAb binding to platelets. Blood was drawn by a 2-syringe technique. The blood from the second syringe was placed in a polypropylene tube containing 10.9 mmol/L sodium citrate. The blood was centrifuged at 750g for 3 minutes to obtain PRP. Platelets were purified from the PRP by centrifugation on a Larex gradient (3 mL 20% Larex and 5 mL 10% Larex). The platelets were diluted to 2 × 10^6/mL in 0.145 mol/L NaCl, 2.7 mmol/L KCl, 3.8 mmol/L HEPES, 0.1% glucose, 0.35% BSA, and 2 mmol/L CaCl₂, pH 7.4. The Fab fragment of MoAbs 185G8 and OKM5, which recognize an 88-kD protein on normal platelets, was used to determine the number of GPIV molecules on normal and Nak" platelets. Concentration-dependent Fab binding to platelets was performed in the presence of EGTA or thrombin-activated platelets. Endogenous thrombospondin surface-expression studies were performed by incubation of 400 µL of nonactivated or thrombin-stimulated platelets with 100 µL of varying concentrations of a radiolabeled MoAb 185G8 specific for thrombospondin; binding was also performed in the presence of EGTA.

The MoAb Fab fragments were labeled with Na^125I by the Pierce iodo-Bead method (Pierce, Rockland, IL). The platelet-bound ^125I MoAb was separated from free antibody by centrifugation on 1 mL of 10% Larex for 3 minutes at 12,000g. Nonspecific binding was determined by adding 100-fold excess of cold MoAbs. The LIGAND program (Unit on Biostatistical Methodology, National Institutes of Health, Bethesda, MD) was used for the calculation of Scatchard plots. The nonspecific binding calculated by the LIGAND program agreed with the nonspecific binding calculated experimentally.

Perfusion studies. Umbilical arteries were dissected free from human umbilical cords. Arterial segments were everted and exposed to air for 5 minutes to remove the endothelial cell layer. The arterial segment was placed in an annular perfusion chamber similar to that developed by Baumgartner. The arterial segment was connected via Silastic tubing to a plastic reservoir and peristaltic pump. Phosphate-buffered saline was perfused across the subendothelial surface for 5

Fig 4. Time course of anti-αβ3 integrin (176D7) inhibition of (●) Nak" and (○) normal platelet adhesion to type I collagen is shown. 176D7 was incubated with Nak" or normal platelets on collagen-coated wells for 1 to 29 minutes. There was no significant difference between Nak" (n = 3) and normal (n = 6) platelet adhesion at any time point (P = .2).
minutes to wash the vessel before blood perfusion. Citrated whole blood (final concentration, 10.9 mmol/L sodium citrate) was perfused and recirculated for 5 minutes at a rate of 300 mL/min at 37°C. The corresponding wall shear rate, 2,600 sec⁻¹, closely approximates conditions physiologically present in the microvasculature.

Cross sections of arterial segments were fixed, dehydrated, embedded in epoxy resin, and stained. Sections were evaluated morphometrically by light microscopy according to Baumgartner and Muggli.² Platelet-subendothelium interaction was categorized as C, contact platelets attached but not spread on the surface, or S, platelets spread on the surface. Platelet adhesion is defined as C + S, and each parameter is expressed as percentage of the total number of points counted (approximately 700) per vessel segment.

RESULTS

In platelet aggregation studies, the Nak⁺⁺' patients had normal or decreased lag times, normal or enhanced total platelet aggregation, and normal ATP release to several doses of type I collagen (6.25 to 100 µg/mL; Fig 1). The Nak⁺⁺ individuals also showed normal platelet aggregation and ATP release to other agonists including arachidonic acid, thrombin, ristocetin, adenosine diphosphate (ADP), and epinephrine.

Adhesion to BSA-coated wells was between 1% and 2% in normal and Nak⁺⁺ platelets. Platelets from the Nak⁺⁺ individuals adhered to types I, III, and IV collagen in the presence of 2 mmol/L Mg²⁺ to the same or to a slightly greater extent than did normal platelets at all time points, from 4 to 60 minutes (P = 0.1; Fig 2). In the presence of 100 µM EDTA, normal and Nak⁺⁺ platelet adhesion was decreased at all time points compared with adhesion in the presence of Mg²⁺; however, no significant differences were observed in adherence between normal and Nak⁺⁺ platelets at any time (P = .05). In the experiments using type I collagen with Nak⁺⁺ platelets n = 6, and with normal platelets n = 4; in experiments using types III or IV collagen with Nak⁺⁺ platelets n = 2, and with normal platelets n = 5.

The 176D7 antibody inhibited platelet adhesion to type I, type III, and type IV collagen when added at concentrations of 0.2 to 20 µg/well with both normal and Nak⁺⁺ platelets (Fig 3). In the time course of adhesion, 176D7 was allowed to incubate with normal or Nak⁺⁺ platelets on type I collagen-coated wells for 1 to 29 minutes. The 176D7 caused progressive inhibition of normal and Nak⁺⁺ platelet adhesion from 1 to 29 minutes of incubation. There was no significant difference between normals (n = 6) and Nak⁺⁺ platelets (n = 3) in the percentage of adhesion at any time point (P = 0.2; Fig 4). No significant difference was noted in the amount of adhesion at any time point between normal and Nak⁺⁺ platelets incubated with saline instead of antibody (P = 0.2).

By flow cytometry the mean number of positive cells with OKM5 in Nak⁺⁺ platelets was 2.0% compared with 97.0% in normals, and with 18Sg8 was 2.2% compared with 98.4% in normals. However, monocytes from these two patients did express GPIV. The mean number of positive cells was 98.1% compared with 86.6% in normals. The Nak⁺⁺ platelets had normal amounts of GP Ib and GPIb-IIIa by flow cytometry and of αβ₃ integrin by flow cytometry and concentration-dependent binding with the antibody 176D7 (Fig 5). Nak⁺⁺ platelets stimulated with ADP or thrombin expressed normal amounts of thrombospondin, von Willebrand factor, and fibrinogen on their cell surface.

Normal and Nak⁺⁺ platelets activated with thrombin showed no significant difference in the number of thrombospondin molecules detected by the MoAb antibody 181G8. Normal platelets expressed 30,800 molecules of thrombospondin per platelet with a dissociation constant (Kd) of 23 nmol/L. Nak⁺⁺ platelets expressed 30,400 molecules of thrombospondin with a Kd of 81 nmol/L. Similar amounts of thrombospondin were detected on unstimulated or EGTA-treated normal and/or Nak⁺⁺ platelets; approximately 3,000 thrombospondin molecules were detected per platelet.

Concentration-dependent binding of 18Sg8 or OKM5 to normal platelets in the presence of EGTA showed 49,500 sites per platelet with a Kd of 32 nmol/L. In contrast, the Nak⁺⁺ platelets contained only 500 sites per platelet and a Kd of 2 nmol/L. Thrombin activation of normal or Nak⁺⁺ platelets did not alter the number of sites expressed or the Kd.

Perfusion studies of platelet adhesion at high shear rates showed no significant differences between Nak⁺⁺ platelets and normal platelets in the number of contact or spread platelets. Examination of the vessel segments showed about 30% of the subendothelial surface to be covered with platelets with the majority of this coverage composed of a mono-
layer of spread platelets. Less than 3% of the surface was covered by platelets that had contacted the surface but had not spread, and less than 1% of the surface was occupied by platelet thrombi.

**DISCUSSION**

We have studied two Nak- Japanese women whose platelets contain 1% to 2% of GPIV who have not experienced any hemostatic problems. GPIV has been proposed as a receptor for platelet adhesion to collagen and as a collagen and thrombospondin binding site; however, in our studies which are in agreement with those of Kehrel et al and Tandon et al, stimulated platelets deficient in GPIV have the same capacity as normal platelets to bind endogenous thrombospondin. From these studies in individuals who lack platelet adhesion to collagen and in collagen-induced platelet aggregation, over compensates for their lack of GPIV.

Collagen-induced platelet aggregation and ATP release were normal or enhanced in the individuals with GPIV deficiency, which has also been reported by Yamamoto et al. Perhaps, Nak- platelets use alternative pathways for some platelet functions that, in the case of collagen-induced platelet aggregation, over compensates for their lack of GPIV.

Nak- platelets were indistinguishable from normal platelets in the extent of adherence to purified type I, III, or IV collagen in the presence of Mg ++ or a low concentration of EDTA at all time points from 4 to 60 minutes. The a2β1 integrin appears to be primarily responsible for platelet aggregation to collagen and static platelet adhesion to collagen in our system. Nak- platelets had normal amounts of a2β1 integrin and, accordingly, were inhibited by the anti-a2β1 integrin 176D7 to the same extent as normal platelets in both platelet aggregation to collagen and in collagen-induced platelet aggregation. From these studies in individuals who lack GPIV, we cannot identify a specific role for GPIV in static platelet adhesion to collagen or high shear force platelet adhesion to the subendothelium.

In a recent publication, Diaz-Ricart et al have reported that platelets lacking CD36 showed significantly decreased adhesion to collagen in a flowing whole blood system at 2 and 10 minutes of perfusion time. We have not performed time course experiments in our perfusion system, but there are several differences between our perfusion system and the perfusion system used by Diaz-Ricart et al. Our perfusions are performed at a shear rate of 2,600-1 second approximates the shear force in arterioles or arteries with a high degree of stenosis. Their perfusions were performed at a shear rate of 800-1 second that more closely approximates the shear force in larger vessels such as veins and arteries. We used umbilical arterial segments in our system, whereas Diaz-Ricart et al used plastic coveslips coated with equine tendon collagen.

Perhaps, the differences in methods used also play a role in the discrepancies between our results in static adhesion and the results obtained by other investigators. For example, we labeled platelets with 11Cr as opposed to 51Cr, and we used bovine collagen as opposed to equine collagen. The methods we have used have not provided us with evidence that GPIV plays a role in collagen-induced platelet aggregation, in platelet adhesion to collagen in either a static or high shear force system, or in the endogenous or exogenous thrombospondin binding to platelets. These studies do not exclude other role(s) for GPIV in other aspects of platelet physiology or pathology.

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