Platelet Adhesion to Collagen Type IV Under Flow Conditions


Collagen type IV is a sheet-forming collagen and a major constituent of the vessel wall. To find out which conditions are important for platelet adhesion to collagen type IV, we performed perfusion studies with anticoagulated blood in parallel plate perfusion chambers. The role of divalent cations was investigated by using plasmas with variable concentrations of Mg\(^{2+}\) and Ca\(^{2+}\) ions. When Mg\(^{2+}\) concentration was decreased from 2.00 mmol/L to 0.25 mmol/L at a fixed Ca\(^{2+}\) concentration of 1.25 mmol/L, platelet coverage on the collagen type IV surface decreased from 22.8% ± 1.8% (n = 4) to 4.6% ± 0.6% (n = 4) at a shear rate of 1,800 s\(^{-1}\). Also, platelet aggregate formation on collagen type IV was strongly impaired. A monoclonal antibody against the glycoprotein (Gp) Ib receptor and von Willebrand factor (vWF)-depleted plasma reduced the platelet coverage to collagen type IV to, respectively, 10% and 45% of the control value. Electron microscopy showed that vWF was only present between platelets and between the platelet and the collagen type IV surface, but did not bind elsewhere to collagen type IV. These data indicate that collagen type IV is a reactive collagen for platelets. Differences in physiologic plasma magnesium concentrations may in part explain the differences in platelet reactivity to collagen type IV between individuals, and perhaps contribute to differences in the risk for thrombosis.

© 1996 by The American Society of Hematology.

Materials and Methods

**Adhesive surfaces.** Calfskin collagen type I and human placenta collagen types I, III, and IV were obtained from Sigma (St Louis, MO) and solubilized in 50 mmol/L acetic acid (1.4 mg/mL). To assess the quality of the collagens and their potential contamination by other collagens and/or plasma proteins, we tested the collagen preparations by enzyme-linked immunosorbent assay (ELISA) for the presence of collagen types I, III, IV, and V and of both vWF and fibronectin (FN). No contamination (ie, <0.5% wt/wt) by other collagens could be detected in the different collagen preparations with polyclonal antibodies directed against collagen types I and V (Southern Biotechnology Associates Inc, Birmingham, AL), a monoclonal antibody (MoAb) directed against collagen type III (Heyl & Co, Berlin, Germany), and an MoAb against collagen type IV (MoAb 1048; courtesy of Dr J. Cleutjens, University of Limburg, Limburg, The Netherlands). Also, no apparent contamination (ie, <7 ng/mg collagen) with vWF or FN was detected by ELISAs using a polyclonal antibody against vWF (Dakopatts, Glostrup, Denmark) and an MoAb directed against FN (CLB-HEC 140; from Dr J.A. van Mourik, CLB, Amsterdam, The Netherlands). The solubilized collagens were sprayed onto clean glass cover slips (Menzel, Braunschweig, Germany) with a retouching airbrush (Badger model 100; Badger Brush Co, Franklin Park, IL) to a surface density of 30 µg·cm\(^{-2}\) supporting optimal platelet coverage.\(^4\)\(^5\) After spraying, the cover slips were blocked by incubation with a 1% human albumin solution (Behringwerke, Marburg, Germany) in HEPES-buffered saline (HBS) 10 mmol/L, HEPES/150 mmol/L, NaCl, pH 7.35) for 30 minutes at room temperature.

Human vascular endothelial cells derived from umbilical veins were isolated according to the method of Jaffe et al\(^{16}\) with some modifications.\(^17\) The cells were cultured in RPMI 1640 containing 20% pooled human serum. Endothelial cells of passage three were
PLATELET ADHESION TO COLLAGEN TYPE IV

used. After the cells had grown to confluence on glass cover slips, matrices were isolated by exposing the cells to 0.1 mol/L NH₄OH. This step was followed by three washes with phosphate-buffered saline (PBS) 10 mmol/L sodium phosphate/150 mmol/L NaCl, pH 7.4).

Plasmas with different Mg and Ca concentrations. Normal citrated plasma to which 33 U/mL low-molecular-weight heparin (LMWH Fragmin; Kabí Pharma, Stockholm, Sweden) was added was dialyzed against HBS containing 5 mmol/L d-glucose, 0.25 to 2.00 mmol/L Mg²⁺, and 1.25 mmol/L Ca²⁺. To study the effect of Ca⁴⁺ ions, plasmas were dialyzed against HBS containing 0.25 to 2.00 mmol/L Ca²⁺ and 1.00 mmol/L Mg²⁺. Total magnesium and calcium concentrations were measured with an Ektachem Analyzer (Eastman Kodak Co, Rochester, NY), and free calcium was assayed with an ABL 505 Analyzer (Radiometer, Copenhagen, Denmark). Free magnesium concentrations were not measured. However, we could deduce the free magnesium concentrations from the total magnesium concentrations in the HBS buffer. The final pH of the plasmas was between 7.35 and 7.55.

vWF- and Fn-depleted plasma. vWF-depleted plasma was obtained by affinity chromatography with MoAb RU-1 (MoAb RU-1 was raised against human vWF in our laboratory, and its epitope was mapped to the A2 domain of vWF; University Hospital Utrecht, Utrecht, The Netherlands) coupled to CNBr-Sepharose 4B (Pharmacia, Uppsala, Sweden). The final vWF content was less than 0.4 µg/mL as determined by ELISA; the level of FN was unchanged. FN-depleted plasma was obtained by passing normal plasma over a gelatin-Sepharose column as previously described by Houdijk and Sixma. The final FN content was less than 3 µg/mL as determined by ELISA; the level of vWF was unchanged. The citrated plasmas were subsequently treated like the Mg/Ca plasmas (dialysis against HBS containing 5 mmol/L d-glucose, 1.00 mmol/L Mg²⁺, and 1.25 mmol/L Ca²⁺).

vWF and Fn. vWF was purified from human cryopreparates by polyethylene glycol precipitation and gel filtration, and vWF antigen levels and vWF ristocetin cofactor activity were measured. FN was isolated from normal human plasma by passage over a gelatin-Sepharose column. The purity of the preparations was tested by ELISA. To study the role of plasma proteins in platelet adhesion to collagen type IV, purified vWF (10 µg/mL plasma) or purified FN (300 µg/mL plasma) were added to vWF- or Fn-depleted plasma.

MoAbs. MoAb AK2 against the vWF binding site of GpIb was kindly provided by Dr M.C. Berndt (Frahman, Australia). MoAb CLB-RAG35 against the GpIb binding domain on vWF was kindly provided by Dr J.A. van Mourik (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). This antibody has been described previously. Both MoAbs were used as ascites and the amount necessary to completely inhibit ristocetin-induced platelet aggregation was determined. A 10-fold higher concentration was added to the perfusate 30 minutes before perfusion.

Patients. To study the involvement of vWF in platelet adhesion to collagen type IV, one patient with severe von Willebrand’s disease (vWD) and one patient with vWD type I platelet-low was tested. The severe vWD patient had no vWF in the platelets and plasma (<0.01 U/mL vWF:Ag), and the patient with vWD type I platelet-low had no vWF in the platelets and a low vWF level in the plasma (0.04 U/mL ristocetin cofactor activity and 0.1 U/mL vWF:Ag).

Perfusions. Fresh blood from normal donors who denied having taken aspirin in the preceding 10 days was anticoagulated either with 1/100 vol 200-µM Fragmin (final concentration, 20 U/mL) or with 1/100 vol 110-mmol/L trisodium citrate. The platelets and red blood cells of the citrated blood (blood group O) were used for preparation of reconstituted blood. Platelets and red blood cells were washed by centrifugation as previously described and then resuspended in the vWF-depleted plasmas, FN-depleted plasmas, or Mg/Ca plasmas. Platelet counts were standardized at 200,000/µL (final concentration), and the hematocrit was between 0.37 and 0.40. Platelet deposition under nonpulsatile flow conditions was performed using parallel plate perfusion chambers as previously described. The chambers used had a slit width of 10 mm and slit heights of 0.6 mm (shear rate, 1,600 s⁻¹) or 1 mm (shear rate, 300 s⁻¹), corresponding to flow rates of, respectively, 56 and 36 mL/min. Whole blood or reconstituted blood (15 mL) was prewarmed at 37°C for 10 minutes, duplicate cover slips were inserted into the perfusion chamber, and the blood was then circulated through the perfusion chamber for 5 minutes. The system was rinsed with HBS after each perfusion. Perfusions were also performed in a modified parallel plate perfusion chamber with a slit height of 0.1 mm and a slit width of 2 mm (Fig 1), corresponding to flow rates of 60 µL/min (shear rate, 300 s⁻¹) and 320 µL/min (shear rate, 1,600 s⁻¹). The inlet and outlet were constructed with special care to avoid any possible disturbance in laminar flow. Blood was drawn through the perfusion chamber by a Harvard infusion pump (pump 22, model 2400-004; Natick, MA).

Evaluation. The cover slips from the perfusion chamber were removed and rinsed with HBS, fixed in glutaraldehyde (0.5% in PBS), and stained with May-Grünwald/Giemsa as described previously. Platelet deposition was quantified, with a light microscope (magnification X1,000) coupled to a computerized image analyzer (AMS 40-10; Saffron, Walden, UK). Three lines perpendicular to the flow direction were evaluated: one line in the center of the cover slip and two lines 3 mm to the right and 3 mm to the left of center. Platelet deposition was expressed as the percentage of the surface covered with platelets and platelet aggregates.

Binding studies. Purified vWF was radiolabeled with Na¹²⁵I (Amersham, Buckinghamshire, UK) using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) containing lactoperoxidase/glucose oxidase according to the instructions of the manufacturer. Noncovalently linked¹²⁵I was removed by dialysis against Tris-buffered saline (TBS) 50 mmol/L Tris/100 mmol/L NaCl, pH 7.4). Radiolabeling did not affect binding as tested by dilution with unlabeled vWF. Results were obtained in two independent experiments performed with different radiolabeled preparations (specific activities, 211 and 382 cpm/ng).

Cover slips sprayed with human placenta collagen types I, III, and IV were blocked with TBS plus 3% bovine serum albumin (BSA Sigma) and 0.1% Tween, pH 7.4 (1 hour at room temperature). They were subsequently incubated with 70 µL of the radiolabeled vWF solution (1 to 8 µg/mL for 2 hours at room temperature). After incubation, the cover slips were washed and counted in a gamma counter. Results were expressed as specific binding (total binding minus nonspecific binding with an excess of 40-fold unlabeled vWF).

Confocal laser scan microscopy. After perfusion, the cover slips were rinsed with HBS and fixed and permeabilized in PBS containing 3% paraformaldehyde, 0.025% glutaraldehyde, and 0.5% Triton X-100 for 15 minutes at room temperature. They were then rinsed with PBS and blocked with, respectively, 0.15 mmol/L glycine in TBS (20 minutes at room temperature) and 3% BSA + 0.1% Tween 20 in TBS (30 minutes at room temperature).

Platelets were visualized by staining F-actin with FITC-conjugated phallolidin (Molecular Probes, Eugene, OR), whereas vWF was visualized with a polyclonal antibody against vWF (Dakopatts, Glostrup, Denmark) and a TRITC-conjugated goat antirabbit F(ab)² fragment (Southern Biotechnology, Birmingham, AL). After mounting, the cover slips were visualized with the Bio-Rad MRC-1000 Laser Scanning Confocal Imaging System (Bio-Rad Microscience, Herts, UK).
Electron microscopy. Melanine-coated cover slips were prepared as described by Westphal et al. Collagen type IV was dissolved in 50 mmol/L acetic acid and sprayed on the melanine-coated cover slips. After perfusion at a shear rate of 1,600 s⁻¹, samples were fixed in 2% paraformaldehyde and 0.2% glutardialdehyde in 0.1 mol/L phosphate buffer, pH 7.4. After rinsing with PBS/0.15 mmol/L glycine, the melanine foil was removed from the glass cover slips with 0.8% hydrofluoric acid at 4°C. Melanine foils were used for ultrathin cryosectioning and transmission electron microscopy (JEOL, Tokyo, Japan). vWF was visualized with a polyclonal antibody against vWF (Dakopatts) and protein-A gold. The polyclonal antibody against vWF did not bind to collagen type III. Incubation with protein-A gold alone resulted in low background staining.

Flow cytometry. Fixed platelets were washed twice with PBS to which 5 mmol/L EDTA was added (PBS/EDTA) and diluted to a concentration of 5 × 10⁹/mL. Ten microliters of platelet suspension was incubated with 5 µL FITC-conjugated anti-GpIb MoAb (5 µg/mL) and 5 µL biotinylated MoAb (5 µg/mL) for 30 minutes at room temperature, followed by a second incubation step with phycoerythrin-conjugated streptavidin (Becton Dickinson, San Jose, CA). Subsequently, the platelets were washed twice with PBS/EDTA and resuspended in 2 mL PBS for analysis. Platelets were analyzed in a FACScan flow cytometer (Becton Dickinson) at a wavelength of 488 nm. Platelets were distinguished from other cells using the anti-GpIb MoAb. FACScan data were analyzed with PC-LYSIS software (Becton Dickinson).

The following antibodies were used: MoAb 6.20, specific for GpIb; CD62 MoAb RUU-SP 2.17; CD63 MoAb RUU-SP 2.19; MoAb RUU-SP 1.77, specific for GMP-33, an α-granule membrane protein; CD51 MoAb RUU-PL 7E8; MoAb RUU-PL 7F12, specific for Gp IIIa, and MoAb 2.41, an activation-dependent MoAb for GpIb-IIIa.

β-Thromboglobulin. Measurement of β-thromboglobulin (β-TG), a release product of α-granules and a marker for platelet activation, was performed using a commercially available ELISA (Asserachrom β-TG; Diagnostica Stago, Asnieres-sur-Seine, France).

Statistical analysis. Results are expressed as the mean ± SEM for data obtained from different experiments, or as the mean ± SD for data obtained from different cover slips within one experiment. Student's t-test was used to test for significant differences between groups. P values less than .05 were considered significant.

RESULTS

Platelet adhesion to collagen type IV. Platelet adhesion to collagen type IV was tested in both the recirculating perfusion system and the single-pass perfusion system. A collagen type IV density of 30 µg/cm² resulted in homogenous platelet coverage, consisting of aggregates and occasional single dendritic platelets (Fig 2A and B). Platelet adhesion was increased with increasing shear rate in both systems (Fig 3A and B). The percent platelet coverage reached a maximum at a shear rate of 1,000 s⁻¹ in the recirculating perfusion system, whereas the increase in platelet coverage continued up to a shear rate of 2,000 s⁻¹ in the single-pass perfusion system. A sharp, donor-dependent decline above a shear rate of 2,000 s⁻¹, as shown in Fig 3C for the single-pass perfusion system, was observed in both systems. Significant differences between both perfusion systems were observed for platelet deposition on collagen type IV at a shear rate of 1,600 s⁻¹ (Fig 4). No significant differences were observed between the recirculating and single-pass perfusion systems for platelet deposition on collagen type I or collagen type III (results not shown).

To find an explanation for the increased platelet adhesion
PLATELET ADHESION TO COLLAGEN TYPE IV

Fig 2. Light micrographs of platelets adhering to collagen type IV. Whole low-molecular-weight heparin-anticoagulated blood was perfused over collagen type IV in the recirculating perfusion system (A) or the single-pass perfusion system (B) for 5 minutes at 1,600 s⁻¹ (original magnification 600×; direction of flow from right to left).

In the recirculating perfusion system, an increase in β-TG release of 35.2% was observed (Fig 5). The β-TG content of plasma immediately before perfusion was between 130 and 200 ng/mL.

Role of divalent cations. In the present study, blood was anticoagulated with Fragmin (a low-molecular-weight heparin), since we have observed that platelet adhesion to collagen type IV was almost absent in citrated blood at high shear rate. Plasmas with varying concentrations of Mg²⁺ and Ca²⁺ ions were used to investigate the role of magnesium and calcium in platelet deposition on collagen type IV in the recirculating perfusion system. The results were compared...
Fig 3. Shear rate dependence of platelet adhesion to collagen type IV. Whole blood was anticoagulated with low-molecular-weight heparin and perfused for 5 minutes over collagen type IV. (A) Perfusion performed in the recirculating perfusion system; (B) perfusions performed in the single-pass perfusion system. Values are the mean ± SEM obtained in 3 independent experiments with blood from 3 different donors. (C) Perfusion performed in the single-pass perfusion system. Values are the mean ± SD of 3 independent cover slips obtained in 1 experiment with blood from a typical donor, which shows a sharp decline at a shear rate \(>2,250\) s\(^{-1}\).

Fig 4. Platelet adhesion to collagen type IV in the recirculating perfusion system (■) compared with the single-pass perfusion system (□) at shear rates of 300 and 1,600 s\(^{-1}\). Whole low-molecular-weight anticoagulated blood was perfused for 2, 5, or 10 minutes. Data points represent a typical experiment and are the mean ± SD of 3 independent cover slips. For the perfusion assays performed in the recirculating perfusion system, only the first cover slip was sprayed with collagen type IV; the second cover slip was coated with human albumin. * \(P < .05\) v recirculating perfusion system.

with platelet deposition onto collagen types I and III and the extracellular matrix of human endothelial cells (ECM).

The effect of Mg\(^{2+}\) ions on platelet deposition was investigated in the presence of 1.25 mmol/L Ca\(^{2+}\) ions. Ionized (free) magnesium levels between 0.25 and 2.00 mmol/L corresponded with mean total plasma magnesium levels between 0.43 and 2.78 mmol/L as measured with the Ektachem Analyzer E700 XR. Platelet deposition on all collagen types studied and to the ECM was strongly dependent on the concentration of Mg\(^{2+}\) ions (Fig 6A through D). The effect was most pronounced at the high shear rate of 1,600 s\(^{-1}\). For the collagens, both the aggregate size and number of aggregates per surface area were increased with increasing Mg\(^{2+}\) concentration.

Platelet adhesion to collagen type IV at shear rate 1,600 s\(^{-1}\) was strongly dependent on Mg\(^{2+}\) concentration in the plasma. Aggregate formation was strongly impaired at a low Mg\(^{2+}\) concentration of 0.25 mmol/L. Platelet coverage on the collagen type IV surface decreased from 22.8% ± 1.8% to 4.6% ± 0.6% (mean ± SEM, \(n = 4\)) when the free magnesium concentration decreased from 2.00 to 0.25 mmol/L (Fig 6A). The Mg\(^{2+}\) dependence of platelet adhesion to collagen type IV at a low shear rate of 300 s\(^{-1}\) was much smaller than at shear rate 1,600 s\(^{-1}\), especially for Mg\(^{2+}\) concentrations between 0.25 and 1.00 mmol/L.
Platelet adhesion to collagen type IV

Fig 5. β-TG in plasma immediately before perfusion (■) and after perfusion (▲) over collagen type IV at a shear rate of 1,600 s⁻¹ in the single-pass and recirculating perfusion systems. Plasma β-TG before perfusion was set at 100% (control); plasma β-TG after perfusion was calculated as a percent of the control value. Values are the mean ± SD of 8 independent perfusion runs performed in 2 independent experiments with low-molecular-weight anticoagulated whole blood of 2 different donors. For perfusion assays performed in the recirculating perfusion system, only the first cover slip was sprayed with collagen type IV; the second cover slip was coated with human albumin. *P < .05 v before perfusion in the recirculating perfusion system; **P < .05 v after perfusion in the single-pass perfusion system.

In comparison, platelet coverages on collagen types I and III decreased from, respectively, 15.9% ± 0.7% to 7.0% ± 0.7% (mean ± SEM, n = 5) and from 30.9% ± 6.8% to 11.2% ± 2.8% (n = 4) when the free magnesium concentration decreased from 2.00 to 0.25 mmol/L at a shear rate of 1,600 s⁻¹ (Fig 6B and D). In contrast to collagen types IV and I, aggregate formation on collagen type III was only slightly impaired at a Mg²⁺ concentration of 0.25 mmol/L, resulting in relative good aggregate formation in citrated blood (results not shown). The ECM was used as a more physiologic surface for studying the role of Mg²⁺ ions in platelet adhesion to the vessel wall. At a shear rate of 1,600 s⁻¹, platelet adhesion decreased from 34.9% ± 2.7% to 19.2% ± 2.9% (mean ± SEM, n = 5) when Mg²⁺ concentration decreased from 2.00 to 0.25 mmol/L. No dependence on Mg²⁺ concentration was observed at a shear rate of 300 s⁻¹ (Fig 6C).

To study the effect of variable Ca²⁺ concentrations on platelet deposition on collagen type IV, plasmas were dialyzed against HBS containing 0.25 to 2.00 mmol/L Ca²⁺ and 1.00 mmol/L Mg²⁺. No effect of Ca²⁺ concentration was observed on platelet deposition. To study whether the ratio between Ca²⁺ and Mg²⁺ could influence platelet deposition on collagen type IV, plasmas were dialyzed against HBS containing 0.25 to 2.00 mmol/L Mg²⁺ in the presence of either 0.50 or 1.25 mmol/L Ca²⁺. No differences were observed between Ca²⁺ concentrations in the percent platelet coverage or in the morphology of platelets that adhered to collagen type IV.

Role of vWF and FN. The role of vWF and FN in platelet deposition on collagen type IV was studied by comparing the platelet deposition in blood reconstituted with vWF-depleted or FN-depleted plasma with the platelet deposition in the same blood samples to which purified vWF (10 μg/mL) or FN (300 μg/mL) had been added. The lack of FN in plasma did not result in a decreased platelet coverage of...
collagen type IV. Neither was any effect observed when collagen type IV was preincubated with purified FN.

The lack of vWF in vWF-depleted plasma resulted in a significantly decreased platelet coverage that was 45% of control values (45% ± 7.3%, n = 4 independent experiments) at a shear rate of 1,600 s⁻¹, whereas no effect was observed at a shear rate of 300 s⁻¹. The MoAbs AK2 (against the vWF binding site of GpIb) and CLB-RAG35 (against the GpIb binding site on vWF) significantly reduced platelet adhesion to collagen type IV by 90% at a shear rate of 1,600 s⁻¹ in both the recirculating perfusion system and the single-pass perfusion system.

The importance of vWF in platelet adhesion to collagen type IV was confirmed by the results in vWD patients. Addition of purified vWF to the severe vWD blood at a final concentration of 10 μg/mL did not result in a significant increase of platelet coverage at a shear rate of 300 s⁻¹ (platelet coverage, 8.9% ± 0.6% [n = 3]; platelet coverage without vWF, 9.4% ± 0.7% [n = 3]). Addition of purified vWF to the blood of the vWD type I patient at a shear rate of 1,600 s⁻¹ resulted in an increase of platelet coverage of greater than 200% (platelet coverage, 14.1% ± 3.4% [n = 4]; platelet coverage without vWF, 4.0% ± 0.5% [n = 4]).

The above-described experiments clearly showed that platelet adhesion to collagen type IV at a shear rate of 1,600 s⁻¹ depended on the presence of vWF. However, when sprayed collagen type IV was incubated for 2 hours with a radiolabeled vWF solution, almost no binding of vWF to collagen type IV could be demonstrated (Fig 7). This result was confirmed under flow conditions. With the use of a polyclonal antibody against vWF and electron microscopy (Fig 8A and B) or confocal laser scan microscopy (results not shown), we found that vWF was only present between the platelets and between the platelet and collagen type IV surface, and was not bound elsewhere to collagen type IV.

**DISCUSSION**

**Platelet adhesion to collagen type IV.** In this report, we have studied the adhesive properties of collagen type IV under flow conditions using low-molecular-weight heparin as anticoagulant. In contrast to platelet coverage on collagen types I and III at 1,600 s⁻¹, platelet coverage on collagen type IV at 1,600 s⁻¹ was significantly higher in the single-pass perfusion system compared with the recirculating perfusion system. Differences in platelet adhesion to collagen type IV between both systems were also observed with Orgaran (a heparinoid) or with unfractionated heparin-anticoagulated blood (results not shown). At present, the reason for this difference is not clear. Plasma β-TG was slightly increased in the recirculating perfusion system, suggesting that platelet activation may affect platelet adhesion to collagen type IV. However, it must be noted, that the increase in β-TG during perfusion is minimal (maximal β-TG 70 ng/mL plasma, corresponding to ±1% of the total β-TG content of 1 mL platelet-rich plasma).

**Role of divalent cations.** The role of divalent cations was investigated using plasmas with concentrations of Mg⁺⁺ and Ca⁺⁺ ions ranging from 0.25 to 2.00 mmol/L. In our experiments, plasmas dialyzed against HBS with 0.25 to 2.00 mmol/L Mg⁺⁺ and 1.25 mmol/L Ca⁺⁺ showed total plasma magnesium concentrations of 0.43 to 2.78 mmol/L, corresponding to ionized magnesium percentages of 60% to 70%, also found by others. All the collagens tested and the ECM showed a strong dependence on Mg⁺⁺ ions at high shear rate (shear rate, 1,600 s⁻¹; Fig 6).

Platelet-collagen adhesion can be divided into divalent cation–independent adhesion and Mg⁺⁺-dependent adhesion. In the current study, it seems that for collagen type IV at high shear rate, the Mg⁺⁺-dependent mechanism is the most important mechanism for platelet adhesion. Since platelet adhesion to the ECM (Fig 6C) and to collagen type III (Fig 6D) was relatively good at low Mg⁺⁺ concentrations, these surfaces are probably less dependent on the Mg⁺⁺-dependent mechanism. It is likely that for platelet adhesion to the ECM (at low shear rate), other proteins are involved in optimal platelet adhesion to collagen type IV in flow conditions.

The results described herein open the intriguing possibility that differences in plasma magnesium concentrations in the physiologic range (0.35 to 0.85 mmol/L) but also under pathophysiologic circumstances may dramatically increase or decrease the platelet-collagen interaction. However, poor platelet adhesion to collagen types I and IV at high shear rate (>1,000 s⁻¹) in blood from some donors was not always due to low plasma magnesium. Even at total magnesium concentrations above 1.00 mmol/L, some donors adhered poorly to collagen types I and IV, and were for that reason excluded. A possible explanation is that the number of GpIa-IIa receptors on the platelet surface is also involved in optimal platelet adhesion to collagen.

**Role of vWF and FN.** Platelet adhesion to collagen type
IV was not dependent on plasma FN. We cannot exclude an effect of FN released from platelets and it cannot be studied, since no platelets exist that lack FN. From the literature, it is well known that FN has an enhancing effect on platelet aggregation.

Perfusion experiments with vWF-depleted plasma, MoAbs against the vWF binding site of GpIb and the GpIb binding site of vWF, and in vWD patients all suggest that platelet adhesion to collagen type IV at shear rate 1.600 s⁻¹ is dependent on the GpIb-vWF interaction. As already described for the other collagen types, no vWF dependency was observed at a low shear rate of 300 s⁻¹. With the use of an antibody against vWF and electron microscopy or confocal laser scan microscopy, we found that vWF was only present between the platelets and between the platelet and collagen type IV surface, and was not elsewhere bound to the collagen type IV surface. In contrast to collagen types I and III, it seems that the presence of platelets is a prerequisite for vWF binding to collagen type IV. The experiments performed with vWF-depleted plasma and with vWF-deficient platelets showed that both platelet vWF and exogenously added vWF participate in platelet adhesion. The experiments performed with vWF-deficient platelets open the possibility that platelets adhere to collagen type IV via GpIIIa and trap exogenously added vWF necessary to stabilize the primary platelet-collagen interaction.

Collagen type IV is not the only adhesive protein that shows vWF dependence without direct vWF binding. A comparable situation was described for FN and fibrinogen. As already speculated for FN, platelets may release an unknown factor or protein that is able to cross-link vWF and collagen type IV. However, it is also possible that direct binding occurs, but that the affinity of vWF for collagen type IV is too low to detect it.
To conclude, our data indicate that collagen type IV is a reactive collagen for platelets that can play an important role in hemostasis and thrombosis. Mg$^{2+}$ ions play an important role in platelet adhesion to collagen type IV, and at high shear rate the adhesion is dependent on the GP Ib-IX complex. Differences in physiologic plasma magnesium concentrations may in part explain the differences in platelet reactivity to collagen type IV between individuals, and perhaps contribute to differences in the risk for thrombosis.

ACKNOWLEDGMENT

We acknowledge the technical assistance of Annelarie M. van de Hoeven in culturing the cells, Dr Harry F.G. Heynen for technical assistance in the electron microscopy studies, and the Red Cross Bloodbank Utrecht for the blood supply.

REFERENCES

15. Houdijk WPM, Sixma JJ: Fibronectin in artery subendothelium is important for platelet adhesion. Blood 65:598, 1985
Platelet adhesion to collagen type IV under flow conditions

G Henrita van Zanten, EU Saelman, KM Schut-Hese, YP Wu, PJ Slootweg, HK Nieuwenhuis, PG de Groot and JJ Sixma