Studies of Adhesion-Dependent Platelet Activation
Distinct Roles for Different Participating Receptors Can Be Dissociated by Proteolysis of Collagen

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Abstract—The molecular differences between native-type collagen type I fibrils (NC) and their pepsinated monomers (PC) were used to uncover receptors involved in platelet-collagen interaction along the adhesion-activation axis. The platelet-depositing capacity of NC and PC under blood flow and their adhesive properties and respective morphologies, aggregation, procoagulant capacity, and tyrosine phosphorylation were compared under different cationic milieus, including or excluding the glycoprotein (GP) Ia/IIa. NC was consistently a more preferable and activating substrate than PC during flow (5 minutes) and in platelet aggregation. In PPACK-treated blood, both NC (3.3-fold) and PC (2.7-fold) increased platelet attachment on elevation of the shear rate from 500 to 1640 s⁻¹, whereas in citrated blood, adhesion and thrombus growth on PC were negligible under the high shear rate, unlike on NC (1.9-fold increase). The complete lack of platelet deposition on PC in citrated blood could be overcome by restoring physiological Mg²⁺ concentration, and in contrast to NC, platelets interacting with PC were highly dependent on Mg²⁺ during adhesion, aggregation, and procoagulant response. Monoclonal antibody (mAb 131.7) against GP IV inhibited platelet deposition to NC in citrated blood (2 minutes) by 49%, which was not further increased by coincubation with mAb against GP Ia (6F1). These results stress the importance of GP Ia/IIa in shear-resistant platelet deposition on collagen monomers. In native fibers, however, the preserved quaternary structure with telopeptides activates additional platelet receptors capable of substituting GP Ia/IIa and GP IV. (Arterioscler Thromb Vasc Biol. 1999;19:3033-3043.)

Key Words: hemostasis ■ platelet ■ collagen ■ receptor ■ divalent cations

On exposure of subendothelial collagen to flowing blood, adhering platelets, with their subsequent activation and procoagulant capacity, initiate hemostasis and thrombosis. Pathophysiological platelet-collagen interactions include bleeding disorders due to either defective receptors or autoantibodies or to impaired collagen synthesis, indicating several functional determinants on both the platelets and collagen.

Thus far, glycoprotein (GP) Ia/IIa, integrin α₃β₁, has been recognized as the major platelet receptor for collagen in primary adhesion, and accordingly, dysfunctional GP Ia/IIa associates with prolonged bleeding tendencies in patients. Although GP IV has been implicated in the very early phases of adhesion, the impact of GP IV, as well as the other receptor candidates, on hemostasis has remained unsettled. GP VI is involved in collagen-induced platelet activation, evidenced both by a poor aggregation response in patients lacking this receptor and by collagen-related peptides that likely induce strong platelet aggregation in normal platelets via this receptor. However, patients lacking GP VI have only mild bleeding tendencies, and under blood flow, the collagen-related peptides are unable to retain firm adhesion. Qualitative differences in collagen preparations complicate the identification of the determinants in platelet-collagen interaction. The term “native collagen fibers” has been used to cover a spectrum of preparations, from suspensions of native fibrils (eg, Horm) to dialysed fibrils consisting of salt-soluble tropocollagens, but also “fibrils” formed after dialysis of protease-extracted collagen monomers. However, the removal of telopeptides, eg, by pepsin, prevents the formation of fibrils, resulting instead in small nonbanded fibrous aggregates, and the severity of the impaired fibril formation depends on the extent of the pepsin digestion. Thus, the structural differences in collagen preparations are at least partly responsible for the inconclusive data on the determinants of the platelet-collagen interaction. Moreover, studies focusing on these effects under blood-flow conditions have been limited. Most of the studies having used static adhesion and traditional platelet aggregation techniques.

To obtain more information about the platelet-recruiting capacity and activating potential of type I collagen, the collagen monomers derived from fibrils by extensive pepsin treatment (eliminating telopeptides and quaternary structure) were used as a tool to investigate the responsible determinants. These fibrils and monomers were compared as substrates in experiments that dissociated the hemostatic events...
evolving from the platelet-collagen interaction under flowing blood: adhesion, aggregate formation, tyrosine phosphorylation, and procoagulant activity.

Methods

5-Hydroxytryptamine creatinine sulfate (5-HT) was obtained from Amershams International plc. Aurin tricarboxylic acid (ATA, MW 473.4) and Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) peptide were from Sigma. Horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-human von Willebrand factor (vWF), HRP-conjugated goat anti-mouse IgG antibodies, and monoclonal antibody (mAb) AN51 (against GP Ib) were from Dakopatts A/S; mAbs Gi9 (against GP Ia), S22 (against GP Ib), and SZ22 (against GP IIb) were from Immunotech SA; and 4G10 (against phosphotyrosine) was from Upstate Biotechnology, Inc. S-2238 was from Chromogenix AB and Thromborel S from Behringwerke AG. Bovine α-thrombin was from Dade (Baxter Healthcare Co.), mAbs 131.7 (against GP IV) and 6F1 (against GP Ia, α1) were kind gifts from Dr Narendra Tandon (Otsuka America Pharmaceutical, Inc, Rockville, Md) and Dr Barry Coller (Mount Sinai Medical Center, New York, NY), respectively, and vWF was obtained from Dr Thierry Burnouf (Centre Regional de Transfusion Sanguine, Lille, France).

Preparation of Collagen

Collagen type I fibrils from bovine Achilles’ tendon were extracted with 0.5 mol/L acetic acid and salt-precipitated with 1.7 mol/L NaCl, essentially as previously described.26 To obtain collagen monomers (PC) devoid of quaternary structure and telopeptides, native-type collagen fibrils (NC) were extensively treated with pepsin [3 additions of pepsin (Sigma Chemical Co), 1 mg/mL, rotation for 3 weeks at 4°C] and salt-precipitated. After the collagen stock solutions were centrifuged at 100,000g for 1 hour at 4°C, only 2% of NC (acid-soluble collagen) but >90% of PC (acid-soluble collagen) remained in the supernatant. The collagen concentrations were determined by a modified hydroxyproline assay27 or with Sircol dye from Biocolor Ltd. The structural properties of the collagen preparations were analyzed by transmission electron microscopy (TEM) (JEOL 1200EX). Collagen stocks were immobilized onto gold grids supported with carbon-coated polyvinyl formal plastic film, and negatively stained with 1% potassium phosphotungstic acid, pH 7.3, for 1 minute at 22°C.

Blood Collection

The study was approved by an institutional review board. Blood was obtained from healthy volunteers who had not used any medication during the previous 10 days. Nine volumes of free-flowing blood were collected into 1 volume of either (1) 30 mmol/L (final concentration) d-phenylalaninyl-l-prolyl-l-arginine chloromethyl ketone (PPACK) (Calbiochem-Novabiochem Corp); (2) 90 mmol/L sodium citrate, additionally adjusted depending on the donor’s hematocrit28; or (3) acidic citrated dextrose, pH 6.5, for gel-filtered platelets (GFPs). The anticoagulants were chosen to yield various concentrations of divalent cations that affect the function of GP IIb/IIIa activity.

Blood samples were fixed with 2.5% phosphate-buffered glutaraldehyde at 35°C to induce in situ fibril formation.32 For observation under light microscopy, the coverslips were stained with Coomassie brilliant blue R250.

Platelet Deposition on Immobilized Collagen Under Flow Conditions in Whole Blood

To study platelet deposition (adhesion-dependent platelet aggregate formation), whole blood was perfused in parallel-plate perfusion chambers with defined rheological characteristics.31,33,34 After the PRP was labeled, the blood was reconstituted without adjustment of the platelet counts. When indicated, the PRP was incubated with mAb 6F135 (final concentration, 10 µg/mL) and/or mAb 131.736 (5 µg/mL) for 20 minutes at 22°C before the blood was reconstituted and then allowed to stabilize for 30 minutes. The prewarmed (37°C) blood aliquots were recirculated over coverslips for 5 minutes at shear rates of 500 or 1640 s⁻¹. Then the samples were briefly perfused and rinsed with PBS, and the deposited ³H activity was measured in a liquid scintillation counter (1414 Rackbeta, Wallac), as described previously.31 The perfusions were completed within 3.5 hours of blood collection.

Scanning electron microscopy (SEM; JEOL SEM-820) was used to analyze the morphology of platelet deposition and adhesion. The samples were fixed with 2.5% phosphate-buffered glutaraldehyde at 22°C for 2 hours. After rinsing with PBS, the samples were dehydrated, critical point–dried under CO₂, and sputter-coated with gold or platinum.

Platelet Adhesion on Immobilized Collagen

GFPs (100×10⁶/mL) were labeled as described. Collagen-coated coverslips were placed in 24-well flat-bottom multidisks (NUNC) precoated with 2% human serum albumin, and GFPs (0.5 mL) were incubated stationary for 30 minutes at 22°C in the presence of 2 mmol/L of either Ca²⁺ or Mg²⁺. Afterwards, the coverslips were rinsed in PBS and subjected to scintillation counting. This platelet density and stationary conditions were chosen to promote adhesion without inducing platelet-platelet interaction. When indicated, GFPs were preincubated for 30 minutes with mAb Gi9 (20 µg/mL) or 6F1 (10 µg/mL), which had been dialyzed to remove NaN₃.

Platelet Aggregation

Aggregation of platelets (300×10⁶/mL in suspension or in PRP anticoagulated with either acidic citrated dextrose, pH 6.5, or PPACK) was measured turbidimetrically in a dual-channel aggregometer (Payton Associates Inc). The samples were stirred at 700 rpm and preincubated for 1 minute at 37°C before the addition of collagen. The rates of primary aggregation (min⁻¹) and maximal aggregation (%) at 5 minutes were assessed.

Tyrosine Phosphorylation

GFP suspensions (500×10⁶/mL), preincubated with 300 µmol/L of KQAGDV, were stimulated in an aggregometer with 1.5 µg/mL of NC or preformed PC “fibrils” (HEPES buffer, 1.5 hours, 35°C) in the presence of 2 mmol/L of either Ca²⁺ or Mg²⁺. At selected time points, an equal volume of 2% SDS LaemmlI sample buffer with 3 mmol/L sodium orthovanadate was added. The samples (20 µL) were electrophoresed on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Problin 45, Pharmacia). After blocking, the membranes were incubated with 0.2 µg/mL of 4G10 and HRP-conjugated goat anti-mouse antibody. The tyrosine phosphorylation patterns were visualized with the enhanced chemiluminescence technique (ECL kit, Amersham). The blots were stripped and then reprobed with SZ22 to control the platelet amounts on different lanes.
Assessment of Procoagulant Activity of Collagen-Adherent GFPs

After adhesion of GFPs on either NC or PC in the presence of 2 mmol/L of either Ca$^{2+}$ or Mg$^{2+}$, the coverslips were washed and incubated for 10 minutes with PBS containing 0.9 mmol/L CaCl$_2$. Then a 1:20 dilution of platelet-poor plasma (PPP) was added, and the generation of thrombin was enhanced by trace amounts of tissue thromboplastin (1:5000 final dilution of Thromborel S) under agitation for 10 minutes at 37°C. From each well, 180 μL of supernatant was reacted with a chromogenic substrate, S-2238 (200 μmol/L), for 6 minutes. Absorbance was measured at 405 nm (Labsystems Multiscan MCC). A control was included by incubating PPP and thromboplastin over collagen-coated coverslips without adhered platelets. This control value was deducted from the values obtained with platelet-adhered coverslips. The thrombin amounts formed were calculated by use of known concentrations of bovine α-thrombin (Dade) in a standard curve.

Statistics

The data are presented as mean±SD and were analyzed with a paired Student’s t test. Regression analysis was applied to study the association between the adhesion results. All assays were done in duplicate, and n refers to the number of donors. Because of the great interindividual differences in platelet responses to collagen,38 all comparisons between NC and PC in a given experiment were performed in samples from the same donor.

Results

Collagen Structure

The Coomassie blue–stained collagen-coated coverslips were viewed by light microscopy after the treatment to induce fibrils. These were thick bundles of collagen fibrils that clearly differed from segmented strands of collagen monomers (Figure 1, A and B). The collagen preparations were negatively stained and analyzed for periodicity in TEM to verify that the native type I collagen was in the form of true fibrils. Indeed, this preparation displayed periodic fibrils, whereas the PC monomers failed to do so (Figure 1, C and D), even after the treatment to induce “fibrils.”

Whole-Blood Perfusion Studies

To dissociate the involvement of different receptors during platelet-collagen interaction, the platelet-recruiting capacity of NC and PC was compared under various shear rates and anticoagulants: a low to intermediate shear rate of 500 s$^{-1}$ and a high shear rate of 1640 s$^{-1}$ and blood anticoagulated with either citrate (86 μmol/L Ca$^{2+}$, 8 μmol/L Mg$^{2+}$) or 30 μmol/L PPACK (normal cations; 1.37 mmol/L Ca$^{2+}$, 0.62 mmol/L Mg$^{2+}$), respectively. Under the perfusion conditions tested, platelets predominantly preferred NC over PC (Figure 2A).

In PPACK-anticoagulated blood, platelet deposition was not significantly larger on NC than on PC at the low shear rate of 500 s$^{-1}$ (5.4±2.7 versus 3.7±1.7×10$^6$/cm$^2$), but at the high shear rate of 1640 s$^{-1}$, almost 2 times more platelets deposited on NC than on PC (17.8±10.3 versus 10.0±6.8×10$^6$/cm$^2$) (Figure 2A). The shear-dependent increase in platelet deposition was of similar magnitude on both substrates: 3.3-fold on NC and 2.7-fold on PC, suggesting that the vWF-mediated platelet deposition was not markedly affected by the differences between NC and PC. This result was further confirmed by use of ATA as an inhibitor for vWF-mediated platelet

Figure 1. Light micrographs of collagen-coated coverslips (A and B) and TEM micrographs of negatively stained collagen preparations (C and D), illustrating the differences in collagen structure. The native-type collagen I fibrils (A and C) expressed periodic structure (C), which was lacking in monomers (B and D). Despite the fibril-forming treatment, the monolayers remained as nonbanded fibrous aggregates (D). Light microscopy magnification ×20, bar=10 μm; TEM magnification ×40 000, bar=200 nm.
deposition on collagen. At the high shear rate, ATA inhibited platelet deposition in a similar manner for both PC and NC, by \( \approx 40\% \) (data not shown).

In citrated blood devoid of \( \text{Mg}^{2+} \), which is important for GP Ia/IIa activity, the total platelet deposition diminished on both substrates (Figure 2A), and the platelet-recruiting differences between NC and PC were accentuated. Over 3-fold more platelets deposited on NC than on PC at the shear rate of 500 s\(^{-1}\) (3.9 \pm 0.7 versus 1.2 \pm 0.4 \times 10^6/cm\(^2\)). The difference between the collagens was even more profound when the shear rate was elevated to 1640 s\(^{-1}\) (15 times larger platelet deposition on NC than on PC (7.6 \pm 3.1 versus 0.5 \pm 0.2 \times 10^6/cm\(^2\)). NC showed a shear-induced increase in platelet deposition, in contrast to PC, on which platelet adhesion and subsequent aggregation were severely impaired at the high shear rate. This effect was also clear in the SEMs of the perfusion channel on PC under the stationary conditions and flow. Detectected with HRP-conjugated mAb, NC bound more vWF than PC under stationary conditions. However, in flow under high-shear-rate conditions, vWF binding from citrated PPP with washed and glutaraldehyde-fixed red blood cells did not differ (data not shown). Furthermore, incubation of the PC surface with vWF (10 mg/mL) before the perfusion did not improve the defective platelet deposition (data not shown). However, when the physiological level of \( \text{Mg}^{2+} \) cations was restored in citrated blood (by addition of 7 mmol/L \( \text{MgCl}_2 \), Microlyte 6 analyzer), platelet deposition on PC was corrected, reaching up to 84\% (66 \pm 20\%, \( n=4 \)) of that displayed in PPACK-treated blood under the high-shear-rate conditions. The restoration of physiological magnesium concentration allowed platelet deposition on PC to reach 61\% and 83\% of the NC-induced platelet deposition at the high and the low shear rates, respectively (\( n=2 \)). In PPACK blood at 3 minutes of perfusion, 6F1 dropped the deposition to PC by 92\% (\( n=1 \)). Interestingly, additional determinants of NC could circumvent the functionally restricted GP Ia/IIa, as evidenced by the moderate platelet deposition in citrated blood at both shear rates. Furthermore, at the low shear rate, the platelet deposition on NC in citrated blood did not
essentially differ from platelet deposition in PPACK blood \((P=0.16)\). Finally, the inhibitory effects of mAbs against GP IV and GP Ia were tested on platelet deposition to NC after 2 minutes of perfusion. mAb 131.7 inhibited 49±16\% \((n=5)\) of platelet deposition on NC at high shear rate in citrated blood. Under these conditions, the addition of 6F1 did not further inhibit platelet deposition \(\text{(data not shown)}\).

SEM supported the results of \(^{[3]}\)H-5-HT-labeled perfusions, illustrating both the surface coverage and the morphology of deposited platelets \((\text{Figure 3})\). At the low shear rate, irrespective of the collagen surface, and in the same anticoagulant, platelets and aggregates were fairly evenly distributed, but the size of the aggregates varied \((\text{Figure 3, A through D})\). The high shear rate increased the morphological differences, which depended on both the anticoagulant and the substrate \((\text{Figure 3, E and F})\). The relatively low PPACK concentration was chosen to allow adhesion-dependent activation events and possible formation of local fibrin. In PPACK-anticoagulated blood, platelet deposition on NC ranged from single platelets to multilayered aggregates \((\text{Figure 3E})\), whereas platelet deposition on PC seemed more uniform \((\text{Figure 3F})\). Strand-like structures, most likely representing locally formed fibrin, were found between the aggregates. However, it has been shown previously that at this PPACK concentration, circulating thrombin–antithrombin III complexes were not elevated after the perfusion.\(^{31}\) In citrated blood, platelet recruitment on NC resembled that on PC in PPACK blood \((\text{Figure 3G})\). Contrasting the evenly covered blood, platelet recruitment on NC resembled that on PC in citrated blood, whereas it was not mandatory for NC. Unlike PC, NC possessed the ability for adhesion and activation-related morphological changes in the presence of Ca\(^{2+}\).

Adhesion Studies

Because platelet deposition depended on the shear-resistant buildup of the thrombus on adhesion, we next studied the primary adhesion of platelets on PC and NC in the presence of 2 mmol/L Mg\(^{2+}\) or Ca\(^{2+}\) and specifically the role of GP Ia/Ia therein.\(^{10}\) In the presence of Mg\(^{2+}\), PC was consistently a significantly preferable substrate for platelet adhesion than NC \((\text{Figure 4})\). The adhesion to PC correlated well with the adhesion to NC \((r=0.83, P<0.005)\), indicating the involvement of the same platelet receptor, ie, GP Ia/Ia, in adhesion to both PC and NC. On the contrary, switching the cations from Mg\(^{2+}\) to Ca\(^{2+}\) strongly decreased platelet adhesion to PC, resulting in 2.4 times larger adhesion to NC than to PC, probably because of the closure of GP Ia/Ia for collagen.\(^{40}\) In sharp contrast to PC, switching the cations from Mg\(^{2+}\) to Ca\(^{2+}\) enhanced platelet adhesion on NC \((\text{Figure 4})\). Ca\(^{2+}\)-dependent platelet adhesion to NC did not correlate with Mg\(^{2+}\)-dependent adhesion to PC \((r=0.41)\), but it did correlate with the adhesion to NC in the presence of Mg\(^{2+}\) \((r=0.76, P<0.01)\), suggesting distinct receptors for NC under the differing divalent cation milieu as well as an interplay between GP Ia/Ia and another receptor(s).

Blocking GP Ia with mAbs \((6F1 \text{ or Gi9, both IgG1 subclasses})\) decreased Mg\(^{2+}\)-dependent platelet adhesion to PC \((63\% \text{ and } 52\%, \text{ respectively})\) \((\text{Figure 5A})\). The extent of this inhibition was similar to that caused by the switching of Mg\(^{2+}\) to Ca\(^{2+}\), and in the presence of Ca\(^{2+}\) 6F1 did not further inhibit adhesion to PC. The mAbs did not inhibit platelet adhesion on NC when either Mg\(^{2+}\) or Ca\(^{2+}\) was provided \((\text{Figure 5B})\). In contrast, in the presence of Mg\(^{2+}\), 6F1, but not Gi9, rather increased adhesion on NC. As a control, mAbs against GP Ib did not affect adhesion on either PC or NC \((\text{data not shown})\).

SEM revealed interesting cation-dependent aspects of platelet morphology and confirmed that the selected assay conditions favored adhesion of single platelets \((\text{Figure 6})\). In the presence of Mg\(^{2+}\), PC- and NC-adherent platelets appeared similar, showing many pseudopods and full spreading of the cytoplasm \((\text{Figure 6, A and C})\). In the presence of Ca\(^{2+}\), only a few platelets adhered on PC, and they retained a round shape with a few projections \((\text{Figure 6B})\). In contrast, platelets on NC projected pseudopods and were still able to spread \((\text{Figure 6D})\). In addition, a notable proportion of "spongy"-appearing platelet ghosts was detected, which had swollen and no longer remained spread or had pseudopods.

The adhesion results confirmed the blood flow studies in that GP Ia/Ia was essential for primary platelet adhesion to PC, whereas it was not mandatory for NC. Unlike PC, NC possessed the ability for adhesion and activation-related morphological changes in the presence of Ca\(^{2+}\).

Aggregation Studies

Because of the better aggregate-forming capacity of NC during blood flow, platelet aggregation was studied with PPACK-treated and citrated PRP and GFPs in the presence of divalent cations. Here, the platelet preference for NC over PC was also more obvious in citrated PRP, in which a 4-fold higher concentration of PC was needed to induce maximal aggregation of an extent similar to that with NC, but the lag time still remained twice as long \((\text{Table 1})\). In PPACK PRP, the 4-fold higher concentration of PC resulted in a compatible lag time, rate, and maximal aggregation. Although during the NC-induced aggregation the lag time did not vary with the anticoagulation, the rate was enhanced, benefiting from the presence of cations. The same cation-dependent enhancement was observed with PC. To exclude the role of fibril formation in the extended lag time, PC was preincubated at 35°C for 2 hours in a neutralizing buffer. Although this treatment improved the aggregating capacity of PC, the lag time, rate, and maximal aggregation all failed to match aggregation induced by the corresponding concentrations of NC \((\text{data not shown})\). 6F1 affected PC significantly more than NC by prolonging lag time and reducing the rate and maximal aggregation in citrated PRP, and these effects were even more pronounced in PPACK PRP \((\text{data not shown})\). The aggregation data of GFPs were complementary to those of PRP \((\text{Table 2})\). The aggregating capacity of PC was severely impaired when Mg\(^{2+}\) was omitted and Ca\(^{2+}\) was present. This also prolonged the lag time of NC-triggered aggregation, but once the aggregation
started, its rate and maximum were the same irrespective of the ionic environment. In contrast to plasma milieu, fibril-forming treatment improved the PC-induced aggregation response of GFPs, especially in the presence of Mg$^{2+}$ (data not shown).

**Figure 3.** SEMs illustrating the differences in coverage and morphology of platelet depositions after blood perfusions over NC and PC under various anticoagulants and shear rates: A, NC, 30 μmol/L PPACK, 500 s$^{-1}$; B, PC, 30 μmol/L PPACK, 500 s$^{-1}$; C, NC, citrate, 500 s$^{-1}$; D, PC, citrate, 500 s$^{-1}$; E, NC, 30 μmol/L PPACK, 1640 s$^{-1}$; F, PC, 30 μmol/L PPACK, 1640 s$^{-1}$; G, NC, citrate, 1640 s$^{-1}$; and H, PC, citrate, 1640 s$^{-1}$. Magnification ×1050; bar=10 μm. Note the very few platelets in H located at the channel edge.

Studies on Tyrosine Phosphorylation as a Marker of Activation

Time-dependent tyrosine phosphorylation was also assessed to capture the differential NC- and PC-induced activation of
platelets. To discard the phosphorylation events dependent on GP IIb/IIIa activation, an inhibitory peptide (KQAGDV) was used. In the presence of Mg\(^2+\), both NC and prefibrillated PC induced similar patterns of tyrosine-phosphorylated proteins, but with NC, the kinetics of phosphorylation was slightly faster than with PC (Figure 7). The differences in phosphorylation patterns were accentuated in the presence of Ca\(^2+\), when several bands were not visible during PC induction or appeared only at the latest time points. The switch of cations also changed the NC-induced phosphorylation: in the presence of Ca\(^2+\), several bands appeared slowly or their intensity decreased.

Studies of Procoagulant Activity on Collagen-Adherent Platelets

Finally, the procoagulant activity of NC- and PC-adherent platelets was assessed by measurement of thrombin formation. Platelets adhering on NC had consistently increased procoagulant capacity compared with platelets adhering on PC, and platelets, which originally adhered to NC in the presence of Ca\(^2+\), generated the most thrombin (Figure 8). However, if the GFPs were first allowed to adhere in the presence of Mg\(^2+\) and the procoagulant activity was then developed by the addition of external Ca\(^2+\), significant thrombin formation was observed on PC, albeit still less than on NC when related to the number of bound platelets. Thrombin formation (\(\mu\)U \(\times 10^6\) GFPs\(^{-1}\) \(\times\) min\(^{-1}\)) was 11±4 (PC, Mg\(^2+\)) < 18±11 (NC, Mg\(^2+\)) < 30±15 (NC, Ca\(^2+\)), n=4.

Discussion

Structural differences resulting from the removal of collagen telopeptides and native quaternary structure (Figure 1) lead to reduced activation potential of the collagen, whereas the adhesive capacity remained, as illustrated by relatively similar surface coverages on both the collagen fibrils and monomers during perfusion (Figures 2 and 3) (except at the high shear rate in citrated blood) and adhesion experiments with GFPs in the presence of Mg\(^2+\) cations (Figure 4). As previously reported, adhesion to monomeric collagen (1) was Mg\(^2+\)-dependent and mediated by GP Ia/IIa,\(^10\) (2) was inhibited by Ca\(^2+\) cations,\(^10,29\) and (3) resulted in complete spreading of platelets\(^41\) (Figures 4, 5, and 6).

Our most interesting finding was the complete cation-dependence of the shear force–resistant thrombus formation on PC, in contrast to NC, which were capable of inducing thrombus formation cation-independently. Compatible with our other findings, this result manifests the importance of GP Ia/IIa as a mandatory primary receptor of platelet interaction with the collagen triple helix, as also reported by others.\(^42\) In support of our results, it was recently highlighted that GP Ia/IIa is irreplaceable in resisting shear forces.\(^20,43\) Being a poor adhesive substrate for GFPs in the presence of Ca\(^2+\) or in the absence of divalent cations in blood flowing at the high shear rate, monomers revealed the importance of the first collagen-adherent platelets in thrombus formation.\(^37\) Collagen monomers may offer a way to study the role and donor variability of GP Ia/IIa, which are likely to be associated with certain receptor polymorphisms and even thrombotic events.\(^44,45\)

The differences between native-type collagen fibrils and monomers cannot be explained by differential vWF-binding...
capacity, because (1) vWF binding to collagen has been reported to be cation-independent,46 (2) the vWF-dependent increase in platelet deposition was observed on both substrates in PPACK-anticoagulated blood, (3) it was similarly inhibited by ATA,39 and (4) incubation of the monomer surface with vWF before perfusion (citrated blood, high shear rate) was unable to correct for the lacking platelet deposition, in contrast to restoration of the physiological Mg\(^2+\) concentration.

Adhesion to monomers correlated strongly with adhesion to fibrils in the presence of Mg\(^2+\), whereas Mg\(^2+\)-dependent adhesion on monomers did not correlate with adhesion on fibrils in the presence of Ca\(^2+\), suggesting that at least 2 different receptors mediate platelet adhesion to collagen, depending on the experimental conditions and the substrate. Existence of additional collagen receptor(s) capable of re-

**TABLE 1. Comparison of NC- and PC-Induced Aggregation in Citrated and PPACK-Treated PRP**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Lag Time, s</th>
<th>Rate, min(^{-1})</th>
<th>MA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrated PRP</td>
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<tr>
<td>NC, 2.5 (\mu)g/mL</td>
<td>60±18</td>
<td>1.9±0.7</td>
<td>76±25</td>
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<tr>
<td>PC, 10 (\mu)g/mL</td>
<td>121±18</td>
<td>2.5±1.0</td>
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<tr>
<td>PPACK-treated PRP</td>
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<td></td>
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<tr>
<td>NC, 2.5 (\mu)g/mL</td>
<td>48±6</td>
<td>3.4±0.7</td>
<td>89±3</td>
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<tr>
<td>PC, 10 (\mu)g/mL</td>
<td>50±5</td>
<td>4.5±1.0*</td>
<td>90±4</td>
</tr>
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</table>

Data represent mean±SD; n=4.

\(^*\)P<0.05. MA indicates maximal aggregation.

**TABLE 2. Comparison of NC- and PC-Induced Aggregation of GFP in the Presence or Absence of Mg\(^{2+}\)/Ca\(^{2+}\)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Lag Time, s</th>
<th>Rate, min(^{-1})</th>
<th>MA, %</th>
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<tbody>
<tr>
<td>2 mmol/L Mg(^{2+})</td>
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<tr>
<td>NC, 1.5 (\mu)g/mL</td>
<td>32</td>
<td>2.3</td>
<td>78</td>
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<tr>
<td>PC, 7.5 (\mu)g/mL</td>
<td>36</td>
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<tr>
<td>2 mmol/L Ca(^{2+})</td>
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<tr>
<td>NC, 1.5 (\mu)g/mL</td>
<td>64</td>
<td>2.6</td>
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<tr>
<td>PC, 20 (\mu)g/mL</td>
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<td>2 mmol/L Ca(^{2+}), 1 mmol/L Mg(^{2+})</td>
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<tr>
<td>NC, 1.5 (\mu)g/mL</td>
<td>36</td>
<td>2.4</td>
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<tr>
<td>PC, 7.5 (\mu)g/mL</td>
<td>42</td>
<td>2.6</td>
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</table>

Representative example of 4 different donors.
placing GP Ia/IIa was further supported by the ability of collagen fibrils to withhold thrombus formation under the high shear force (Figures 2 and 3) and the unchanged adhesion under the conditions in which GP Ia/IIa–inhibiting antibodies and Ca\(^{2+}\) cations were present (Figures 4 and 5). In the presence of GP Ia– and GP IV–blocking antibodies, ‘50% of the platelet deposition remained in citrated blood at high shear rate, compatible with an unknown platelet adhesion receptor for native-type collagen fibrils.

In addition to containing the molecular determinants for GP Ia/IIa– and GP IV–independent adhesion, collagen fibrils induced platelet activation more efficiently than monomers, as also recently suggested by increased thromboxane A\(_2\) production triggered with acid-insoluble collagen. The better platelet-binding and aggregate-forming capacity and independence of divalent cations of fibrils observed during perfusions were supported by the aggregation studies, in which \(\approx 50\%\) of the platelet deposition remained in citrated blood at high shear rate, compatible with an unknown platelet adhesion receptor for native-type collagen fibrils.

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In the presence of Ca\(^{2+}\), augmented platelet activation by fibrils was also evidenced by the tyrosine-phosphorylation events (Figure 7) and enhanced procoagulant capacity (Figure 8). Conversely, the similarities in tyrosine phosphorylation induced by NC and PC in the presence of Mg\(^{2+}\) and the distinct NC-induced tyrosine phosphorylation patterns in different cationic milieus (Figure 7), as well as the equal procoagulant responses of NC and PC if the platelets were first allowed to bind in the presence of Mg\(^{2+}\) (Figure 8), all suggest that GP Ia/IIa also participates in platelet activation. Recently, the interplay in the GP Ia/IIa–GP IIb/IIIa axis was disturbed by mast cell–derived heparin proteoglycans, which strongly inhibited aggregate formation subsequent to adhesion on collagen.

The identity of the hypothetical collagen receptor(s) acting on determinants on native-type fibrils was not elucidated by these experiments. The activating receptor is likely to be GP VI, which is reported to induce potent signal transduction by collagen or by agents acting solely through it. In this study, GP VI may participate, because NC induced faster tyrosine phosphorylation patterns, as well as a procoagulant response, which could accord with the morphological changes of platelet deposition on NC in the presence of Ca\(^{2+}\) (Figure 6). However, it has been reported that under blood flow, GP VI alone is incapable of retaining adhesion, so it is unlikely that it acts as a primary adhesive receptor. We found that in citrated blood, an mAb against GP IV (131.7) was able to inhibit platelet deposition on NC in the early adhesive phase by 49%, as reported previously. Thus, it is possible that when GP Ia/IIa and GP IV are eliminated in our perfusion experiments and when vWF is unable to firmly stop platelets, a third, as yet unidentified receptor plays a necessary part in the early platelet interaction with native collagen fibrils.

In the light of the present understanding of platelet-collagen interaction, our results suggest that (1) GP Ia/IIa is an absolute requirement for the collagen triple helix to support shear-resistant thrombus growth, and thus monomers

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**Figure 7.** Time-dependent (0, 15, 30, and 45 seconds and 1, 3, and 5 minutes) tyrosine phosphorylation patterns induced by stimulation of GFPs with 1.5 \(\mu \)g/mL of NC and of prefibrillated PC in the presence of 2 mmol/L Mg\(^{2+}\) (A) or Ca\(^{2+}\) (B) and KQAGDV. The control samples were stirred at 37°C for 1 minute. Example is representative of 3 separate donors.

**Figure 8.** Procoagulant activity generated by platelets that had first adhered on NC (hatched bars) or PC (solid bars) in the presence of 2 mmol/L Mg\(^{2+}\) or Ca\(^{2+}\). Thrombin-forming capacity was assessed in the presence of diluted plasma and tissue thromboplastin. Samples of supernatants were reacted with the chromogenic thrombin substrate S-2238, and their absorbance was measured at 405 nm. A reagent control was subtracted from the values. Data represent mean \pm SD; \(n=4\); \(*P<0.01\).
provide a tool to study GP Ia/Illa activity in individual donors; (2) the native fibril structure of the collagen molecule is involved in platelet-activating events such as aggregate formation, tyrosine phosphorylation signaling, and development of procoagulant activity; (3) an unknown receptor acting via quaternary structure/telopeptides is capable of replacing GP IV–, GP Ia/Illa–, and cation-dependent functions in primary adhesion; and (4) when functional, GP Ia/Illa also participates in the events leading to platelet activation.

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Studies of Adhesion-Dependent Platelet Activation: Distinct Roles for Different Participating Receptors Can Be Dissociated by Proteolysis of Collagen
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