Haematologica 2000; 85:280-288
original paper

Hemostasis & Thrombosis

Thrombin facilitates primary platelet adhesion onto vascular surfaces in the absence of plasma adhesive proteins: studies under flow conditions

MARIBEL DÍAZ-RICART, EVA ESTEBANELL, MIGUEL LOZANO, JOSÉ AZNAR-SALATTI,* JAMES G. WHITE,° ANTONIO ORDINAS, GINÉS ESCOLAR

*Servicio de Hemoterapía-Hemostasia, Hospital Clinic, Universidad de Barcelona, Spain; *Centeón S.A., Barcelona, Spain; °Department of Pediatrics, University of Minnesota, Minnesota, USA*

ABSTRACT

***Background and Objectives*. The effect of local and circulating thrombin on platelet adhesion onto vascular surfaces was explored in the absence of plasma adhesive proteins using flow conditions.**

***Design and Methods.* To study the local effects of thrombin, denuded rabbit aorta segments were incubated with thrombin concentrations of 0.001, 0.01 and 0.1 U/mL. To evaluate the effects of circulating thrombin, the same concentrations were added to perfusates consisting of washed platelets and washed red blood cells suspended in a human albumin solution (5%). In some experiments, purified von Willebrand's factor (vWF) (Haemate-P) was added to the perfusates (0.8 U/mL of vWF, final concentration). A humanized chimeric antibody to the GPIb-IIIa complex (Reopro) was used to determine the role of this glycoprotein on platelet adhesion under the conditions described. The effect of blocking GPIb was also assessed. Perfusions were carried out at 800 s^{-1} for 10 min. The interaction of platelet with the vessel surface was morphometrically evaluated and expressed as percentage of surface coverage (%SC). Changes in the surface expression of the major platelet antigens were also analyzed by flow cytometry.**

***Results.* Incubation of subendothelial surfaces with thrombin enhanced platelet deposition with respect to control levels (increases in SC of 64%, 79% and 86% with 0.001, 0.01 and 0.1 U/mL of thrombin, respectively). Low concentrations of thrombin (0.001 and 0.01 U/mL) incorporated in the perfusates resulted in a similar pro-adhesive effect (increases in SC of 64% and 71%, respectively) while the highest concentration (0.1 U/mL) failed to produce a pro-adhesive effect due to the augmented formation of platelet aggregates with subsequent thrombocytopenia (15±1 vs. 160±5 x10^9 plt/L in the perfusates). Similar results were obtained when VWF was present in the perfusate. Reduction of platelet deposition by blockade of GPIIb-IIIa (to 5.3±0.7%) was partially restored by thrombin. Blockade of GPIb prevented platelets from adhering even when thrombin was present (%SC of 2.0±0.8%). No significant changes in the distribution of platelet membrane glycoproteins during perfusion experiments were detected.**

***Interpretation and Conclusions.* Our results suggest that thrombin facilitates primary platelet adhesion onto vascular surfaces even in the absence of plasma adhesive proteins. This effect seems to be mainly dependent on the GPIb/vWF axis.**

Key words: thrombin, platelet adhesion, GPIIb-IIIa, GPIb, von Willebrand's factor

Correspondence: Maribel Díaz-Ricart, M.D., Servicio de Hemoterapia y Hemostasia, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain. Phone: international +34-3-2275400 Ext.: 2034 or 2307 – Fax: international +34-3-2275448 – E-mail: mdiaz@clinic.ub.es

Vascular subendothelium is a collagen-rich adhesive surface onto which platelets adhere, spread and aggregate. The initiation of these events implies the presence of functional glycoproteins in the platelet membrane. Studies with circulating blood have provided evidence that GPIb, through its interaction with subendothelial von Willebrand's factor (vWF), plays a fundamental role by supporting platelet adhesion.[1,2] GPIIb-IIIa participates in platelet spreading[3] and platelet aggregate formation.[4] Once activated, platelets provide a procoagulant surface on which thrombin is generated, stabilizing the growing thrombi.[5-7]

The major body of evidence arising from studies into thrombosis models, or from investigations of experimental perfusion systems with circulating blood, suggests that thrombin facilitates platelet interaction with the damaged subendothelium. Subendothelial tissue factor promotes platelet adhesion and hemostasis by increasing local thrombin formation at sites of vascular damage.[8,9] Local thrombin synthesis facilitates platelet recruitment on collagen surfaces[10] or damaged vessel walls[11] and fosters platelet thrombus growth.[7] Thrombin binds to the extracellular matrix,[12] remains biologically active and would act as a potent inducer of platelet deposition and thrombus formation in a non-anticoagulated whole blood perfusion system.[13] It has been suggested that an increased formation of fibrin could play a role in the increased platelet deposition mediated by thrombin.[13,14]

In contrast, investigations performed on washed platelet suspensions have generated evidence of a decreased presence of GPIb on the platelet surface after activation by thrombin.[15,16] According to these *in vitro* studies, exposure of platelets to thrombin

results in the mobilization of 60 to 70% of the GPIb receptors from the surface towards the internal membranes of the open canalicular system.[17-19] The internalization of GPIb by thrombin could be a mechanism through which thrombin downregulates platelet adhesion.[20,21] The decreased platelet adhesion predicted from *in vitro* studies on platelet suspensions is in sharp contrast to the fostering effects of thrombin on platelet adhesion suggested from studies with circulating blood. Therefore, it would be useful to evaluate the consequences that a diminished surface expression of GPIb, supposingly produced by thrombin, may have on platelet adhesion.

The present study was designed to evaluate the specific effects of local and circulating thrombin on platelet adhesion onto subendothelial surfaces. To investigate the basic mechanisms involved in the effects of thrombin, blood was experimentally depleted of plasma adhesive proteins and coagulation factors. In some experiments, an intermediate-purity factor VIII concentrate (Haemate-P) was added to the perfusates, as previously described,[22] in order to assess the effect of vWF as a main adhesive protein. To determine the roles of GPIb and GPIIb-IIIa receptors, studies were also performed in the presence of specific monoclonal antibodies. Modifications in the presence of platelet membrane glycoproteins GPIb, GPIIb-IIIa, GPIV and platelet activation markers during the perfusion studies were monitored with flow cytometry techniques.

Design and Methods

Experimental design

Studies were designed to evaluate the specific effects of thrombin on platelet adhesion to exposed subendothelial surfaces in flowing blood. For this purpose, experiments were performed with blood which had been experimentally depleted of coagulation factors. To test the local effects of thrombin, vascular segments were incubated with different concentrations of thrombin (human plasma thrombin, Sigma Chemical Co, St. Louis, MO, USA), 0.001, 0.01 and 0.1 U/mL, for 15 minutes before being perfused with reconstituted blood samples. To test the effects of circulating thrombin, thrombin was added to the perfusates, within 1 min of initiating the perfusion, at the same concentrations as used previously. In some experiments, purified vWF (Haemate-P, Centeon GmbH, Germany) was added to the perfusates (0.8 U/mL of vWF, final concentration). The effect of blocking GPIb and GPIIb-IIIa was evaluated in both experimental situations.

Preparation of the perfusates

Blood was obtained by venipuncture from healthy volunteers who had not taken any drug known to affect platelet function for at least ten days. Blood samples were collected in citrate/phosphate/dextrose buffer (CPD) at a final concentration of 9 parts of blood to 1 part of anticoagulant. Platelet-rich plasma (PRP) was separated by centrifugation of citrate-anticoagulated blood for 20 minutes at 100× g. Plasma was discarded and platelets were washed twice with equal volumes of citrate/acid citric/dextrose (93 mM sodium citrate, 7 mM citric acid and 140 mM dextrose) pH 6.5, containing 5 mM adenosine and 3 mM theophylline. The final pellet was resuspended in Hanks' balanced salt solution (136.8 mM sodium chloride, 5.3 mM potassium chloride, 0.6 mM sodium hydrogen phosphate anhydrous, 0.4 mM potassium dihydrogen phosphate, 0.2 mM sodium dihydrogen phosphate 2-hydrate, 0.37 mM magnesium sulphate heptahydrate, 0.5 mM magnesium chloride hexahydrate, 1.26 mM calcium chloride). Red cells were washed three times with saline buffer containing glucose. Perfusates consisted of washed platelets suspended in a 5% human albumin solution,[22] at a final concentration of 160×10^9 plt/L, and washed red blood cells (RBC) adjusted to give a rise in hematocrit equivalent to 40%.

When required, and before adding the platelet suspension and the RBC volume, purified vWF (Haemate-P, Centeon GmbH, Germany) was added to achieve a final concentration of vWF of 0.8 U/mL, as previously described.[22]

Effect of antibodies to GPIb or GPIIb-IIIa

In some experiments, platelets were incubated with a monoclonal antibody (AP1) to block GPIb. The platelet suspension was incubated before reconstitution with a concentration of AP1 of 1 µg/mL, which was able to block completely ristocetin-induced platelet aggregation.

The effect of incubating platelets with an anti-GPIIb-IIIa monoclonal antibody (7E3, Reopro, Lilly) was assessed. When required and before reconstitution, platelet suspensions were incubated with Reopro, for 30 minutes before reconstitution, at a final concentration of 4 µg/mL in blood. This concentration was calculated from the therapeutic dose of Reopro used in humans.

Perfusion studies

Perfusions were performed through an annular chamber over rabbit aorta segments previously everted and denuded.[23] In some experiments, the vascular segments were previously incubated with different concentrations of thrombin for 15 minutes before initiating the perfusion. In those experiments performed to test the effect of circulating thrombin, the different concentrations of thrombin were added to the perfusates 1 minute after initiating the perfusion. Flow was maintained by means of a peristaltic pump (Watson-Marlow, 502S) at a shear rate of 800 sec^{-1} for 10 minutes at 37°C. In some experiments aliquots of perfused blood samples were collected, for flow cytometry analysis, at different time points during the perfusion and fixed with paraformaldehyde (0.3% final concentration). After perfusions, segments were removed, fixed with 2.5% glutaraldehyde, embedded in JB-4 compound (Polysciences, Warrington, PA), thin-sectioned for light microscopy and stained with a 0.02% solution of toluidine blue.[24]

Morphometric evaluation

Interaction of platelets with subendothelial surfaces was evaluated according to Baumgartner and Muggli.[25] Platelets or groups of interacting platelets were classified as follows:[26] Contact (C), platelets

that were attached but not spread on the subendothelium; Adhesion (A), platelets that were spread on subendothelium or formed layers of less than 5 µ in height; and Thrombi (T), platelet aggregates of 5 µ or more in height. Under the experimental conditions used in these studies in which no plasma proteins were present, the formation of large aggregates was practically absent. Therefore, the total surface covered (SC) by platelets was obtained by adding C+A, and expressed as a percentage with respect to the total surface of the vessel screened.

Flow cytometry studies

A study of platelet surface glycoproteins and activation-dependent antigens was carried out on 500 µL aliquots of perfused blood samples collected in paraformaldehyde (final concentration 0.3%) at different times during the perfusion (before starting and at 1, 2, 5 and 10 minutes).

Immunolabeling of platelets with monoclonal antibodies (MoAbs) was performed in blood samples using dual-color analysis.[27] Briefly, after collection of samples, 5 µL of blood were added to polypropylene tubes preloaded with 50 µL phosphate buffered saline (PBS). The following MoAbs (purchased from Immunotech, Marseille, France) were used for labeling: anti CD41a-PE, anti CD62P-FITC, anti CD63-FITC, anti CD36-FITC, anti CD42b-FITC and a IgG1-FITC as a negative control. Samples for dual-color analysis were first incubated for 15 min with saturating concentrations of anti CD41a-PE in the dark and at room temperature, without stirring, followed by the addition of FITC-conjugated MoAbs and incubation for 15 minutes. Samples were then diluted with 1 mL PBS and analyzed.

Blood samples were analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) at an excitation wavelength of 488 nm. Fluorescence and scatter signals were calibrated with 2 mm Calibrite beads (Becton-Dickinson). FITC and PE fluorescences were detected with a 530/30 and 575/26 nm band pass filter, respectively, and overlap in the emission spectra of FITC and PE was corrected. Blood samples were diluted in such a way that the flow rate through the laser beam was less than 3,000 events per second. Platelets were differentiated from red and white cells by their specific membrane immunofluorescence.

Histograms were composed from fluorescence data obtained in the logarithmic mode; 5,000 particles were analyzed in each sample. Mean fluorescence intensities (MFI) corresponding to antibody binding (GPIb, GPIIb-IIIa and GPIV) were expressed using LYSYS II 1.1 conversion software (Becton-Dickinson) in an arithmetic mode on a Hewlett-Packard H-P 217 computer (Palo Alto, CA, USA). In order to analyze the data corresponding to MoAbs anti CD42b, anti CD41a and anti CD36, the mean fluorescence intensity during the perfusion was expressed as a percentage of the value existing at the beginning of the perfusion. To analyze CD62P and CD63, results were expressed as the percentage of positive platelets for these MoAbs. An analytical marker was set in the green fluorescence channel to define the 2% of the platelet population with the highest membrane fluorescence at the baseline level. This marker was used as a threshold to determine the proportion of platelets exhibiting immunofluorescence above this level in all subsequent samples.[28]

Statistics

Statistical analysis was performed using paired Students' t-test and a $p<0.05$ was considered statistically significant.

Results

Effect of thrombin bound to subendothelium on platelet interactions

Control experiments consisted of blood perfusates containing adjusted numbers of platelets (160×10^9 plt/L) and washed red blood cells (40% hematocrit) which were perfused at 800 sec^{-1}, for 10 min, through an annular chamber containing denuded rabbit aorta segments (Figure 1A). Results of these control studies showed an average surface covered by platelets (%SC) equivalent to 11.7±1.2% (mean±SEM, n=8). Platelet counts at the end of the perfusion studies were slightly below the initial values, but differences

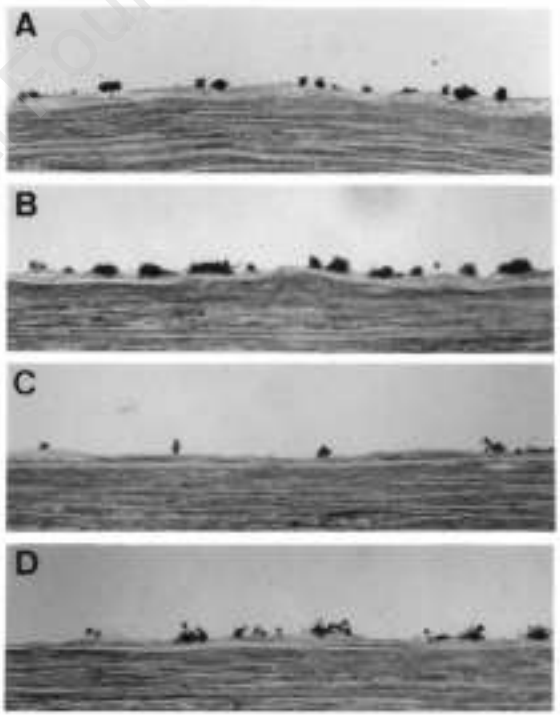

Figure 1. Micrographs (x400) showing platelet adhesion onto subendothelium of vascular segments perfused at 800 s^{-1} for 10 min. Perfusates consisted of washed platelets (160×10^9 plt/L) and red blood cells (40% hematocrit) resuspended in human albumin solution (5%). Images correspond to perfusions of reconstituted blood samples over subendothelial surfaces (A) non-treated or (B) previously incubated with 0.1 U/mL of thrombin; in some experiments perfusates containing platelets previously incubated with Reopro (anti GPIIb-IIIa chimeric antibody) were perfused over subendothelial surfaces (C) non-treated or (D) previously incubated with 0.1 U/mL of thrombin.

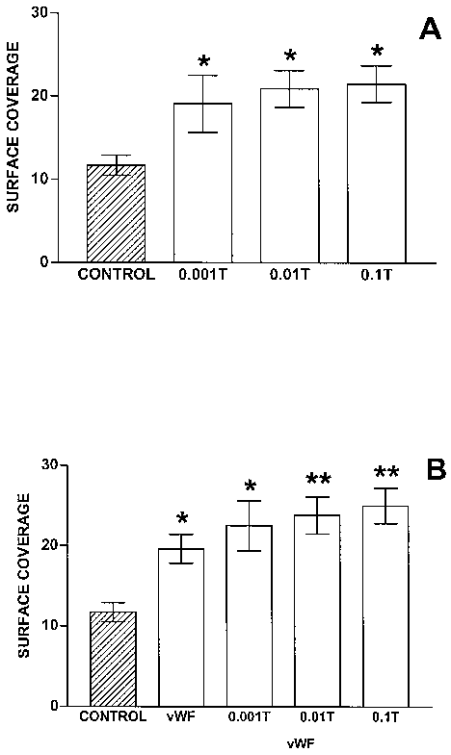

Figure 2. Bar diagrams show the effect of thrombin bound to subendothelium on platelet interactions. Subendothelial surfaces were incubated for 15 min before initiating perfusions with increasing concentrations of thrombin (T): 0 (CONTROL), 0.001, 0.01 and 0.1 U/mL. Perfusates consisted of washed platelets (160×10^9 plt/L) and red blood cells (40% hematocrit) resuspended in human albumin solution (5%) in the absence (A) and in the presence (B) of purified von Willebrand's factor (vWF) (0.8 U/mL, final concentration). Perfusions were carried out at 800 s^{-1} for 10 min at 37°C. Data are expressed as percentages with respect to the total surface screened. Mean±SEM, n=8, *p<0.01 and **p<0.001 vs. control data.

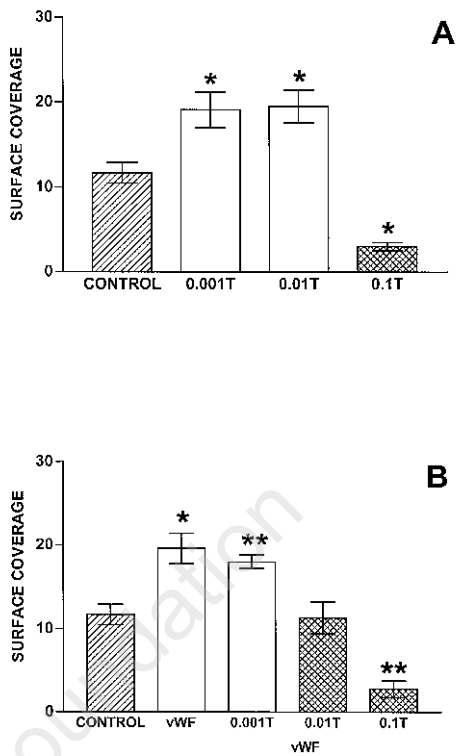

Figure 3. Bar diagrams show the effect of circulating thrombin on platelet-subendothelium interactions. Concentrations of thrombin (T) of 0 (CONTROL), 0.001, 0.01 and 0.1 U/mL were added to the perfusates after 1 min of initiating the perfusion over non-treated subendothelial surfaces. Perfusates consisted of washed platelets (160×10^9 plt/L) and red blood cells (40% hematocrit) resuspended in human albumin solution (5%) in the absence (A) and in the presence (B) of purified von Willebrand's factor (VWF) (0.8 U/mL, final concentration). Double-dashed bars indicate a decrease in platelet counts below 50%. Perfusions were carried out at 800 s^{-1} for 10 min at 37°C. Data are expressed as percentages with respect to the total surface screened. Mean±SEM, n=8, *p<0.01 and **p<0.001 vs. control data.

never reached levels of statistical significance.

As shown in Figure 2A, incubation of vascular segments with increasing concentrations of thrombin (0.001, 0.01 and 0.1 U/mL) resulted in an increase in the surface covered by platelets (64%, 79% and 86% of increase respectively, considering control values as 100%). The increase in the surface coverage promoted by thrombin bound to subendothelium was statistically significant with respect to controls (p<0.001) (Figure 2A), although it seemed to reach a plateau with no statistically significant differences among the different concentrations of thrombin used (%SC of 19.1±3.4%, 20.9±2.2% and 21.5±2.2%, respectively). Incubation of vascular segments with the different concentrations of thrombin did not significantly alter platelet counts with respect to the initial values in reconstituted blood samples before perfusion.

Addition of vWF to the perfusates significantly increased the percentage of surface covered by platelets from 11.7±1.2% (in controls) to 19.6±1.8% (p<0.01) (Figure 2B). In this experimental situation, when vascular segments were incubated with 0.001, 0.01 and 0.1 U/mL of thrombin, values of %SC increased to 22.5±3.1% (p<0.01), 23.8±2.3% (p<0.001) and 25.0±2.2% (p<0.001), respectively (see Figure 2B).

Effect of circulating thrombin on platelet-subendothelium interactions

In parallel experiments the effects of circulating thrombin (0.001, 0.01 and 0.1 U/mL of perfusate) on platelet adhesion were also evaluated. In these experiments, thrombin was added 1 min after the perfusion was initiated. The addition of the lower concentrations of thrombin, 0.001 and 0.01 U/mL, significantly increased platelet deposition onto the subendotheli-

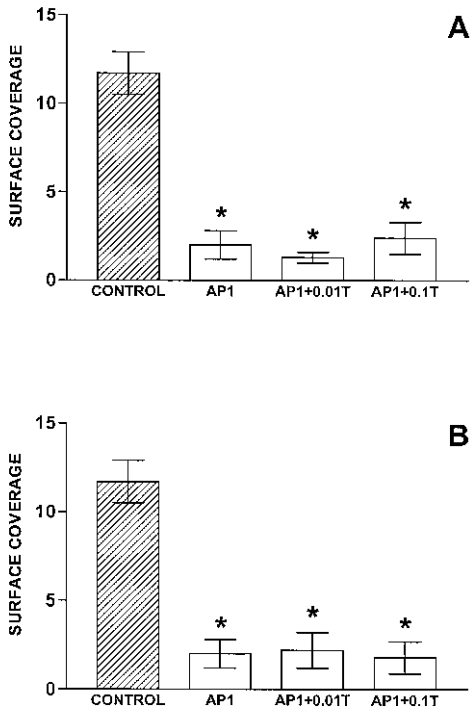

Figure 4. Bar diagrams represent surface covered by platelets on subendothelial surfaces perfused with reconstituted blood samples containing platelets before (CONTROL) and after treatment with a monoclonal antibody to GPIb (AP1). In A) experiments were performed using subendothelial surfaces previously incubated with 0, 0.01 or 0.1 U/mL of thrombin for 15 min. In B) non-treated subendothelial surfaces were perfused with blood samples to which 0, 0.01 or 0.1 U/mL of thrombin were added 1 min after initiating the perfusion. Results are expressed as percentages of surface covered by platelets with respect to the total surface screened. Mean±SEM, n=4; *p<0.001.

um with values of surface coverage equivalent to 19.1±2.1% (mean±SEM) and 19.5±1.9%, respectively (p<0.001). These values represent relative increases of 64% and 71% with respect to the control (Figure 3A). Platelet counts after perfusion were slightly below those obtained in the initial sample (157±10 and 149±5×10^9 plt/L, with thrombin 0.001 and 0.01 U/mL, respectively) though differences did not reach the levels of statistical significance. The addition of 0.1 U/mL of thrombin dramatically reduced the degree of platelet deposition onto the perfused subendothelial surfaces. The %SC dropped to values of 3.01±0.5% (relative decrease of 75% with respect to the control values; p<0.001). A dramatic reduction in the platelet count (15±1×10^9 plt/L) was observed in the post-perfusion samples with this high concentration of thrombin (0.1 U/mL).

In the presence of vWF (Figure 3B), addition of 0.001 U/mL of thrombin to the perfusates increased the surface coverage with respect to control values (18.0±0.8% vs. 11.7±1.2%, p<0.001), although the coverage did not differ significantly when compared with the %SC reached in the presence of vWF itself (%SC of 19.6±1.8%). However, higher concentrations of thrombin, of 0.01 and 0.1 U/mL, did cause a drop in the platelet counts from 160×10^9 (in controls) to 75±15 and 13±2×10^9 plt/L, respectively. The decrease in platelet counts observed resulted in a reduction of the surface coverage to 11.3±1.9% and 2.8±1.0%, respectively.

Effect of local or circulating thrombin on platelet adhesion in the presence of specific antibodies to glycoprotein Ib or IIb-IIIa

Blockade of GPIb resulted in an almost complete inhibition of platelet adhesion (2.0±0.8%, mean± SEM, n=4, p<0.001 vs. control) (Figures 4A and 4B). Incubation of segments with thrombin or the addition of thrombin to the perfusates did not modify the results obtained with the anti-GPIb antibody.

Platelet membrane GPIIb-IIIa was blocked by incubation of platelet suspensions with 4 mg/mL of Reopro (final concentration in the perfusates). Blockade of GPIIb-IIIa by the specific chimeric antibody reduced platelet deposition in control experiments (Figure 1C) (from 11.7±1.2% to 5.3±0.7%, p<0.05). This inhibitory effect (about 50% with respect to control values) was partially restored by thrombin, either locally incubated on the subendothelium (Figure 1D) or added to the perfusate. Results of these studies, using the same experimental design described before, are summarized in Figures 5A and 5B. The concentration of 0.001 U/mL of thrombin was not used in these experiments since its effect either incubated on the vascular segments or added to the perfusates was similar to that observed with the concentration of 0.01 U/mL.

Perfusion of denuded vascular segments, previously incubated with 0.01 and 0.1 U/mL of thrombin, with perfusates containing the anti-GPIIb-IIIa resulted in %SC of 7.5±1% and 9.9±1.7%, respectively, reaching %SC values of 70% and 89% with respect to control values, considered as 100% (Figure 5A).

Addition of 0.01 and 0.1 U/mL of thrombin 1 minute after initiation of perfusion to the perfusates, previously incubated with the anti GPIIb-IIIa antibody, restored platelet deposition onto the subendothelial surface to values of 9.4±1.5% and 7.8±1.3% (85% and 70% of control values) (Figure 5B). The decreases in platelet counts observed in the post-perfusion samples exposed to circulating thrombin (0.01 and 0.1 U/mL) were partially corrected when the anti GPIIb-IIIa antibody was incorporated in the perfusates, but remained low (35±5×10^9 plt/L) with the highest concentration of thrombin (0.1 U/mL).

Changes in the expression of platelet membrane glycoproteins during perfusion experiments

There were no changes in the expression of the antigens studied during control perfusions. However, in experiments in which thrombin was added to the perfusates at a final concentration of 0.1 U/mL we observed significant changes in the expression of

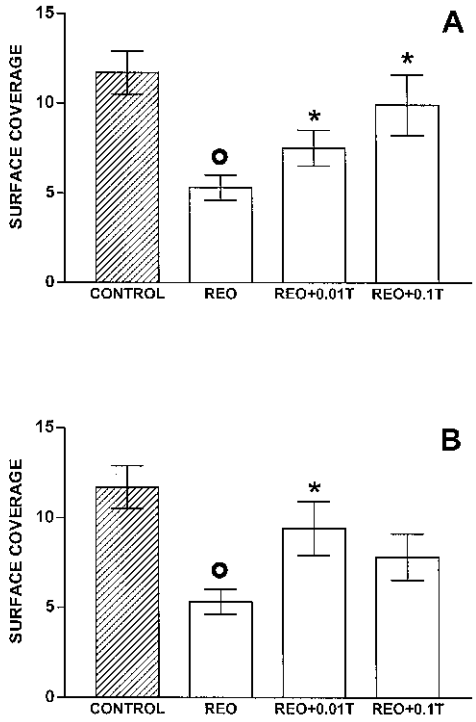

Figure 5. Bar diagrams represent surface covered by platelets on subendothelial surfaces perfused with reconstituted blood samples containing platelets before (CONTROL) and after treatment with a chimeric anti GPIIb-IIIa antibody (REO). In A) some experiments were performed using subendothelial surfaces previously incubated with 0, 0.01 or 0.1 U/mL of thrombin for 15 min. In B) non-treated subendothelial surfaces were perfused with blood samples to which 0, 0.01 or 0.1 U/mL of thrombin were added 1 min after initiating the perfusion. Results are expressed as percentages of surface covered by platelets with respect to the total surface screened. Mean±SEM, n=4; ○ = $p<0.05$ Reopro vs. Control, *$p<0.05$ vs. Reopro.

most of the antigens analyzed. After 1 minute of adding thrombin, the percentage of platelets expressing activation-dependent antigens (CD62P and CD63) increased from 2±0.8% and 2.2±0.3% (initial values) to 18.9±2.6% and 19.5±1.3%, respectively. In the same samples, we detected increases in the intensity of fluorescence for CD36 and CD41a of 164±5% and 34.6±4.1%, respectively. No changes were observed in the expression of CD42b. The presence of 0.1 U/mL of thrombin in the perfusates induced platelet aggregation with an important decrease in the platelet counts, which caused a reduction in platelet adhesion, as mentioned before.

We did not observe significant changes in the expression of the antigens analyzed in those experiments in which vessel segments were incubated with thrombin. A slight increase of 6.5±0.1% was observed for GPIIb-IIIa expression.

Discussion

Results of the present study indicate that thrombin promotes platelet adhesion to subendothelial surfaces under flow conditions, and this effect also occurs in the absence of plasma adhesive proteins. Previous studies have shown that thrombin binds to the extracellular matrix and remains biologically active. After binding, thrombin seems to undergo configuration changes that prevent antithrombin III/heparin from affecting its catalytic activity.[12] It has been suggested that thrombin would cause internalization of GPIb and subsequent reduction in platelet adhesion. However, our present data, obtained under experimental conditions in which adhesion was mainly dependent on the GPIb-vWF axis, suggest that thrombin increases platelet adhesion. Moreover, thrombin was capable of moderately improving adhesion in experiments in which GPIIb-IIIa receptors had been blocked by specific antibody fragments. The latter evidence suggests that other receptors or adhesive proteins expressed during platelet activation could partially overcome the blocking effects of anti-GPIIb-IIIa therapy.

Several studies have suggested that GPIb becomes internalized in the open canalicular system when isolated platelets are exposed in suspension to thrombin.[16,17,19,21] This clearance of GPIb from the external membranes has been proposed as a mechanism to regulate platelet adhesive function.[20] A similar effect of thrombin on GPIb receptors could be expected locally at the subendothelial environment when platelets initiate primary hemostasis.[15] In our experiments, incubation of vascular segments with low concentrations of thrombin (0.001 to 0.01 U/mL) was followed by a marked increase of platelet attachment onto the perfused subendothelial surface. The fact that an antibody to GPIb inhibited the pro-adhesive effect of thrombin indirectly suggests that this glycoprotein was mainly responsible for the adhesion of platelets observed under our experimental situation.

Our experiments demonstrate that thrombin, local or circulating, has a pro-adhesive effect on platelets. Should thrombin decrease surface GPIb, this phenomenon will have little relevance on platelet adhesion onto subendothelium under flow conditions.[29] It should be noted that in the vast majority of experiments in which an important down-regulation of GPIb was seen, platelets had been exposed to high concentrations of thrombin while suspended in buffers containing EDTA.[15,16,19,20,21] Although a certain degree of internalization has been observed with concentrations of thrombin equivalent to 0.05 U/mL,[16] the down-regulation seems to be maximal with concentrations ≥ 1 U/mL.[17] The internalization of GPIb seems to be quantitatively lower in the presence of Ca^{2+}, as measured by flow cytometry,[21] although differences in the extent of this down-regulation between different laboratories persist.[30] Our present data suggest that the negative effects of thrombin on platelet adhesion predicted in previous studies[20] may not be relevant when studies are performed under more physiologic conditions (i.e shear stress and thrombin concentrations).

Platelet adhesion decreased significantly after the

addition of higher concentrations of thrombin to the perfusates. Data from our flow cytometry studies suggest that the decrease in platelet adhesion observed under such experimental conditions is likely related to the severe thrombocytopenia caused by recruitment of platelets into aggregates[31] or to platelet microvesiculation. This pro-aggregating effect of thrombin was also seen in the presence of vWF although this phenomenon occurred with a ten-fold lower concentration of thrombin. We should consider that perhaps one of the problems of the flow cytometric determinations performed in our study is that on many occasions they are performed on platelets that have failed to interact with the subendothelium or with other platelets and, therefore, they are not representative. However, results obtained from experiments in which blood platelets suspended in buffers are exposed to high concentrations of thrombin, while subsequent platelet aggregating responses are chemically blocked,[16-20] produce an even more dramatic distortion of normal platelet function. Therefore, conclusions in our study are mainly derived from those results obtained from the adhesion studies.

Chimeric antibodies to GPIIb-IIIa have been successfully used to prevent vascular restenosis.[32,33] Our present studies confirm that such a therapeutic approach effectively inhibits platelet deposition, probably by blocking residual platelet spreading supported by GPIIb-IIIa receptors. However, in our studies thrombin could partially overcome the effect of GPIIb-IIIa blockade. Studies by different groups have suggested that vWF and fibrinogen[34,35] released during the activation of platelets binds, to GPIIb-IIIa present in the open canalicular system. Internal pools of glycoproteins located in the open canalicular system or α-granules would be less available to therapeutic antibodies present in the plasma environment. Another possible explanation is that thrombin could act synergistically with the collagen-rich subendothelial surface thus facilitating platelet adhesion. Glycoprotein IV (GPIV or CD36) accelerates platelet interactions with collagen surfaces[36] and it has been suggested that thrombin increases the expression of this glycoprotein.[37]

Data from this study may have therapeutic implications. The controversy about the relative roles of antiplatelet or antithrombin strategies is still ongoing.[38-42] In those situations in which deep vascular damage occurs (e.g. angioplasty), there is exposure of tissue factor resulting in thrombin generation. In fact, the experimental conditions used in the present study resemble those occurring in deep vascular damage.[43] The thrombogenicity of the collagen-rich surface resulting from enzymic digestion of a vascular segment in this study was further enhanced by exposure to thrombin. Our data support the concept that generation of thrombin at sites of deep vascular damage facilitates platelet interaction. Therapeutic approaches with anti-thrombin drugs have been shown to be effective in preventing platelet deposition in experimental thrombosis models.[38,43]

Modulation of the vWF/GPIb axis is perceived as a promising therapeutic approach aimed at preventing excessive platelet interactions with damaged vessels.[44-47] If GPIb disappeared from the platelet surface, as predicted from studies on platelets activated in suspension, the rationale for strategies targeting the vWF/GPIb axis would be lost. Our present results indirectly suggest that the beneficial effects of therapeutic strategies aimed at modulating GPIb/vWF, or perhaps GPIa-IIa, GPIV and GPVI/collagen should be explored in the near future.

In summary, data from the present study show that preactivation by thrombin facilitates primary platelet adhesion onto collagen-rich vascular surfaces in an experimental system which is mainly dependent on the GPIb/vWF axis. Though GPIIb-IIIa/fibrinogen mechanisms may play a role, the effects of thrombin are clearly manifested in the absence of fibrinogen or are still noticeable when GPIIb-IIIa receptors are specifically blocked.

Contributions and Acknowledgments

All the authors participated in the conception and design of the present study, in the analysis and interpretation of data, and in drafting and revising the manuscript. MD-R was primarily responsible for the conception of this investigation and production of the article. EE and JA-S carried out the perfusion and blocking experiments and ML performed the flow cytometry analyses. JGW and AO participated in the discussion of the results. GE was involved in the conception of the study, discussion of the data and correction of the manuscript. The order of authorship has been a joint decision of the co-authors based on their participation in the design of the study, the experimental tasks and in the discussion of the results.

Funding

This work was supported by Fondo de Investigaciones Sanitarias de la Seguridad Social, Ministerio de Sanidad (FIS 98/321, 99/0110, 99/0106); Dirección General de Investigaciones Científicas y Técnicas, Ministerio de Educación y Ciencia (DGICYT, PM95/0103), and CIRIT (SGR97-133), Spain.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received June 24, 1999; accepted November 25, 1999.

Potential implications for clinical practice

- It has been suggested that thrombin causes internalization of platelet GPIb. However, exposure of platelets to surface-bound thrombin does not reduce platelet interaction but it promotes adhesion and aggregation. This effect is not blocked by antiplatelet strategies directed at GPIIb-IIIa.

References

1. Weiss HJ, Tschopp TB, Baumgartner HR. Impaired interaction (adhesion-aggregation) of platelets with the subendothelium in storage-pool disease and after

aspirin ingestion. A comparison with von Willebrand's disease. N Engl J Med 1975; 293:619-23.
2. Bolhuis PA, Sakariassen KS, Sander HJ, Bouma BN, Sixma JJ. Binding of factor VIII-von Willebrand factor to human arterial subendothelium precedes increased platelet adhesion and enhances platelet spreading. J Lab Clin Med 1981; 97:568-76.
3. Weiss HJ, Turitto VT, Baumgartner HR. Further evidence that glycoprotein IIb-IIIa mediates platelet spreading on subendothelium. Thromb Haemost 1991; 65:202-5.
4. Baumgartner HR, Sakariassen KS. Factors controlling thrombus formation on arterial lesions. Ann NY Acad Sci 1985; 454:162-77.
5. Weiss HJ, Baumgartner HR, Turitto VT. Regulation of platelet-fibrin thrombi on subendothelium. Ann NY Acad Sci 1987; 516:380-97.
6. Weiss HJ, Turitto VT, Vicic WJ, Baumgartner HR. Fibrin formation, fibrinopeptide A release, and platelet thrombus dimensions on subendothelium exposed to flowing native blood: greater in factor XII and XI than in factor VIII and IX deficiency. Blood 1984; 63:1004-14.
7. Gast A, Tschopp TB, Baumgartner HR. Thrombin plays a key role in late platelet thrombus growth and/or stability. Effect of a specific thrombin inhibitor on thrombogenesis induced by aortic subendothelium exposed to flowing rabbit blood. Arterioscler Thromb 1994; 14:1466-74.
8. Weiss HJ, Lages B. Evidence for tissue factor-dependent activation of the classic extrinsic coagulation mechanism in blood obtained from bleeding time wounds. Blood 1988; 71:629-35.
9. Weiss HJ, Turitto VT, Baumgartner HR, Nemerson Y, Hoffmann T. Evidence for the presence of tissue factor activity on subendothelium. Blood 1989; 73:968-75.
10. Wagner WR, Hubbell JA. Local thrombin synthesis and fibrin formation in an in vitro thrombosis model result in platelet recruitment and thrombus stabilization on collagen in heparinized blood. J Lab Clin Med 1990; 116:636-50.
11. Badimon L, Badimon JJ, Lassila R, Heras M, Chesebro JH, Fuster V. Thrombin regulation of platelet interaction with damaged vessel wall and isolated collagen type I at arterial flow conditions in a porcine model: effects of hirudins, heparin, and calcium chelation. Blood 1991; 78:423-34.
12. Bar-Shavit R, Eldor A, Vlodavsky I. Binding of thrombin to subendothelial extracellular matrix. Protection and expression of functional properties. J Clin Invest 1989; 84:1096-104.
13. Salatti JA, Fenton J, Anton P, Sakariassen KS. Alpha-thrombin bound to extracellular endothelial matrix induces pronounced fibrin deposition and platelet thrombus growth in flowing non-anticoagulated human blood. Blood Coagul Fibrin 1994; 5:561-6.
14. Orvim U, Roald HE, Stephens RW, Roos N, Sakariassen KS. Tissue factor-induced coagulation triggers platelet thrombus formation as efficiently as fibrillar collagen at arterial blood flow conditions. Arterioscler Thromb 1994; 14:1976-83.
15. George JN, Torres MM. Thrombin decreases von Willebrand factor binding to platelet glycoprotein Ib. Blood 1988; 71:1253-9.
16. Michelson AD. Thrombin-induced down-regulation of the platelet membrane glycoprotein-Ib-IX complex. Semin Thromb Hemost 1992; 18:18-27.
17. Michelson AD, Ellis PA, Barnard MR, Matic GB, Viles AF, Kestin AS. Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, adenosine diphosphate, or an in vivo wound. Blood 1991; 77:770-9.
18. Michelson AD, Barnard MR. Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb-IIIa complex. Blood 1987; 70:1673-8.
19. Hourdille P, Heilmann E, Combrié R, Winckler J, Clemetson KJ, Nurden AT. Thrombin induces a rapid redistribution of glycoprotein Ib-IX complexes within the membrane systems of activated human platelets. Blood 1990; 76:1503-13.
20. Hourdille P, Gralnick HR, Heilmann E, et al. von Willebrand factor bound to glycoprotein Ib is cleared from the platelet surface after platelet activation by thrombin. Blood 1992; 79:2011-21.
21. Nurden P. Bidirectional trafficking of membrane glycoproteins following platelet activation in suspension. Thromb Haemost 1997; 78:1305-15.
22. Aznar-Salatti J, Escolar G, Arnau C, et al. An intermediate-purity factor VIII concentrate supports platelet adhesion under flow conditions. Hemophilia 1997; 3:14-20.
23. Baumgartner HR. Platelet interaction with collagen fibrils in flowing blood. I. Reaction of human platelets with α-chymotrypsyin-digested subendothelium. Thromb Haemost 1977; 37:1-16.
24. Escolar G, Bastida E, Ordinas A, Castillo R. Interaction of platelets with subendothelium in humans treated with aspirin and dipyridamole alone or in combination. Thromb Res 1985; 40:419-24.
25. Baumgartner HR, Muggli R. In: Gordon JL, ed. Adhesion and aggregation: morphological demonstration and quantitation in vivo and in vitro; Platelets in Biology and Pathology. Amsterdam: North Holland Biomedical Press; 1976. p. 23-60.
26. Escolar G, Bastida E, Castillo R, Ordinas A. Development of a computer program to analyze the parameters of platelet-vessel wall interaction. Haemostasis 1986; 16:8-14.
27. Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987; 70:307-15.
28. Scharf RE, Tomer A, Marzec UM, Teirsten PS, Ruggeri ZM, Harker LA. Activation of platelets in blood perfusing angioplasty-damaged coronary arteries. Flow cytometric detection. Arterioscler Thromb 1992; 12:1475-87.
29. van Zanten GH, Heijnen HF, Wu Y, et al. A fifty percent reduction of platelet surface glycoprotein Ib does not affect platelet adhesion under flow conditions. Blood 1998; 91:2353-9.
30. Rao GH, Peller JD, White JG. Influence of ionized calcium on thrombin-induced down regulation of GPIb/IX receptors on human platelets. Thromb Res 1997; 85:23-31.
31. Lozano M, Bos A, de Groot PG, et al. Suitability of low-molecular-weight heparin(oid)s and a pentasaccharide for an in vitro human thrombosis model. Arterioscler Thromb 1994; 14:1215-22.
32. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. The EPIC Investigation. N Engl J Med 1994; 330:956-61.
33. Lefkovits J, Ivanhoe RJ, Califf RM, et al. Effects of platelet glycoprotein IIb/IIIa receptor blockade by a chimeric monoclonal antibody (abciximab) on acute and six-month outcomes after percutaneous transluminal coronary angioplasty for acute myocardial infarction. Am J Cardiol 1996; 77:1045-51.
34. Adelman B, Carlson P, Powers P. von Willebrand factor is present on the surface of platelets stimulated in plasma by ADP. Blood 1987; 70:1362-6.
35. Gralnick HR, Williams S, McKeown L, et al. Endoge-

nous platelet fibrinogen surface expression on activated platelets. J Lab Clin Med 1991; 118:604-13.
36. Díaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA. Platelets lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. Blood 1993; 82:491-6.
37. Michelson AD, Wencel-Drake JD, Kestin AS, Barnard MR. Platelet activation results in a redistribution of glycoprotein IV (CD36). Arterioscler Thromb 1994; 14:1193-201.
38. Heras M, Chesebro JH, Webster MW, et al. Hirudin, heparin, and placebo during deep arterial injury in the pig. The in vivo role of thrombin in platelet-mediated thrombosis. Circulation 1990; 82:1476-84.
39. Ip JH, Fuster V, Israel D, Badimon L, Badimon J, Chesebro JH. The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. J Am Coll Cardiol 1991; 17:77B-88B.
40. Chesebro JH, Zoldhelyi P, Badimon L, Fuster V. Role of thrombin in arterial thrombosis: implications for therapy. Thromb Haemost 1991; 66:1-5.
41. Badimon JJ, Weng D, Chesebro JH, Fuster V, Badimon L. Platelet deposition induced by severely damaged vessel wall is inhibited by a boroarginine synthetic peptide with antithrombin activity. Thromb Haemost 1994; 71:511-6.
42. Lefkovits J, Plow EF, Topol EJ. Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. N Engl J Med 1995; 332:1553-9.
43. Lam JY, Chesebro JH, Steele PM, et al. Antithrombotic therapy for deep arterial injury by angioplasty. Efficacy of common platelet inhibition compared with thrombin inhibition in pigs. Circulation 1991; 84:814-20.
44. Ruggeri ZM. Receptor-specific antiplatelet therapy. Circulation 1989; 80:1920-2.
45. Weinstein M, Vosburgh E, Phillips M, Turner N, Chute-Rose L, Moake J. Isolation from commercial aurintricarboxylic acid of the most effective polymeric inhibitors of von Willebrand factor interaction with platelet glycoprotein Ib. Comparison with other polyanionic and polyaromatic polymers. Blood 1991; 78:2291-8.
46. Prior C, Chu V, Holt J, et al. Production and functional characterization of a recombinant fragment of von Willebrand factor (vWF): antagonist to platelet receptor GPIb. Biotechnology 1992; 10:66-73.
47. McGhie I, McNatt J, Ezov N, et al. Abolition of cyclic flow variations in stenosed, endothelium-injured coronary arteries in nonhuman primates with a peptide fragment (VCL) derived from human plasma von Willebrand factor-glycoprotein Ib binding domain. Circulation 1994; 90:2976-81.