original paper
Haematologica 1997; 82:566-571

INHIBITION OF FIBRIN DEPOSITION ON THE SUBENDOTHELIUM BY A MONOCLONAL ANTIBODY TO POLYMORPHONUCLEAR LEUKOCYTE INTEGRIN CD11b. STUDIES IN A FLOW SYSTEM

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ABSTRACT

Background and Objective. The development of prothrombotic and procoagulant states may be regulated by direct platelet-leukocyte contact mediated by membrane receptors. We have investigated the role of CD11b integrin in polymorphonuclear leukocytes (PMN) on fibrin formation and platelet reactivity with vascular subendothelium.

Methods. Studies were carried out following a well-established perfusion model, employing either citrated blood, where fibrin formation is blocked, or blood anticoagulated with low molecular weight heparin, which allows thrombin and fibrin formation. Isolated PMN or platelets were treated with specific monoclonal antibodies to CD11b or to CD62P, respectively, and incorporated in reconstituted blood.

Results. Treatment of PMN with anti-CD11b significantly decreased the percentage of surface covered with a thick layer (>5 μm) of fibrin (34.8±3.3% vs 53.3±4.9% in control, $p<0.05$); it also reduced the average height of fibrin layer and the number of adherent leukocytes (7.9±1.2 μm vs 10.6±1.4 μm in control, $p<0.05$; and 87±8 PMN/mm^2 vs 186±25 PMN/mm^2 in control, $p<0.05$) respectively. Treatment of PMN with CD11b did not significantly affect the attachment of platelets onto the subendothelium when using citrated blood, though a slight decrease in platelet adhesion was observed in the heparinized samples. Treatment of platelets with anti-CD62P did not significantly modify any of the parameters studied.

Interpretation and Conclusions. Our results indicate that PMN have a role in promoting fibrin deposition under flow conditions, through the participation of CD11b integrin. Under our experimental conditions, this effect does not seem to be influenced by CD62P expressed on activated platelets.

Key words: endothelium, polymorphonuclear leukocytes, CD11b, platelet, fibrin, blood flow

Polymorphonuclear leukocytes (PMN) interact extensively with other blood cells. PMN are believed to play an important role in hemostatic and pathological processes such as inflammation, thrombosis or injury of the myocardium after ischemic reperfusion.[1,2] Treatment strategies involving the mechanical removal or inhibition of PMN activation have proved successful in ameliorating reperfusion-mediated injury.[3-5]

It has been reported that receptors on leukocytes, platelets and endothelium promote cellular interactions at sites of inflammation, thrombosis and vascular injury.[6] The adhesion of activated platelets to neutrophils is an important mechanism in hemostasis and inflammation, and provides a procoagulant surface which amplifies thrombin generation and thrombus formation.[7,8] A variety of complex interactions between blood platelets and leukocytes that influence cell reactivity have been reported, some of them referring to the ability of platelets to elicit several PMN responses, such as enhancing adhesiveness,[9] promoting PMN lysosomal enzyme release,[10] and increasing their aggregating capacity.[11] It is known that the activation of leukocytes leads to the up-regulation of the adhesion receptor CD11b, which binds to endothelial cell ligand ICAM-1 and mediates PMN homotypic interaction.[12]

Monocytes have been described as being very effective in activating blood clotting, and they also carry the CD11b integrin.[13] However, the physiological significance of the coagulant properties of PMN, although assumed, has not been sufficiently investigated in flowing human blood. It has been reported that PMN activate factor X bound to the CD11b on their surface and express a factor V/Va-like binding site for factor Xa, thus organizing a functional prothrombinase complex.[13,14] Furthermore, CD11b is a multifunctional adhesion receptor capable of binding a wide range of soluble proteins,[15] fibrinogen

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Received April 21, 1997; accepted August 7, 1997.

being the most relevant.

Because of the wide range of ligands and the multiple mechanisms involving leukocyte CD11b which may interfere in interaction with platelets and coagulation mechanisms, in the present study we have considered the role of PMN CD11b mediating both the reactivity of platelets and fibrin deposition onto a damaged vessel segment. For this purpose, isolated PMN and platelets were treated with specific monoclonal antibodies (MoAb) to CD11b and to CD62P (P-selectin), respectively, and incorporated into reconstituted blood. The blood was perfused at a relatively low shear rate through perfusion chambers containing denuded arterial segments. At the end of the perfusion period, platelet and fibrin deposition on the perfused subendothelium were morphometrically analyzed. Our results indicate that PMN have a role in promoting fibrin deposition under flow conditions, but this phenomenon did not seem to be influenced by P-selectin.

Materials and Methods

Blood collection

Blood was obtained from healthy volunteers who had not ingested drugs affecting platelet function in the previous 10 days. Two aliquots of blood were separated from each donor, the first was anticoagulated with 110 mM trisodium citrate 1:10 (vol/vol) and the second with low molecular weight heparin (LMWH) (Fragmin; Kabi, Pharmacia) at a concentration equivalent to 20 U/mL. Low doses of LMWH such as the one we used have been demonstrated to prevent blood coagulation for prolonged periods without impairing thrombin generation when the blood is exposed to a thrombogenic vessel surface.[16]

Preparation of plasma and cell fractions

To prepare platelet-poor plasma, the blood was centrifuged (1000×g, 20 min, 22°C). Washed platelets were prepared as previously described[17] and resuspended in Hanks' buffer, pH 7.2. A fraction of sedimented red cells was washed 3 times by centrifugation (1000×g, 20 min, 4°C) with 0.9% NaCl containing 5 mM α-glucose.

PMN were prepared according to a previously described method with minor modifications.[18] After a 60-minute sedimentation with 10% Dextran (vol/vol) (Sigma Chemical, St. Louis, MO, USA), the resulting leukocyte suspension was submitted to an osmotic shock to eliminate contaminant erythrocytes, and the red cell-free cell fraction was then washed 3 times by centrifugation (1000×g, 10 min, 4°C) in Hanks'-HEPEs (HH) buffer, pH 7.2 to which 5 mg/mL of bovine albumin (Sigma) were added. Two vol of the leukocyte suspension were recovered and layered on 1 vol of Ficoll-Paque (Pharmacia, Uppsala, Sweden). After centrifugation (1000×g, 10 min, 4°C), the bottom layer was resuspended in HH buffer (final concentration 5×10^7cells/mL). Potentially contaminating platelets were checked for in each experiment and they were never observed to be higher than 30 platelets per 100 PMN. The percentage of other leukocytes in PMN preparations was routinely determined by May-Grünwald-Giemsa staining, and was verified to be under 5%.

To study the effect of anti-CD11b and anti-CD62P, 2 mL samples of PMN or platelet suspensions were incubated respectively with 50 µL MoAb against leukocyte antigen CD11b (Becton Dickinson, San Jose, CA, USA) or with a MoAb to CD62P (Serotec, Oxford, England) for 20 min at room temperature. PMN or platelet suspensions incubated with an equivalent dose of an isotypic IgG antibody (Immunotech, Marseille, France) were also prepared to be used as controls.

Preparation of perfusates and perfusion experiments

The perfusates, of a total volume of 20 mL, were prepared by resuspending 9 mL of washed red blood cell fraction in 11 mL of platelet-poor plasma to which washed or MoAb-treated PMN and platelets had been previously added. In the reconstituted blood fractions, the final hematocrit was 40% and the platelet and PMN counts were 2×10^8 and 5×10^6 cells/mL, respectively. The perfusates were left at 37°C for 5 min before starting the perfusion. Perfusions were carried out in annular chambers containing a damaged vascular segment, according to the method described by Baumgartner.[19] Blood was recirculated through the chamber for 10 min at the shear rate of 600 s^{-1} using a peristaltic pump. This intermediate shear rate value allowed for simultaneous quantification of both platelet interaction and fibrin deposition. High shear rates promote platelet interaction but interfere with fibrin formation, while lower ones have the opposite effect.[20]

Preparation of vascular segments

Rabbit abdominal aorta segments were obtained from 2.5-2.8 kg-rabbits from New Zealand. Arteries for perfusion studies were prepared and processed as previously described.[19] Complete de-endothelialization was achieved by overnight incubation of vessel segments with α-chymotrypsin. Enzymatic digestion ensured total removal of endothelial cells, preventing the potential release of prostacyclin and allowing a homogeneous exposure of the subendothelial structures.

Processing of vessel segments for morphometric evaluation

At the end of the perfusions, the segments were rinsed with 20 mL of phosphate buffered saline, pH 7.2. The rods were removed from the chamber and the segments sliced off and washed with Milloning's buffer, pH 7.2, containing 4% formal-

dehyde and 1% glutaraldehyde. The fixed segments were histologically processed as described to observe platelet interaction[19] and fibrin deposition onto subendothelium.[20]

Morphometric evaluation

Platelet interaction with the subendothelium was evaluated using a light microscope equipped with a split prism. Thanks to this prism, the virtual image of the preparation formed in the microscope (platelets interacting with the vessel) is superimposed onto the real image of an electronic pen. Digital signals that are generated when the electronic pen is displaced on the surface of a digitizing table are sent to a computer and converted into parameters (i.e. length, height). A specially devised program automatically classifies the structures into the different morphometric categories. Details of this method have been provided elsewhere.[19,21]

The morphometric classification was performed according to the basic criteria described by Baumgartner,[19] with minor modifications. In general, platelets or groups of platelets were classified as follows: adhesion (%A) represented platelets that have spread out on the surface and that may form aggregates of less than 5 µm in height; thrombus (%T) included platelets that may form aggregates of 5 µm or more in height; total covered surface (%C.S.) was obtained by adding %A+%T.

A similar method was applied to evaluate fibrin deposition onto subendothelium.[20] The percentage of subendothelial surface covered with fibrin and the average thickness of the fibrin layer were measured. Additional information was obtained on fibrin strand bunches attached to the subendothelium, which were classified into two categories according to their thickness: over 5 µm in height and less than 5 µm in height.

PMN attachment was also quantified, and data were expressed as number of PMN per mm^2 of subendothelial surface.

Data analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed by a paired two-tailed Student's t-test. Differences between experimental groups were considered significant if $p \leq 0.05$.

Results

Effect of anti-CD11b on platelet-subendothelium interaction

Incubation of PMN with anti-CD11b did not significantly modify platelet-subendothelium interaction either in citrated blood or in heparinized blood. Samples of citrated blood prepared with MoAb-treated PMN showed a covered surface of 21.8±2.7%, while percentages of thrombus and adhesion were 10.8±2.1 and 11.0±1.2 respectively. Very similar values were found in controls (see Figure 1).

Studies performed using heparinized blood with MoAb-treated PMN showed a decrease in the percentages of covered surface, thrombus and adhesion, when compared to the controls (%C.S.: 11.1±3.4% versus 18.9±2.6%; %T: 5.4±1.3 versus 9.8±3%; %A: 5.1±2.0% versus 8.8±4.0%). These differences, however, did not reach statistical significance. All of these results are summarized in Figure 2.

Effect of anti-CD11b on fibrin deposition and PMN adhesion

Analysis of results obtained from perfusions performed with blood anticoagulated with LMWH showed an overall reduction in the amount of fibrin deposition on perfused subendothelium. Although the total surface covered with fibrin was not statistically reduced, the average thickness of the fibrin layer was significantly decreased in blood samples containing anti-CD11b treated PMN (7.9±1.2 µm versus 10.5±1.6 µm in controls; $p<0.05$). Moreover, the percentage of subendothelial surface covered with a thicker fibrin layer was also significantly reduced (34.8±3.3% versus 53.3±4.9% in controls; $P<0.05$). These data are summarized in Figure 3.

Patterns of interaction obtained with heparinized blood were different to those observed in citrated blood. PMN were very rarely observed attached on the subendothelium or associated with platelets, but were often observed trapped inside the fibrin strand network, as assessed by microscopic observation. PMN presented a high degree of activation, frequently showing degranulation phenomena. When the cells were treated with anti-CD11b, degranulated PMN were less frequently observed and the number of interacting PMN was significantly reduced (87±8 PMN/mm^2 vs 186±25 in control, $p<0.05$). Selected micrographs illustrating some of these features are shown in Figure 4.

Effect of anti-CD62P on fibrin deposition and PMN adhesion

Treatment of platelets with a MoAb to CD62P had no significant action on % of fibrin deposition on the subendothelium (43.3±7.7 vs 56.1±5.7). These data are summarized in Figure 3. Neither was the number of adherent PMN on the subendothelium modified by anti-CD62P (234±26 vs 186±25 in control).

Discussion

It has been suggested that an elaborated *cross-talk* between platelets and neutrophils participates in the development of inflammatory and thrombotic diseases.[22,23] Up-regulation of PMN and monocyte receptors induced by interaction with platelets may have important implications in prothrombotic states. Thus, strategies targeting leukocyte antigens with specific monoclonal antibodies appear to be a major objective. In the present study we have

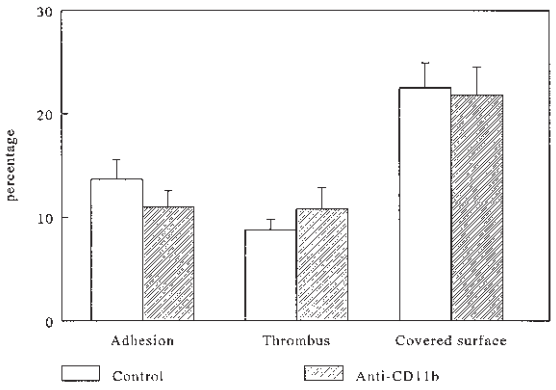

Figure 1. Percentage of platelet interaction with subendothelium using blood anticoagulated with citrate. (Mean±SEM in 6 independent experiments).

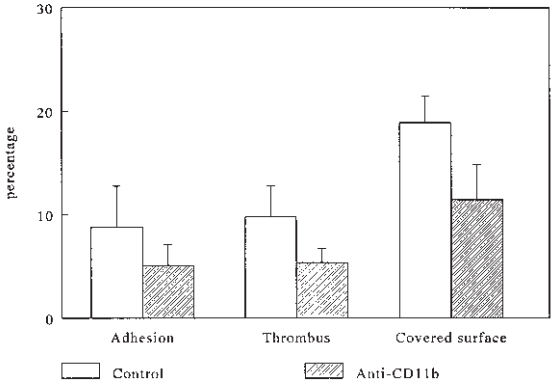

Figure 2. Percentage of platelet interaction with subendothelium using blood anticoagulated with LMWH. (Mean±SEM in 6 independent experiments).

explored the role of the PMN CD11b integrin influencing PMN adhesiveness, interaction of platelets, and fibrin deposition on the subendothelium under flow conditions, as well as exploring the role of platelet activation-dependent antigen CD62P in the process. Our results support the participation of the PMN CD11b integrin in coagulation processes and leukocyte adhesiveness under flow conditions, but this role does not seem to be mediated by CD62P.

Studies employing a perfusion system have demonstrated that platelet number and shear rate contribute to fibrin deposition onto damaged vascular surfaces,[24,25] and have proved that tissue factor activity in the subendothelium promotes fibrin formation.[26] Although adhesion between platelets and neutrophils can increase vascular occlusive and thrombotic events, it may be beneficial in amplifying hemostatic and inflammatory functions. However, the physiological significance of the coagulant properties of PMN has not been sufficiently investigated in flowing human blood. It is known that neutrophils attach to activated platelets[7] through selectins or integrins, regulating intercellular communications.[27] After attachment, neutrophils become activated and release numerous mediators which may induce local tissue injury or platelet activation.[28] This process will lead to thrombin formation and fibrin deposition at the sites of vascular damage.[22,29] Our results showed a decrease in the amount of fibrin formation after incubation of PMN with a MoAb to CD11b. These results would support the participation of this integrin in coagulation processes.[30] Moreover, careful microscopical observation of both control and anti-CD11b treated samples reveals the implication of PMN in platelet/fibrin masses, which seems to be associated with CD11b-dependent cellular adhesion. However, treatment of PMN with anti-CD11b in citrated blood did not significantly modify the attachment of platelets to the subendothelium at the shear rate employed, and had only minimal influence in samples anticoagulated with LMWH.

In vivo interaction will occur in whole blood under the action of circulatory flow forces. Thus, studies which take rheological factors into account are particularly desirable.[31] In the present study, a well-established perfusion model[19,32] was used to investigate the role of PMN CD11b integrin mediating the interactions of platelets-subendothelium and fibrin deposition. Our results demonstrate that PMN indirectly participate in the formation of fibrin, and

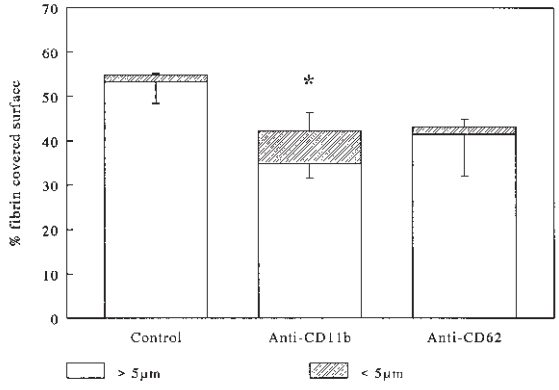

Figure 3. Percentage of fibrin deposition onto perfused subendothelium, in blood with anti-CD11b treated PMN and CD62P treated platelets compared to control. Vessel surface covered with a thick layer (> 5 µm; empty bar) or a thin layer (< 5 µm; dashed bar) of fibrin as well as the total covered surface are represented. (Mean±SEM in 6 independent experiments; the symbol * denotes statistical significance with respect to whole blood, $p < 0.05$).

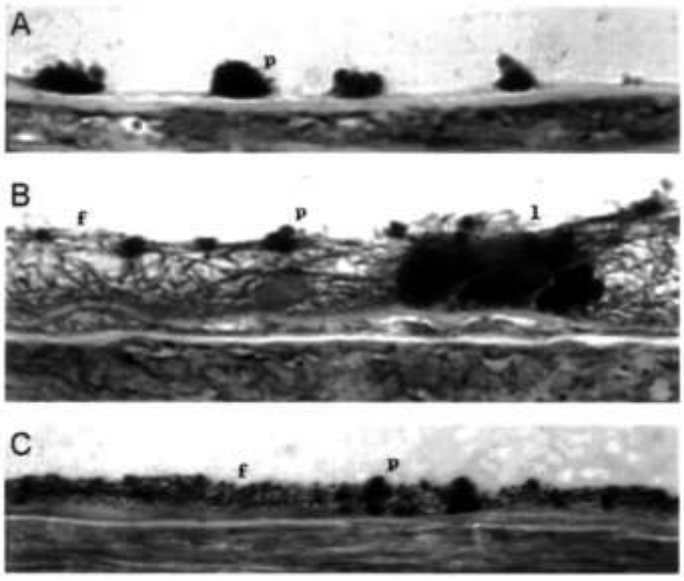

Figure 4. Computer-enhanced digital micrographs (×900) taken in cross-sections of everted rabbit-aorta segments after perfusion (10 min; shear rate: 600 sec^{-1}).
A: blood anticoagulated with citrate;
B: blood anticoagulated with LMWH;
C: blood anticoagulated with LMWH containing anti-CD11b treated PMN.
Signaled details: groups of interacting platelets (p), degranulated leukocytes (l) and fibrin strands (f) are clearly visible.

that the mechanism is mediated by the PMN CD11b integrin, but not by CD62P. According to previous studies,[14] this mechanism probably operates through the activation of factor X. PMN are able to activate factor X bound to the CD11b on their surface and express a factor V/Va-like binding site for factor Xa, thus organizing a functional prothrombinase complex.[13,14] Blockade of factor X binding to CD11b by the specific antibody could explain the subsequent inhibition of fibrin formation observed in our studies. The blockade of CD62P is known to inhibit fibrin deposition promoted by leukocyte accumulation *in vivo*.[29] We could not confirm this effect in our system, although we observed a non-significant decrease in the percentage of fibrin deposition.

Results obtained in the present study illustrate the influence of cellular interactions mediated by integrins expressed on PMN in the coagulation mechanisms. Several leukocyte antigens have been targeted with specific monoclonal antibodies. However, the clinical implications and potential limitations of these strategies have not been evaluated in homologous systems. Prevention of neutrophil activation may be a target for pharmacological modulation under some particular clinical conditions.[31] Our results further reinforce the involvement of leukocytes in thrombotic processes[32] and give experimental support for therapeutical approaches designed to prevent leukocyte activation. The actual effectiveness of such therapeutical approaches should be evaluated in specific clinical trials.

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