Platelet Adhesion to Vascular Cells

The Role of Exogenous von Willebrand Factor in Platelet Adhesion

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Platelet deposition on cultured fibroblasts and on their extracellular matrix (FBM) was investigated in a flow system with citrated blood and was compared with platelet deposition on cultured endothelial cells, smooth muscle cells, and their extracellular matrices. Platelet deposition was present at all surfaces except on intact endothelial cells. Deposition on FBM consisted of contact platelets, spread platelets, and a few small aggregates. On intact fibroblasts cells, the surface coverage was lower, and platelets formed aggregated. Factors involved in primary hemostasis, particularly the wall shear rate, von Willebrand factor (vWF), and fibronectin, were investigated on FBM. The reactivity of FBM was determined by the passage number of the cultured cells. The vWF was involved in platelet adhesion on FBM at only the high shear rate (>800 s⁻¹). Platelet deposition was independent of plasma fibronectin at all shear rates tested. Matrix-associated fibronectin was involved in adhesion at low and high wall shear rates. We conclude that FBM can be used as a platelet adhesive surface especially to study the contribution of exogenous vWF to platelet adhesion because FBM does not contain vWF. (Arteriosclerosis 10:462-469, May/June 1990)

Blood vessels are lined with a monolayer of endothelial cells, which prevent platelet deposition and clot formation. The endothelial cells produce extracellular matrix, which makes up the subendothelium. Loss of endothelial cells or contraction of these cells upon injury results in a rapid adhesion of platelets to the exposed subendothelium; subsequently these platelets undergo degranulation. Injuries penetrating the subendothelium and going through the media and/or adventitia lead to hemostatic plugs. So in contrast to superficial injuries, which result in a thrombotic response, deep injuries show a hemostatic response.

In recent years, extensive use has been made of the extracellular matrix of vascular endothelial cells (ECM) as a model for the vessel wall in perfusion studies with a rectangular perfusion chamber. Platelet adhesion and thrombus formation on the connective tissue matrix, which represents the deeper layers of the vascular wall, have been less well documented. In this study, we focus on the hemostatic response of fibroblasts and smooth muscle cell (SMC) cultures and their extracellular matrices in comparison to endothelial cell cultures and their matrices using the flow model with citrated blood. Important parameters for platelet adhesion, such as von Willebrand factor (vWF) and fibronectin, were studied on fibroblast matrix (FBM). FBM was preferred because of its uniform thickness as compared with smooth muscle cell matrix (SMCM), which varies in thickness. A special point of interest was investigating the usability of the FBM as a surface for the study of the role of exogenous vWF in platelet adhesion. Many studies on vWF dependency have been performed with isolated vessel wall components. To study the vWF dependency in a more natural condition, it was necessary to block the vWF present in the matrix itself. Since the FBM lacks endogenous vWF, it is well suited for a study on the vWF dependency on native vessel wall components.

Methods

Cell Culture and Isolation of Extracellular Matrix

Human vascular endothelial cells derived from umbilical veins were isolated and cultured as described. Fibroblasts derived from human fetal lung tissue or human skin were cultured in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Human SMC were derived from human aorta and cultured as described by Loesberg et al. Experiments with endothelial cells and SMC were carried out with cells of the third passage. Fibroblasts were used from passages 3 to 23. To isolate the extracellular matrix, cells grown to confluence were exposed to 0.1 M NH₄OH for 30 minutes at room temperature with gentle shaking. The cell layer was completely removed by this procedure. The extracellular matrix was washed three times with phosphate-buffered saline (PBS) before use. In some experiments, the FBM was incubated at 37°C in 10 μl of Krebs-Ringer buffer, pH 7.35 (with 2.5 mM CaCl₂ and 19 mM citrate) containing vWF (0.5, 1, 2, 3, or 5 U vWF/RiCoF) for 2 hours at room temperature. Controls were incubated without buffer only. In another set of experiments, FBM was incubated with antifibronectin F(ab')₂ fragments or nonimmune rabbit F(ab')₂ fragments. Incubations were performed with 0.78 mg/ml antibody in PBS overnight at room temperature.

Immunofluorescence Studies

Immunofluorescence studies were performed as described by Houdijk et al. The rabbit polyclonal anti-
bodies against collagen type I and type III were a generous gift of Jürgen Rautenberg (Münster, FRG). The antibodies against collagen type IV and V, laminin, and nidogen were a generous gift of Rupert Timpl (Max Planck Institute, Martinsried, FRG).

**Purification of von Willebrand Factor and Characteristics of Anti-von Willebrand Factor**

The vWF was purified from human cryoprecipitate as described before. The monoclonal antibody CLB-RAg 35 is directed against the platelet binding domain of human vWF (generous gift from Jan A. van Mourik, CLB, Amsterdam) and inhibits the interaction of vWF with glycoprotein Ib.

**Purification of Fibronectin and Characteristics of Anti-fibronectin**

Fibronectin was isolated from human plasma by affinity chromatography on gelatin-Sepharose as previously described.

F(ab’)2 fragments of rabbit antiserum against human plasma fibronectin were purchased lyophilized (Cappel Laboratories, Cochranville, PA) and were reconstituted in 0.02 M PBS (pH 7.3). The protein concentration was 7.8 mg/ml.

**Von Willebrand Disease**

Characterization of the diverse types of von Willebrand disease (vWD) was performed according to the criteria recently described by Ruggeri.

**Perfusion Chamber**

Perfusions were carried out in a rectangular perfusion chamber containing two knobs, a modification of the chamber of Sakariassen et al. Preparation of Perfusates

Blood from normal healthy donors or patients with vWD was anticoagulated with one-tenth vol 110 mM trisodium citrate. Part of the perfusates were performed with whole blood and part with reconstituted blood. Reconstituted blood was prepared as follows. Platelet-rich plasma (PRP) was obtained from whole blood by centrifugation (10 minutes at 200 g, 20°C). Aspirin (10 μM) was added to the PRP to prevent aggregate formation on surfaces during perfusion without influencing adhesion to subendothelium or to collagen. To one volume of PRP, one volume of Krebs-Ringer buffer (4 mM KCl, 107 mM NaCl, 20 mM NaHCO3, and 2 mM Na2SO4) containing 19 mM citrate (pH 5.0) was added, with a final pH of about 6. A platelet pellet was obtained by centrifugation (10 minutes at 500 g, 20°C). Platelets were resuspended in Krebs-Ringer buffer containing 19 mM citrate and 5 mM glucose (pH 6.0). For each wash step, 100 ml of resuspension buffer was used, and the platelets were washed twice by centrifugation (10 minutes at 500 g, 20°C). After the second wash, platelets were resuspended to a platelet count of 190 000/μl in plasma, human albumin solution (HAS) composed of 4% (wt/vol) human albumin in Krebs-Ringer buffer containing 19 mM citrate, 2.5 mM CaCl2, and 5 mM glucose (pH 7.35), or HAS with the addition of purified fibronectin in plasma concentration (300 μg/ml). Platelet-poor plasma (PPP) was obtained from PRP by centrifugation (10 minutes at 2000 g, 20°C). The plasma pH was adjusted with 1 mM HCl to 7.35. Plasma contamination of red cells was avoided by washing them three times in saline containing 5 mM glucose (2000 g, 20°C, twice for 5 minutes, the last time for 15 minutes). The washed red cells were added to a hematocrit of 0.4 at 15 minutes before perfusion. Perfusates were pre-warmed just before perfusion at 37°C for 10 minutes. Perfusions were performed at wall shear rates ranging from 100 s⁻¹ to 1300 s⁻¹, for 1, 3, 5, or 10 minutes. Before and immediately after perfusion, the system was rinsed with 20 ml of 10 mM HEPES, 0.154 mM NaCl buffer (pH 7.35).

**Evaluation**

Platelet deposition was determined morphologically with a light microscope connected to an image analyzer (QuantaMet 720, Imago, Royston, U.K.) as described before. For this purpose, coverslips covered with platelets were fixed in 0.5% glutaraldehyde in PBS immediately after perfusion at room temperature for at least 30 minutes and subsequently in a refrigerator (6° to 8°C) overnight. They were washed with PBS once and stained with May-Grünwald (Merck, Darmstadt, FRG; 1:1 in distilled water) and Giemsa (Merck, 1:4 in distilled water) for 5 and 15 minutes, respectively. The coverslips were washed with distilled water at least three times to avoid staining contaminations. They were air-dried and enclosed.

**Electron Microscopy**

For electron microscopic evaluation, fibroblasts were cultured on Thermanox coverslips (Miles, Naperville, IL). After perfusion, fibroblasts or FBM were fixed with 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7) for 30 minutes at room temperature followed by fixation in the refrigerator overnight. They were embedded in epon as described for vessel segments with some modifications. After osmium tetroxide fixation (1% in distilled water) and dehydration as described, vessel segments were totally embedded in epon, whereas in the case of the coverslips, only the perfusate surface was covered with epon. This allowed separation between the coverslips and the embedded cells or matrix after polymerization of the epon. Separation was attained by a difference in shrinkage between the two materials caused by heating the embedded coverslip on a hot plate (70°C) followed by a rapid transfer to liquid nitrogen. Epon-embedded cells and matrices were treated with a mixture of 1% osmium tetroxide and 1% ruthenium red (ICN Biochemicals, Plainview, NY) in distilled water for 30 minutes at room temperature before re-embedding. Ultrathin sections were stained with uranyl acetate and Reynolds lead citrate and were viewed in a JEOL 1200-EX electron microscope.

**Statistical Analysis**

The significance of differences between the means was calculated with Student’s t test.
Figure 1. Morphology (en face) of platelet deposition on coverslips with cultured cells or their extracellular matrices. Perfusions were performed with whole blood at a wall shear rate of 1300 s⁻¹ for 5 minutes. After perfusion, the coverslips were fixed and stained as previously described. A. Endothelial cell matrix. B. Smooth muscle cell matrix. C. Fibroblast matrix. D. Endothelial cells. E. Smooth muscle cells. Arrow indicates platelet aggregate. F. Fibroblasts. Single arrow indicates spread platelets, double arrow, platelet aggregates. × 1000
PLATELET ADHESION TO VASCULAR CELLS

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Results

Morphology of Platelet Deposition on Endothelial Cells, Smooth Muscle Cells, Fibroblasts, and Their Extracellular Matrices

The extracellular matrices of confluent vessel wall cells were exposed to flowing blood at a wall shear rate of 1300 s\(^{-1}\) for 5 minutes. On ECM, platelets were spread on the surface with little or no aggregate formation (Figure 1A). Adhesion to SMCM (Figure 1B) and FBM (Figure 1C) was lower than to ECM. On SMCM, however, platelets were less spread out than on ECM. On FBM, contact platelets or platelets which begin to spread, spread platelets, and small aggregates were found, the deposition appearing more patchy than on ECM and SMCM. The absolute values of deposition on FBM were dependent upon the number of passages of the cells in culture, as will be described later.

Confluent cell cultures were exposed to flowing blood at a wall shear rate of 1300 s\(^{-1}\) for 5 minutes. Platelet deposition was observed on SMC (Figure 1E) and on fibroblasts (Figure 1F), but not on endothelial cells (Figure 1D).

Electron microscopic evaluation of cross-sectioned fibroblasts showed an abluminal matrix only. Therefore, platelet deposition was due to direct deposition on the fibroblasts (Figures 2A and 2B). Ultrathin cross-sections of FBM showed deposition of small aggregates and spread platelets (Figure 2C).

Characterization of Fibroblast Matrix

The presence and distribution of different matrix proteins in the isolated extracellular matrix of confluent cultures of fibroblasts was studied by indirect immunofluorescence. With the use of specific antibodies, a strong fibrillar fluorescence pattern was seen for collagens type III, type IV, and type VI and fibronectin. The matrices also gave a positive fluorescence for collagen type I. The matrices were negative for vWF, laminin, nidogen, and collagen type V (data not shown).

Quantification of Platelet Deposition on Fibroblasts Compared to Their Matrix

Platelet deposition on fibroblasts and FBM was determined morphologically after perfusions at wall shear rates of 300 s\(^{-1}\) and 1300 s\(^{-1}\) for 1, 3, 5, and 10 minutes. The platelet surface coverage was less on fibroblasts than on FBM under all conditions (Table 1).

Effect of Passage Number on Fibroblast Matrix

The shear-rate dependence of FBM was determined by the passage of the cultured cells. Early passages (3 to 10) produced a very reactive matrix. Optimal adhesion was already obtained at 300 s\(^{-1}\) and decreased slightly at higher shear rates. FBM derived from cells with higher passage numbers (10 to 20) gave lower absolute adhesions. The shear-rate dependence was more or less hyperbolic and leveled off at 1300 s\(^{-1}\). Higher passages produced a hardly reactive matrix.

Von Willebrand Factor Dependence for Platelet Deposition on Fibroblast Matrix

Three approaches were used to study the role of exogenous vWF for platelet deposition on FBM.

To study the shear-rate dependence of platelet deposition on vWF, monoclonal antibody CLB:RAg:35, which is directed against the platelet binding site of vWF,\(^1\) was added to unfractionated blood. Perfusions were performed at wall shear rates of 100 s\(^{-1}\), 300 s\(^{-1}\), 800 s\(^{-1}\), and 1300 s\(^{-1}\) for 5 minutes. Significant inhibition of platelet deposition by CLB:RAg:35 was obtained at 800 s\(^{-1}\) and 1300 s\(^{-1}\) but not at the lower wall shear rates (Table 2).

The concentration dependence for platelet adhesion on the amount of exogenous vWF was investigated by the addition of purified vWF to reconstituted blood prepared with HAS and by performing perfusions for 5 minutes. Perfusion with HAS alone gave about 60% of the adhesion obtained with normal plasma (surface coverage HAS=7.6%±0.7%, normal plasma=12.7%±1.3%). Adhesion after the addition of 1 U purified vWF/ml of HAS was higher than in normal plasma (surface coverage 24.0%±2.1%). The addition of 2 or 3 U vWF/ml did not give significantly higher levels than 1 U vWF/ml.

The ability of the FBM to bind functional vWF was investigated by pre-incubation of the matrix with vWF and perfusions in the absence of exogenous vWF (Table 3). Perfusions were performed with reconstituted blood containing human albumin solution as a plasma substitute at a wall shear rate of 1300 s\(^{-1}\) for 5 minutes. An increase in platelet deposition was shown with increasing amounts of vWF for pre-incubation reaching a maximum after incubation of the FBM with 2 U vWF/ml. Above this concentration, platelet deposition remained the same.

Fibronectin Dependence for Platelet Deposition on Fibroblast Matrix

Perfusions performed with HAS on FBM pre-incubated with a control antibody resulted in normal adhesion at 300 s\(^{-1}\) and decreased adhesion at 1300 s\(^{-1}\) of 26%. The decreased adhesion was not normalized by the addition of purified fibronectin to HAS (in plasma concentration) (Table 4), indicating that plasma fibronectin was not involved. Thus, the inhibition was totally due to lack of exogenous vWF as shown above.

Matrix-associated fibronectin, however, was involved in platelet adhesion at both shear rates. This is shown in the perfusions performed with HAS. Incubation of the FBM with anti-fibronectin F(ab')\(_2\) fragments resulted in a decrease in platelet deposition at 300 s\(^{-1}\) (surface coverage control, 16.0%±1.0%; anti-fibronectin, 9.9%±0.8%, p<0.05) and 1300 s\(^{-1}\) (surface coverage control, 11.1%±0.9%; anti-fibronectin, 7.7%±0.8%, p<0.05).

Von Willebrand Disease

To test the FBM as a model for study of the functional role of mutated vWF, we studied platelet adhesion to FBM in blood of patients with vWD (Table 4). Platelet adhesion to fibroblast matrices was impaired in whole blood from all patients.
Figure 2. Transmission electron microscopic cross-section of perfused fibroblasts (A, B) or fibroblast matrix (C) (1300 s⁻¹, 5 minutes).
A. Platelet aggregate (T) on fibroblast (F). ×14 000. B. Detail from inset in A. Interaction between thrombus and fibroblast. ×110 000
C. Small platelet aggregates on fibroblast matrix indicated by arrows. × 9300
Table 1. Time Dependence of Platelet Deposition on Fibroblasts and Their Extracellular Matrix

<table>
<thead>
<tr>
<th>Perfusion time (min)</th>
<th>Percentage surface coverage (300 s⁻¹)</th>
<th>Percentage surface coverage (1300 s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FB</td>
<td>FBM</td>
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<tr>
<td>1</td>
<td>0.8±0.0</td>
<td>5.9±0.8</td>
</tr>
<tr>
<td>3</td>
<td>5.7±1.3</td>
<td>9.2±1.2</td>
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</tr>
<tr>
<td>10</td>
<td>7.0±0.3</td>
<td>46.5±2.4</td>
</tr>
</tbody>
</table>

The values are means±SEM (n=3).

Perfusions were performed at wall shear rates of 300 s⁻¹ and 1300 s⁻¹ at the indicated perfusion times. Platelet deposition on fibroblasts (FB) (passage number 6) or fibroblast extracellular matrix (FBM) was evaluated morphologically.

Discussion

Previous observations of platelet aggregate formation on damaged vessel walls as studied in biopsy material indicated that the platelet response to vascular injury in vivo depended on the depth of the vascular injury and the type of exposed cells and their matrices. To gain more insight into platelet adhesion to vessel wall components, we used a rectangular perfusion chamber in which corks containing cultured vessel wall cells or their extracellular matrices were exposed to blood under well-defined flow conditions. In the past few years, much knowledge has been obtained about endothelial cells and ECM. In the study presented here, perfusion experiments were expanded to cultured cells from deeper layers of the vessel wall and their extracellular matrices, that is, fibroblasts and SMC. By using citrated blood, platelet deposition was mainly restricted to primary adhesion, which is the first step of a thrombotic or hemostatic response.

The results of perfusions on vessel wall cells in culture appear to match very well with their in vivo localization. On fibroblasts and SMC, platelet aggregates were deposited, as appeared in en face evaluation (Figures 1B and 1C), and this is in accordance with the observation of thrombi formed in larger injuries in biopsy sections. Direct fibroblast-platelet interaction was shown in electron microscopic cross-sections (Figure 2). On endothelial cells, no platelet deposition was observed (Figure 1A) as has been previously described. This is in agreement with their localization as a lining of the vasculature with antithrombotic and antiaggregatory properties.

Platelet deposition was present on all the extracellular matrices of the cells mentioned above. Deposition on FBM was unevenly distributed and consisted of contact platelets, spread platelets, and a few small aggregates, while on ECM and SMCM, coverage was more homoge-
neous, and aggregates were absent. The deposition on FBM and SMCM was lower than expected on the basis of their in vivo localization. This was not due to partial coverage of the coverslips with matrix as was shown in the immunofluorescence studies. This indicates that FBM and SMCM composition from cells in culture may differ from their connective tissue matrix in vivo, e.g., in collagen composition. Preliminary data have shown that the addition of vitamin C to culture medium influences synthesis and post-translational modification of collagen and results in aggregate formation on FBM and SMCM. Optimal conditions approximating the in vivo situation are difficult to determine, which implies that direct extrapolation to the in vivo situation is not allowed. Reactivity of FBM was also influenced by the passage number of the cells; the higher the passage number, the lower the platelet deposition. This indicates that FBM composition may alter with increased passage number and means that experiments can only be compared when fibroblasts originate from the same passage and are grown under similar conditions. On an endothelial cell matrix, platelet adhesion levels off after 5 minutes, while on FBM there is a pronounced increase in platelet coverage at 10 minutes compared to 5 minutes, although the absolute amount of platelets adhering after 5 minutes on ECM is the same as after 10 minutes on FBM. The slower adhesion to FBM is probably due to the absence of vWF in the FBM. This might also explain the difference in the shear-rate dependence of platelet adhesion between FBM and ECM. Due to the presence of vWF in the matrix, there is a stronger increase in platelet adhesion with increasing shear rate to ECM than to FBM because vWF determines the extent and rate of platelet adhesion at higher shear rates.

The absence of vWF in FBM makes this surface very well suited for a study of the role of exogenous vWF. SMCM, on the other hand, contains some vWF obtained from the culture medium, which contributes to platelet adhesion (De Groot PG, personal communication). Furthermore, FBM was preferred to SMCM because fibroblasts grow in a monolayer, whereas SMC form hills and valleys, causing a matrix of variable thickness after harvesting that makes en face evaluation on this surface unreliable. Platelet adhesion on FBM was dependent on vWF and fibronectin, as has been previously shown for platelet adhesion on subendothelium, ECM, and purified vessel wall collagens type I and III.

vWF was involved in platelet adhesion on FBM at high shear rates only (800 s⁻¹, 1300 s⁻¹), as determined by the addition of an antibody against the platelet binding site for vWF (CLB-RAg-35) to the perfusate (Table 2). This resembles the situation for subendothelium and purified collagen and is different from ECM, where involvement of vWF was also present at a lower wall shear rate. vWF dependence was also apparent in perfusion with HAS containing purified vWF at a shear rate of 1300 s⁻¹ and after pre-incubation of FBM with purified vWF and perfusions with HAS (1300 s⁻¹, Table 3). Adhesion with purified vWF in HAS was about twice as high as compared with the same amount of vWF in normal plasma, indicating that vWF in plasma works less well. A possible explanation may be the competition of vWF with other plasma components for platelet adhesion on FBM. Platelet adhesion on FBM after perfusion with HAS was higher as compared with ECM incubated with anti-vWF. This indicates that the reactivity of the FBM is higher as compared with ECM without available vWF. In a preliminary experiment, FBM was perfused with whole blood of patients with different forms of vWD. No correlation was found between the amount of plasma vWF and the number of platelets adhering to FBM. This indicates a prominent role of platelet vWF in some types of vWD. This is currently under investigation.

Platelet deposition on FBM was dependent on matrix-associated fibronectin at low and high shear rate (300 s⁻¹, 1300 s⁻¹) and not on plasma fibronectin, as shown by omission of fibronectin from the perfusate and incubation of the FBM with antifibronectin F(ab)² fragments. Thus, the quantity of fibronectin in FBM was sufficient for normal adhesion, as shown previously for subendothelium and in contrast to ECM, where plasma fibronectin was also required. However, on subendothelium vessel wall, fibronectin was only necessary at high wall shear rates, whereas on FBM and ECM, involvement of matrix-associated fibronectin was also present at a low shear rate.

We conclude that the FBM can be used as a platelet adhesion model based on morphological observations and dependence on factors involved in platelet adhesion. The absence of vWF in the FBM matrix but dependence on vWF in plasma makes this an attractive model for use in studies in which exogenous vWF is a variable, for example, in various types of vWD or with mutant vWF of site-mutated mRNA.

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