Role of von Willebrand Factor Associated to Extracellular Matrices in Platelet Adhesion

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The respective role of plasmatic and endothelial extracellular matrix (ECM)-associated von Willebrand factor (vWF) in platelet adhesion was investigated at a high shear rate using a parallel-plate perfusion chamber. Incubation of the endothelial ECM with a monoclonal antibody (MoAb) to vWF, which specifically blocks vWF binding to platelet GP Ib (MoAb 322), inhibited 45% of platelet adhesion. Complete inhibition was achieved by incubating both plasma and endothelial ECM with MoAb 322 at concentrations that blocked only about 50% of adhesion when added separately. The effect of ECM-associated vWF was further demonstrated when a fibroblastic ECM, normally devoid of vWF, was coated with purified plasmatic vWF. Matrix-associated vWF was able to significantly enhance platelet adhesion in both the presence and the absence of plasmatic vWF. In contrast, this effect was not seen on endothelial ECM. Binding of exogenous vWF to the ECM was specific and dose dependent, reached the same value (500 ng/cm²) on both fibroblastic ECM and endothelial ECM, but exhibited a threefold-lower apparent dissociation constant (Kₐ) on fibroblastic than on endothelial ECM. Our studies suggest that vWF deposited by endothelial cells in the ECM may be the most active form in platelet adhesion, whereas plasmatic vWF may only play a secondary role.

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VON WILLEBRAND factor (vWF) is a multimeric glycoprotein (GP) with a molecular mass ranging from 0.5 to over 15 × 10⁶ daltons.¹ The vWF synthesized by endothelial cells is released into the plasma as well as deposited in the subendothelium.² vWF initiates the hemostatic process by mediating platelet adhesion to the subendothelium, which is exposed when the continuous endothelial cell layer is disrupted.³ This function occurs through the binding of vWF to collagenous components of the subendothelium and to additional noncollagenous site(s) for vWF in the subendothelium whose nature is still unknown,⁴ as well as to two distinct platelet receptors, GP Ib and GP Iib/IIIa.⁵ In vitro the GP Ib-vWF interaction requires the presence of the nonphysiologic agent ristocetin.⁶ The physiologic counterpart of ristocetin may be related to the shear rate,⁷ the importance of which was demonstrated by studies of platelet-vessel wall interaction under standardized flow conditions.⁸

Whole blood perfusion studies have been developed to investigate the mechanism of action of vWF in platelet adhesion and activation.⁹,¹⁰ According to current knowledge, the binding of vWF to the subendothelium induces a change of vWF conformation which then exposes its GP Ib binding site, allowing platelet adhesion.¹¹ Subsequent platelet spreading and aggregate formation are mediated by the GP Iib/IIIa.¹² Perfusion studies using blood from patients with severe von Willebrand disease have confirmed the involvement of vWF as a mediator of platelet adhesion at high shear rates (> 800 s⁻¹).¹³ Recent evidence indicates that high shear rates favor not only the interaction of vWF with subendothelium but also platelet activation.¹⁴

The respective involvement in platelet adhesion of vWF localized in plasma, platelets, and subendothelium is not completely understood. We have previously shown that, in addition to plasmatic vWF, platelet vWF is involved in platelet adhesion to purified collagen at a high shear rate.²⁰ However, such a model does not reflect the complex composition of the subendothelium. In contrast, the extracellular matrix (ECM) produced by human endothelial cells in culture contains the main constituents of the subendothelium in vivo (collagens, fibronectin, and proteoglycans), as well as vWF.²¹ Moreover, the endothelial ECM does not induce such a strong aggregate formation as collagen and appears as a relevant model to study platelet adhesion to the subendothelium.²²

In this study, we investigated the respective role of plasmatic vWF and endothelial ECM-associated vWF in platelet adhesion on ECM using perfusion chambers. To examine the specificity of the function of endothelial ECM-associated vWF compared with that of plasmatic vWF, we blocked the interaction of the platelets with either plasmatic vWF or endothelial ECM-associated vWF by a monoclonal antibody (MoAb) to vWF that inhibits the GP Ib-vWF interaction, and measured the resulting inhibitory effect on platelet adhesion. Our results show that ECM-associated vWF is involved in platelet adhesion at high shear rates. An increase in platelet adhesion was observed when fibroblastic ECM, but not endothelial ECM, were coated with purified vWF, thus confirming the involvement of ECM-associated vWF in platelet adhesion. Binding studies were performed to quantitate the respective interaction of purified vWF with fibroblastic ECM and with endothelial ECM.

MATERIALS AND METHODS

Culture of endothelial cells and fibroblasts. Human umbilical vein endothelial cells were isolated and cultured in alpha modification of Eagle’s minimum essential medium (aMEM) (Eurobio, Les Ulis, France) supplemented with 20% heat-inactivated human pooled sera.²³ At confluence, cells from a first or second passage

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were subcultured (2 to 3 x 10^3/cm^2) on gelatin-coated coverslips (Thermanox; Miles Laboratories, Naperville, IL) pretreated with 0.05% glutaraldehyde (Merck, Nogent-sur-Marne, France). For binding studies, cells were subcultured in gelatin-coated microtiter wells (Remova-strips; Dynatech, Marnes-la-Coquette, France) pretreated in the same way. Human bone marrow fibroblasts were obtained as previously described and cultured in aMEM containing 10% fetal calf serum (Boehringer, Meylan, France). They were passaged three to four times before use to eliminate contaminating cell types. Subcultures were performed as described for endothelial cells.

Preparation and solubilization of ECM. ECM were prepared from both fibroblastic endothelial cell monolayers 2 days postconfluence by exposing the cells to 0.1 mol/L NH_4OH for 2 to 10 minutes. ECM were then extensively washed in phosphate-buffered saline (PBS: 137 mmol/L NaCl, 2.7 mmol/L KCl, 8.8 mmol/L NaH_2PO_4, 1.5 mmol/L KH_2PO_4, pH 7.4). The integrity of ECM was checked by phase-contrast microscopy. ECM-coated coverslips, wells, or dishes were stored at 4°C in PBS and used within a week.

To assess their total protein, fibronectin, or vWF content, ECM were scraped off coverslips or 60-mm polystyrene tissue culture dishes with a rubber policeman, solubilized in 8 mol/L urea, 2 mmol/L dithiothreitol (DTT), pH 8.4, and dialyzed in 10 mmol/L Tris-HCl buffer, pH 8, containing 1 mol/L urea, 2 mmol/L DTT, and 1 mmol/L EDTA for 1 hour at 4°C and overnight in 10 mmol/L Tris-HCl buffer, pH 8, containing 1 mmol/L EDTA. Solubilized ECM were stored at ~80°C until the following assays were performed. The total protein content was assessed by the method of Lowry et al. VWF was measured by radioimmunoassay using polyclonal rabbit IgG against human vWF. Fibronectin was measured by enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit IgG against human fibronectin (kind gift of Diagnostica Stago, Franceville, France). Because the detergent used to dissolve the cell layer may affect the composition of the matrix, we compared, in 60-mm dishes, the total protein, vWF, and fibronectin content of solubilized endothelial ECM prepared by either 0.1% Triton X-100 (BDH Chemicals Ltd, Poole, England) or 0.1 mol/L NH_4OH. ECM prepared with Triton X-100 contained more protein than those prepared with NH_4OH (1,267 ± 141.5 ng/cm^2 and 1,005 ± 169.5 ng/cm^2, respectively, three paired experiments). vWF represented less than 1% of the total protein content of endothelial ECM, compared with fibronectin, which represented about 60%. The amounts of endogenous fibronectin were similar in the ECM prepared by either treatment (640 ± 120 ng/cm^2). Although there was a trend toward higher vWF content in endothelial ECM treated with NH_4OH (3.1 ± 0.6 ng/cm^2) than with Triton (1.8 ± 0.5 ng/cm^2), this difference was not statistically significant. Compared with endothelial ECM, the total protein content of fibroblastic ECM prepared by NH_4OH treatment was 2.5-fold higher (2,566.7 ± 272.8 ng/cm^2), whereas the fibronectin content was 1.5-fold higher (970 ± 202 ng/cm^2). NH_4OH treatment was selected throughout this study to prepare ECM.

Purification and labeling of vWF. vWF was purified from plasma cryoprecipitates (Centre National de Transfusion Sanguine, Paris, France) according to the method of Thorrell and Blombäck. Briefly, the cryoprecipitate was dissolved in 20 mmol/L imidazole-HCl buffer, pH 6.8, 150 mmol/L NaCl, and dialyzed against 22 mmol/L Tris-HCl, pH 6.8, 260 mmol/L NaCl, and 2 mol/L glycine. The precipitate was centrifuged at 10,000 g for 20 minutes at 4°C. vWF was purified from the supernatant by gel filtration on Sepharose CL-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden), concentrated by precipitation with 40% saturated (NH_4)_2SO_4, and stored at ~8°C. Purified vWF contained approximately 180 vWF antigen U/mg protein and less than 1% fibrinogen and 0.1% fibronectin.

For binding studies, vWF was labeled with ^125^I (Amersham International, Amersham, England) using Iodo-Gen (Pierce Chemical Co, Rockford, IL) as described. Specific radioactivity of vWF varied from 1 to 5 μCi/μg of protein unless the use of high vWF concentrations required a lower specific activity (0.05 to 0.2 μCi/μg). ^125^I-vWF was stored at 4°C and used within 3 days.

Antibodies. Mouse MoAbs to human vWF were prepared in the laboratory and used as purified IgG. In perfusion studies we used an MoAb (MoAb 322) that specifically blocks the binding of vWF to ristocetin-treated platelets and recognizes the vWF-GP Ib binding site. A monospecific polyclonal rabbit antihuman vWF IgG was used in the multimeric analysis of vWF. Controls included nonimmune IgG from mouse ascitic fluid.

Perfusion studies. Interactions of platelets with ECM were studied in citrated blood at shear rates ranging from 100 to 2,600 s^-1 using parallel-plate perfusion chambers. Details of the dimensions of the chambers and the corresponding flow rates have been published recently.

Perfusates (15 mL) of reconstituted normal human blood were mixed to obtain 1.5 × 10^8 platelets/mL, 40% hematocrit, and 20 mmol/L citrated plasma. For plasma-free studies, platelets were washed and resuspended in Tyrode's solution containing 4% bovine serum albumin (BSA; Calbiochem, La Jolla, CA) as described, except that BSA was substituted for human albumin. Perfusions were performed at 37°C for 5 minutes under pulsatile blood flow conditions using an occlusive roller pump (Perpex Jubilee; LKB Instruments AG, Luzern, Switzerland) and siliconized tubings of 3-mm inner diameter. After perfusion, coverslips were fixed in 2.5% glutaraldehyde/0.1 mol/L sodium cacodylate, pH 7.4, at 4°C for 2 hours.

Staining of the preparations was performed with toluidine blue (0.75%) for 2 minutes and basic fuchsin (0.01%) for 30 seconds. Platelet interaction with ECM was evaluated as the percentage of surface covered with platelets. In the Results section we will refer to that as a measurement of platelet adhesion. Evaluation was performed on a light microscope interfaced with an image analyzer (Quantimet 720; Cambridge Instruments, Cambridge, England) connected to a motorized stage that allowed automatic measurements of platelet surface coverage. The average of 90 values was calculated on three rows of 30 joining fields located parallel to the flow at a constant distance of 1, 4.5, and 10 mm from the proximal end of the coverslip. Correction of the data for artifacts was included in the computer program.

Some perfusion experiments were performed in the presence of MoAb 322 at a shear rate of 1,600 s^-1. ECM-coated coverslips were preincubated with 400 μL MoAb 322 for 1 hour at 20°C and washed in Tyrode's solution. Alternatively, perfusions were performed in the presence of MoAb 322 in the perfusate. Tyrode's solution or nonimmune IgG from mouse ascitic fluid were used as controls.

Binding of ^125^I-vWF to ECM. ECM were prepared from 2-days' postconfluence fibroblasts or endothelial cells subcultured in gelatin-coated microtiter wells. The total protein content of the matrices deposited in wells was estimated in a pool of solubilized ECM from 70 wells (1.4 μg/cm^2 endothelial ECM, 2.2 μg/cm^2 fibroblastic ECM). Binding of ^125^I-vWF was assessed on ECM-coated microtiter wells in the absence of flow. The wells were incubated with 2% BSA in 300 μL of 25 mmol/L Tris-HCl buffer, pH 7.4, 100 mmol/L NaCl (Tris buffer saline; TBS) for 1 hour at 20°C. The wells were then washed and incubated with 100 μL of various concentrations of ^125^I-vWF in TBS, 1% BSA overnight at 20°C. After the incubation period, ECM were washed twice with 100 μL TBS and
ROLE OF ENDOTHELIAL ECM-ASSOCIATED vWF

dried. Total binding was measured as the radioactivity associated to the wells and counted in a 1260 multigamma II counter (LKB Instruments SA). Nonspecific binding was estimated as the radioactivity bound to plastic wells coated with BSA. Specific binding was defined as total minus nonspecific binding. Each aliquot was measured in duplicate.

Some binding experiments were performed on ECM-coated coverslips, which were preincubated with 400 μL 125I-vWF for 14 hours at 20°C and washed in Tyrode’s solution before the perfusion run at 1,600 s⁻¹ as described above.

Multimeric analysis of matrix vWF. Endothelial cells were grown to confluence in 60-mm dishes, the supernatant of the cell cultures was collected, and ECM prepared as described above. ECM were then incubated in TBS, pH 6.8, containing 6 mol/L urea, 1% sodium dodecyl sulfate (SDS), for 30 minutes at 20°C. Samples from solubilized ECM, the supernatant of endothelial cells, and a normal pool plasma diluted to 1/20 with 10 mmol/L Tris-HCl buffer, pH 8.8 containing 2% SDS were layered on the side of 1.4% agarose gels. Agarose (HTG; Seakem) and Gel Bond film were from Marine Colloids (Rockland, ME). Gel electrophoresis was run horizontally at a constant voltage of 200 V, as described in detail.24 The gel was fixed in 25% isopropanol and 10% acetic acid for 2 hours and rinsed. Multimers of vWF were then revealed by incubation of the wet gel with a 125I-labeled monospecific polyclonal rabbit antihuman vWF IgG and visualized by autoradiography.

In another set of experiments, 125I-labeled purified plasmatic vWF was allowed to bind to fibroblastic ECM, and the bound material was eluted in TBS, pH 6.8, containing 6 mol/L urea, 1% SDS for 30 minutes at 20°C. The multimeric composition of the eluted sample was compared with that of the starting 125I-labeled purified plasmatic vWF on a 1.4% agarose gel. In that case, the gel was fixed after electrophoresis and dried, and the multimers were visualized by autoradiography.

Statistical analysis. Statistical significance of differences between means was evaluated using the Student’s t-test for paired samples.

RESULTS

Effect of shear rate on platelet adhesion. Because the function of vWF in platelet adhesion to subendothelium or to collagen is known to be shear-rate dependent, we determined the effect of shear rate on platelet adhesion to endothelial or fibroblastic ECM after perfusion with reconstituted blood. Figure 1 shows the effect of shear rate on the variations of the percent surface coverage of endothelial and fibroblastic ECM. On endothelial ECM, platelet adhesion was clearly shear-rate dependent as shown by the threefold increase in percent surface coverage (19.4 ± 3 to 54.9 ± 1.8) between 100 s⁻¹ and 1,600 s⁻¹ (Fig 1). In contrast, increasing the shear rate from 100 s⁻¹ to 2,600 s⁻¹ did not significantly alter the adhesion values on fibroblastic ECM (percent surface coverage ranging from 17.9 ± 2.8 to 15.6 ± 2.9). In addition, microscopic examination of the coverslips after the perfusions at high shear rates showed qualitative differences in the pattern of platelet deposition on the endothelial ECM compared with the fibroblastic ECM (Fig 2). Endothelial ECM were homogeneously covered with large areas of platelets, suggesting that platelet deposition respected the fibrillar network of the matrix (Fig 2A). In contrast, platelet deposition on fibroblastic ECM followed an ordered, linear organization crossing the ECM fibrillar network and parallel to the flow (Fig 2B).

This aspect was not seen at low shear rate (100 s⁻¹), where the pattern of adhesion was essentially the same on endothelial (Fig 2C) and fibroblastic ECM (Fig 2D).

Because perfusions on both ECM were run in the presence of plasmatic vWF, the differences observed suggest that endothelial ECM-associated vWF is involved in the shear-rate dependent increase of platelet adhesion to endothelial ECM. To have a sensitive method to detect the effect of vWF on platelet adhesion, we performed subsequent perfusion experiments at a shear rate of 1,600 s⁻¹.

Comparison of the effect on platelet adhesion of inhibition of plasmatic and matrix-associated vWF. To determine the respective role in platelet adhesion of vWF circulating in plasma and of vWF associated to the endothelial ECM, we perfused reconstituted blood at 1,600 s⁻¹ in the presence of MoAb 322, which inhibits the vWF-GP Ib interaction. The effect of vWF inhibition after the addition of various concentrations of MoAb 322 to perfuse (Fig 3A), endothelial ECM (Fig 3B), or both (Fig 3C) was measured by the variations of surface coverage relative to a reference value in the presence of control IgG. No inhibition was observed when buffer or control IgG was added. When MoAb 322 was added to the perfusate, a dose-dependent inhibition of platelet adhesion to endothelial ECM was obtained (Fig 3A); 50% inhibition was obtained on addition of 2.5 μg/mL MoAb 322 and 88% inhibition for a concentration of 20 μg/mL MoAb 322.

We next incubated endothelial ECM with increasing concentrations of MoAb 322 (25 to 200 μg/mL) and measured the inhibition of platelet adhesion. As shown in Fig 3B, platelet adhesion was incompletely inhibited (45% inhibition relative to the control value in the absence of MoAb) even when the concentration of MoAb 322 was increased to 200 μg/mL.

To assess whether blocking both plasmatic and endothelial ECM-vWF may further affect adhesion, we performed
experiments in the presence of MoAb 322 both in plasma and on endothelial ECM (Fig 3C). Increasing concentrations of MoAb 322 were incubated on endothelial ECM whereas the concentration of MoAb 322 in the perfusate was kept constant at 2.5 μg/mL. This concentration of MoAb 322 was selected because it only partially blocks platelet adhesion as previously shown in Fig 3A. In these conditions (Fig 3C), a complete inhibition was obtained compared with the only partial inhibition obtained when MoAb 322 was added separately either in the plasma or to the ECM.

In addition, when endothelial ECM-associated vWF was blocked, the pattern of platelet adhesion appeared as a linear organization parallel to the flow and strikingly resembled the pattern observed on fibroblastic ECM. These qualitative changes in the pattern of platelet adhesion were not observed when plasmatic vWF was inhibited, thus suggesting a specific role of endothelial ECM-associated vWF.

Control experiments were performed using fibroblastic ECM. As for endothelial ECM, 50% inhibition was obtained when 2.5 μg/mL MoAb 322 was added to the perfusate and 20 μg/mL MoAb 322 resulted in an almost complete inhibition. In contrast, no inhibition of platelet adhesion to fibroblastic ECM was seen when MoAb 322 (25 to 500 μg/mL) was incubated with fibroblastic ECM (not shown).

These results clearly show that both plasmatic vWF and ECM-associated vWF are involved in platelet adhesion to endothelial ECM.

Different effects on platelet adhesion to endothelial and fibroblastic ECM of incubation of the matrix with purified vWF. We next addressed the question whether purified plasmatic vWF coated on a fibroblastic ECM will mediate platelet adhesion to the same extent as the endogenous vWF of the endothelial ECM. To this end endothelial and fibroblastic ECM were preincubated with purified plasmatic 125I-vWF at a concentration of 15 to 20 μg/mL, close to the plasmatic concentration. ECM were washed and perfused at 1,600 s⁻¹ with reconstituted blood containing either normal plasma or an albumin solution. The values of platelet adhesion were similar to those obtained when unlabeled vWF was substituted for labeled vWF.

Figure 4 shows that in the presence of plasmatic vWF, platelet adhesion on fibroblastic ECM preincubated with 125I-vWF was significantly enhanced (33.0% ± 2.9% surface coverage) compared to preincubation with BSA only (16.2% ± 2.5% surface coverage). Interestingly, no enhancement of platelet adhesion was observed on endothelial ECM preincubated with 125I-vWF in the same conditions.
ROLE OF ENDOTHELIAL ECM-ASSOCIATED VWF

ECM was able to enhance platelet adhesion even in the absence of plasma vWF. This indicates that endogenous vWF of endothelial ECM is probably present in sufficient amounts to fully support platelet adhesion at high shear rate.

Multimeric composition of plasma and endothelial ECM-associated vWF. We next addressed the question of whether the function of matrix-associated vWF was related to a structural property different from that of plasmatic vWF. To this end, we analyzed, on 1.4% agarose gels, the multimeric structure of vWF from the solubilized endothelial ECM and the supernatant of endothelial cell cultures by reference to pooled plasmatic vWF. vWF released in the supernatant of endothelial cells contained the whole set of multimers with high, intermediate mol wt multimers and dimers. Matrix-associated vWF had the same multimeric distribution as endothelial cell supernatant vWF with some even higher mol wt multimers. Both endothelial supernatant and ECM-associated vWF had higher mol wt multimers than plasmatic vWF (Fig 5).

In another set of experiments, the multimeric composition of radiolabeled sample eluted after its binding to the fibroblastic ECM was compared with that of purified 125I-vWF. Identical results were obtained on 1.4% agarose gels, indicating that the binding procedure did not affect the vWF structure (data not shown).

Binding of purified plasmatic vWF to the ECM. Because ECM-associated vWF was able to support platelet adhesion at high shear rate, it was of interest to investigate the kinetics of interaction between exogenous vWF and the ECM. The binding of purified vWF to the endothelial ECM and to the fibroblastic ECM was studied in the absence of flow, using microtiter wells coated with ECM. As controls, polystyrene wells coated with BSA were used.

Total binding of purified 125I-vWF to ECM reached an equilibrium in 14 hours that remained stable until 18 hours. Nonspecific binding on control wells presented the same kinetics and did not exceed 30% of total binding (data not shown).

In equilibrium conditions, specific binding of vWF to ECM-coated wells was concentration dependent, with similar values of maximal binding obtained on both ECM. However, half-saturation was reached at lower concentrations of 125I-vWF on fibroblastic ECM than on endothelial ECM (Fig 6A). Replot of the data according to Scatchard equation (Fig 6B) indicated a single class of binding sites and a threefold-lower Kd value on fibroblastic ECM (Kd = 224 µmol/L) than on endothelial ECM (Kd = 630 µmol/L). This value was obtained using mol wt = 275 Kd for the vWF subunit. The number of sites estimated from the Scatchard plot was similar on both ECM. However, when the binding data were normalized for the amount of protein estimated in the ECM of each cell type, the number of sites per microgram of protein was 1.5 higher in endothelial ECM compared with fibroblastic ECM.

The binding of 0.5 µg/mL 125I-vWF to endothelial and fibroblastic ECM was 50% inhibited by 25 µg/mL vWF and completely inhibited by an 800-fold excess of unlabeled vWF (Fig 7).

Fig 3. Effect of MoAb 322 on platelet adhesion to endothelial ECM. MoAb 322 was added to perfusates (A), or preincubated with endothelial ECM for 1 hour (B), or added simultaneously to the perfusate and the endothelial ECM (C). Reconstituted blood perfusions were run at 1,600 s⁻¹. After the perfusion platelet surface coverage was assessed as described in the legend to Fig 1. Results are expressed as variations of adhesion relative to a reference value (27.24% ± 2.66% surface coverage) in the presence of control IgG. (□), Control IgG; (■), MoAb 322. Mean ± SEM of three experiments in duplicate.

This strongly suggests that vWF adsorbed to the ECM was active in platelet adhesion. When the experiment was performed in a plasma-free system, similar results were found (Fig 4), thus indicating that vWF associated to the
Fig 4. Effect of {superscript}125I-{vWF} on platelet adhesion to ECM. Endothelial ECM (A) and fibroblastic ECM (B) were preincubated with {superscript}125I-{vWF} (solid bars) or BSA (hatched bars) before perfusion at 1,600 s⁻¹ with reconstituted blood (with plasma) or in 4% BSA solution (without plasma). After the perfusion, platelet surface coverage was assessed as described in the legend to Fig 1. Mean ± SEM of four experiments in duplicate; Student’s t-test for paired samples (without v with incubation of vWF). NS, non significant; *P < .0125; **P < .02.

To obtain more information on the nature of the vWF-ECM interaction, we studied the effect of increasing salt concentrations. The binding was inhibited as the NaCl concentration was increased from 0.1 mol/L to 0.6 mol/L, showing that the vWF-ECM interaction was very sensitive to ionic strength variations in the physiologic range. It is unlikely that the ligand of vWF was dissociated from the ECM by increasing ionic strength, because the same amount of {superscript}125I-vWF was bound to endothelial and fibroblastic ECM that had been incubated overnight in a 25 mmol/L Tris-HCl buffer, pH 7.4, containing either a low (100 mmol/L NaCl) or a high salt concentration (2 mol/L NaCl) before the binding assay. The latter was performed by incubating the ECM with {superscript}125I-vWF in a 25 mmol/L Tris-HCl buffer, pH 7.4, 100 mmol/L NaCl (data not shown).

These results show that the interaction of vWF with ECM is concentration dependent, saturable, and specific. They suggest that the interaction of vWF with its matrix ligand(s) is predominantly of an ionic nature. The interaction of plasmatic {superscript}125I-vWF with endothelial ECM is characterized by a threefold-higher Kᵦ value than that with fibroblastic ECM.

DISCUSSION

The function of plasmatic vWF as a mediator of platelet adhesion at high shear rates has been clearly shown.\textsuperscript{14,17,18} vWF associated to the ECM of endothelial cells\textsuperscript{15,31} has been used as a model to study the function of subendothelial vWF. However, in the presence of plasmatic vWF, conflicting data have been reported, claiming that subendothelial vWF was able to mediate platelet adhesion at high shear rate,\textsuperscript{36,37} whereas endothelial ECM-associated vWF was not.\textsuperscript{38} The latter was only able to fully support platelet adhesion in the absence of plasmatic vWF.\textsuperscript{39} In an attempt to clarify the role of ECM-associated vWF in platelet adhesion, we have devised a model of ECM-coated coverslips or microtiter plates that allows comparative studies of vWF binding and of platelet adhesion to endothelial ECM that contains vWF and to fibroblastic ECM that is devoid of vWF.

Our results clearly show that ECM-associated vWF plays an essential role in the interaction of platelets with the ECM. In the presence of plasmatic vWF in the perfusate, a shear-rate-dependent increase of platelet adhesion was observed on endothelial ECM but not on fibroblastic ECM. The distribution of platelet adhesion to endothelial ECM at high shear rate was homogeneous, suggesting that endothelial ECM-associated vWF provided platelet binding sites throughout the endothelial ECM. No change in the surface coverage values was seen when endothelial ECM were
coated with exogenous vWF, indicating that endothelial ECM-associated vWF by itself supports platelet adhesion, as was previously demonstrated in the absence of plasmatic vWF. In contrast, platelet interactions with fibroblastic ECM were precoated with exogenous vWF. Interestingly, the enhancing effect on adhesion of the coating of vWF on fibroblastic ECM occurred both in the presence and in the absence of circulating plasma vWF, showing that ECM-associated vWF mediated platelet adhesion in both conditions. However, platelet adhesion to fibroblastic ECM coated with purified vWF was still morphologically different from the adhesion to endothelial ECM, indicating that ECM-associated exogenous vWF did not readily substitute for endogenous vWF.

Platelet adhesion to endothelial ECM preincubated with an MoAb to vWF, which specifically blocks vWF binding to platelet GP Ib (MoAb 322), was inhibited to a plateau of 40%. This finding was in agreement with the inhibition of platelet adhesion to the subendothelium in whole blood perfusion studies by a polyclonal antibody to vWF, or an anti-vWF MoAb that blocks the binding of vWF to platelet GP Ib (MoAb CLB RAg 35). However, our results contradict those obtained by Houdijk et al, who did not observe any inhibition when whole blood was perfused over an endothelial ECM preincubated with MoAb CLB RAg 35. This discrepancy may well be explained by differences between MoAb CLB RAg 35 and MoAb 322 in their concentration or affinity for vWF. The incomplete inhibition may be explained by the function of plasmatic vWF responsible for the remaining adhesion after blocking ECM-associated vWF by MoAb 322. This complementary role of plasmatic vWF was shown by the almost complete inhibition of adhesion on endothelial ECM when the effect of plasmatic vWF was neutralized by MoAb 322. It was suggested by Houdijk et al that the addition of MoAb CLB RAg 35 to the plasma could also inhibit matrix vWF. To avoid this problem, we selected concentrations of MoAb 322 leading to only partial inhibition of either plasmatic or endothelial ECM-associated vWF, and found an additive effect when incubated in each compartment leading to complete inhibition of adhesion. However, it is also possible that the effect of MoAb 322 on plasmatic vWF inhibits both its functions in the initial platelet attachment to the ECM and the subsequent thrombus formation.

vWF containing a higher multimeric organization than plasmatic vWF has an increased capacity to bind to the platelets and is a better hemostatic agent. We found that vWF deposited in endothelial ECM consists of higher mol wt multimers than plasmatic vWF, as reported recently. This finding is in agreement with the report that the largest multimers of vWF bind preferentially to a fibroblast ECM. When we compared the binding of increasing concentrations of purified plasmatic vWF to endothelial ECM with that to fibroblastic ECM, a single class of low-affinity binding sites and similar saturation values were found per square centimeter of ECM of each cell type. A threefold-lower Kₐ value was obtained for the interaction of vWF with the fibroblastic ECM than with the endothelial ECM. If the binding data were expressed as a fraction of the total protein content, the apparent saturation value became 1.5-fold lower on fibroblastic ECM than on endothelial ECM, indicating a smaller number of sites per microgram of protein of the fibroblastic ECM. However, this ratio is only a rough estimate of the matrix composition, and we have no indication at the moment that the specific, yet unknown, matrix ligand of vWF is identical and present in similar amounts in both ECM. Apparently, the amount of plasmatic vWF required to saturate the endothelial ECM or the fibroblastic ECM (500 ng vWF/cm²) was much higher than the amount of endogenous vWF associated to the endothelial ECM (3 ng/cm²), which was capable by itself to support platelet adhesion. Thus, endothelial ECM-associated vWF cannot be readily substituted by exogenous vWF.
for its interaction with the ECM and its effect on platelet adhesion at high shear rate.

Our results indicate that both pools of plasmatic and endothelial ECM-associated vWF are involved by different mechanisms in platelet adhesion. vWF deposited by the endothelial cells in the ECM may be the most active form in platelet adhesion, whereas plasmatic vWF may only play a secondary role and bind when ECM-associated vWF is functionally or quantitatively impaired. This hypothesis may lead to the description of specific abnormalities of platelet adhesion secondary to a qualitative or quantitative defect of subendothelial vWF.

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Role of von Willebrand factor associated to extracellular matrices in platelet adhesion

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