Platelet Adhesion to Exposed Endothelial Cell Extracellular Matrixes Is Influenced by the Method of Preparation


The relative thrombogenicity of extracellular matrixes (ECMs) produced by cultured human umbilical endothelial cells (ECs) was studied under flow conditions. ECMs were prepared using a number of physical and chemical methods, and their reactivity toward platelets was morphometrically evaluated. von Willebrand factor (vWF), fibronectin (FN), and 13-hydroxy-9-cis,11-trans-octadecadienoic acid (13-HODE) were also determined. We found that platelet adhesion to ECMs differed significantly, both quantitatively and qualitatively, with the method of ECM preparation. Mechanically prepared ECM exposed a less thrombogenic surface compared with ECM prepared by chemical methods (platelet-covered surface of 20% and 50%, respectively). Evaluation of the ECM components vWF, FN, and 13-HODE showed significant changes, both in their concentrations and distribution patterns, depending on the method of ECM preparation. The decrease measured in the levels of ECM-associated vWF (from 108 to 9.2 ng/10^4 cells) and the minor changes observed in the distribution pattern of subendothelial FN did not appear to be sufficient to explain the altered platelet adhesion observed in our model. This suggests that the amount of 13-HODE probably associated to the remaining ECs present in the mechanically exposed ECM could be one factor that specifically contributed to the nonthrombogenic state of these preparations. We conclude that the degree of ECM reactivity toward platelets is dependent on the method of ECM preparation and that this is related to the removal of specific EC/ECM components that modulate their thromboresistant/thrombogenic properties. This fact should be taken into account when ECMs produced by cultured ECs are used in platelet adhesion studies. (Arteriosclerosis and Thrombosis 1991;11:436–442)

Healthy endothelium is nonreactive to circulating blood cells, but on removal of the endothelial cell (EC) layer, the exposed extracellular matrix (ECM) is highly reactive to platelets. The interaction of platelets with this exposed surface is of critical importance for primary hemostasis.1,2 The nonreactivity of the endothelium is thought to be mediated by components released by the ECs,3–5 while the reactivity of the ECM seems to be contributed to by different types of collagen, von Willebrand factor (vWF), fibronectin (FN), and other adhesive proteins.6–8

Recently, it has been reported that ECs metabolize linoleic acid via the lipoxygenase enzyme to 13-hydroxy-9-cis,11-trans-octadecadienoic acid (13-HODE) under basal conditions, and it has been postulated that 13-HODE facilitates the thromboreistant property of the endothelium by regulating the expression of adhesive moieties on the surface of the ECs.9 It has also been reported that 13-HODE renders the underlying basement membrane nonreactive to circulating platelets.10 These latter observations are in contrast to other studies, in which the ECM was found to be highly reactive to circulating platelets and 13-HODE was considered not to be a major metabolite involved in platelet-subendothelium interactions.11,12

This apparent inconsistency raises the possibility that the different subendothelial structures are not equally reactive to platelets and that the thromboreistance of the ECM may vary depending on the
severity or nature of EC removal. In this study, the reactivity to platelets of ECM prepared by different methods was measured under flow conditions. Furthermore, the relation between the thromboreactivity of the ECM and the amounts and distribution patterns of vWF, FN, and 13-HODE present in the ECM were examined. Our results indicate that platelet adhesion to ECM is markedly influenced by the method of EC removal and subsequent ECM exposure.

Methods

Blood Collection and Platelet-Free Reconstituted Perfusate

Blood obtained from healthy volunteers who had not ingested aspirin in the previous 15 days was collected into citric acid/citrate/dextrose-containing sterile tubes (20 mM) for the perfusion experiments. Other blood samples were processed to prepare the perfusates for exposure of the ECM to shear stress. The blood was centrifuged (150g, 10 minutes, 22°C) to remove platelet-rich plasma. Platelet-free plasma was prepared by centrifugation (1,300g, 20 minutes, 22°C) and stored at 4°C until use. The remaining red blood cells were washed three times in 0.9% NaCl containing 10 mM a-D-glucose. Platelet-free plasma and red blood cells were reconstituted to a final hematocrit of 47±2% to obtain the platelet-free reconstituted perfusate.

Human Endothelial Cell Culture

ECs were isolated from human umbilical cord veins according to a previously described method. Basically, ECs were harvested with collagenase treatment (2% in phosphate-buffered saline [PBS], 15 minutes, 37°C; Boehringer-Manheim, Mannheim, F.R.G.) and were maintained and subcultured in Eagle’s minimal essential medium supplemented with 1 mM glutamine, 2 mM N-(2-hydroxyethyl)piperezine-N’-(2-ethanesulfonic acid) (HEPES), 100 units/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, Irving, Scotland), and 20% pooled human sera. The ECs were grown at 37°C in a 5% CO2 humidified incubator. ECs previously identified by immunologic criteria were subcultured in their second passage on 1% gelatin-precoated glass or plastic coverslips (Thermanox, Miles Laboratories, Naperville, Ill.) and were used for adhesion assays and vWF, FN, and 13-HODE determinations.

Preparation of Extracellular Matrix

The underlying ECM of 7-day cultured ECs was exposed according to one of the following methods.

1) Shear stress. EC-coated coverslips inserted into the flat chamber (described below) were perfused with platelet-free reconstituted perfusate at a shear rate of 1,600 sec⁻¹ for 15 minutes to eliminate the ECs. The coverslips were then removed from the chamber and washed with 0.15 M PBS, pH 7.4.

2) Nitrogen drying. The chamber was perfused with a flow of nitrogen (40 ml/min) for 10 minutes at 37°C.

The dehydrated ECs were then dislodged by rinsing with PBS.

3) Cellulose stripping. Strips of cellulose acetate were laid flat on the EC monolayers for 2 seconds and then peeled off, thus removing the ECs. This method has been described in detail elsewhere.

4) Ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid treatment. EC-coated coverslips were incubated with 1 ml 2% ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) (37°C, 30 minutes) causing the ECs to detach from the surface.

5) Ammonia exposure. EC-coated coverslips were incubated with 0.1 N NH₄OH (37°C, 30 minutes). The supernatant and lysed ECs were then removed. ECM-coated coverslips obtained by any of these procedures were washed and stored for 60 minutes in PBS until use.

Perfusion Adhesion Assay

Plastic coverslips coated with the ECM produced by EC cultures processed as previously described were inserted into flat chambers according to Sakai et al. Anticoagulated blood samples were then recirculated for 5 minutes at 37°C. Flow was maintained by a peristaltic pump (model 502S, Watson Marlow, Cornwall, England) and adjusted to obtain a shear rate of 1,300 sec⁻¹, which corresponds to the shear rate of the microvasculature.

Morphometric Evaluation

The exposed ECM surface and the interaction of platelets with the ECM preparations were evaluated morphometrically by two different methods.

1) En face. After perfusion, the coverslips were removed from the chamber, rinsed with PBS, and then fixed in 0.5% glutaraldehyde in PBS (2 hours, 4°C). The coverslips were dehydrated in a graded series of ethanol and stained with May-Grunwald and Giemsa (Merck, Darmstadt, Germany) as previously described. The percentage of exposed ECM and the degree of platelet interaction with the ECM were evaluated by means of a semiautomated method. The exposed ECM surface was expressed as the percentage of the total coverslip area devoid of ECs. Profiles of single or grouped platelets were projected with an electromagnetic pen to a manual optical analysis system (MOP20, Kontron, Zurich, Switzerland) connected to a personal computer (IBM XT, 286). This system automatically integrates areas and expresses them as the percentage of the total ECM screened.

2) Cross section. Once the en face evaluation was performed, the plastic coverslips were dehydrated in a graded series of ethanol and embedded in JB-4 solution (Polysciences Inc., Warrington, Pa.). Infiltration of the samples was performed in catalyzed JB-4 solution (2 hours, 4°C). Coverslips were embedded in flat molds (Histomold, LKB, Bromma, Sweden). The perfused side of the plastic coverslips was laid on top of the mold filled with catalyzed
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Arachidonic acid did not. These data indicate that the contrast, the addition of 75 μM of 5-, 12-, or 15-
hydroxyeicosatetraenoic acid or 100 μM of linoleic or arachidonic acid did not. These data indicate that the detection of 13-HODE in the preparations. In controls. The primary or secondary antibody was sorbed with the corresponding antigens were used as primary antibodies. Ten micrograms of purified vWF (Dako, A082), FN (Dako, A245), and 13-HODE were used as primary antibodies. Ten micrograms of purified 13-HODE added to undiluted antiserum neutralized the detection of 13-HODE in the preparations. In contrast, the addition of 75 μg of 5-, 12-, or 15-
hydroxyeicosatetraenoic acid or 100 μg of linoleic or arachidonic acid did not. These data indicate that the antiserum were specific for the 18-carbon metabolites, the majority of which was 13-HODE as confirmed by high-performance liquid chromatography (HPLC).

von Willebrand Factor Determinations
ECMs obtained by the different methods of preparation were extracted with 6 M urea and 1% Triton X-100 in PBS for analysis of vWF. vWF antigen was determined by an enzyme-linked immunosorbent assay (ELISA) as previously described. Pooled normal human plasma containing 10 μg/ml vWF was used as a standard. Rabbit polyclonal antibodies, monospecific for vWF, served as the solid phase, and a mixture of the murine monoclonal antibodies CLB-Rag 35 and CLB-Rag 50 was used as an indicator, in combination with peroxidase-labeled sheep antirabbit IgG (Institute Pasteur, Marnes-la-Garenne, France). The response of serial dilutions of ECM samples paralleled that of purified vWF. Dissolving the vWF in extraction buffer followed by dialysis did not alter the results found in the ELISA.

13-Hydroxy-9-cis,11-trans-octadecadienoic Acid Determinations
Other EC- or ECM-coated glass coverslips were extracted with methanol for an estimation of their monohydroxy fatty acid content. The amount of fatty acid metabolites extracted into methanol was quantified by reverse-phase HPLC according to the method described. Briefly, the methanol extracts were blown to dryness with a flow of nitrogen, and the residues were then resuspended in 0.9 ml 40% acetonitrile, pH 3.5. The samples were then injected into a NOVA PACK C18 cartridge (3 μM, 5 mm x 10 cm) using a μBondapack C18 guard column (Water Scientific, Toronto, Canada). An acetonitrile gradient (from 40% to 80%) was used to separate the metabolites, which were measured at ultraviolet absorbances of 234 and 254 nm. The sensitivity and variability in 13-HODE analysis by HPLC was 2±0.1 ng.

Statistical Analysis
Results of the experiments are expressed as mean±SEM. They were analyzed with the Wilcoxon test for paired data. A level of p<0.05 was considered statistically significant.

Results
Exposed Extracellular Matrix Surface After Different Treatments
The percentage of the area of exposed ECM varied according to the different methods of preparation. In untreated EC coverslips, 6.7±1.3% of the area was exposed ECM (Figure 1), and with NH4OH EC lysis of the EC-coated coverslips, there was almost complete removal of the ECs, exposing 99.1±0.4% of ECM. The amount of ECM exposed with the other methods of preparation varied between these values (Figure 1).
Platelet Adhesion Under Flow Conditions

The interaction of platelets with the ECM was assessed morphometrically. The total surface area covered with platelets as measured en face (Figure 1) on ECMs prepared by shear stress was 14.5%. The total surface area covered with platelets onto ECMs prepared by cellulose acetate stripping or nitrogen flow was 22–23%. Low platelet coverages were generally found in those preparations in which ECs were mechanically removed. In contrast, the total surface area covered by platelets on the EGTA- and NH₄OH-prepared ECMs was approximately 50% (Figure 1).

In cross sections, the qualitative evaluation of platelets adherent to the ECM displayed a varying degree of platelet activation, depending on the method of ECM preparation used (Figure 2). Thus, the majority of platelets associated with the shear stress preparation were mainly in contact (>80%), and only a few platelets had been stimulated enough to spread or to form aggregates (Figure 3), indicating that this preparation rendered a less adhesive surface. A similar pattern of interaction was generally observed in those coverslips in which ECs had been removed by mechanical methods (nitrogen drying and cellulose stripping). In contrast, the majority of the platelets associated with the EGTA and NH₄OH preparations were spread and/or aggregated (>50%), suggesting that the last methods expose a more reactive surface.

Statistical comparisons performed between preparations that resulted in the exposure of a considerable surface of ECM (cellulose stripping, EGTA, and NH₄OH) showed significant differences in their reactivity toward platelets. As shown in Figure 3, a statistical increase in the presence of spread platelets was observed in EGTA- and NH₄OH-treated ECs (p<0.01 vs. cellulose stripping). A similar increase in the presence of aggregates was also observed (p<0.05 vs. cellulose stripping).
Histoimmunolocalization of von Willebrand Factor, Fibronectin, and 13-Hydroxy-9-cis,11-trans-octadecadienoic Acid

Paraformaldehyde-fixed coverslips containing ECMs from which ECs had been removed were labeled for vWF and FN localization. In control preparations, the pattern of staining for vWF followed the regular distribution pattern as previously described. In the mechanically exposed ECMs, remaining ECs showed a punctate pattern of staining corresponding to the vWF located in Weibel–Palade bodies. ECM–vWF showed a punctate pattern of staining following fibrillar structures. In the ECMs prepared with NH₄OH and EGTA, the fluorescence was located on the fibrillar structures of the matrix.

Control samples incubated with FN antibodies showed fluorescent labeling following fibrillar arrangements defining the limits of the cells (Figure 4). Mechanically exposed ECM showed homogeneous distribution of FN with some areas devoid of fluorescent labeling, corresponding to the damaged zones. Chemical methods exposed a very homogeneously distributed staining for FN fibrils.

13-HODE staining was detected in ECM preparations in which there were remaining ECs and also in

**FIGURE 3.** Bar graph of cross-sectional morphometric evaluation of platelet interaction with ECMs (a, contact; b, spread; c, aggregate) under flow conditions. Evaluation of platelet interaction is expressed as the percentage of total covered surface (mean±SEM). *p<0.05, **p<0.01 in comparison with cellulose stripping; n=7. S.S.; shear stress, 1,600 sec⁻¹, 5 minutes; Nit., nitrogen drying; Strip., cellulose stripping; EGTA, 2% ethylene glycol-bis[β-aminoethyl ether]N,N',N'-tetraacetic acid treatment; NH₄OH, ammonia exposure.

**FIGURE 4.** Photomicrographs of histoimmunofluorescent images of different ECM preparations. A: Untreated ECs in which 1) vWF was detected in the ECM and localized in cell bodies; 2) FN was localized only in the ECM; and 3) intracellular amounts of 13-HODE were detected. ×250; B: ECM exposed by mechanical methods, in which 1) vWF fluorescence appeared inside some remaining ECs and was also observed over the ECM, following fibrillar arrangements; 2) FN staining showed a homogeneous distribution and some damaged areas devoid of fluorescent labeling; and 3) variable amounts of 13-HODE were detectable in remaining ECs. ×650; C: Chemically exposed ECM, in which 1) only extracellular vWF was stained, confirming that there were no ECs in those preparations; 2) a very homogeneous mesh of FN staining was observed; and 3) lack of fluorescent staining indicates absence of 13-HODE; ×650. ECM, extracellular matrix; EC, endothelial cell; vWF, von Willebrand factor; FN, fibronectin; 13-HODE, 13-hydroxy-9-cis,11-trans-octadecadienoic acid.
Table 1. von Willebrand Factor and 13-Hydroxy-9-cis,11-trans-octadecadienoic Acid

<table>
<thead>
<tr>
<th>Condition</th>
<th>vWF (ng/10^6 cells)</th>
<th>13-HODE (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECs</td>
<td>108.0±12.6</td>
<td>12.60±1.0</td>
</tr>
<tr>
<td>Shear stress</td>
<td>70.4±8.5</td>
<td>...</td>
</tr>
<tr>
<td>Cellulose acetate stripping</td>
<td>41.6±4.5*</td>
<td>ND*</td>
</tr>
<tr>
<td>2% EGTA, 30 minutes</td>
<td>28.7±3.5*</td>
<td>ND*</td>
</tr>
<tr>
<td>0.1N NH₄OH</td>
<td>9.2±1.1*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

vWF, von Willebrand factor; 13-HODE, 13-hydroxy-9-cis,11-trans-octadecadienoic acid; EC, endothelial cell; EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; NH₄OH, ammonia treatment; ND, not detectable.

Values are mean±SEM, n=7.

*p<0.01.

those that were mechanically exposed. In any of the ECMs obtained with NH₄OH and EGTA, 13-HODE was detected (Figure 4).

Analysis of von Willebrand Factor and 13-Hydroxy-9-cis,11-trans-octadecadienoic Acid

The amount of vWF bound to the intact ECs was 108±12.6 ng/10⁶ ECs. There was 76.2±5.4 ng/equivalent area in ECs prepared by shear stress and 41.6±4.5 ng/equivalent area in ECs exposed by cellulose acetate stripping. The amount of vWF in ECMs prepared by chemical methods ranged from 28.7±3.5 (EGTA) to 9.2±1.1 (NH₄OH) ng/equivalent area as shown in Table 1.

The amounts of 13-HODE associated with the ECM preparations are shown in Table 1. There was 12.6±1.0 ng 13-HODE/10⁶ ECs associated with intact ECs. There was no 13-HODE detected in the ECMs exposed by cellulose acetate, EGTA, and NH₄OH.

Discussion

A number of studies have demonstrated that a variety of components of the ECM underlying the endothelium modulate platelet–ECM interactions. Among others, these components include several types of collagen and the noncollagenous proteins vWF and FN. Recently, it has been suggested that the EC-derived linoleic acid lipoxigenase metabolite 13-HODE is also important in the regulation of platelet–ECM interactions, although the role of this metabolite in platelet subendothelium is still not well elucidated and remains a matter of controversy.

In this study, we have explored the importance of ECM preparation with regard to its thrombogenic properties under flow conditions. We have focused on the changes in the ECM concentration and distribution of two adhesive proteins, vWF and FN, and of 13-HODE, the lipoxigenase metabolite involved in the thromboresistant properties of ECM.

The results of our study indicate that the thrombogenic or thromboresistant properties of the ECM underlying the endothelium, examined under flow conditions, are influenced by the method of EC removal and subsequent ECM exposure. Morphometric analysis of treated ECMs showed significant differences in the extent of exposed ECM after the different procedures of EC removal. Nonchemical procedures were less effective in exposing the matrix than those that required chemical treatment of the ECs. When the ECs were placed in the chamber and perfused with anticoagulated whole blood under the same conditions, the percentage of exposed ECM covered with platelets was not proportional to the extent of exposed subendothelial material, indicating that the reactivity of the ECM is dependent on the method of its preparation. Histoimmunolocalization studies showed that vWF and FN were present in the differently prepared ECMs. Conversely, immunofluorescent localization of 13-HODE in the ECM exhibited marked differences in the amounts of the metabolite present. Quantification of the levels of vWF by an ELISA and of 13-HODE by HPLC confirmed these observations. The levels of vWF were significantly reduced in the preparations in which most of the cells were eliminated. It is important to point out that in all cases studied, the levels of this adhesive protein exceeded the minimum amount that, according to Sixma et al., is necessary to support platelet adhesion. It is also interesting to consider that although vWF and FN in the subendothelium are important in supporting platelet deposition, in both cases plasma provides an alternative source of these proteins, and this fact may minimize the effects of their removal from the ECM. On the contrary, according to our current knowledge, 13-HODE is present only inside ECs; thus, the remnant 13-HODE found inside the remaining ECs may cause the low thrombogenicity found in mechanically exposed ECM.

Not only were there quantitative differences in the interactions of platelets with the ECM but also there were qualitative differences in the nature of the interactions of platelets with the different ECM preparations. The platelet interactions with the ECMs exposed by mechanical methods were only contact interactions, indicating lower thrombogenicity. Conversely, the platelets analyzed in the sections prepared from ECMs exposed by chemical methods were mostly spread on the surface or aggregated, suggesting that these methods markedly affect the composition of the resting endothelium/subendothelium.

Our microscopic examination shows that a variable number of ECs remain attached to the ECM after the different methods of exposure. It is clear that this fact may also influence ECM reactivity; thus, the possibility that the remaining ECs are still metabolically active, thereby synthesizing prostacyclin, endothelium-derived relaxing factor, or even 13-HODE itself, all inhibitors of platelet clumping, cannot be ruled out.

In summary, the data we report here indicate that under dynamic conditions, the reactivity of the ECM toward platelets depends on the procedure of EC removal, which directly influences the levels of different ECM components. ECM is a widely used...
substratum for platelet adhesion experiments; the results of our investigations suggest that it is important to consider the method of preparation to avoid spurious results in the studies concerning platelet-ECM interactions.

Acknowledgments

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References


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