Localization of Factor-VIII-Related Antigen in Human Vascular Subendothelium

By Jacob H. Rand, Ira I. Sussman, Ronald E. Gordon, Sadie V. Chu, and Vera Solomon

Factor-VIII-related antigen has previously been shown to be synthesized by vascular endothelial cells. Using both an immunofluorescent staining technique and electron microscopy, we have demonstrated the presence of factor-VIII-related antigen in human vascular subendothelium. This finding may have implications in the mechanism of platelet adhesion to deendothelialized blood vessel surfaces.

Factor-VIII-related antigen (factor-VIII-r:ag) appears to play a role in the adhesion of platelets to the deendothelialized vascular surface. While its specific role in the process is not clear, there is evidence supporting the idea that factor-VIII-r:ag present in the vessel wall mediates platelet adhesion. Factor-VIII-r:ag is present in human vascular endothelium, and endothelial cells grown in tissue culture synthesize this material and secrete it into nutrient medium. Also, vessels of patients with severe von Willebrand's disease lack factor-VIII-r:ag. It has recently been shown that following deendothelialization, arterial subendothelium binds added labeled factor-VIII-r:ag and that the amount of labeled material bound correlates with the quantity of platelets subsequently adhering to the surface. In the present study, we were able to determine that factor-VIII-r:ag is already present in subendothelium prior to the removal of endothelial cells.

MATERIALS AND METHODS

Vessels

Human umbilical cords were obtained following birth. Surplus saphenous veins from patients undergoing saphenous vein bypass graft procedures were obtained after surgery. Umbilical veins and saphenous veins were treated in the same manner. The vessels were cannulated and rinsed thoroughly with phosphate-buffered saline (PBS), pH 7.2, or with Tris-calcium acetate buffer, pH 7.4 (0.025 M Tris, 0.33 M calcium acetate). Equivalent results were obtained with both calcium and non-calcium containing buffers. A small segment was then removed and set aside in cold buffer. The remainder of each vessel was divided into 2-3-cm segments that were treated in the following manner.

One set of segment was deendothelialized using a variation of the method described for in vivo use by Baumgartner. A Fogarty 2F arterial balloon embolectomy catheter (V. Mueller, Linden, N.J.) was passed uninfused through the lumen of the segment. The catheter was inflated to a diameter slightly larger than that of the vessel segment used and withdrawn through its entire length. This process was repeated twice, following which the vessels were rinsed thoroughly with PBS. The effluent was examined with phase-contrast microscopy to confirm the presence of endothelial cells.

The lumens of a second group of segments were filled with a solution of 0.05% collagenase (Sigma, St. Louis, Mo.). Both ends of each segment were clamped and the vessels were incubated without agitation for differing periods in a 37°C waterbath. The incubation intervals for our experiments were chosen after preliminary studies with intervals between 5 and 45 min. They showed that at 10 min the majority of endothelium was removed but the internal elastic lamina was still present, while at 30 min, all endothelial cells were removed along with internal elastic lamina. At the end of each incubation interval, the segments were washed with cold PBS. The effluent was examined for endothelial cells.

In a third group of vessel segments, studies were performed to assess the effects of collagenase digestion on vascular subendothelium. In these studies, segments were incubated with a highly purified preparation of collagenase without contaminating protease activity (Advanced Biofactures, Lynbrook, N.Y.). Since highly purified collagenase preparations are known to be less effective in removing endothelial cells than preparations that are contaminated with other proteases, these segments were mechanically deendothelialized, as described above, prior to incubations. The segments were then incubated with Tris-calcium acetate buffers containing collagenase concentrations of 0, 75, 150, 300, and 500 U/ml at 37°C for intervals of 10, 30, 60, and 120 min.

After preparation, all segments, whether untreated, balloon catheterized, or collagenase digested, were frozen by immersion in a mixture of dry ice in methylbutane. Four-micron cross-sections were made of each segment and mounted on glass slides. Sections were then fixed in acetone at room temperature for 10 min, allowed to dry, and stored in a freezer at –60°C.

Preparation of Rabbit Antisera

A high purity factor-VIII concentrate was prepared according to the method of Shapiro et al., the starting material being fresh-frozen plasma. Factor-VIII antisera was prepared and absorbed according to the method of Zimmerman, Ratnoff, and Powell. A crude globulin extract was derived from the absorbed serum by precipitation with sodium sulfate, and further purification was achieved by DE 52 ion-exchange chromatography (Whatman, Ltd., Clifton, N.J.). By immunodiffusion, this anti-factor-VIII antibody reacted with purified factor VIII and produced one band against plasma, but by immunoelectrophoresis, three immunoprecipitants bands were observed. This antibody showed no reaction against fibrinogen or purified cold-insoluble globulin (CIG). It neutralized procoagulant factor VIII in an inhibitor assay system and was a potent inhibitor or ristocetin-induced platelet agglutination.

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to insure specificity of the globulin fraction, the preparation was diluted 1:50 in plasmas from 3 patients with severe von Willebrand’s disease. Following this last absorption, the antibody retained its biologic neutralizing activity to both procoagulant factor VIII and ristocetin cofactor and provided one band on immunoelectrophoresis. This globulin fraction was also diluted 1:50 in PBS and in plasmas from 3 normals and 3 severe hemophiliacs. Antibody diluted in plasma was incubated for 1 hr at 37°C prior to use.

Anti-human cold-insoluble globulin was generously provided by Dr. Dean Mosher, University of Wisconsin, Madison, Wisc. and was diluted 1:20 in PBS.

Anti-human fibrinogen and anti-human albumin were obtained from a commercial source (Behring, Somerville, N.J.) and were diluted 1:100 and 1:10 in PBS, respectively.

**Immunofluorescent Staining**

Sections of vessels were washed 3 times for 5 min in phosphate-buffered saline (PBS), pH 7.2.

Excess buffered saline was removed and a drop of each diluted rabbit antiserum was added. The slide was incubated at room temperature for 45 min in a moisture chamber and was then washed 3 times for 5 min in PBS. The section was then incubated for 45 min at room temperature with FITC-conjugated goat anti-rabbit globulin (Meloy, Springfield, Va.) using a 1:50 dilution, and the washing procedure was repeated.

A drop of mounting fluid consisting of glycerol 10%, PBS 90%, and 0.25 mg/ml propidium iodide (Calbiochem, La Jolla, Calif.) for counterstaining nuclei was added. The slides were examined under a light microscope. The presence and distribution of dense precipitate was observed.

**Electron Microscopic Localization**

Upon receipt of the umbilical vessels, they were fixed and immersed in an aqueous solution of 2% paraformaldehyde with 0.037 M sodium phosphate buffer adjusted to pH 7.2. After 1 hr of fixation, the vessels were washed for 12 hr in PBS to which 10% sucrose had been added.

The washed tissues were frozen by immersion in liquid nitrogen and then thawed as a means of mildly disrupting the cells so that antisera and solutions could penetrate them. The vessels were transferred to a 1:40 PBS dilution of the rabbit antibody to factor-VIII-rg sera and incubated at 37°C for 1 hr. Specimens were removed from the diluted antiserum and washed for 30 min in 3 changes of PBS sucrose. The specimens were then transferred to a 1:40 dilution of sheep Fab anti-rabbit IgG (SARG; 1.25 μg/ml) coupled to horseradish peroxidase (HRP) (glutaraldehyde; Pasteur Institute, Garches, France) and incubated for 1 hr at 37°C. After 3 10-min washes in PBS 10% sucrose, the tissues were treated with 3% glutaraldehyde in PBS 10% sucrose for 30 min to fix the antibodies to the tissue sites. The fixative was thoroughly washed out with three changes of PBS-sucrose for periods of excess of 1 hr. The tissues were then incubated for 7–10 min in a 0.5% solution of 3,3-diaminobenzidine tetrahydrochloride (DAB) to which H2O2 had been added to make a final concentration of 0.01% treated with 1% unbuffered osmium tetroxide to render the DAB reaction product electron dense, dehydrated with ethanol followed by propylene oxide, and embedded in Epon 812.

Control specimens consisted of: (A) vessels incubated with normal rabbit serum (NRS) rather then specific antiserum against factor-VIII-rg and then sequentially treated with SARG and DAB; (B) vessels treated with SARG followed by DAB; (C) vessels treated with DAB alone; and (D) vessels treated with anti-factor-VIII-rg, which had been absorbed with plasmas of patients with severe von Willebrand’s disease and plasmas from patients with hemophilia, as described above. After incubation with the absorbed primary antiserum, the tissue was sequentially treated with SARG and DAB.

Sections, 1-μm thick, were cut from all blocks and examined with a light microscope. The presence and distribution of dense precipi-

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**Table 1. Immunofluorescent Localization Studies in Umbilical Veins**

<table>
<thead>
<tr>
<th>Vessel Treatment</th>
<th>Antiserum</th>
<th>Diluent*</th>
<th>Immunofluorescence†</th>
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<tbody>
<tr>
<td>Control</td>
<td>Anti-factor-VIII-related antigen</td>
<td>von Willebrand’s disease plasma</td>
<td>Specific endothelial layer staining</td>
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<tr>
<td></td>
<td></td>
<td>Hemophilic plasma</td>
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<tr>
<td></td>
<td></td>
<td>PBS</td>
<td>Specific endothelial layer staining</td>
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<td></td>
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<td>Normal plasma</td>
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<tr>
<td></td>
<td>Anti-CIG</td>
<td>PBS</td>
<td>Diffuse staining in all vessel layers</td>
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<tr>
<td></td>
<td></td>
<td>von Willebrand’s disease plasma</td>
<td>—</td>
</tr>
<tr>
<td>Deendothelialization‡</td>
<td>Normal rabbit serum</td>
<td>von Willebrand’s disease plasma</td>
<td>Specific endothelial layer staining</td>
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<tr>
<td></td>
<td>Anti-factor-VIII-related antigen</td>
<td>Hemophilic plasma</td>
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<td>von Willebrand’s disease plasma</td>
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<td>Anti-fibrinogen</td>
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<td>Anti-albumin</td>
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<tr>
<td></td>
<td>Normal rabbit serum</td>
<td>PBS</td>
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*See text for dilutions.

†All sections were incubated with FITC-conjugated goat anti-rabbit globulin.

‡Umbilical veins and saphenous veins deendothelialized with 10-min collagenase incubation or mechanically with balloon catheter.
tates in vessels were evaluated under double-blind conditions. Representative areas of labeled cells were sectioned for electron microscopy, as were random areas from blocks in which no precipitate was discerned with the light microscope. The sections were examined without staining with heavy metal salts.

RESULTS

Immunofluorescent Staining

A summary of our findings is presented in Table 1. In vessels stained with anti-factor-VIII-r:ag, luminal fluorescence was associated with endothelial cells. The effect was blocked by dilution of the antibody in normal and hemophilic plasmas (Fig. 1E) but not by plasmas of patients with severe von Willebrand's disease (Fig. 1A and B). In higher power views, there appeared to be a layer of subendothelial fluorescence distinct from endothelial cells (Fig. 1B), and methods were employed to selectively remove the endothelial cell layer. When vessels were deendothelialized by either short incubations with the less purified collagenase or by balloon catheterization, residual immunofluorescence was consistently noted in the lumen (Fig. 1 C and D). This staining was also blocked by normal and hemophic plasmas but not by von Willebrand's disease plasmas, supporting the specificity of staining for factor-VIII-r:ag. Longer incubation times with the same collagenase resulted in absence of subendothelial immunofluorescence.

This collagenase preparation is known to be contaminated with other proteases. Therefore, in order to assess whether these digestion studies demonstrated binding of factor-VIII-r:ag to subendothelial collagen, further digestion studies were undertaken, employing a purified collagenase preparation. In these studies, subendothelial fluorescence could not be removed using a collagenase concentration of as much as 500 U/ml and an incubation of 2 hr. Thus, specific factor-VIII-r:ag binding to collagen could not be proven.

As mentioned earlier, our anti-factor-VIII-r:ag showed no reaction against purified CIG by immunodiffusion. In order to further test whether the subendothelial fluorescence might be due to the presence of an antibody to CIG contaminating the anti-VIII preparation, which was not absorbed by the von Willebrand's disease plasmas, we treated vessels with an antisera to CIG. In contrast to the luminal staining encountered with anti-VIII, vessels treated with anti-CIG stained diffusely in all layers with brightest staining in the adventitium, in agreement with the finding of Stenman and Vaheri.13 Furthermore, dilution of anti-CIG in von Willebrand's disease plasma resulted in complete blocking of immunofluorescent staining, thus ruling out the possibility that our von Willebrand's disease plasmas might have failed to remove some contaminating anti-CIG. Deendothelialized vessels treated with antisera to fibrinogen and albumin also showed no immunofluorescence. Minimal autofluorescence was appreciated in unstained vessel sections.

Electron Microscope Localization

Electron microscopic examination of thin unstained sections of umbilical vessels showed dense precipitate, which represented localization of factor-VIII-r:ag along the endothelial lining and into the basement membrane. The label was found along the lumenal (Fig. 2A) and intercellular spaces of endothelial cells. It appeared most concentrated in the basement membrane (Fig. 2A) and in the luminal portion of the internal elastic lamina. While this labeling pattern was seen with the antibody preparation absorbed in
FACTOR VIII-ANTIGEN IN SUBENDOTHELIUM

Fig. 2. (A) Electron micrograph of an unstained ultrathin oblique section from an umbilical vessel treated with rabbit anti-factor-VIII-r:ag and then with Fab fragments of SARG coupled to HRP followed by the DAB-H2O2 reaction shows the localization of factor-VIII-r:ag in the vessels. The dense precipitate is located in the lumen and on the lumenal and intercellular plasma membranes (small arrows). The precipitate extends into the basement membrane (BM) (large arrow). Mitochondria (M) and endoplasmic reticulum (ER) are also present in the endothelium. (30,600 x) (B) Electron micrograph of an unstained ultrathin section from an umbilical vessel treated with anti-factor-VIII-r:ag absorbed with plasma from a patient with severe factor-VIII deficiency. After treatment with the absorbed serum, the tissue was incubated with the Fab fragments of SARG coupled to HRP. This tissue showed no precipitate resulting from the reaction product of the HRP, DAB, and H2O2. Normal rabbit serum showed the same result. (34,000 x)

plasmas of patients with severe von Willebrand's disease, all controls, including absorption of the antibody preparation with hemophilic plasmas, showed no evidence of precipitate.

DISCUSSION

Factor-VIII-r:ag appears to play a role in the adhesion of platelets to vascular subendothelium. Bloom et al., using an indirect fluorescent method, found a thin layer of immunoreactive material lining blood vessel endothelium and suggested that this material was adsorbed from plasma. Hoyer et al., in a further study, detected factor-VIII-r:ag throughout the cytoplasm of endothelial cells and suggested that this was consistent with synthesis of factor-VIII-r:ag by these cells, an interpretation supported by the tissue culture work of Jaffe et al.

In the present study, we used a similar immunofluorescent method to demonstrate residual of factor-VIII-r:ag in human vascular subendothelium after selective deendothelialization in a plasma-free system. Utilizing electron microscopy, we were able to demonstrate the presence of factor-VIII-r:ag within the basement membranes of umbilical vessels with intact endothelium. Thus, our data are consistent with the hypothesis that endothelial cells deposit factor-VIII-r:ag into subendothelium and that this material becomes exposed on disruption of the endothelial lining. We considered the possibility that basement membrane collagen might be the subendothelial component to which factor-VIII-r:ag is bound but were unable to demonstrate this, since we could not remove identifiable factor-VIII-r:ag after prolonged incubation with purified collagenase.

Recently, Sakariassen et al., using a Baumgartner perfusion chamber, showed that labeled factor-VIII-von Willebrand's factor complex binds to human renal artery subendothelium and that deposition of labeled platelets correlated with the quantity of labeled factor-VIII-von Willebrand's factor bound. Interestingly, they showed a significant degree of platelet deposition even in the absence of any added labeled complex, a finding that could be explained by preexisting subendothelial factor-VIII-r:ag. Our data, together with the data of Sakariassen et al. and the demonstration of inducible binding sites for factor-VIII-von Willebrand's factor on platelets provide an interesting construct for some of the initial steps in platelet adhesion following vascular injury. Within this construct, endothelial cells produce factor-VIII-r:ag, secreting it both into plasma and into subendothelium where it is bound to some as yet unidentified subendothelial component. Damage to endothelium results in exposure of a subendothelial surface containing factor-VIII-r:ag, which mediates the binding of platelets having its receptor and also additional plasmatic factor-VIII-r:ag.

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