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Co-Localization of von Willebrand Factor and Type VI Collagen in Human Vascular Subendothelium

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The binding of von Willebrand factor (vWF) to subendothelium constitutes an important initial step in the process of platelet adhesion to exposed subendothelium following blood vessel injury. We previously demonstrated that vWF is present in human vascular subendothelium and recently found that a 150 kd vWF-binding protein, which we extracted from subendothelium, is type VI collagen. Although we have established that vWF and type VI collagen bind* in vitro, *it is not known whether these two proteins are associated in the vascular subendothelium* in situ. *We, therefore, 1) investigated the morphological effects of our biochemical extraction procedure on human umbilical veins by scanning and transmission electron microscopy, 2) analyzed the subendothelial extract by immunofluorescence for the presence of vWF and collagens and by electron microscopy for morphological characteristics, and 3) localized vWF and type VI collagen in subendothelium by immunofluorescence and by single- and double-label immunoelectron microscopic studies with protein A–conjugated gold particles. We found that the surface exposed following de-endothelialization is composed of microfibrils and contains very little fibrillar collagen. The subendothelium is stripped after sodium dodecyl sulfate–urea extraction, and the extract itself contains immunoreactive vWF and type VI collagen but no immunoreactive type I or III fibrillar collagens. Immunofluorescence and immunoelectron microscopic studies showed that vWF and type VI collagen are both present in subendothelium, where both co-localize to microfibrils. In conclusion, vWF that binds to type VI collagen* in vitro, *also co-localizes with type VI collagen in subendothelium, where both are associated with microfibrils. Type VI collagen, therefore, appears to serve as a biologically significant binding site for vWF* in vivo *and may thereby play a role in mediating platelet adhesion to exposed subendothelium following vascular injury. (Am J Pathol 1993, 142:843–850)

Von Willebrand factor (vWF), a multimeric glycoprotein (for recent review, see ref. 1) promotes the adhesion of platelets to the vascular subendothelium following injury to the vascular endothelium in high shear rate conditions[2] by serving as a bridge between platelets and the subendothelium. With respect to the platelet surface, two vWF receptors have been recognized, GP Ib (for review, see ref. 3) and the GP IIb-IIIa integrin complex.[4] The binding site(s) for vWF within vascular subendothelium, however, have not been conclusively identified.

We previously demonstrated that vWF is present in human vascular subendothelium[5–7] and showed that a 150 kd vWF-binding protein extracted from subendothelium is a component of type VI collagen,[8] a unique form of collagen with globular and fibrillar domains (for reviews, see ref. 9, 10). Furthermore, we showed that purified type VI collagen binds vWF. However, it has not been established whether vWF and type VI collagen are associated *in situ* nor how the putative complex is localized within the de-endothelialized vascular surface.

We, therefore, proceeded to investigate the following: 1) the effects of our biochemical extraction procedure on the morphology of the blood vessel wall, 2) whether vWF and collagens are present in the

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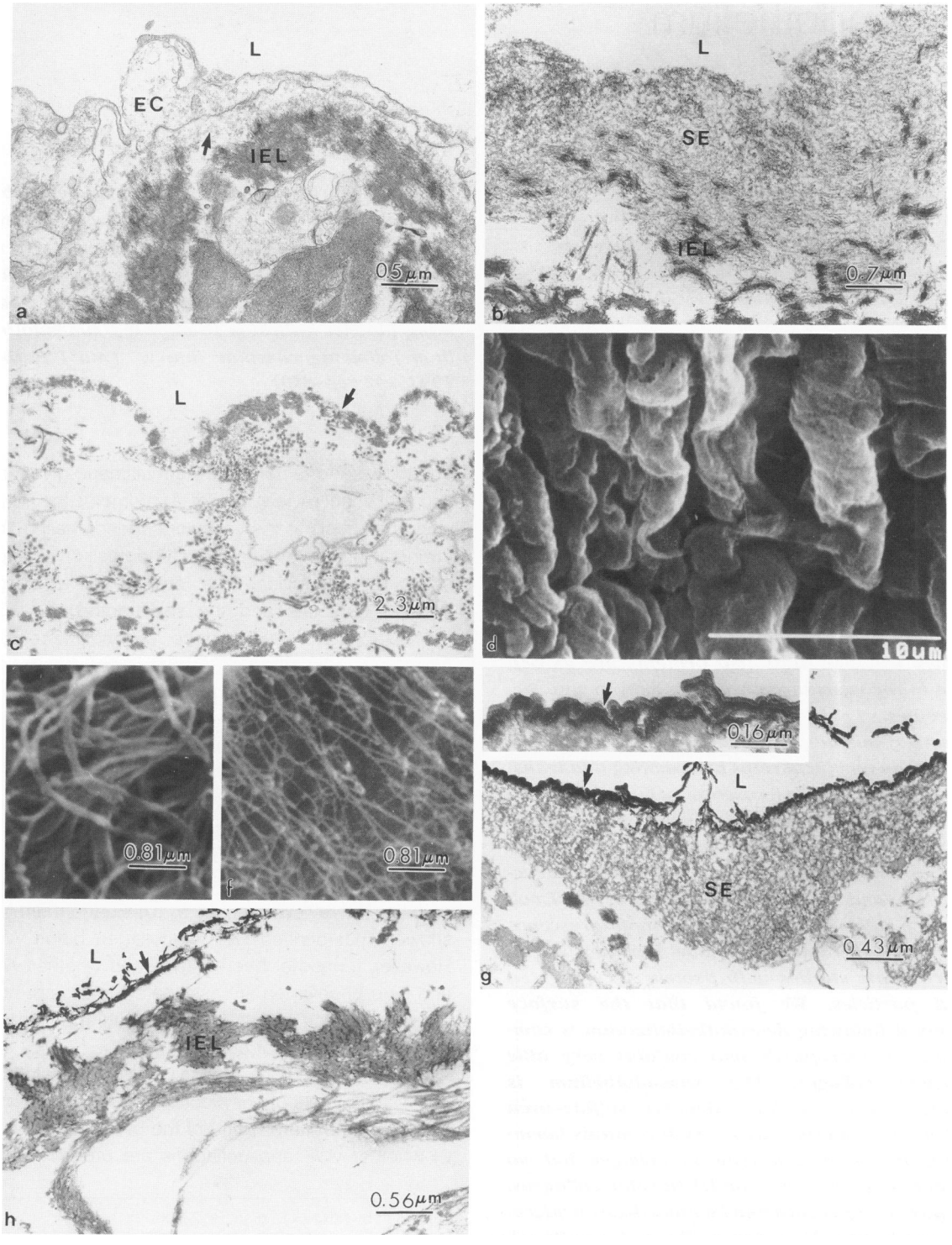

Figure 1. *Corresponding transmission and scanning electron micrographs of intact, de-endothelialized and SDS-urea-extracted umbilical veins.* **a:** *TEM of umbilical vein fixed in 3% glutaraldehyde and embedded in Epon shows an intact, endothelialized umbilical vein. A subendothelial layer (arrow) containing microfibrils is seen beneath the endothelial cells (EC) and above the internal elastic lamina (IEL) (×29,900).* **b:** *Subendothelium (SE) of umbilical vein following balloon catheter de-endothelialization. Microfibrils in this zone are above the internal elastic lamina (IEL) and facing the lumen (L) (×21,580).* **c:** *The effects of the SDS-urea extraction method on the subendothelium, with virtually complete stripping of the microfibril-containing subendothelium, leaving a bare surface above the internal elastic lamina (arrow). There are scattered residual collagen fibers*

extracts, and 3) whether vWF and type VI collagen co-localize in the vascular subendothelium.

Materials and Methods

Collection of Umbilical Blood Vessels

Human umbilical cords were collected following birth, and the veins were rinsed with iso-osmolar phosphate-buffered saline (PBS), pH 7.4. Segments of vein measuring approximately 3 cm were removed for processing (see below), and the remainder of the vessel was de-endothelialized as previously described.[8] The tissues were then treated as follows.

Extraction of Subendothelium

Each of the de-endothelialized veins was divided into two equal segments, usually approximately 5 to 6 cm long. The lumen of one half of each vein was filled with an extraction buffer solution containing 8mol/L urea–2% sodium dodecyl sulfate (SDS) and a cocktail of protease inhibitors in PBS, pH 7.4, as described,[8] while the other half of each vein was filled with iso-osmolar PBS, pH 7.4. The ends were ligated and the vessels were incubated in a 37 C waterbath for 90 minutes. The solutions in the lumens were harvested after incubation, dialyzed against distilled water at 4 C for 24 to 48 hours, and then centrifuged at 3500*g* for 10 minutes at 10 C. The pellets of the precipitated subendothelial extract were collected and kept at −80 C for immunofluorescence and electron microscopic studies. The post-extraction veins were rinsed with PBS and cut into blocks ranging from 1.0 mm to 1.0 cm in thickness.

Electron Microscopic Studies

For transmission electron microscopy (TEM), the blocks and the pellets were fixed in 3% gluteraldehyde in 0.2 mol/L sodium cacodylate buffer for 1 hour, postfixed in 1% osmium tetroxide, dehydrated, and then embedded in Epon 812. One-micron-thick sections were cut and stained with methylene blue to orient regions for thin sectioning. Ultrathin sections were cut and counterstained with uranyl acetate and lead citrate and examined with a 100 CX electron microscope (JEOL, Ltd., Tokyo, Japan).

For scanning electron microscopy (SEM), the tissues were critical-point dried through liquid CO_2 after dehydration. Samples were mounted on silver blocks, sputter-coated in an argon atmosphere with gold using a sputter coater, and then examined and photographed at 15Kv in the upper stage of a Hitachi S-530 scanning electron microscope. Following this, half the samples were cut from the silver blocks, treated with 100% alcohol and propylene oxide, and embedded in Epon 812 for TEM.

Negative Staining Electron Microscopy and Immunofluorescence Studies of Precipitated Subendothelial Extract

In addition to TEM, described above, negative staining was performed as follows. The pellets of the precipitated subendothelial extract were resuspended and diluted 1:2, 1:4, and 1:8 in distilled water. Carbon-coated, 400-mesh grids were floated, with the support film side facing down, for 2 minutes on a drop of the resuspension of the pelleted materials. After drying with a piece of filter paper, the grids were negatively stained with 5% uranyl acetate in distilled water for 30 seconds, viewed, and photographed under the same conditions.

For immunofluorescence studies, the precipitated subendothelial extracts were snap frozen in OCT compound (Miles Laboratories, Naperville, IL). Four micron cryostat sections were cut and mounted onto glass slides. The mounted sections were washed with 3 changes of iso-osmolar PBS, pH 7.4, for 5 minutes each, and incubated for 45 minutes at room temperature in a moisture chamber with the primary antibodies. The primary antibodies included rabbit polyclonal anti-vWF antiserum (Janssen Biochimica, Piscataway, NJ), at a final dilution of 1:100 after absorption in von Willebrand disease plasma, as described,[5] and 1:100 dilutions of monospecific rabbit polyclonal anti-human type I, III, and VI collagens (Chemicon, Temecula, CA). Equivalent concentrations of non-immune rabbit serum were used as controls. The sections were then washed for 15 minutes in the iso-osmolar PBS and incubated with a

remaining (×6,600). **d**: *SEM of umbilical vein shows the carpeted folds of the endothelialized surface in an intact, endothelialized umbilical vein (×6,000).* **e**: *Corresponding to TEM of* **(b)**, *is an SEM of the de-endothelialized subendothelial surface of the vein covered with a meshwork of fibers covering the internal elastic lamina (×72,000). Following SDS-urea extraction,* **(f)** *shows the removal of the bulk of fibers seen in the pre-extracted ubendothelium, corresponding to TEM of* **(c)**. *A fine reticular network of fibers remains over the exposed internal elastic lamina (×72,000).* **g**: *TEMs of the same specimen as* **(e)**, *with inset showing a higher magnification of the material corresponding to the meshwork observed in the SEM. Note the microfibril-laden subendothelium is seen beneath the coating gold (arrows) (×35,100, inset: ×91,000).* **h**: *The same specimen as shown in* **(f)**, *observed here by TEM (×27,000). Note the stripping of the microfibrillar subendothelium beneath the gold coating (arrow) and above the elastic lamina (IEL), with scattered collagen fibers remaining.*

1:100 dilution of FITC-conjugated goat anti-rabbit IgG (Cooper Biomedical, West Chester, PA). They were again washed with 3 changes of iso-osmolar PBS and mounted with a drop of glycerol diluted 1:10 in iso-osmolar PBS, pH 7.4. The sections were viewed with a Nikon Optiphot epifluorescence microscope (Nippon Kogaku, Garden City, NY), utilizing a super high-pressure mercury lamp and B-mode excitation with a 515 W absorption filter and a 496 auxiliary filter, and photographed under identical conditions.

Immunolocalization Studies of Umbilical Vein

Immunofluorescence studies were performed on the de-endothelialized umbilical veins. Cross-sections of de-endothelialized vein measuring 0.3 to 0.5 cm were snap frozen in OCT compound. Four-micron-thick cryostat sections were cut and mounted onto glass slides. The mounted sections were washed with 3 changes of iso-osmolar PBS, pH 7.4, for 5 minutes each and then incubated for 45 minutes at room temperature in a moisture chamber with the same primary antibodies described above for the precipitated subendothelial extract. Again, equivalent concentrations of non-immune rabbit serum were used as controls. The sections were then washed for 15 minutes in iso-osmolar PBS and incubated with a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG, following which they were again washed, mounted, viewed, and photographed as described above.

Immunoelectron microscopic studies were performed as follows. Freshly obtained umbilical veins were rinsed with iso-osmolar PBS, pH 7.4, and perfusion fixed with 2% paraformaldehyde in iso-osmolar PBS, pH 7.4, for 5 minutes. The veins were snap frozen in OCT compound. Twenty to thirty micron cryostat sections were cut and washed with 3 changes of iso-osmolar PBS, pH 7.4, for 15 minutes. For single-antigen labeling, the sections were incubated with the primary antibodies (anti-type VI collagen or anti-vWF, both 1:5 diluted in the iso-osmolar PBS, pH 7.4) in a moisture chamber at room temperature overnight. After 5 changes of iso-osmolar PBS, pH 7.4, at 4 C overnight, the sections were incubated with staphylococcal protein A–colloidal gold complexes (size 5 nm or 10 nm, 1:2 dilution, Amersham, Arlington Heights, IL) at room temperature overnight. Double labeling studies were performed by a modification of the procedure of Roth et al.[11] The sections were first incubated with the anti-type VI collagen antiserum and then with 5 nm staphylococcal protein A–colloidal gold complexes at a 1:2 dilution, followed by 2 hours incubation with normal goat serum. The sections were then incubated with the anti-vWF followed by 15 nm staphylococcal protein A–colloidal gold complexes at a 1:2 dilution. Equivalent concentrations of non-immune rabbit serum were used as controls. The sections were dehydrated with ethanol and embedded in Epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined with the electron microscope.

Results

Transmission and Scanning Electron Microscopic Studies of Pre- and Postextraction Blood Vessels

By TEM, an intact endothelial cell layer was observed in the untreated blood vessels situated above the subendothelium (Figure 1a), whereas SEM showed a ruffled carpet of endothelium (Figure 1d). Following de-endothelialization, TEM revealed the remaining subendothelium to be rich in microfibrils, with very little fibrillar collagen present (Figure 1b), whereas the corresponding SEM showed a surface covered with fibers that were 70 to 90 nm in diameter (Figure 1e). TEM of the identical SEM specimens (Figure 1g with inset), showed that the zone underneath the sputter coating contained microfibrils.

Subsequent extraction of subendothelium with the buffer containing 2% SDS–8 mol/c urea resulted in the removal of the subendothelium over the internal elastic lamina, as observed by TEM (Figure 1c). Occasional collagen fibers were noted above the internal elastic lamina. By SEM, the meshwork of fibers that had been observed before subendothelial extraction was removed (Figure 1f). Fine fibers with a diameter of 45 to 55 nm, which represent the same collagen fibers seen by TEM, remained over the internal elastic lamina. TEM of the identical SEM specimens (Figure 1h) revealed an absence of the microfibrils but occasional strands of remaining fibrillar collagen in the area between the sputter-coated microfibrils and the internal elastic lamina.

Immunofluorescence and Morphological Analysis of Subendothelial Extract

Immunofluorescent analysis of the precipitated SDS-urea subendothelial extract (Figure 2, a–c) showed that vWF and type VI collagen were localized in this precipitate, whereas type I and III fibrillar collagens were not localized in the precipitate. Nega-

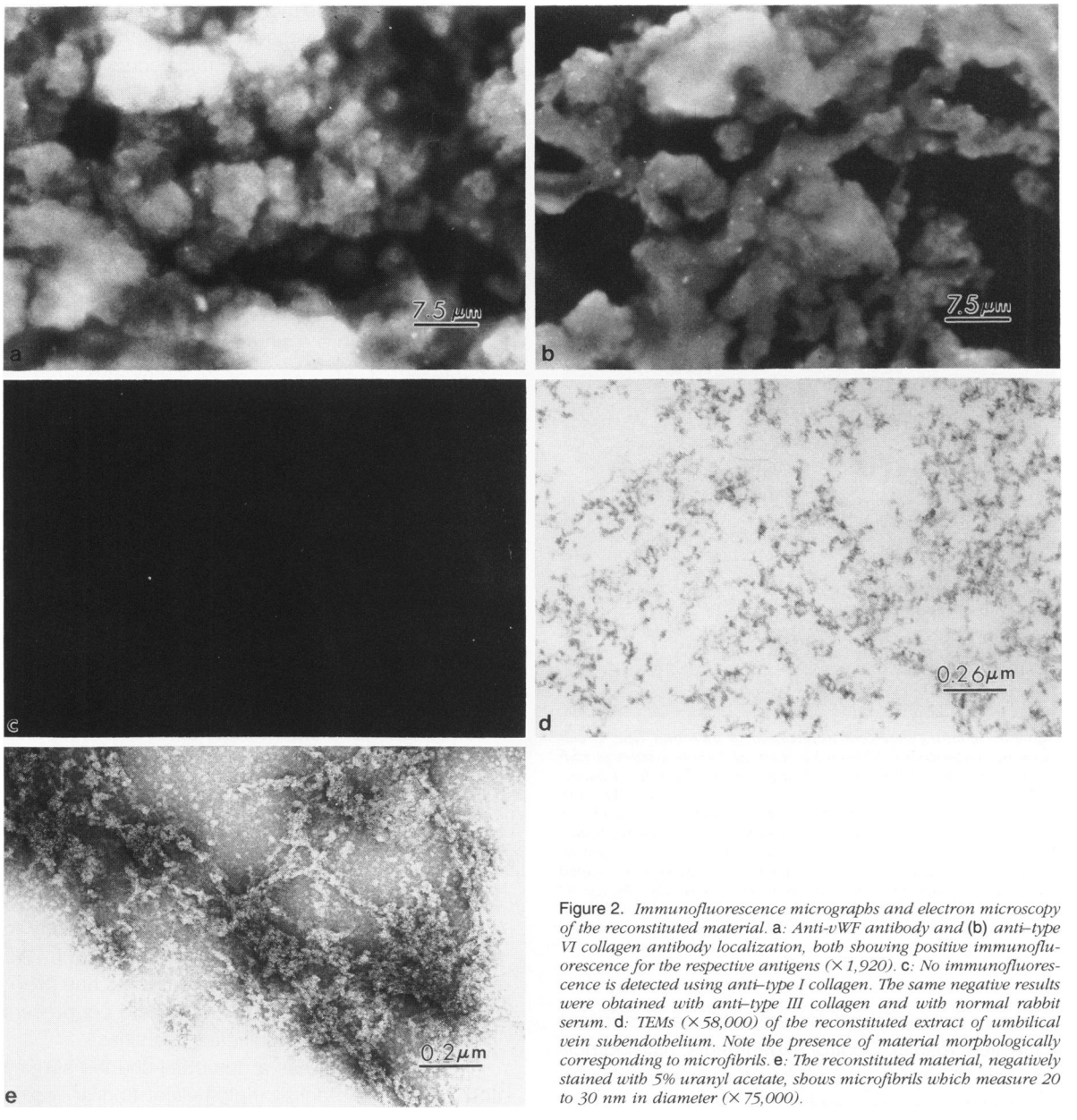

Figure 2. *Immunofluorescence micrographs and electron microscopy of the reconstituted material.* a: *Anti-vWF antibody and* (b) *anti-type VI collagen antibody localization, both showing positive immunofluorescence for the respective antigens (×1,920).* c: *No immunofluorescence is detected using anti-type I collagen. The same negative results were obtained with anti-type III collagen and with normal rabbit serum.* d: *TEMs (×58,000) of the reconstituted extract of umbilical vein subendothelium. Note the presence of material morphologically corresponding to microfibrils.* e: *The reconstituted material, negatively stained with 5% uranyl acetate, shows microfibrils which measure 20 to 30 nm in diameter (×75,000).*

tive staining of the precipitated SDS-urea extract revealed a composition that morphologically resembled microfibrils (Figure 2, d and e).

Immunofluorescence and Immunoelectron Microscopic Localization Studies of Subendothelium

Immunofluorescent studies of de-endothelialized umbilical vein (Figure 3, a and b) localized both vWF and type VI collagen to the subendothelium above the internal elastic lamina. The distribution of vWF was clearly limited to the subendothelium. Although by immunofluorescence there was a major concentration of type VI collagen in the subendothelium, a lesser intensity of type VI collagen was noted in the deeper portions of the blood vessel wall.

Immunogold electron microscopic studies confirmed the presence of vWF and of type VI collagen in the subendothelium, where both were associated with microfibrils (Figure 4, a–c). The localization studies were performed with both antibodies separately (Figure 4, a and b) and with double labeling (Figure 4c). The double label studies were performed with both possible sequences—i.e., anti-vWF first, followed by anti–type VI collagen as well as anti–type VI

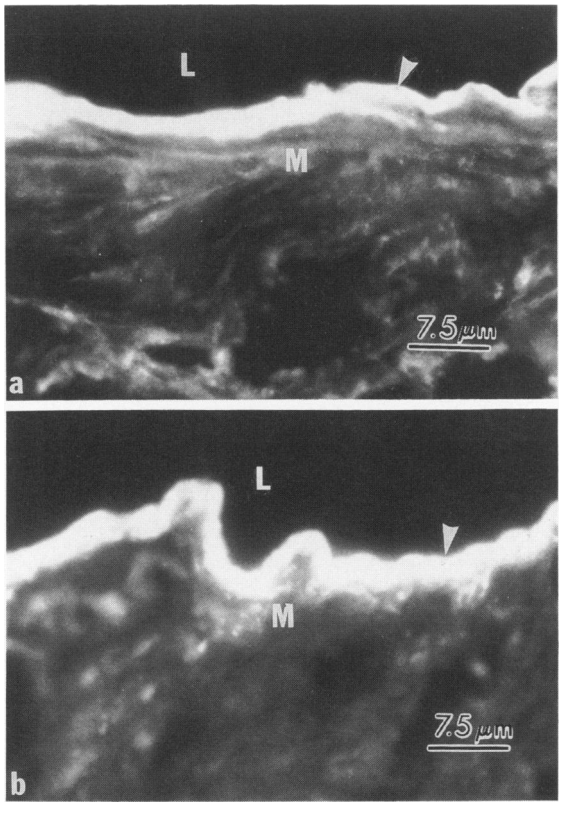

Figure 3. *Immunofluorescent localization of vWF and type VI collagen in cross-sections of de-endothelialized human umbilical vein subendothelium (×1,920).* a: *Localization of vWF by indirect immunofluorescence using monospecific rabbit polyclonal anti-vWF antibodies followed by FITC-conjugated goat anti-rabbit IgG. The vWF is predominantly localized to the vascular subendothelium (arrowhead). The lumen (L) and vascular media (M) are labeled for orientation.* b: *Similarly, localization of type VI collagen using monospecific rabbit polyclonal anti-type VI collagen as the primary antibody. The type VI collagen is predominantly localized to the vascular subendothelium (arrowhead). The lumen (L) and vascular media (M) are labeled for orientation.*

collagen first, followed by anti-vWF—and showed equivalent results. Control experiments, utilizing normal rabbit serum at equivalent concentrations to those of the primary antibodies showed no significant deposits of gold particles on the same tissues (Figure 4d).

Discussion

The subendothelial surface, which is exposed to flowing blood following damage to endothelium, may serve to modulate the hemostatic response following vascular injury. We previously extracted subendothelium with SDS-urea and identified a binding site for vWF and type VI collagen.[8] In the current study, we found that the subendothelial zone contains predominantly microfibrillar material, that the subendothelial extract is immunoreactive for vWF and type VI collagen and has the morphology of microfibrils, and that, by immunofluorescence and both single and double label immunoelectron microscopy, vWF and type VI collagen both co-localize to the microfibrils of the subendothelium. Taken together with our previous work, these data are consistent with the hypothesis that type VI collagen serves as a binding site for vWF *in situ* and is in a position where this complex can mediate platelet adhesion to exposed subendothelium following vascular injury.

We utilized umbilical veins for our studies because they contain abundant vWF in their subendothelium and were therefore a prime model for studying the mechanism of vWF binding to extracellular matrix and because human umbilical vein endothelial cells are the most frequently used source for umbilical cell culture. Although our findings need to be extended to study other blood vessels, others have found microfibrils in the subendothelial zones of various blood vessels.[12–15]

The method we describe removes subendothelium and leaves a residual surface devoid of microfibrils. Therefore, this extraction method may also be useful for studying the composition of vascular subendothelium. The postextraction subendothelial surface may provide a useful model for reconstituting subendothelial components in studying their roles in the hemostatic events that follow vascular injury.

Subendothelial vWF, as distinct from plasma vWF, contributes substantially to platelet adhesion.[16–18] Whereas most studies investigating vWF binding to collagen have used type I and III fibrillar collagens as a substrate,[19–23] these collagens are unlikely to constitute the subendothelial binding site for vWF because, as we have shown here and as has previously been demonstrated,[24–26] they are not present in subendothelium in significant quantities.

Type VI collagen has a dumbbell-shaped subunit and contains a central triple-helical fibrillar region comprising less than half of the molecule and 2 adjoining globular regions at its amino- and carboxyl-termini (for reviews see refs. 9 and 10). Interestingly, type VI collagen contains repetitive domains that are highly homologous to the A domains of vWF, and it has been proposed that these two molecules are members of a superfamily of A-like domain–containing proteins that, thus far, also include members of the integrin and complement systems and cartilage matrix protein.[10] Although the functional significance of these homologies is not yet understood, it has been proposed that this domain structure is involved in specific adhesive interactions.[10] Type VI collagen has a microfibrillar morphology[9,15] and is soluble in urea.[27]

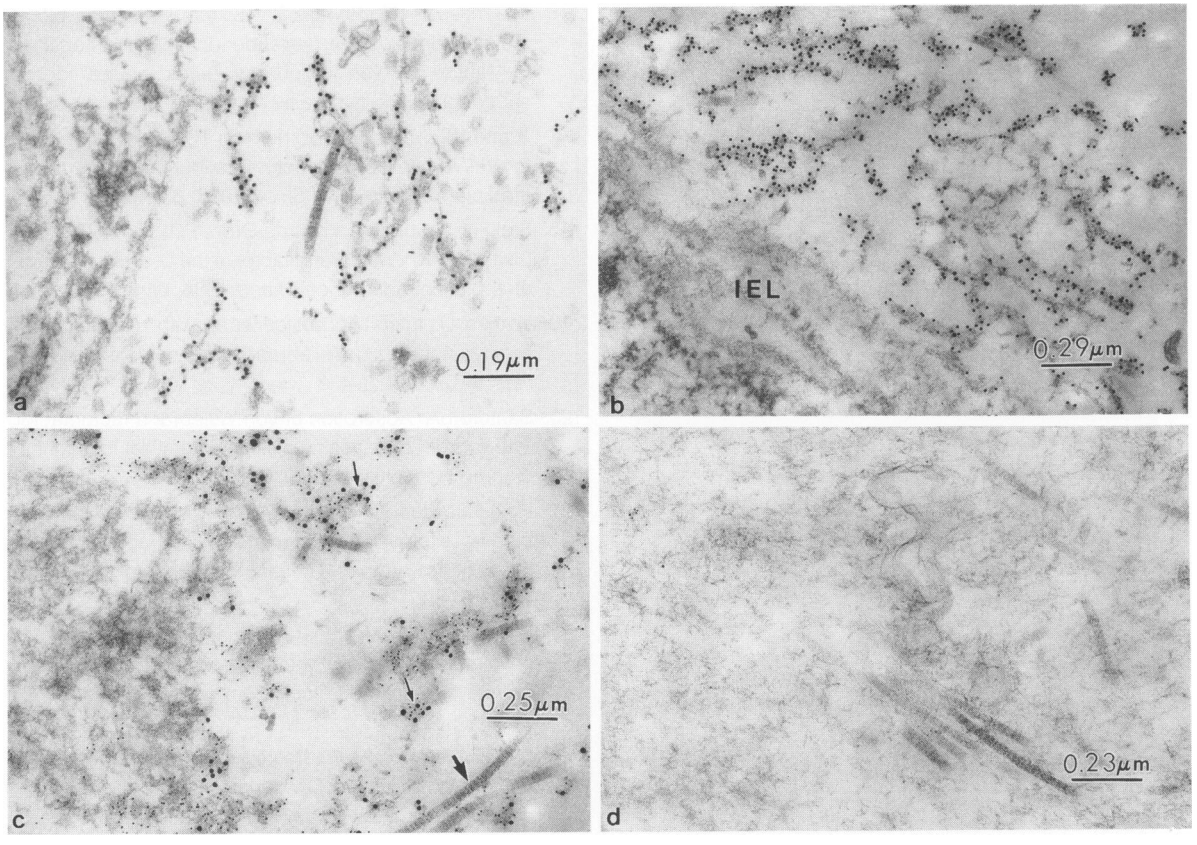

Figure 4. *Immunoelectron microscopic localization of vWF and type VI collagen in human umbilical vein subendothelium, labeled with anti-vWF and anti-type VI collagen antibodies, detected with immunogold-conjugated protein A. All of the figures have been oriented similarly, with the superficial, luminal portion of the subendothelium to the right of the figure and the deeper portions toward the left.* **a:** *Single labeling of vWF (×80,000) and* **(b)** *single labeling of type VI collagen (×52,800), with gold particles (size 10 nm). Both vWF and type VI collagen are present in the subendothelium, above the internal elastic lamina, where they are associated with microfibrils. Virtually no gold particles were observed along the thick collagen fibrils and in the elastic fibers.* **c:** *Double labeling, after the method of Roth et al,[11] of vWF (size 15 nm) and type VI collagen (size 5 nm). Note gold particles, representing the localizations of both proteins, are deposited on microfibrillar structures (×59,400).* **d:** *Negative control using same double labeling procedure as in* **(c)**, *with substitution of non-immune rabbit serum at dilutions equal to the specific antisera (×65,000).*

Morphologically, the subendothelium has previously been shown to be rich in structures known as microfibrils.[12] We have previously observed, using immunoperoxidase in conjunction with TEM, an association between vWF and the subendothelial microfibrils above the internal elastic lamina,[4] however, since those studies utilized the immunoperoxidase reaction, we could not distinguish the actual sites of localization with certainty at the ultrastructural level because of the possible diffusion of the peroxidase reaction product. Based upon studies utilizing microfibrils extracted from bovine aorta, Legrand and Fauvel with co-workers[28–30] had found that microfibrils bind vWF and that they contain thrombospondinlike structures. More recently, Arbeille et al[14] showed that exogenously added vWF binds to the microfibrils of porcine arterial subendothelium, where it co-localizes with thrombospondin. Thus, previous studies are highly suggestive of a microfibril binding site for vWF. Recently, Katsuda et al[15] reported the presence of immunoreactive type VI collagen in microfilaments in human blood vessels. The presence of type VI collagen in microfibrils and the *in vitro* affinity of vWF for type VI collagen[8] make it the most plausible candidate for the vWF binding material.

In conclusion, we have found that vWF and type VI collagen are both present in subendothelium, where they both localize to the microfibrils. Thus, type VI collagen and vWF are situated in an anatomic position where the complex of two proteins may serve as physiologically relevant attachment sites for platelets following vascular injury.

Acknowledgments

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