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Morphological Relationships of von Willebrand Factor, Type VI Collagen, and Fibrillin in Human Vascular Subendothelium

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von Willebrand factor (vWF) plays an important role in the process of platelet adhesion after endothelial injury by serving as a bridge between constituents of the vascular subendothelium and platelet membrane receptors. We previously presented evidence that type VI collagen microfibrils serve as a binding site for vWF in human vascular subendothelium. However, others have proposed that vWF is not associated with type VI collagen but rather with the thicker elastin-associated microfibrils, which contain several proteins including fibrillin. We therefore investigated the relationships among vWF, type VI collagen, and fibrillin in human vascular subendothelium by immunoelectron microscopy using single- and double-labeling immunogold localization techniques. In addition, we observed the three-dimensional ultrastructure of vWF-microfibril complexes by stereo paired micrographs and stereo viewer. We found that vWF co-localizes only with the type VI collagen microfibrils in subendothelium but not with fibrillin microfibrils or striated collagen. The vWF is present in subendothelium in the form of electron-dense aggregates having diameters varying between 65 and 80 nm that are closely associated with, and enmesh, the type VI collagen microfibrils and have structural similarities to intracellular Weibel-Palade bodies. The occasional co-localization of type VI collagen and fibrillin adjacent to internal elastic lamina was observed. These results are consistent with the hypothesis that type VI collagen, but not fibrillin-containing microfibrils, serves as a physiologically relevant binding site for vWF in the vascular subendothelium, where the type VI collagen-vWF complex may play an important role in modulating the hemostatic response to vascular injury. (Am J Pathol 1996, 149:283–291)

After vascular injury, von Willebrand factor (vWF) plays a role in the process of platelet attachment to exposed subendothelium by bridging between platelet membrane receptors and the subendothelium (for review see Ref.1). vWF is present in the subendothelium,[2–4] and this pool of vWF is known to play an important role in the process of platelet adhesion.[5–7] It would therefore be important to elucidate the subendothelial binding site(s) for this protein.

We previously showed that vWF co-localizes with type VI collagen microfibrils in human vascular subendothelium.[8] However, other authors have reported that vWF binds to thrombospondin-containing elastin-associated microfibrils[9–11] and that, in vascular subendothelium, vWF co-localizes with the thicker thrombospondin-containing microfibrils but not with type VI collagen microfibrils.[12] As elastin-associated microfibrils are known to contain fibrillin,[13,14] antibodies against this protein together with antibodies against vWF and type VI collagen could be used to resolve the relationships among these constituents of the vascular subendothelium.

We therefore systematically investigated the relationships of these subendothelial components using single- and double-label immunogold transmission

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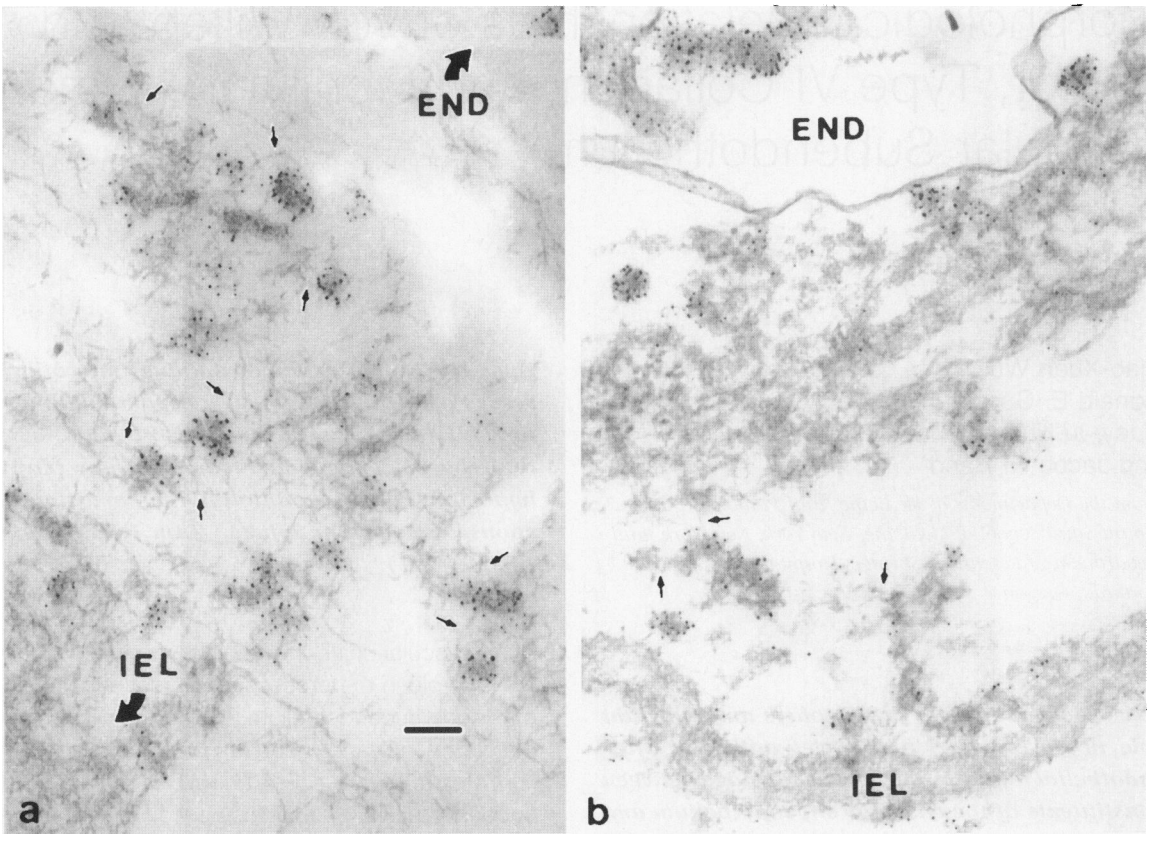

Figure 1. *Immunogold labeling of vWF in the subendothelium of vein* (a) *and artery* (b). *The gold particles label the electron-dense aggregates associated with thin microfibrils* (small arrows). *END, endothelial cells. Magnification, ×70,000; bar, 107 nm.*

electron microscopic techniques. In addition, as antigens that appear to co-localize by conventional two-dimensional immunoelectron microscopy may, in fact, be present in different planes, we observed the three-dimensional ultrastructure of the vWF-microfibril complexes in subendothelium by stereo paired micrographs.

Materials and Methods

Antibodies

Rabbit polyclonal antibody to human vWF (Janssen Biochimica, Piscataway, NJ), mouse monoclonal fibrillin antibody (MAb 69),[13] and mouse monoclonal type VI collagen (MAb VI)[15] antibody were used as the primary antibodies. Goat anti-rabbit and goat anti-mouse IgG coupled to 5- or 15-nm colloidal gold particles or staphylococcal protein-A-gold complexes with 5- or 15-nm gold particles (Amersham, Arlington Heights, IL) were used as the secondary antibodies for the immunolocalization of vWF, type VI collagen, and fibrillin. MAb 69 and MAb VI were lyophilized in hybridoma cell culture medium and were reconstituted in an appropriate volume of distilled water before use. MAb 69 has been previously characterized and shown to be specific for the pepsin-resistant PF3 domain of fibrillin and reacts with native human fibrillin in tissue sections.[13] MAb VI was made by mixing monoclonal antibodies V1–1, H-VI-2, N3-VI-2, CI,2-VI-1, and CI,2-VI-2, which have been characterized previously; all react with native type VI collagen in tissue sections.[15]

Electron Microscopic Immunolocalization

Human umbilical cords were collected immediately after delivery. The vessels were rinsed with iso-osmolar phosphate-buffered saline (PBS), pH 7.4, and perfusion fixed with 2% paraformaldehyde in PBS for 5 minutes. The tissue was then snap-frozen in OCT compound (Miles Laboratories, Naperville, IL). Thirty- to forty-micron cryostat sections were cut and washed with three changes of iso-osmolar PBS, pH 7.4, for 15 minutes. Pre-embedding immunolabeling was then performed according to previously established procedures with minor modification.[8] For single-antigen immunogold labeling, the sections were

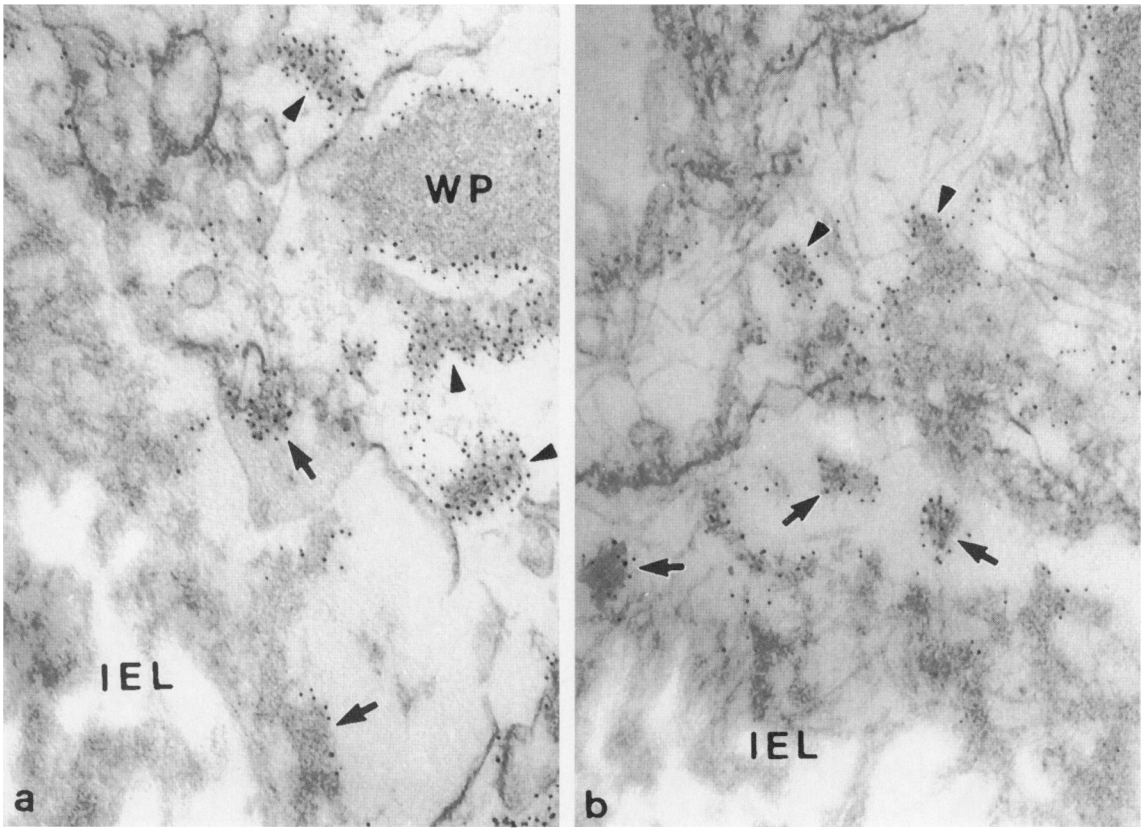

Figure 2. *Immunogold labeling of vWF in the subendothelium of vein. Note that the fragments of Weibel-Palade bodies (WP;* **arrowheads**) *located adjacent to the basal cytoplasmic membrane of endothelial cells* (**a**) *have a similar profile in electron density, shape, and size to the vWF-labeled electron-dense aggregates deeper in the subendothelium* (**b**; **arrows**). *Magnification, × 89,100; bar, 85 nm.*

incubated at 4°C overnight with primary antibodies (anti-vWF 1:5 diluted in PBS and MAb 69 and MAb VI undiluted) and then with appropriate goat anti-rabbit or goat anti-mouse 5-nm gold conjugate (Amersham) at a 1:2 dilution in PBS. Sections were washed with five changes of PBS for 6 hours at 4°C after the antibody incubation steps. For the detection of double antigens, the sections were first incubated with MAb VI or MAb 69 and then with goat anti-mouse 5-nm gold conjugate or protein-A-gold 5-nm complexes followed by 2 hours of incubation with normal goat serum. The sections were then incubated with anti-vWF or MAb 69 followed by goat anti-rabbit 15-nm gold conjugate or protein-A-gold 15-nm complexes at a 1:2 dilution. Equivalent concentrations of non-immune rabbit and mouse IgGs were used as controls. The vessel sections were then immersed in 3% glutaraldehyde in PBS for 1 hour, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Sections of approximately 80 to 100 nm thickness were cut and stained with uranyl acetate and lead citrate and examined with a JEOL 100 CX electron microscope (JEOL, Tokyo, Japan).

Stereoscopic Three-Dimensional Observations

To observe the three-dimensional relationships of the antigens that had been found to co-localize, grids were also examined using the transmission electron microscope equipped with a goniometer stage and rotating specimen holder (JEOL). Stereo paired micrographs were taken at 7° angles or tilt from one another.

Results

Single-Antigen Labeling Studies

Immunogold single-antigen labeling confirmed the presence of vWF and type VI collagen in subendothelium of human vessels, where they both localize to microfibrils. Interestingly, gold labeling of vWF in subendothelium in all vessels examined was conspicuously localized on round, oval, or irregularly shaped, electron-dense aggregates having a diameter of 65 to 80 nm (Figure 1). These aggregates revealed a similar profile in electron density, shape,

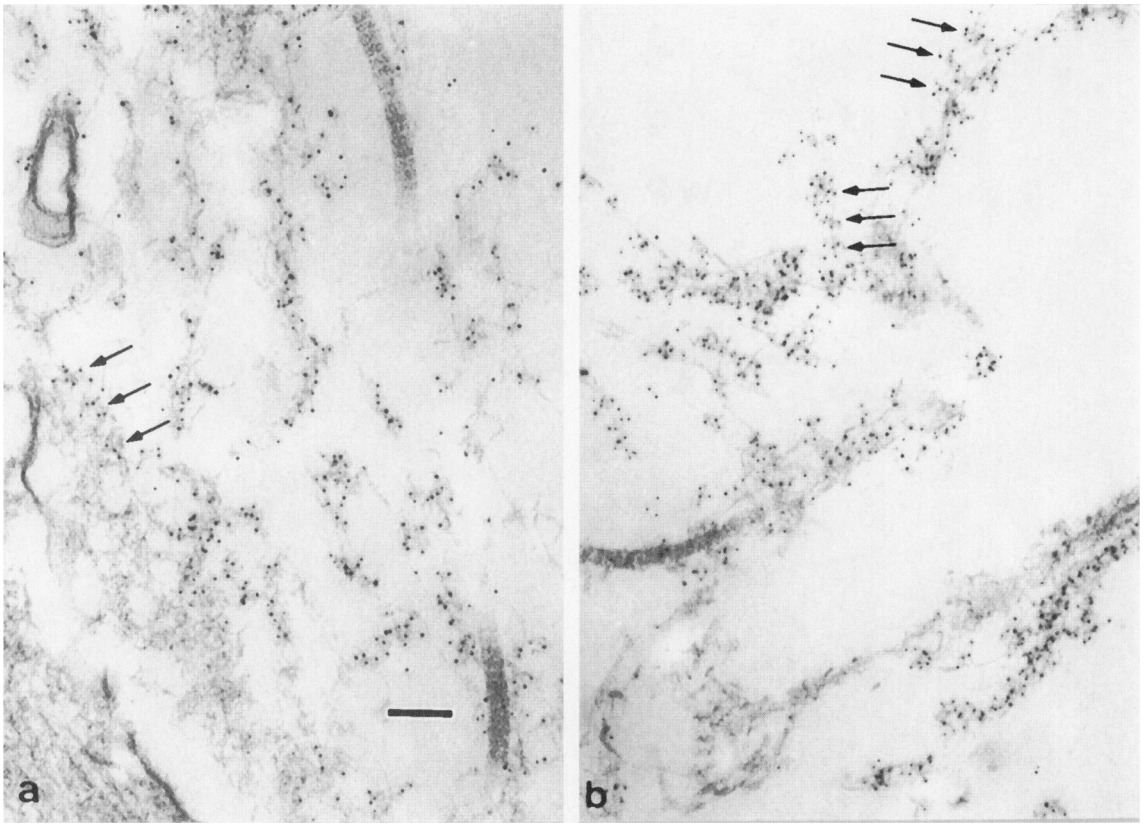

Figure 3. *Immunogold labeling of type VI collagen of vein* (a) *and artery* (b). *The gold particles are seen in the subendothelium; they label thin microfibrils* (*with a mean diameter of 5 nm*) *and cluster at intervals* (arrows) *of 40 to 100 nm. Magnification,* ×*92,500; bar, 81 nm.*

and size to the Weibel-Palade bodies located close to the basal cytoplasmic membrane of endothelial cells (Figure 2). These vWF aggregates were surrounded by fine microfibrils with an approximate mean diameter of 5 nm (Figure 1). The microfibrils were decorated by gold particles labeling for type VI collagen, which were present in nonrandom repeating clusters at intervals of 40 to 100 nm (Figure 3).

Vessels incubated with fibrillin antibodies labeled thicker microfibrils approximately 10 nm in diameter and have a periodicity of approximately 50 nm as previously reported.[16] Immunogold-labeled fibrillin microfibrils were often seen around amorphous cores of internal elastic lamina (IEL; Figure 4). In addition, fibrillin antibodies were also observed to bind to small bundles of microfibrils without amorphous cores in subendothelium, which sometimes appeared to intersect IEL at the subendothelium-IEL junction (Figure 4b). The patterns of localization for all three antigens were similar in both umbilical veins and arteries.

Double-Antigen Labeling Studies

To investigate the inter-relationships among vWF, type VI collagen, and fibrillin, the three possible combinations of double-labeling studies were performed: 1) vWF and type VI collagen, 2) vWF and fibrillin, and 3) type VI collagen and fibrillin.

Double-labeling studies for vWF and type VI collagen showed the co-localization of the two antigens on microfibrillar structure in human vascular subendothelium. We observed gold particles labeling for type VI collagen on the microfibrils surrounding vWF-labeled aggregates (Figure 5). Labeling for type VI collagen was also observed within the vWF aggregates (Figure 5). In contrast to the observations for type VI collagen, there was no association found between the gold particles labeling for vWF and fibrillin (Figure 6). Interestingly, there was also no significant association of labeling for vWF with morphologically detectable fibrillar collagen in the subendothelium (Figure 6).

The localizations of type VI collagen and fibrillin were usually distinct, although in some areas, adjoining the IEL, the co-localization of gold particles labeling for both type VI collagen and fibrillin was observed (Figure 7). Results for all of the double-antigen labeling studies were observed to be similar in both umbilical veins and arteries.

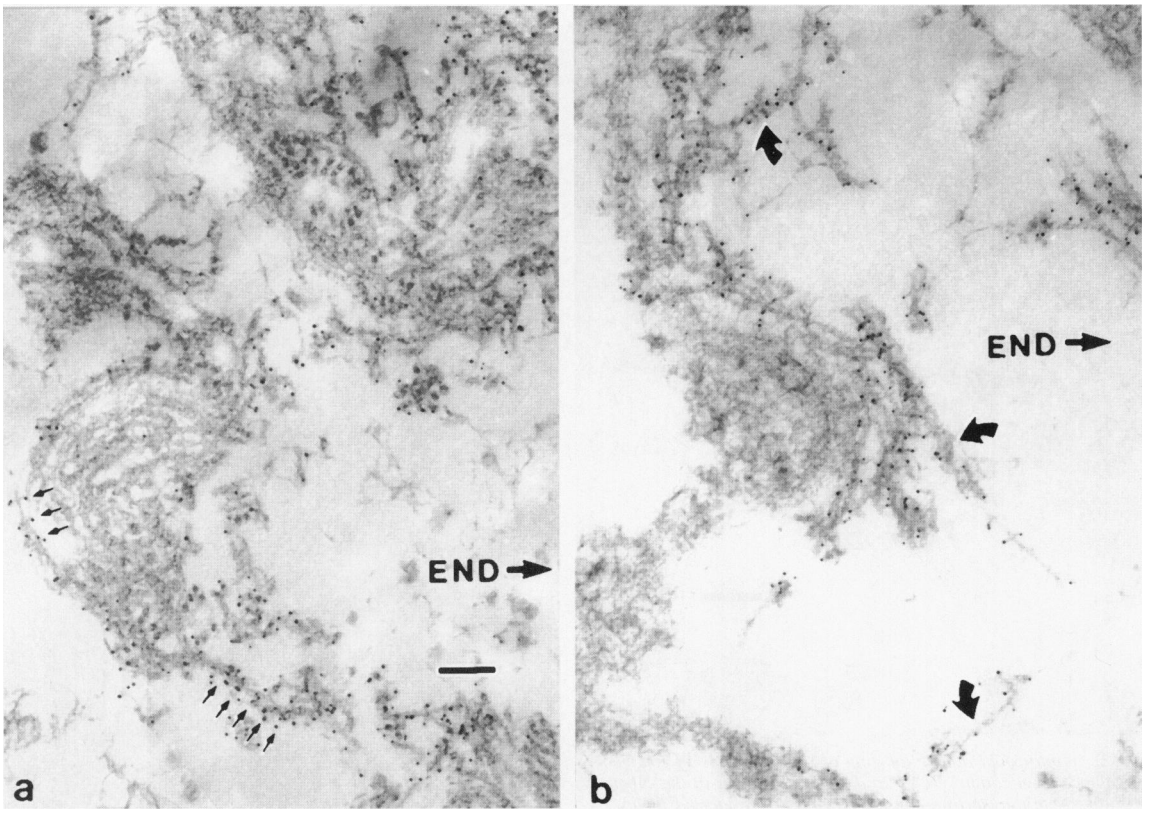

Figure 4. *Immunogold labeling of fibrillin in the subendothelium.* a: *The gold is seen concentrated at 50-nm intervals* (small arrows) *on microfibrils (with a mean diameter of 10 nm).* b: *Small bundles of microfibrils without amorphous cores* (curved arrows) *intersecting IEL at subendothelium-IEL junction. Magnification, × 70,000; bar, 107 nm.*

Three-Dimensional Observations

To further elucidate the relationship of vWF and type VI collagen and how these two components are organized within the vWF aggregates, we examined the three-dimensional ultrastructure of type VI collagen and vWF aggregates with stereo paired electron micrographs taken from thin sections of double-labeled samples. We found that the vWF aggregates in subendothelium are composed of loosely coiled thin strands that aggregate in a spiral pattern. Because of overlap of structures in two-dimensional images, these structures appear as electron-dense granules as described above. Type VI collagen microfibrils extend along these thin strands into the vWF-labeled aggregates. This gives the aggregates a laminated appearance with a less dense layer of type VI collagen between two dense layers of vWF (Figure 8). Labeling of vWF and type VI collagen on two-dimensional representations showed an alignment pattern indicating localization of both on the same structure. However, when viewed in three dimensions, it is clear that, although both may label the same structures, the two antigens may also be present in close association in different planes (Figures 8 and 9).

Discussion

vWF is present in subendothelium,[2–4] where it serves to promote platelet adhesion.[5–7] Type VI collagen, which is present in subendothelium,[17] binds vWF[17,18] and supports platelet adhesion at low shear rate conditions.[19,20] The current results extend our previous finding that vWF co-localizes with microfibrils of type VI collagen in subendothelium.[8] They further clarify the relationship between these two proteins and definitively show that vWF is not associated with elastin-associated microfibrils containing fibrillin.

An interesting aspect of our study is the finding that vWF in subendothelium is present as insoluble aggregates that are morphologically similar to intracellular Weibel-Palade bodies. These vWF particles are closely associated with thin type VI collagen microfibrils, which, by three-dimensional stereo paired electron micrographs, are seen to adjoin and also penetrate the vWF aggregates. The vWF clusters are not associated with the thicker elastin-associated fibrillin microfibrils. Fibrillin and type VI collagen microfibrils are associated with each other at the interface between the IEL and the subendothelium.

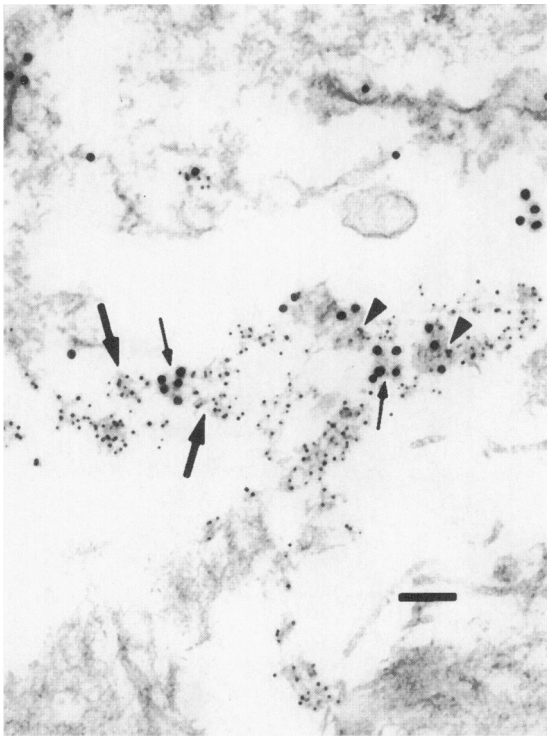

Figure 5. *Immunogold double labeling of vWF and type VI collagen shows the co-localization of vWF and type VI collagen in the subendothelium. Note the electron-dense aggregates were labeled for vWF with 15-nm gold particles* (small arrows) *and surrounded by type VI collagen-labeled microfibrils with 5-nm gold particles* (large arrows). *Labeling for the type VI collagen is also observed within these vWF aggregates* (arrowheads). *Magnification, ×92,400; bar, 80 nm.*

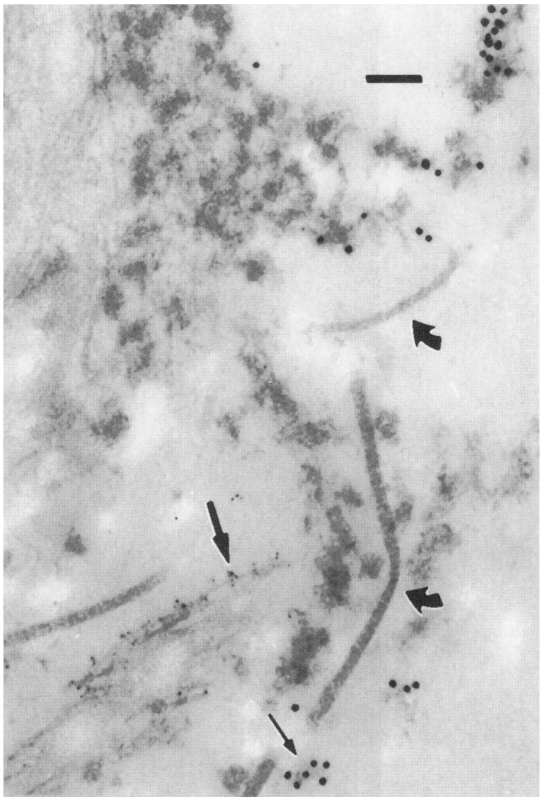

Figure 6. *Immunogold double-labeling of vWF (15-nm gold particles;* small arrow) *and fibrillin (5-nm gold particles;* large arrow) *shows no association between vWF and fibrillin in the subendothelium. Magnification, ×67,500; bar, 111 nm.*

Weibel-Palade bodies are intra-endothelial storage granules that contain the largest forms of vWF that are released in response to physiological stimuli such as thrombin and fibrin.[21–23] As these are considered to be solely intracytoplasmic structures, we found it notable that extracellular deposits of vWF aggregates in the subendothelium had morphological appearances similar to these bodies. This suggests that the vWF packed into Weibel-Palade bodies may, after its release into the subendothelium and binding to type VI collagen microfibrils, retain the intracytoplasmic packing configuration.

A significant aspect of this study is its confirmation of our previous observation of the lack of significant association between vWF and the fibrillar collagen that is present in the subendothelium. This is in contrast to the knowledge that purified fibrillar collagen binds vWF by recognizing specific domains on that molecule (for review see Ref. 1). We speculate that the lack of observable binding of vWF to subendothelial fibrillar collagen *in situ* may be due to shielding of this collagen by other elements such as proteoglycans, as had been demonstrated by Zucker-Franklin and Rosenberg.[24]

The present data, taken together with the functional observations on the interaction between type VI collagen and platelets,[19,20,25] suggest the following model. vWF is secreted abluminally from endothelial cells'

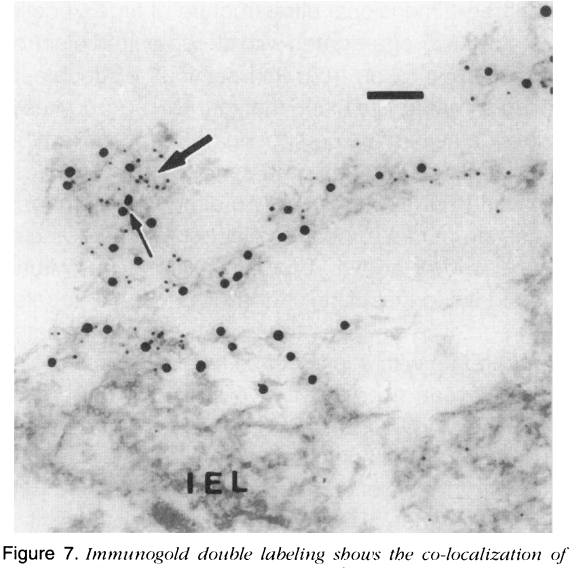

Figure 7. *Immunogold double labeling shows the co-localization of type VI collagen (5-nm gold particles;* large arrow) *and fibrillin (15-nm gold particles;* small arrow) *in an area adjoining the internal elastic lamina. Magnification, ×90,000; bar, 83 nm.*

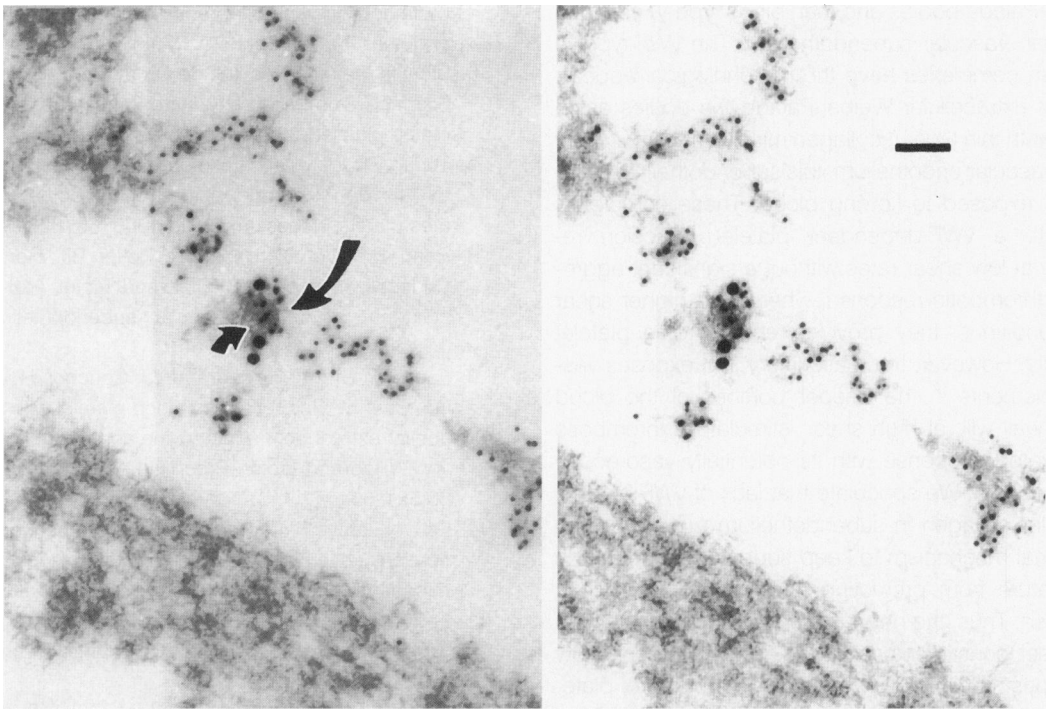

Figure 8. *The three-dimensional ultrastructure of type VI collagen and vWF aggregates. The paired tilted micrographs are positioned so that an observer can see the image in three dimensions by adjusting the height and angle of the stereo viewer over the micrographs until the images are fused or overlapped. Note that the vWF aggregates are composed of loosely coiled thin strands* (large arrow). *Microfibrils of type VI collagen extend into the vWF aggregates* (small arrow). *Magnification, ×132,000; bar, 56 nm.*

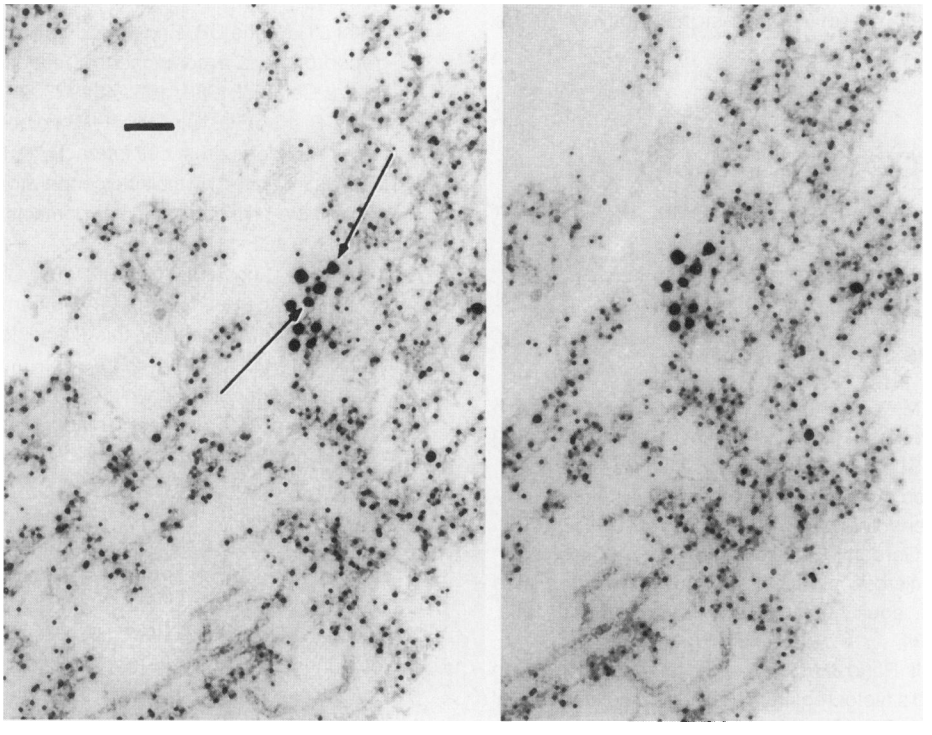

Figure 9. *The three-dimensional ultrastructure of type VI collagen and vWF aggregates. Observe as described in Figure 8. An alignment pattern (between arrows) indicating both localize on the same structure by two dimensions but not three dimensions, where both are present in different planes, associated but segregated. Magnification, ×132,000; bar, 56 nm.*

Weibel-Palade bodies and then binds type VI collagen in human vascular subendothelium. The vWF-type VI collagen complexes have the morphological appearance of extracellular Weibel-Palade-like bodies associated with thin type VI collagen microfibrils. After injury to the vascular endothelium, this subendothelial aggregate is exposed to flowing blood. These complexes stimulate a vWF-dependent platelet adhesion response at low shear rates without a significant aggregatory thrombotic response, whereas at higher shear rate conditions, they provoke relatively little platelet reactivity. However, traumatic injury that exposes vascular elements in the deeper portions of the blood vessel wall will, at high shear, stimulate a thrombotic aggregatory response with its potentially vaso-occlusive sequelae. We speculate that lack of vWF binding to fibrillar collagen in subendothelium may reflect an additional mechanism to keep superficial injury to the vasculature from promoting a significant thrombotic response. Thus, the degree of vascular injury, the type of vessel constituents exposed, and the blood flow conditions may serve to modulate the degree of platelet reactivity from none at all to a limited adhesion response to a full-blown thrombotic response.

In conclusion, vWF is present in subendothelium where it is associated with type VI collagen microfibrils but not with elastin-associated fibrillin-containing microfibrils. It is likely that these type VI collagen-vWF complexes, which are exposed to flowing blood after endothelial injury, play a significant role in the process of platelet adhesion.

Acknowledgments

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