The Ultrastructural Localization of von Willebrand Factor in Endothelial Cells

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Factor VIII-related antigen was localized ultrastructurally in a variety of human tissues (smooth muscle, skeletal muscle, breast, capillary hemangioma) with the use of a low-temperature embedding protein A-gold technique with both polyclonal and monoclonal antisera directed against von Willebrand factor. All endothelial cells examined localized the anti-von Willebrand factor to Weibel–Palade bodies. Cisternae of the endoplasmic reticulum, and cytoplasmic vacuoles were also labeled. These results establish the distribution of factor VIII-related antigen at the subcellular level. The observed distribution suggests that the endothelial cells synthesize von Willebrand factor, store it in Weibel–Palade bodies, and release it by exocytosis. These observations provide *in vivo* confirmation for previous biochemical and immunocytochemical data obtained from studies on cultured endothelial cells. (Am J Pathol 1984, 117:310–315)

FACTOR VIII-related antigen (von Willebrand factor) has been localized to vascular endothelium by immunofluorescence and immunoperoxidase techniques,[1–4] and studies on cultured endothelial cells have suggested that these cells possess the capacity to synthesize Factor VIII.[5–8]

A recent light- and electron-microscopic study of cultured human umbilical cord endothelium has demonstrated that the granular pattern of staining for Factor VIII-related antigen observed by both immunoperoxidase and immunofluorescence techniques corresponds to localization of this antigen in Weibel–Palade bodies.[4] These structures are unique to endothelial cells, but their precise nature and function was previously unknown.

In this paper, we employed a low-temperature embedding colloidal gold immunochemical technique to examine the subcellular localization of Factor VIII–related antigen in endothelium from a variety of human tissues. We confirm that Factor VIII-related antigen localizes Weibel–Palade bodies, and present ultrastructural evidence of its intracytoplasmic synthesis.

Materials and Methods

Normal human tissue from smooth muscle, skeletal muscle, breast, and a capillary hemangioma was obtained promptly after surgical removal. The tissues were minced into 1-mm cubes and fixed in a variety of fixatives. The fixatives included a mixture of 3% paraformaldehyde and 0.1% glutaraldehyde; 1% glutaraldehyde; and Karnovsky's fixative. The tissue was fixed from 1 to 4 hours. Following fixation, the tissue was rinsed in phosphate-buffered saline (PBS), pH 7.4, and reacted with 0.5 M NH_4Cl to quench unreacted aldehyde groups. The tissue was again washed in PBS.

For low-temperature embedding a Lowicryl K4M (Balzers, Hudson, NH) protocol was employed.[8,9] Briefly, after dehydration in increasing concentrations of ethanol, 30%, 50%, 70%, 95%, and 100%, at −20 C, the tissue was infiltrated with increasing concentrations of Lowicryl at −35 C. Initial polymerization was performed with ultraviolet light at −35 C. Final polymerization was performed at room temperature. Following polymerization, 1-μ sections were cut and stained with toluidine blue for selection of areas for thin-sectioning; 600- to 888-Å sections were picked up on

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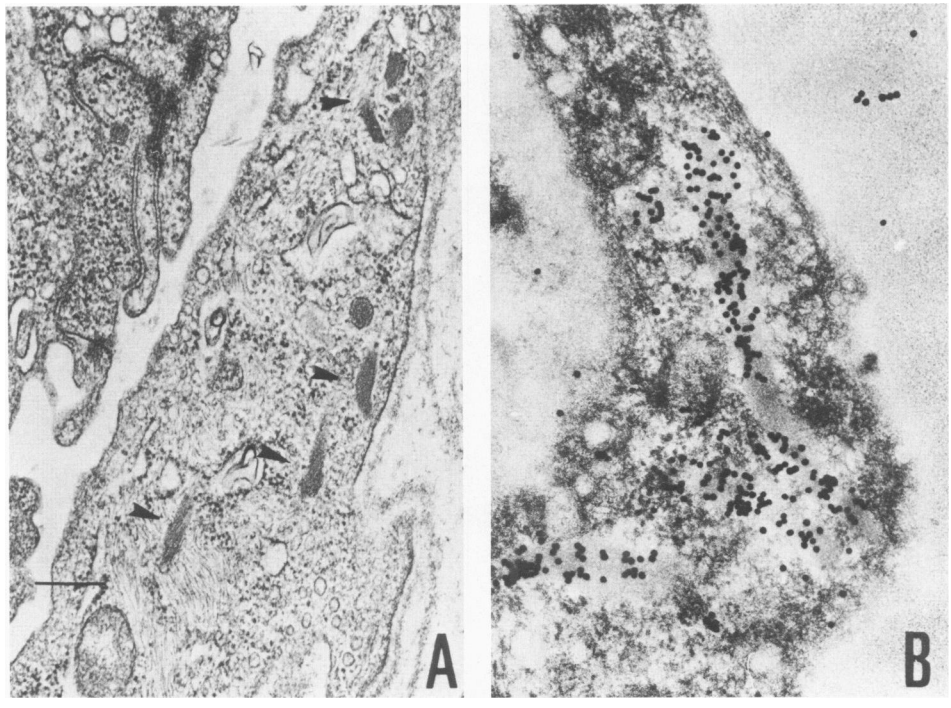

Figure 1 – Hemangioma. **A** – A glutaraldehyde- and osmium-fixed and Epon-embedded endothelial cell with numerous Weibel-Palade bodies (*arrowheads*). (×10,000; bar = 1 μ) **B** – An endothelial cell stained with polyclonal anti-Factor VIII. The numerous Weibel-Palade bodies are heavily labeled with gold particles. (×20,000)

nickel grids coated with Formvar for the immunochemical reactions.

Both polyclonal and monoclonal antibodies against von Willebrand factor were used for the immunochemical reactions. The antisera were obtained from Dakopatts, Santa Barbara, California (United States distributor). With the polyclonal antiserum, a simple two-step reaction was employed. The grids were first reacted with 0.5% egg albumin for 5 minutes. They were then incubated with antiserum in concentrations of 1:100, 1:200, and 1:400 for 1 hour at room temperature. The grids were then washed with PBS and incubated with staphylococcal protein A–colloidal gold complexes for 1 hour at room temperature. The grids were again washed with PBS followed by distilled water and stained with uranyl acetate followed by lead citrate. The grids were then examined on a JEOL JEMS 100 electron microscope.

With the monoclonal antibody a three-step procedure was employed. The initial incubations with egg albumin and primary antiserum were identical to that employed with the polyclonal antiserum. For the monoclonal antisera, dilutions of 1:20 and 1:40 were employed. Following the initial incubation and washing, the grids were incubated with rabbit anti-mouse antiserum for 30 minutes at room temperature. The

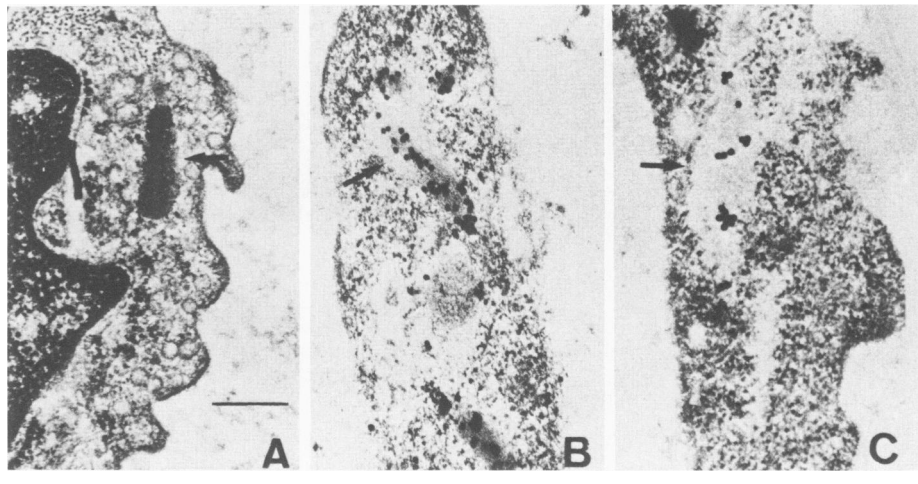

Figure 2 – Capillary within smooth muscle. **A** – This Epon-embedded section demonstrates a characteristic Weibel-Palade body (*arrow*). (×20,000; bar = 0.3 μ) **B** – Polyclonal anti-Factor VIII labels the characteristic Weibel-Palade bodies with gold particles (*arrows*). (×30,000) **C** – A Weibel-Palade body (*arrow*) is labeled with gold particles after staining with monoclonal anti-Factor VIII. (×30,000)

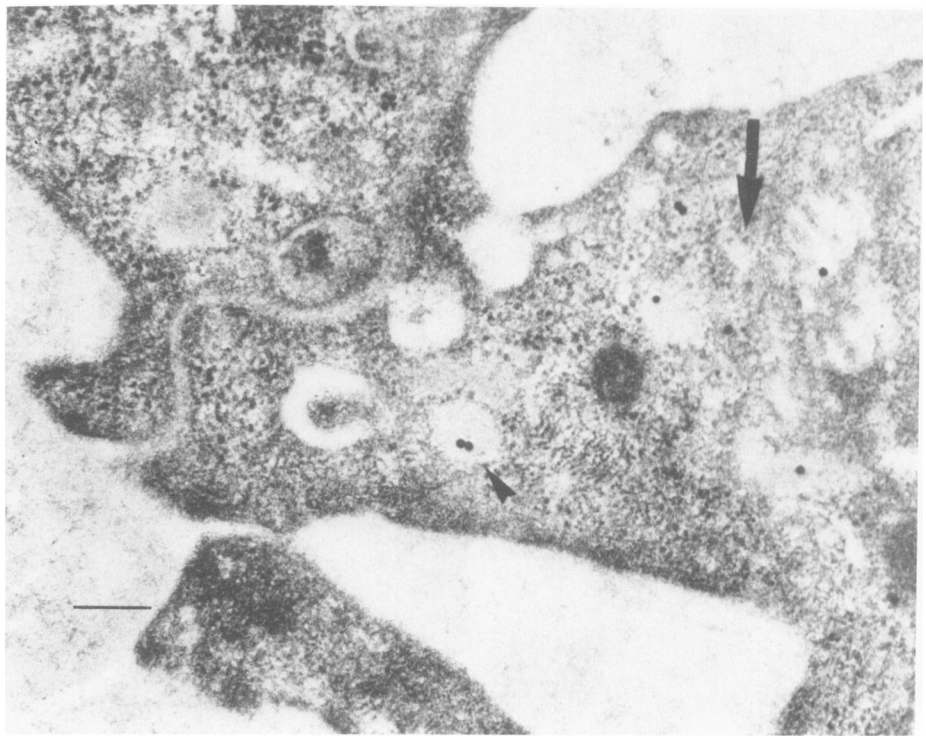

Figure 3 – Hemangioma. There is labeling of the endoplasmic reticulum (*arrow*) and cytoplasmic vacuole (*arrowhead*) (monoclonal anti-Factor VIII). (× 18,000; bar = 0.15 μ)

grids were again washed for PBS and reacted with colloidal gold–protein A and stained in a manner identical to that used with the polyclonal antiserum.

Controls included tissue sections incubated with buffer and nonimmune serum. They were otherwise incubated in an identical manner.

Colloidal gold–protein A complexes were prepared by the sodium citrate method. A 0.1% solution of tetrachloroauric acid in double-distilled water was heated to boiling. To this boiling solution, a 1% solution of sodium tricitrate was added (5 ml/100). Following development of a red/orange color, the pH of the

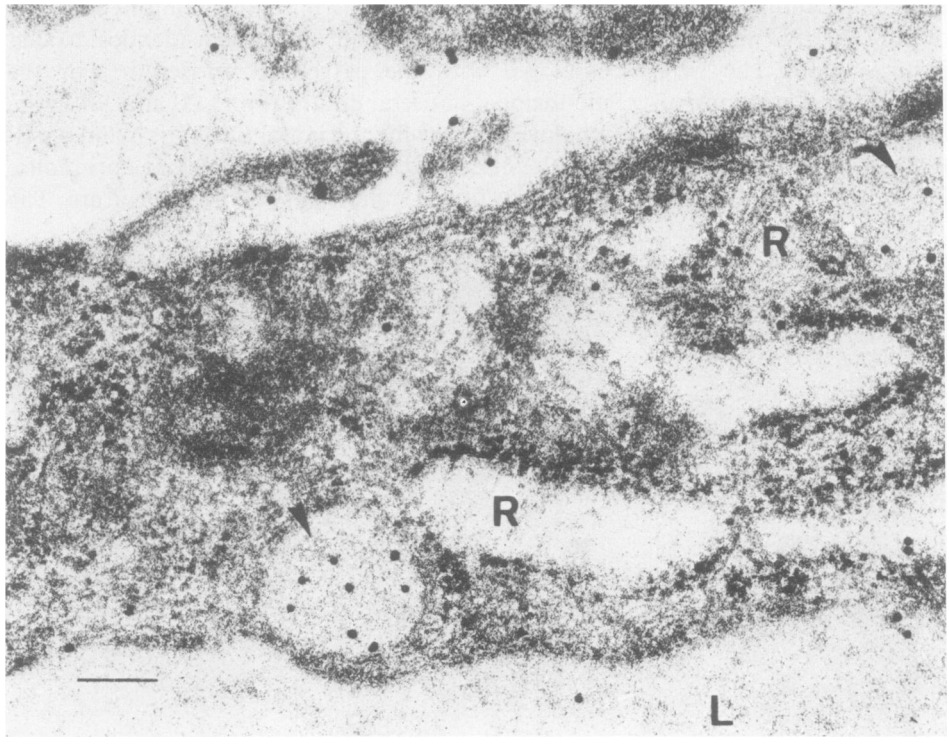

Figure 4 – Capillary in skeletal muscle. Cytoplasmic vesicles (*arrowheads*) containing finely granular material are labeled with polyclonal anti-Factor VIII. These vesicles are immediately adjacent to rough endoplasmic reticulum (*R*). (× 25,000; bar = 0.1 μ)

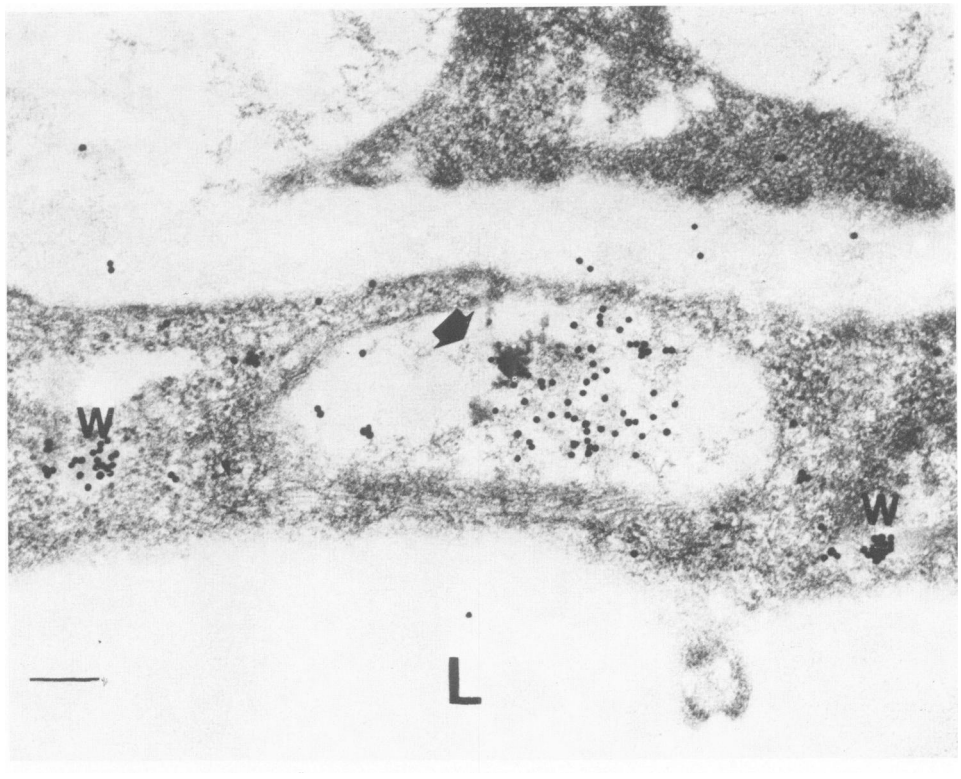

Figure 5—Capillary in smooth muscle. A dilated vesicle (*arrow*) contains amorphous granular material heavily labeled with polyclonal anti-Factor VIII. Weibel-Palade bodies (*W*) are also labeled (×15,000; bar = 0.15 μ)

solution was adjusted to 5.9, and staphylococcal protein A was added (5 μg/1 ml) (Pharmacia, Uppsala, Sweden). The protein A–colloidal gold complexes were collected by centrifugation at 65,000*g* for 45 minutes. The resultant pellet was resuspended in PBS containing 0.2 mg/ml of Carbowax and 0.02% sodium azide. Dilutions of 1:20 of this stock solution were employed for the immunolabeling experiments. This method produces 14-nm gold particles.

Results

Both the monoclonal and polyclonal antisera produce identical patterns of deposition of colloidal gold particles. As might be expected, the intensity of label was greater with the polyclonal antiserum. Nonspecific background staining was more intense with the polyclonal antiserum.

All endothelial cells examined (capillary, venular, and arteriolar) were labeled with anti-Factor VIII antibody. The most intense and consistent labeling was in Weibel-Palade bodies (Figures 1 and 2). As shown in Figure 3, there was also labeling of the cisternae of the endoplasmic reticulum and vesicles in close proximity to the endoplasmic reticulum that contained amorphous granular material (Figure 4). Although the labeling of these structures is sparse, the monoclonal antibody produced highly specific labeling patterns. No discernible background labeling was observed with this antiserum. Not all endothelial cells demonstrated labeling of the endoplasmic reticulum. The one content finding was the labeling of Weibel-Palade bodies. Colloidal gold particles also localized to cytoplasmic vesicles as well as large vacuoles containing amorphous material (Figure 5). In some cells, Weibel-Palade bodies appeared to fuse with the plasma membrane (Figure 6). There were rare colloidal gold particles on the plasma membranes, but cell membranes were largely unlabeled.

Factor VIII-related antigen was not observed in any other cells within the tissues examined. There was no labeling of the extracellular matrix. Controls all failed to exhibit specific staining.

A comparison of fixatives revealed the paraformaldehyde–glutaraldehyde mixture to provide the best preservation of antigenicity, although adequate localization of label was also observed with the other fixatives.

Discussion

Our results demonstrate that Weibel-Palade bodies in normal and neoplastic human epithelium contain Factor VIII-related antigen. The localization of the labeled antibody to these structures was consistent and specific. The labeling of cisternae of endoplasmic reticulum, vesicles and vacuoles, support the data that the antigen is synthesized by endothelial cells. The varia-

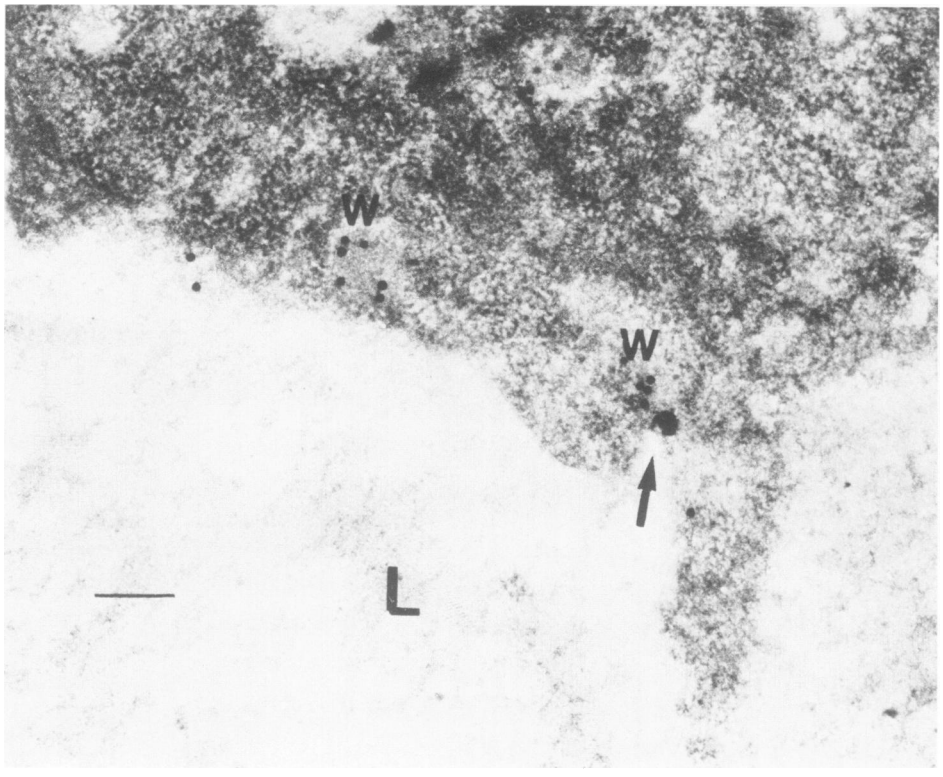

Figure 6—Arteriole in skeletal muscle. There is apparent fusion of a Weibel-Palade body (*W*) labeled with monoclonal anti-Factor VIII with the plasma membrane (*arrow*). (× 15,000; bar = 0.15 μ)

bility of labeling of the endoplasmic reticulum may be a reflection of the different metabolic states of the variety of endothelial cells examined. In an *in vivo* system, one would not expect cells in different organs from different subjects to synchronously synthesize a particular protein. The particularly strong labeling of Weibel-Palade bodies is also consistent with the concept that Factor VIII is "packaged" in these bodies, which then serve as storage vehicles for this substance until it is released into the plasma.

The function of the Weibel-Palade body has been difficult to define. In 1964, while examining endothelial cells of small arteries and capillaries, Weibel and Palade described a rod-shaped structure consisting of a bundle of fine tubules disposed parallel to the long axis of the rod embedded in a dense matrix and surrounded by a unit membrane.[11] They also noted dilated segments of rough endoplasmic reticulum and suggested a functional relationship between these two structures. Subsequent ultrastructural studies by Sengel and Stoebner suggested that these rod-shaped structures were contiguous with and arose from the Golgi complex.[12] These morphologic studies parallel recent investigation indicating that Factor VIII-related antigen is synthesized as a glycoprotein precursor and is reduced in size before secretion.[13]

The endothelial cells might have coagulant activity was suggested by the experiments of Shimamoto and Ishioka on perfused rabbit aortas in which physiologic doses of epinephrine released thromboplastic substances into the vessel lumen.[14] Burri and Weibel demonstrated that epinephrine decreased the density of Weibel-Palade bodies in the perfused vessel segments.[15] They postulated that these organelles might contain a procoagulant substance. The previous immunoelectron-microscopic studies of Wagner and her co-workers indicated that von Willebrand factor was localized to cytoplasmic granular structures which correspond to Weibel-Palade bodies.[4] Our observations demonstrate the localization of Factor VIII-related antigens to these structures *in vivo*.

We did not observe labeling of extracellular structures. Previous studies on tissue cultures of endothelium reported Factor VIII antigenicity on extracellular filamentous structures.[4] This disparity may simply be a reflection of the difference between an *in vivo* and an *in vitro* system. Previous electron-microscopic and light-microscopic immunochemical studies on tissue samples also found that Factor VIII antigenicity was confined to cellular structures with no antigenicity in the extracellular matrix.[1,2] Wagner suggested that the Factor VIII present on extracellular filaments in culture may represent antigen excreted by the endothelial cells. Since *in vivo* this excretion presumably occurs in the plasma, one might not expect labeling of extracellular structures.

Our study also demonstrates the suitability of this

low-temperature embedding technique for the identification of intracellular antigens. This method provides excellent preservation of antigenicity and offers preservation of morphologic features comparable to that with conventional Epon embedding. Not only can antigens be localized by this method, but the biology of the antigen is suggested by the pattern of distribution.

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