Immunolocalization of von Willebrand Protein in Weibel-Palade Bodies of Human Endothelial Cells

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ABSTRACT Immunofluorescence staining of cultured human umbilical vein endothelial cells has shown the presence of von Willebrand protein in the perinuclear region, in small rodlike structures through the cytoplasm, and on filaments of the extracellular matrix. Nonendothelial cells showed no staining with anti-von Willebrand protein antiserum. At the light microscope level, immunoperoxidase treatment of endothelial cells revealed the same pattern and antibody specificity as the fluorescence staining. Thin sections of the peroxidase-stained cells showed decorated filaments close to the substratum and also specific deposits in the endoplasmic reticulum and Weibel-Palade bodies. Control antisera against other selected proteins in endothelial cells failed to stain the Weibel-Palade bodies. These data suggest that the Weibel-Palade bodies of endothelial cells are storage and/or processing organelles for von Willebrand protein.

Von Willebrand protein is a large glycoprotein of complex multimeric structure (1, 2) that mediates attachment of platelets to the subendothelium after vascular injury (3). It is synthesized by megakaryocytes (4), which assure its presence in platelets in granulelike storage compartments (5, 6). After activation, platelets bind both von Willebrand protein released from internal storage sites and protein recruited from plasma to their surface membrane (7). Endothelial cells also synthesize von Willebrand protein (8) and the low concentration present in plasma and the subendothelium is probably derived from this source. We studied the distribution of von Willebrand protein in endothelial cells, in an attempt to detect and identify a storage compartment which would allow rapid release of this protein upon appropriate stimulus or physiologic demand. Using indirect immunofluorescence and immunoelectron microscopy, we determined that von Willebrand protein is concentrated in Weibel-Palade bodies. These are membrane-bound, elongate vesicles of 0.1 × 2–3 μm size that contain regularly spaced tubular structures aligned parallel to the longitudinal axis (9). Our data suggest that Weibel-Palade bodies serve as storage and/or processing vesicles for this protein and provide the first demonstration of unique function for these endothelial cell-specific organelles.

MATERIALS AND METHODS

Cells and Culture Conditions

Endothelial cells were obtained from human umbilical vein using a modification of the method described by Gimbrone et al.(10). Mild proteolytic digestion was carried out with 5 mg/ml of pronase (Calbiochem-Behring, La Jolla, CA) instead of collagenase which increased the yield of cells without producing contamination by smooth muscle cells. Pronase was dissolved in water, incubated for 2 h at 37°C and then dialyzed against 11 mM glucose, 4 mM potassium chloride, 140 mM sodium chloride, 0.87 mM potassium phosphate monobasic, and 3.57 mM sodium phosphate dibasic overnight at 4°C. Cells were cultured in McCoy's 5A medium (Flow Laboratories, McLean, VA) containing 20% fetal bovine serum. The same procedure was followed for obtaining cells from umbilical artery. For metabolic labeling, cells were grown for 3 d in the presence of ^{35}S-methionine (25 μCi/ml, 1445 Ci/mmol) from Amersham Corp. (Arlington Heights, IL).

Antisera

The preparation of antisera against human von Willebrand protein was described previously (11). Monospecific antivimentin (58,000-dalton protein) and antifibronectin antisera were a kind gift from Dr. Richard O. Hynes (Massachusetts Institute of Technology, Cambridge, MA) (12, 13). The antitubulin antiserum was prepared and characterized as described previously (14, 15).

Immunoprecipitation of von Willebrand Protein

Protein A–Sepharose CL-4B (20 mg) (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ) was incubated at room temperature for 15 min with 40 μl anti-von Willebrand protein antiserum and then for 1 h at room temperature with 2 ml of culture medium from ^{35}S-methionine-labeled cells. After extensive washing with radioimmunoprecipitation assay buffer (16), protein A-Sepharose with bound antigen-antibody complex was boiled in electrophoresis sample buffer (17) and the supernatant was analyzed by gel electrophoresis.

Electrophoresis Gels

SDS polyacrylamide slab gels were prepared as described by Laemmli (17) and agarose horizontal slab gels were prepared using a solution of 2% agarose, 0.1% SDS in 0.05 M phosphate buffer at pH 7.0.

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Immunofluorescence

Cells grown on glass cover slips were fixed in 3.7% formaldehyde in PBS for 20 min and, if desired, permeabilized in 0.5% Triton X-100 in PBS for 15 min at room temperature. Incubations with the first rabbit antibody and with goat fluorescein isothiocyanate (FITC) anti-rabbit immunoglobulin (Miles Laboratories, Inc., Elkhart, IN) were for 30 min at 37°C. Our anti–von Willebrand protein antiserum was used at 1/20 or 1/40 dilution and Calbiochem-Behring commercial anti–von Willebrand protein antiserum at 1/40 dilution. All intermediate washes were in PBS. Cover slips were mounted in Gelvatol.

Mitochondrial Staining

Cover slips were incubated with 5 μg/ml of rhodamine 6G (Eastman Kodak Co., Rochester, NY) in culture medium for 10 min. After three 1-min washes in culture medium, cells were photographed live using fluorescence microscopy (18).

Electron Microscopy

Cells grown on plastic Leighton tube cover slips (Costar, Cambridge, MA) were fixed at 4°C in glutaraldehyde in PBS overnight and postfixed for 60 min in osmium tetroxide. Cells were dehydrated through graded alcohols and embedded in durcupan. Thin sections on grids were stained for 20 min with aqueous uranyl acetate and for 3 min in lead citrate. Specimens were examined on a Zeiss 109 electron microscope.

Unlabeled Peroxidase-Anti-Peroxidase Staining

Cells were grown on Leighton tube cover slips, rinsed in PBS and fixed at room temperature in 3.7% formaldehyde in PBS for 20 min. Cells were permeabilized in 0.05% Triton X-100 in PBS for 1.5 min, incubated for 15 min with normal goat serum (1/30 dilution) in PBS, and then treated with a 1/20 dilution of one of the first antisera in PBS containing normal goat serum. Cells were then washed in Tris buffer (50 mM Tris-HCl, pH 7.5). All subsequent incubations were done in Tris buffer and were carried out sequentially as follows: (*a*) goat anti-rabbit immunoglobulin (1/20) (Miles Laboratories) for 30 min; (*b*) horseradish peroxidase anti-peroxidase (PAP) complex (1/20) (Miles Laboratories) in normal goat serum for 30 min; and (*c*) 0.02% 3,3'diaminobenzidine (Sigma Chemical Co., St. Louis, MO), 0.02% hydrogen peroxide for 5 min.

Cells were then washed with water and incubated for 10 min in osmium tetroxide in water. All antibody incubations were done at 37°C. Cover slips were mounted in Gelvatol for light microscope observations or kept overnight in glutaraldehyde in water at 4°C and then embedded for electron microscopy. Thin sections of PAP-treated material were observed with no further staining (19).

RESULTS

Specificity of Anti-vW Antiserum

The monospecificity of the von Willebrand protein antiserum was demonstrated by gel electrophoretic patterns of immunoprecipitated protein obtained from endothelial cell culture medium (Fig. 1). The reduced form migrated as a band of 220,000 mol wt, while the nonreduced protein showed the multimeric pattern characteristic of von Willebrand protein.

Immunofluorescence Staining

Indirect immunofluorescence staining of human umbilical vein endothelial cells with the monospecific antibody showed three distinct patterns, namely bright perinuclear staining, cytoplasmic rodlike structures and filaments (Fig. 2). The filaments formed a veil-like pattern that appeared to be underneath and between cells, and showed little change in distribution upon permeabilization of the cells, further indicating that they were external to the cells. Filaments were present in small, variable amounts in early cultures, but were present consistently in cultures 7 d or older and increased with progressive aging of the cells.

The perinuclear and rodlike patterns were observed only if cells were permeabilized, indicating that these locations of von Willebrand protein were intracellular. The perinuclear pattern

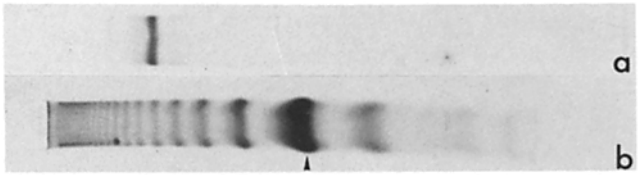

FIGURE 1 Autoradiographs of gels containing immunoprecipitated [^{35}S]methionine-labeled vW protein. vW protein was purified from endothelial cell culture medium using anti-vW protein antibodies bound to protein A Sepharose. Protein migration is from left to right. (*a*) 5% SDS PAGE of reduced vW protein showing the major subunit of 220,000 daltons. (*b*) 2% agarose gel electrophoresis of nonreduced vW protein demonstrating the multimeric composition. The major band (arrowhead) appears to be a dimer, as judged by comparison of its migration with that of fibronectin dimer—460,000 daltons (not shown).

was similar to that seen in studies using antibodies specific for the rough endoplasmic reticulum (20). The number of rods varied from cell to cell within the same culture, from only a few to greater than 100 per cell, regardless of the age of the culture. Although rods were present throughout the cytoplasm, more were located at the periphery of well-spread cells (Fig. 2*b*). The uniform elongate shape of the rods suggested that most were oriented parallel to the substratum. In very well spread cells the fluorescent bodies could be visualized in phase contrast optics as phase-dense rods.

To verify that the fluorescence of rods was due to the localization of von Willebrand protein, and not to nonspecific antibody binding to intracellular organelles, endothelial cells were stained with other antisera. Preimmune serum or immune serum preabsorbed with purified von Willebrand protein showed no staining of rods. Monospecific antisera raised against tubulin, vimentin, and fibronectin produced their respective specific staining patterns, but none stained the rods. Additionally, nonendothelial cultured cells (NIL 8 hamster fibroblasts, HeLa, and GCT fibrous histiocytoma cells) that lack von Willebrand protein showed no staining after incubation with the antiserum against von Willebrand protein. On the other hand, the rods were present in endothelial cells from umbilical arteries. In addition, commercial antibody to von Willebrand protein (Calbiochem-Behring Corp.) produced the same staining pattern. These data all indicate that the rodlike staining seen in endothelial cells is due to the presence of von Willebrand protein in these structures.

To examine whether the antibodies against von Willebrand protein were staining mitochondria, live cells were incubated with rhodamine 6G, a cationic dye that accumulates in mitochondria. As shown in Fig. 3, the endothelial cell mitochondria are very long and ropelike, often spanning the length of the cells. However, the distribution of mitochondria was clearly different from the rod-shaped organelles that stained with the anti-von Willebrand protein antibody (Fig. 2*b*).

Electron Microscope Analyses

The size and shape of the structures stained with anti–von Willebrand protein antibodies and their specific presence in endothelial cells suggested to us that these might be Weibel-Palade bodies. These organelles are found uniquely in endothelial cells and have internal striations that are easily recognizable in the electron microscope. To compare the distribution of the organelles located by immunofluorescence with that of Weibel-Palade bodies, human endothelial cells from umbilical

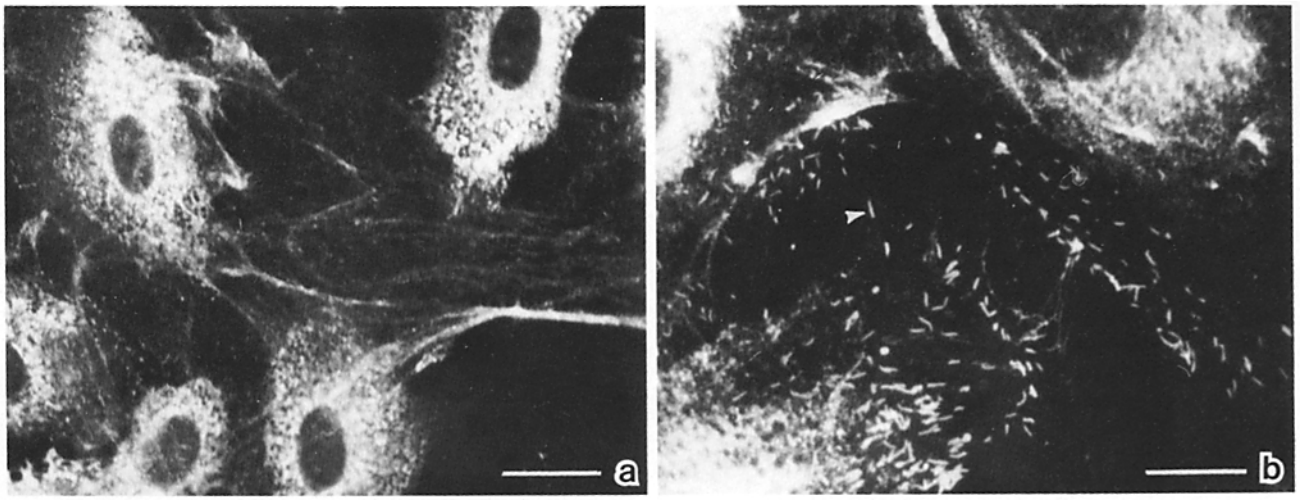

FIGURE 2 Immunofluorescent staining showing the distribution of vW protein in permeabilized endothelial cells. (*a*) 19-day-old culture with many filaments at the flattened regions of the cells. Filaments are also seen around the nucleus, but they are mostly obscured by the bright perinuclear staining. Some rodlike structures are in the plane of focus. Bar, 20 μm. (*b*) 5-d-old culture. The three cells have a large number of the rodlike organelles (arrowhead) but few filaments. Bar, 10 μm.

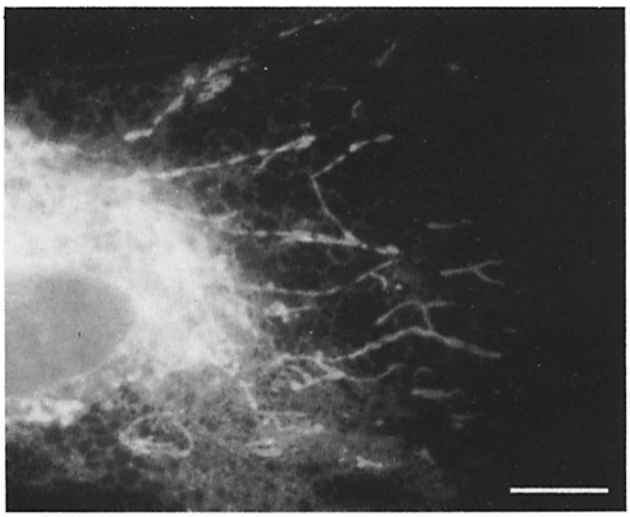

FIGURE 3 Visualization of mitochondria in live endothelial cells. Live cells were stained with rhodamine 6G dye and photographed in the fluorescence microscope. Mitochondria show a ropelike shape and their appearance is distinct from the rodlike bodies shown in Fig. 2 *b*. Bar, 10 μm.

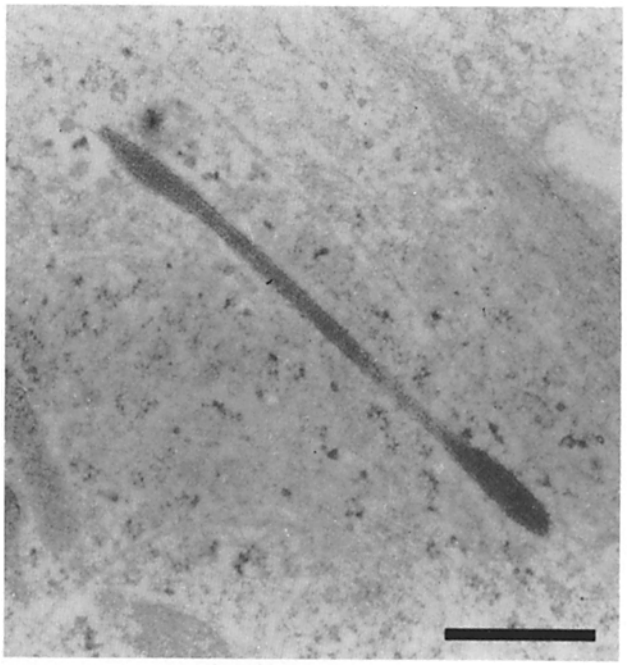

FIGURE 4 Electron micrograph of a Weibel-Palade body. The Weibel-Palade body (2-μm length) is aligned parallel to the edge of the cell and shows typical longitudinal striations. Note the mitochondria and microtubule in the lower left corner. Bar, 0.5 μm.

veins were grown on plastic cover slips and processed for electron microscopy. Thin sections were cut parallel to the substrate. As shown in Fig. 4, densely stained, striated bodies similar to those previously described (9) were found primarily at the periphery of cells. Both the distribution and shape of these Weibel-Palade bodies were reminescent of the patterns obtained for the rod-shaped structures stained with antibody against von Willebrand protein (Fig. 2).

To confirm our hypothesis that von Willebrand protein was concentrated in the Weibel-Palade bodies, endothelial cells were cultured on plastic cover slips and then immunochemically stained by the unlabeled PAP method. After staining was completed, a portion of the cover slip was saved for light microscopy and the remainder processed for electron microscopy. As shown in Fig. 5, the patterns obtained using peroxidase-staining procedures corresponded to those shown for fluorescence. Only antibodies to the von Willebrand protein stained the rodlike structures in the cytoplasm (Fig. 5 *a* and *b*). In thin sections of cells stained with the anti-von Willebrand protein antibodies, the Weibel-Palade bodies had dark deposits on the inside and were easily discernible in the pale background of the surrounding cytoplasm (Fig. 6 *a*). Often crystals were aligned with longitudinal striations (Fig. 6 *b*), a distribution that was similar to that observed for longitudinal spacings seen in Weibel-Palade bodies in conventional thin sections (see Fig. 4). The endoplasmic reticulum was also specifically stained (not shown), supporting the conclusion that the bright perinuclear staining seen by fluorescence microscopy corresponded to the distribution of von Willebrand protein in this organelle. Deposits were also located on long filaments at cell edges and

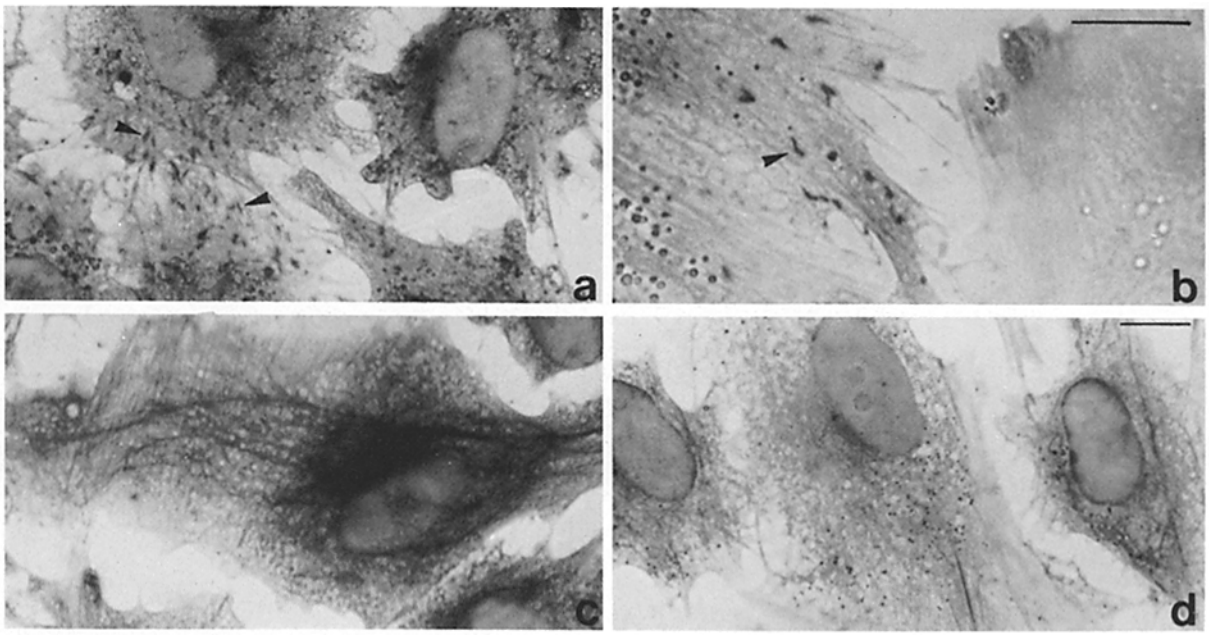

FIGURE 5 Bright-field micrographs of PAP-stained endothelial cells. (*a* and *b*) Staining with anti-von Willebrand protein antibody demonstrating deposits in the perinuclear region and the rod-shaped bodies (arrowheads). Note the variable number of rods present in individual cells. (*c*) The staining pattern obtained with antivimentin (intermediate filament protein) antibodies. No rods are visible, although the specific filament staining is present. (*d*) Staining in the absence of a first antibody. The background staining is weak even though the exposure time was three times longer than in the other panels. No rods are visible. Bars: *a*, *c*, *d*, 10 μm; *b*, 10 μm.

between cells (Fig. 6*c*). The filaments appeared to be close to the substratum.

The degree of background staining of the cytoplasm with peroxidase was generally low, although the external membranes of mitochondria were sometimes stained. This staining was nonspecific because it was also present in parallel controls (Fig. 6*d*) in which primary antisera other than the antibodies to von Willebrand protein were used.

Both the light and electron microscope data were consistent with the conclusion that Weibel-Palade bodies of endothelial cells are sites of concentration of von Willebrand protein and that this protein forms or can be adsorbed onto filaments of the extracellular matrix.

DISCUSSION

We found that von Willebrand protein in human umbilical vein endothelial cells is present in the endoplasmic reticulum, in Weibel-Palade bodies, and also in extracellular matrix filaments. The presence of von Willebrand protein in the endoplasmic reticulum confirms the finding of Jaffe et al. (8) that endothelial cells synthesize this glycoprotein. The perinuclear staining is due to the presence of von Willebrand protein in the endoplasmic reticulum. This staining is the most noticeable and probably is that which is routinely seen when endothelial cells are examined for the presence of von Willebrand antigen by fluorescence.

We have determined that von Willebrand protein is present in the Weibel-Palade bodies by the following criteria: (*a*) The light microscope distribution of rods that are stained immunocytochemically with anti-von Willebrand antiserum was similar to the distribution of Weibel-Palade bodies seen by conventional electron microscopy. (*b*) Thin sections of cells stained with anti-von Willebrand antibody and PAP revealed specific deposits inside the Weibel-Palade bodies, but no staining in other large vesicular structures (Figs. 6*a* and *b*). (*c*) No deposits were found in Weibel-Palade bodies of cells stained with antivimentin or antifibronectin antibodies although the intra- and extracellular filaments formed by the respective antigens were decorated (Fig. 6*d*). In addition, no organelles were stained in the absence of first antibody.

Granular fluorescent staining of endothelial cells has been obtained with anti-von Willebrand protein antibodies using endothelial cells from various bovine tissues (21), pig aorta (22), and human umbilical vein (23). If the staining pattern observed in these studies corresponds to the rodlike staining that we obtained and not to the granular appearance of staining in the rough endoplasmic reticulum, this would represent the distribution of Weibel-Palade bodies in the different tissues.

Until now the role of Weibel-Palade bodies in endothelial cells has been unknown. They have been shown to lack acid phosphatase activity and are therefore thought not to be lysosomes (24). Electron microscope observations suggest that they originate from the Golgi apparatus (25) and their shape and internal striations appear similar to the smaller procollagen secretory granules (26). We have shown that von Willebrand protein is concentrated in the Weibel-Palade bodies. It is possible that the internal tubules observed in Weibel-Palade bodies by electron microscopy are parallel aggregates of von Willebrand protein. This conclusion would be consistent with the peroxidase decoration of these structures (Fig. 6*b*).

As the von Willebrand protein is a large glycoprotein of complex multimeric structure (2, 27) (Fig. 1), its biosynthesis requires several processing steps before secretion. This includes cleavage of a larger precursor to the von Willebrand protein monomer (29; also, Wagner, D. D., and V. J. Marder, manuscript in preparation). It is still unknown whether the Weibel-Palade bodies contain enzymes for processing von Willebrand protein, or whether they serve solely as storage vesicles that are

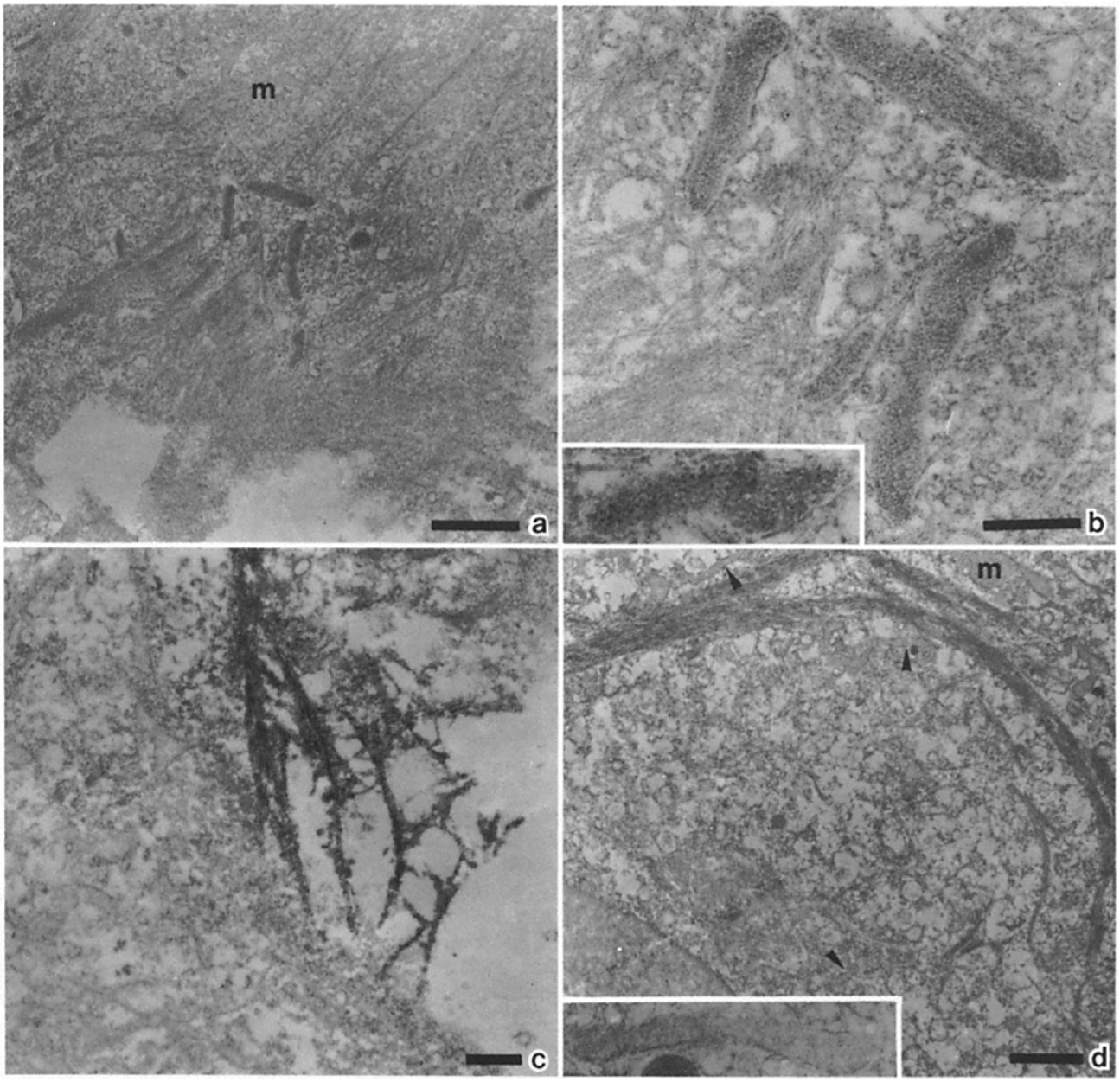

FIGURE 6 Electron micrograph of PAP-stained endothelial cells. In *a*, *b* and *c*, anti-von Willebrand protein antibody was used. (*a*) A cluster of positively stained Weibel-Palade bodies; unstained mitochondria (*m*) are also visible. Bar, 2 μm. (*b*) A higher magnification of the photograph in *a*, showing electron dense deposits located parallel to the longitudinal axes of the organelles. Bar, 0.5 μm. The *inset* shows an unusually shaped Weibel-Palade body from a different cell. × 51,000. (*c*) Strongly decorated extracellular filaments containing von Willebrand protein. Bar, 1 μm. (*d*) Control antisera. The large micrograph shows staining with antivimentin antibody, with positively stained intermediate filaments forming an arc around the nucleus. Mitochondria (*m*) and Weibel-Palade bodies (arrowheads) are unstained. Bar, 2 μm. The *inset* shows an unstained Weibel-Palade body from a cell treated with antifibronectin antiserum. × 28,600.

stimulated to release the protein. It would be of interest to determine whether megakaryocytes, which also synthesize von Willebrand protein, contain organelles similar to the Weibel-Palade bodies.

The filamentous pattern that we observed suggests that at least some of the secreted protein is fixed in vitro onto the subcellular or intercellular matrix. Thus far, the filamentous pattern of von Willebrand protein has been reported only in cultures of endothelial cells from pig aorta (22). Other studies have not described filamentous staining, perhaps because the filaments become prominent only in cultures of 1 wk or older. We conclude that these filaments are external to the cells because they were seen in nonpermeabilized cultures and are adjacent to the cells in thin sections stained with anti-von Willebrand protein and PAP. It is not yet known whether the von Willebrand protein forms filaments by self-assembly or if the protein is adsorbed onto other members of the extracellular matrix.

It remains to be seen whether the subcellular and extracellular distribution is altered in patients with the different variants of von Willebrand's disease. For example, it is possible that affected cells may contain insufficient or defective Weibel-

Palade bodies that process, store, or release von Willebrand protein improperly. In other patients, the von Willebrand protein may be structurally altered, resulting in inadequate association with the subendothelial matrix. Thus, studies on the biosynthesis, cellular distribution, storage, and secretion of von Willebrand protein may provide important information on how this molecule is regulated in both normal and pathologic states.

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