Endothelial Cell Surface Expression and Binding of Factor VIII/von Willebrand Factor

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Factor VIII/von Willebrand factor (FVIII/vWF), a glycoprotein molecular complex found in human plasma, has been demonstrated by cell membrane fluorescence to be present on the surface of cultured human umbilical cord vein endothelial cells. The endothelial nature of these cells was established by electron-microscopic studies that revealed the presence of Weibel-Palade bodies in virtually all cells cultured. A newly developed radioreceptor assay was used to detect FVIII/vWF in the medium taken from these endothelial cell cultures; FVIII/vWF concentration in the medium samples increased with time in culture. FVIII/vWF binding studies showed no significant FVIII/vWF-specific binding to endothelial cell surfaces and did not corroborate a previous report suggesting a FVIII/vWF-specific receptor on human umbilical cord vein endothelium. The presence of FVIII/vWF on endothelial cell membranes and the lack of receptor-mediated binding suggests that the FVIII/vWF either has been absorbed nonspecifically to the cell surface or is an integral part of the endothelial cell membrane. (Am J Pathol 1981, 103: 304–308)

FACTOR VIII/VON WILLEBRAND FACTOR (FVIII/vWF) is a molecular complex having both procoagulant and platelet aggregation activities. Antiserum generated against FVIII/vWF has been used to demonstrate that at least part of the FVIII/vWF complex is synthesized and expressed by vascular endothelia.[1,2] This FVIII/vWF antigen has also been demonstrated in platelets.[3] Only vWF activity (platelet aggregation) and not FVIII activity (procoagulant activity) has been demonstrated in the molecule bound by the anti-FVIII/vWF antibody.

The presence of FVIII/vWF on the surface of vascular endothelial cells may have an important role in intravascular coagulation and platelet aggregation and perhaps in the regulation of FVIII/vWF synthesis. The demonstration of an FVIII/vWF-specific membrane receptor would encourage such a hypothesis. We have demonstrated cell membrane expression of FVIII/vWF on human umbilical cord vein endothelial cells in culture but were unable to show the specific FVIII/vWF membrane receptor binding suggested by others.[4]

Methods and Materials

Antiserum Preparation

FVIII/vWF was isolated from normal human plasma cryoprecipitate by a previously described technique[5] that employed centrifugation, gel filtration, and precipitation with ammonium sulfate. Rabbits were injected subcutaneously on Days 1, 30, and 45 with 250 μl of FVIII/vWF (1.5 absorbance at 280 nm) mixed with 250 μl of complete Freund's adjuvant. When 10 μg of the purified FVIII/vWF used in the immunization protocol was subjected to electrophoresis on a sodium dodecyl sulfate-urea 5% polyacrylamide gel, a single band was noted. The hyperimmune serum, however, showed not only anti-FVIII/vWF activity but also anti-IgG, anti-IgM, and anti-α_2-macroglobulin (α_2M) specificities. Human IgG was isolated by A-50 DEAE Sephadex column chromatography,[6] IgM by euglobulin precipitation,[7] and α_2M by cibacron blue column chromatography.[8] All were assayed for FVIII/vWF contamination by a FVIII/vWF radioreceptor assay (see below). The IgG isolate contained no detectable FVIII/vWF, while the IgM and α_2M isolates had only 0.16% and 0.32% FVIII/

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vWF contamination, respectively. These isolates were used in an exhaustive sequential absorption of the anti-FVIII/vWF serum. Each milliliter of anti-FVIII/vWF was absorbed with a total of 0.13 mg IgG, 0.4 mg IgM, and 0.32 mg α_2M. The absorption was carried out over 5 days; the antigens and antiserum were incubated together for 1 hour at 37 C, then overnight at 4 C, and then centrifuged at 100,000*g* for 1 hour to precipitate the complexes. These absorptions rendered the antiserum FVIII/vWF specific as judged by immunoelectrophoresis and immunodiffusion. Incubation of an aliquot of the absorbed antiserum with FVIII/vWF rendered it completely inactive in immunofluorescence.

Endothelial Cell Culture

The procedure used to harvest the endothelial cells was adapted from Jaffe et al.[1] In brief, human umbilical cords were obtained no later than 3 hours postpartum. The vein was cannulated and Dulbecco's phosphate-buffered saline (PBS) was passed through the vein to wash out free blood and clots. The vein was then filled with 0.05% collagenase (CLS Type I, Worthington Biochemical) and incubated for 15 minutes in a 37 C water bath. After incubation, the umbilical cord was gently kneaded to help free the endothelial cells, and the vein was drained and then washed with PBS. After centrifugation for 5 minutes at 250*g*, the cells were resuspended in Richter's zinc option medium (Grand Island Biological Co.) containing 20% fetal calf serum and 200 μg/ml endothelial cell growth supplement (Collaborative Research, Waltham, Mass) and plated in 35-mm culture dishes (Falcon) that had been vigorously washed with PBS. In order to establish the endothelial nature of the cells harvested in this manner, a 35-mm culture dish containing a confluent layer of cells was fixed in 0.4% glutaraldehyde buffered with 0.1 M sodium cacodylate buffer, postfixed for 1 hour in 1% osmium tetroxide, and embedded in Epon 812. *En face* sections were cut, stained with uranyl acetate and lead citrate, and then examined at 80 kv in a JEOL 100B transmission electron microscope for the presence of Weibel-Palade bodies, endothelial cell-specific cytoplasmic components.[9]

Cell Membrane Fluorescence

First passage cultured endothelial cells was lifted from two culture dishes by incubation with 0.02% EDTA for 3–5 minutes, then centrifuged at 250*g* for 5 minutes. Cells were resuspended in serum-free medium to 4×10^6 cells/ml, and 25 μl of cell suspension was placed in individual wells of U- or V-bottom polystyrene plates (Cooke Laboratory Products). Fifty microliters of primary serum (anti-FVIII/vWF or preimmune) diluted by half $\log_{10}$ dilutions with serum-free medium was introduced into the well and incubated for 20 minutes at room temperature. Each dilution was tested in duplicate. After incubation, the cells were washed twice with serum-free medium, and 50 μl of secondary antiserum (FITC-conjugated goat anti-rabbit IgG diluted 1:16, Cappel Laboratories) was introduced into each well. After 20 minutes' incubation the cells were washed twice, suspended in 8.5% sucrose, and placed on slides for microscopic evaluation. A Zeiss Universal microscope with FITC interference and 50 barrier filters in place was employed. After counting, a fluorescent index (FI) was calculated for each antiserum dilution as follows: $FI = 100 \times (A-B)/A$, where A is the percentage of nonfluorescing cells with preimmune serum and B is the percentage of nonflourescing cells with active antiserum.[10]

Radioreceptor Assay for FVIII/vWF

The FVIII/vW concentration of endothelial cell culture medium was determined by a newly developed radioreceptor assay.[5,11] Briefly, FVIII/vWF specific binding sites on platelet membranes were used to quantitate the ability of samples containing unknown amounts of FVIII/vWF to compete with ^{125}I-labeled FVIII/vWF. Highly purified FVIII/vWF protein was prepared from FVIII/vWF concentrate as reported earlier[5] and was used as a standard for the assay. When the medium from three first-passage endothelial cell cultures obtained from three different umbilical cords was assayed for FVIII/vWF at different volumes (50–200 μl), a parallel relationship between the medium and the standards for the assay was observed. No interfering effect from the medium was detected. Therefore, it is valid to determine medium FVIII/vWF concentration directly by this assay.

Binding of ^{125}I-FVIII/vWF to Endothelial Cells

Endothelial cells from human umbilical cord veins were cultured in 17-mm culture dishes. After the cells formed a confluent monolayer (2.4×10^5 cells), they were used for the study of ^{125}I-FVIII/vWF binding. In each incubation mixture, endothelial cells were incubated in 1 ml of Hanks' balanced salt solution (HBSS) made in 0.1% BSA, pH 7.2, and 0.2 μg ^{125}I-FVIII/vWF (120,000 cpm) in the presence or absence of an excess of unlabeled FVIII/vWF (60 μg) at room temperature or at 37 C. After incubating for 2 hours the medium was aspirated and the cells were immediately rinsed twice with 2 ml of ice-cold HBSS. The cells were solubilized in 0.5 ml of 0.1% Triton X-100 made in 0.1 N

NaOH and 0.4 ml of solubilized endothelial cells was counted in a gamma counter for ^{125}I-FVIII/vWF bound to endothelial cells. The difference of bound counts between incubations with and without an excess of unlabeled FVIII/vWF was defined as specific binding. The binding studies were performed in duplicates. The variation of bound counts between duplicates was less than 5%. The binding studies were also performed in the presence or absence of 1 mg/ml ristocetin, a glycopeptide antibiotic, since ristocetin is essential for FVIII/vWF binding to its receptors on human platelets.[5]

Results

The cell surfaces of cultured human umbilical cord endothelial cells reacted with antihuman FVIII/vWF serum in indirect immunofluorescence. The cells showed characteristic cell surface staining with many punctate spots of fluorescence at the cell membrane. Since cells were viable during exposure to primary and secondary antibody, the cytoplasm, as we viewed it by focusing through it, showed no fluorescence. Two cell lines derived from human gliomas (U 251 MG sp and U 118 MG) and human-fetal skin fibroblasts subjected to the same staining procedure did not stain. Virtually all cells harvested in the manner described from human umbilical cord veins contained Weibel-Palade bodies, thus establishing the endothelial nature of the cells studied (Figure 1).

When the number of FVIII/vWF-expressing endothelial cells was held constant and decreasing amounts of primary antibody were reacted with them, an immunofluorescence titration curve was obtained (Figure 2). At the highest primary antibody concentration, 80–85% of the cells stained positively. The number of cells stained and the intensity of staining of positive cells both decreased as the primary antiserum dilution increased.

As detected by the FVIII/vWF radioreceptor assay, endothelial cell cultures released into the culture medium an average of 0.53 μg/ml FVIII/vWF after 12 hours, 1.53 μg/ml after 24 hours, 3.48 μg/ml after 48 hours, and 5.42 μg/ml after 72 hours (Figure 3).

Studies of binding of radiolabeled FVIII/vWF to cultured endothelial cells showed negligible specific binding both in the presence and absence of ristocetin (190 cpm and 95 cpm bound, respectively, of 120,000 cpm added to 2.4×10^5 cells). Since the amount of specific binding was relatively low in comparison with the nonspecific binding (2500 cpm bound), the specifically bound counts were too low to be correlated with any specific biologic activity. Competition of ^{125}I-FVIII/vWF binding with an excess of control plasma proteins (α_2M and human albumin) resulted in 100 cpm specific binding. Therefore, the bound counts could not be attributed to specific receptor binding.

Discussion

FVIII/vWF has been detected in vascular endothelial cells.[1,2,12] These cells, when in culture, also synthesize and export it into the medium.[2,13]

Using a monospecific anti-FVIII/vWF serum that was exhaustively absorbed with highly purified human α_2M, IgG, and IgM, we have demonstrated the presence of FVIII/vWF on the surface of cultured human umbilical cord vein endothelial cells. When using an antiserum to detect FVIII/vWF, one must be sure that the antiserum has demonstrated monospecificity before use. It has been our experience that very low levels of serum proteins, particularly α_2M, may remain in the "pure" FVIII/vWF preparation and cause

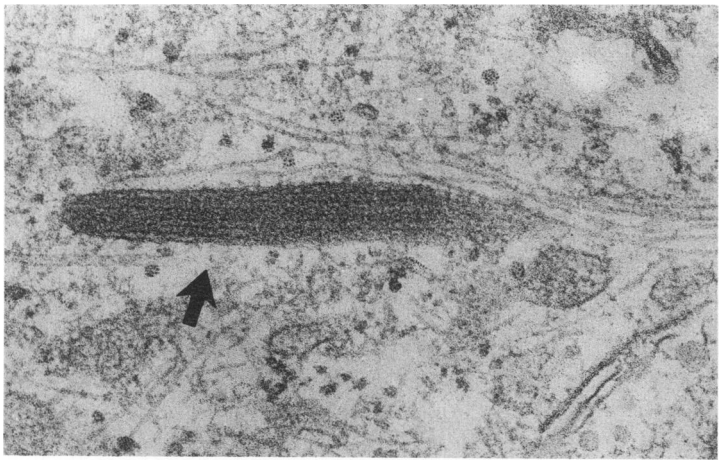

Figure 1—A Weibel-Palade body (*arrow*) is a specific endothelial cell marker visible under the electron microscope. These structures were found in virtually every cultured cell when sectioned *en face*. (× 24,000)

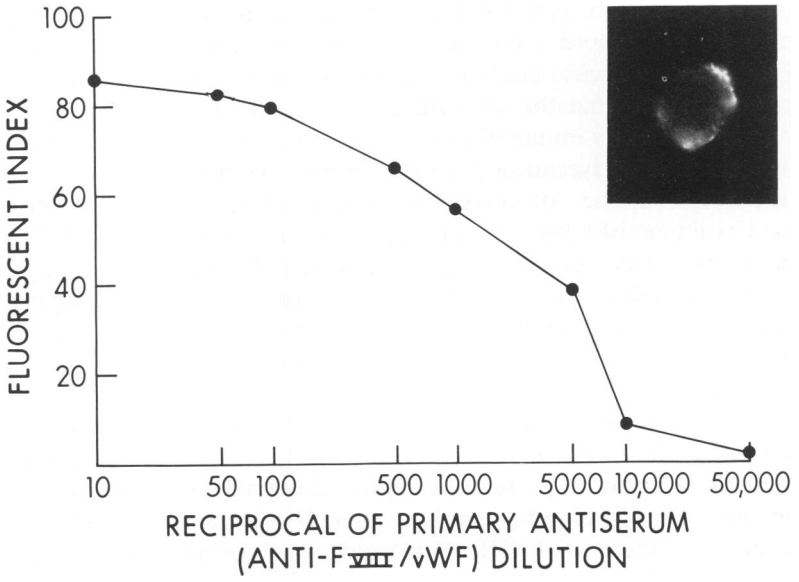

Figure 2—Immunofluorescent staining index plotted against dilution of primary antiserum. **Inset**—Membrane staining pattern obtained with human umbilical cord endothelial cells and antihuman F VIII/vWF serum. (×425)

a significant anti-α_2M response in the immunized animal. It is essential to avoid any anti-α_2M activity in the antiserum, because α_2M has been reported to be present on the surface of endothelial cells.[14]

When samples of spent culture medium were assayed for FVIII/vWF by the use of a sensitive radioreceptor assay for the detection of FVIII/vWF,[5,11] we found low microgram amounts that increased over time. These results agree rather well with those obtained with a ristocetin-dependent platelet aggregation assay. Our methods demonstrated 5.4 μg/ml after 72 hours, while the aggregation assay detected 12.3 ± 1.8 μg/ml.[13]

Because significant amounts of endothelial cell surface-expressed FVIII/vWF were detectable by immunofluorescence, we tested the cells to determine whether a FVIII/vWF-specific receptor was present on the endothelial cell surface and if it was responsible for the FVIII/vWF detected there. There is one previous report in abstract form describing an endothelial cell membrane FVIII/vWF receptor.[4] These investigations suggest that a specific high-affinity receptor exists on endothelial cells. Using ^{125}I-labeled FVIII/vWF, they found its binding capacity was saturated at 1.3×10^{-9} M but 72% of the bound FVIII/vWF could not be competitively released from the cell by addition of unlabeled FVIII/vWF. The endothelial cells internalized only 8% of the bound FVIII/vWF. Also using ^{125}I-FVIII/vWF, we found no specific binding of FVIII/vWF to the membranes of cultured endothelial cells and after 2-hour incubations at 37 C, no uptake of FVIII/vWF by the cells was noted.

The putative receptor previously described in the literature[4] was apparently ristocetin-independent, while the only other described and characterized FVIII/vWF receptor, found on platelet membranes, is ristocetin-dependent.[5] In our hands, the addition of ristocetin (1 mg/ml) did not lead to a significant increase in cell-surface binding to endothelial cells. Because the data supporting the argument for the existence of a specific FVIII/vWF receptor are available in abstract form only,[4] the exact origin of the endothelial cells used and the specifics of their maintenance in culture are unknown. It is conceivable that endothelial cells from adults and from other parts of the vascular tree could express FVIII/vWF in a different fashion. Without a detailed description of the cell culture techniques and assay methods used, it is difficult to evaluate their data critically.

We feel, having demonstrated cell membrane expression and increasing levels of FVIII/vWF in spent tissue culture medium, that there is production, mem-

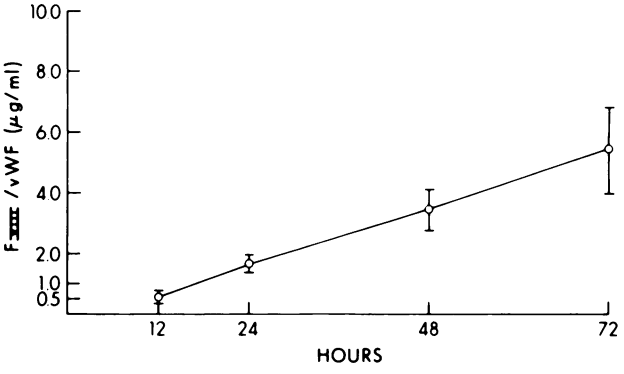

Figure 3—Accumulation of human F VIII/vWF in the medium of cultured human umbilical cord endothelial cells as measured by radioreceptor assay. Vertical lines equal one standard deviation.

brane expression, and export of FVIII/vWF by human umbilical cord vein endothelial cells in culture. The lack of specific binding of radiolabeled FVIII/vWF suggests that the membrane-expressed FVIII/vWF detected by immunofluorescence is present due to nonspecific adsorption of FVIII/vWF to the endothelial cell surface. Although it may be possible to detect by immunofluorescence proteins in the process of being exported via exocytosis, the amount of FVIII/vWF detected on the endothelial cell surfaces is so great that the production rate of FVIII/vWF would have to be much greater and the export rate much lower than suggested by our studies of FVIII/vWF accumulation in the medium of the cultured cells. An alternate explanation, however, is that FVIII/vWF is an integral membrane protein. A report claiming the identification of blood group A, B, and H oligosaccharide structures on FVIII/vWF[15] may have bearing on this hypothesis, because blood group antigens have not yet been demonstrated on any other plasma proteins; these antigens are usually only seen as components of cell membranes.

Note Added in Proof

The binding studies reported in Wall et al[4] are now published in a complete form (Br J Haematol 1980, 46:287–298).

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