In Vitro Cell. Dev. Biol. 26:1167–1172, December 1990
© 1990 Tissue Culture Association
0883-8364/90 $01.50+0.00

ENDOTHELIUM SPECIFIC WEIBEL-PALADE BODIES IN A CONTINUOUS HUMAN CELL LINE, EA.hy926

CORA-JEAN S. EDGELL, JILL E. HAIZLIP, C. ROBERT BAGNELL, JOAN P. PACKENHAM, PAUL HARRISON, BARRY WILBOURN, AND VICTORIA J. MADDEN

Pathology Department 7525, University of North Carolina, Chapel Hill, North Carolina 27599 (C-J. S. E., J. E. H., C. R. B., J. P. P., V. J. M.); and Coagulation Research, The Rayne Institute, St. Thomas' Hospital, London SE1 7EH, England (P. H., B. W.)

(Received 6 August 1990; accepted 17 September 1990)

Summary

Weibel-Palade bodies are ultrastructurally defined organelles found only in vascular endothelial cells. Because endothelium in corpo is very dispersed, isolation and further characterization of this organelle has been dependent on increasing the number of cells in culture. However, primary isolates of endothelial cells have a limited replication potential and tend to senesce in culture. In this report, EA.hy926, a continuously replicating cell line derived from human endothelium, is shown to contain Weibel-Palade bodies. Electron micrographs demonstrate the ultrastructural characteristics of these tissue-specific organelles and their cytoplasmic distribution in EA.hy926 cells. Von Willebrand factor, which has been shown to exist in Weibel Palade bodies, is demonstrated by immunofluorescence in discrete rod-shaped organelles whose size, shape, and distribution are consistent with that of Weibel-Palade bodies in primary endothelial cell cultures. Rapid release of von Willebrand factor can be induced by calcium ionophore, and large multimeric forms of the protein are found in EA.hy926 cells. These two properties are consistent with the function currently ascribed to Weibel Palade bodies: storage of multimerized von Willebrand factor. Thus ultrastructural, immunologic, and functional data establish the existence of this as yet poorly understood tissue-specific organelle in a continuous, vigorously replicating human cell line.

Key words: Weibel-Palade bodies; endothelial cells; EA.hy926 cells; von Willebrand factor.

Introduction

Cytoplasmic rod-shaped organelles consisting of fine tubules within a close-fitting unit membrane were observed in endothelial cells in situ and described by Weibel and Palade in 1964 (31). These Weibel-Palade bodies (WPBs) have been found in no other cell type, and are therefore an excellent marker for endothelium (14).

Von Willebrand factor is one component of these organelles in as much as some of the von Willebrand antigen in cultured human umbilical vein endothelial (HUVE) cells can be localized to WPBs by immunoelectron microscopy (30). Indirect evidence indicates that large multimers of von Willebrand factor are sequestered in these organelles from which they can be released by calcium ionophores and by physiologic stimuli such as thrombin (19,26). Organelles with ultrastructural features similar to WPBs have been demonstrated in homogenates of HUVE cells (12,22,23). Reinders and coworkers (23) have demonstrated that the highest von Willebrand factor specific activity was in the densest fractions of HUVE cell homogenates, that these fractions included only a few proteins in addition to von Willebrand factor in the large molecular weight range, and that these fractions included WPB-like structures. Therefore, von Willebrand factor may be one of few high molecular weight components of WPBs. Cramer et al. (7) have observed that the tubular structures of WPBs are similar to structures in platelet alpha-granules, and have suggested on the basis of immunogold studies that these structures may represent von Willebrand factor itself. A platelet alpha-granule membrane antigen, GMP-140 or PADGEM, has recently been observed in HUVE cells and shown by immunoelectron microscopy to be associated with WPBs (3,16,20). Von Willebrand factor and GMP-140 are the only two proteins currently known to be associated with WPBs.

Isolated endothelial cells appropriate for further characterization of WPBs are not trivial to obtain. Since these cells are dispersed as a monolayer lining the vascular system, only relatively small numbers of cells can be obtained directly. Once isolated, the number of cells can be expanded in culture to a certain extent, but the ratio of normal to senescent cells changes continuously in such primary cultures and the replicative potential of each isolated population can not be predicted. Perhaps even more troublesome is that the expression of some differentiated properties is lost as primary cultures senesce (1,9,21,25).

A clonally pure, vigorously replicating, continuous cell line that maintains differentiated functions of endothelium has been developed in our laboratory. This cell line, EA.hy926 (EA) was derived from a HUVE cell by fusion with an A549/8 cell (10). Many endothelial properties of this cell line have been demonstrated, including von Willebrand factor (10,29), GMP-140 (17), prostacyclin (27), platelet activating factor (5), tissue plasminogen activator and plasminogen activator inhibitor type I (11), thrombomodulin (2,8), vitronectin receptor (13), uptake of modified low density lipoproteins

(2), and other properties (6,24). The possibility that EA cells also produce the endothelium-specific WPBs has been suggested by the intracellular distribution of von Willebrand antigen in our previously published low magnification immunofluorescent micrographs, in which monoclonal antibodies directed against this antigen were shown to bind to fixed-permeabilized EA cells in discrete cytoplasmic foci (10); but these foci are also consistent with Golgi processing of this antigen, receptor mediated or fluid phase endocytosis, etc. The possibility that EA cells contain WPBs is also supported by the more recent demonstration of Johnston et al. (17) that EA cells express the mRNA for GMP-140.

In this report, EA cells that have undergone more than 300 population doublings in culture are shown directly, by electron microscopy, to contain WPBs. A high magnification immunofluorescent micrograph demonstrates von Willebrand antigen in rodlike cytoplasmic organelles of appropriate dimensions, indicating a molecular component that WPBs in EA cells have in common with WPBs in primary endothelial cells. Large multimeric forms and inducible rapid release of von Willebrand antigen from EA cells are demonstrated because storage and inducible release of this protein has been proposed as the function of WPBs.

Materials and Methods

Cell culture. EA cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/liter glucose, 10% fetal bovine serum (Hyclone, Logan, UT), and 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT). These cells were passed at weekly intervals by trypsinization and replated at a lower cell density with a medium change 4 d later. The cells used in this study had been passed more than 80 times, usually at a relative cell density of 1:20, and subcloned 3 times.

Electron microscopy. Postconfluent cultures were rinsed with serum-free medium and fixed with 2% glutaraldehyde in serum-free medium at 37° C for 1 to 2 h. The monolayers were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 1 h and stained with 2% uranyl acetate in 30% ethanol for 30 min. After dehydration at increasing ethanol concentrations, the specimens were embedded in Epon-812. Blocks were sectioned en face and 70-nm sections were mounted on copper grids. The sections were poststained in 6% aqueous uranyl acetate, followed by 0.4% lead citrate. Specimens were observed at 60 kV on a Zeiss 10-A electron microscope.

Immunofluorescence. EA cells were cultured to postconfluency on no. 1 glass cover slips, and rinsed free of culture medium in phosphate buffered saline with physiologic levels of Mg and Ca ions (PBS-Mg,Ca) which was used throughout as diluent. The cells were fixed for 20 min with 3.7% formaldehyde and subsequently permeabilized by treating with 0.5% Triton X-100 for 20 min. The monolayer was exposed to a von Willebrand factor specific mouse monoclonal antibody D7 (4) in PBS-Mg,Ca with 10% normal goat serum and 0.1% NaN_3 for at least 15 min at 37° C and 15 min at room temperature. After careful rinsing, the monolayer was exposed to fluorescein conjugated goat antibodies to mouse IgG heavy and light chains (Hyclone). A microscope with epi-illumination and Leitz H-2 filters was used to visualize and photograph the immunofluorescence.

Induced rapid release of von Willebrand factor. Calcium ionophore A23187 (Molecular Probes, Eugene, OR) was dissolved in dimethylsulfoxide and subsequently diluted 1000-fold to 10 μM in DMEM supplemented with ITS+™ (Collaborative Research, Waltham, MA). Matched cultures of confluent EA cells were carefully rinsed and pairs were treated at 0.02 ml/cm^2 with 10 μM A23187 in DMEM-ITS+, with just 0.1% dimethylsulfoxide in DMEM-ITS+ as a solvent controls, or with DMEM-ITS+ alone as mock treated controls. After 40 min at normal culture conditions, medium was collected from each culture, centrifuged to remove cellular debris, and frozen for subsequent quantitation of von Willebrand antigen.

ELISA for von Willebrand antigen. The amount of von Willebrand antigen released from cells was measured by an enzyme linked immunoabsorbent assay (ELISA) based on a monoclonal antibody. Microtiter wells coated with immunoglobulin from human von Willebrand factor immunized rabbits (DAKO, Carpinteria, CA, no. A082) were used to adsorb the von Willebrand antigen from samples. The retained von Willebrand factor binds a corresponding amount of specific monoclonal mouse antibody D7 (4) supplied in excess. A peroxidase labeled antibody specific for mouse immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, no. 115-035-044) is used along wth TMB (Kirkegaard & Perry Laboratories, Gaithersburg, MD, no. 50-65-00) as substrate to develop the assay colorimetrically. The colorimetric response is calibrated using dilutions of normal human pooled plasma (Pacific Hemostasis, Curtin Matheson Scientific, Houston, TX no. 176-176). The assay is sensitive enough to detect von Willebrand factor at concentrations less than 10^{-4} U/ml. The specificity of the assay is supported by the 40-fold difference it measures between the von Willebrand antigen levels in normal pooled plasma compared to that in plasma from an individual homozygous for von Willebrand disease.

Gel electrophoresis of von Willebrand antigen multimers. A new rapid version (18) of the high resolution agarose electrophoresis technique developed by Zimmerman and colleagues (32) to analyze the range of von Willebrand multimers was used to demonstrate the multimeric state of von Willebrand antigen in an EA cell lysate and in conditioned culture medium from EA cells. Confluent EA cells, that had been trypsinized and washed with soybean trypsin inhibitor at 1 mg/ml, were used to prepare a cell lysate in 2% Nonidet P40 with 4 mM EDTA, 2 mM N-ethylmaleimide, 4 mM phenylmethanesulfonyl fluoride. Culture medium that had been in contact with confluent EA cells at 0.3 ml/cm^2 for 5 d was used to represent constitutively released von Willebrand antigen. Samples were heated in the presence of sodium dodecyl sulfate (SDS) and urea, loaded onto agarose gels, electrophoresed using the PhastSystem (Pharmacia, Piscataway, NJ), blotted onto nitrocellulose, and the von Willebrand antigen visualized immunologically as recently described in detail (18).

Results

Examples of WPBs found in EA cells are shown in Fig. 1. These organelles have the same characteristics in terms of shape, location, size, structural details, orientation, and frequency as WPBs defined originally for endothelial cells in situ (31) and by Haudenschild et al. (15) for cultured endothelial cells.

In EA cells, as in early passage endothelial cells, these rod-shaped organelles are found in the perinuclear and peripheral cytoplasm, close to the culture substratum. The length of the WPBs found in electron micrographs of EA cells range from 0.12 to 1.59

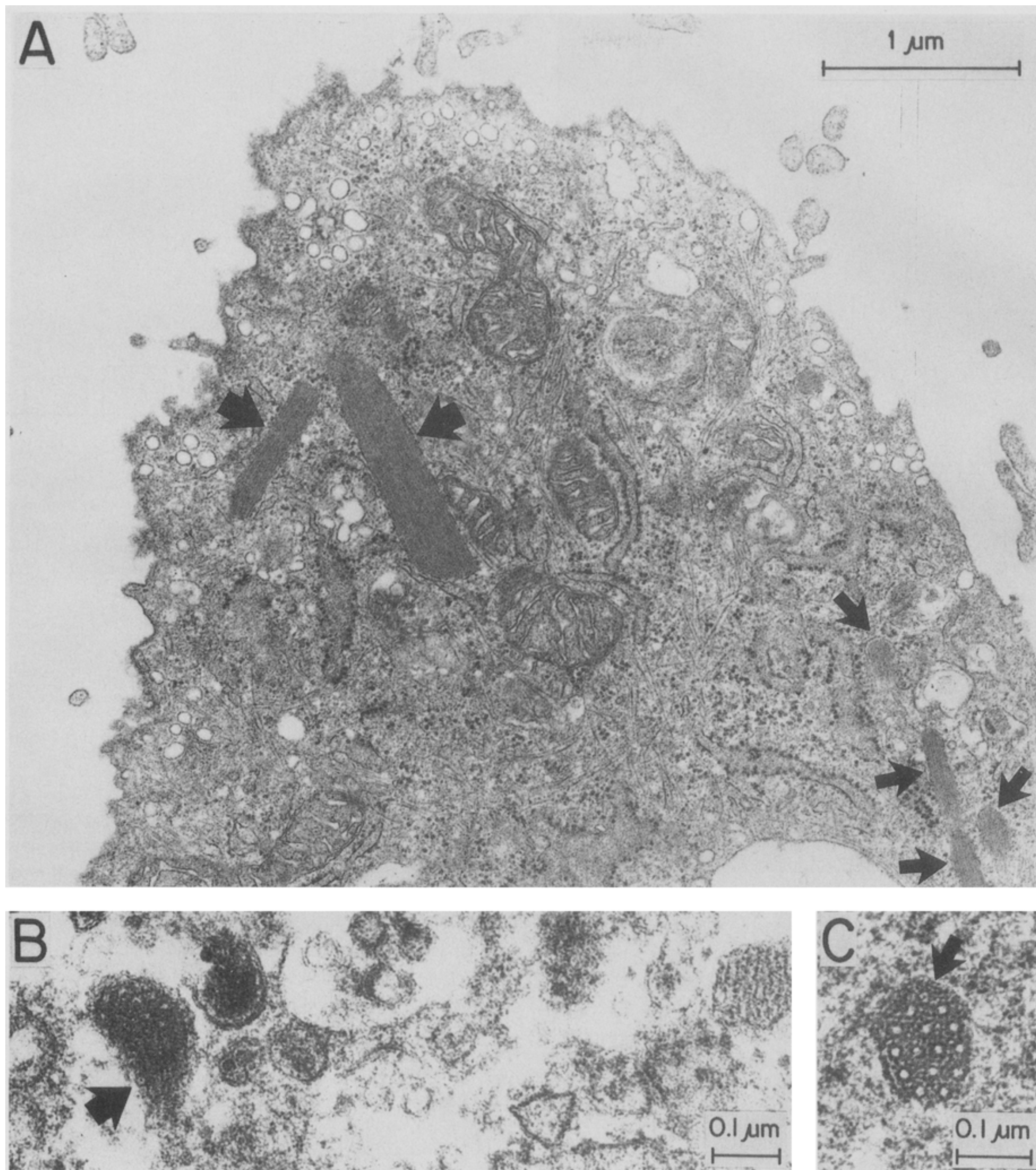

FIG. 1. Electron micrographs of EA cells. *A*, several Weibel-Palade bodies of different sizes are indicated by *arrows; B*, at higher magnification, a tangentially sectioned Weibel-Palade body is shown. *C*, a Weibel-Palade body cross section demonstrates the tubular nature of the component structures and their spacing. *Arrows* in *B* and *C* indicate tubules in which central filaments are discernible.

μm. They consist of parallel tubule arrays within a limiting unit membrane. In cross-sections of these rod-shaped organelles, the tubules have a center-to-center distance of 28 to 35 nm and a diameter of 20 nm. A dot seen in the center of some tubules may indicate a co-axial filament. The cross-sections are encountered infrequently in en face sections, suggesting that the long axes of these organelles are generally oriented parallel to the plane of the culture surface.

The frequency of WPBs in EA cells was evaluated after these cells had undergone three successive clonal isolations and had been in culture for more than 300 population doublings. In a region of highest cell density in a postconfluent culture, WPBs were found in 70% of en face cell sections representing a plane close to the surface on which the cells were grown. A total of 594 WPBs were identified in 86 cell sections for an average of 7 WPBs per cell section. WPBs were less frequent in regions of the culture where the

FIG. 2. Immunofluorescence of von Willebrand antigen in EA cells indicating that much of the antigen is located in rod-shaped organelles of the size expected for WPBs. ×1100.

cell density was not as great. Calcium ionophore treatment resulted in fewer WPBs. After a 1-h treatment with 10 μM A23187, 103 WPBs were identified in 108 cell sections in a region of highest cell density, for an average of 1 WPB per section compared to 7 WPBs per section in the matched untreated culture.

The intracellular distribution of von Willebrand antigen, one of the two known molecular components of WPBs, is demonstrated by immunofluorescence in Fig. 2. Fixed EA cells were treated with monoclonal antibody to von Willebrand factor, which was subsequently visualized by indirect immunofluorescence. Much of this antigen is located in cytoplasmic rod-shaped organelles of appropriate dimensions for WPBs. Up to 90% of the EA cells in postconfluent cultures are positive for von Willebrand factor by immunofluorescence. Controls demonstrate that binding of the secondary antibody is dependent on the presence of the primary monoclonal antibody, and that A549/8 cells are negative.

Von Willebrand antigen is rapidly released into the culture medium when EA cells are treated with calcium ionophore A23187. Figure 3 shows the increased amount of von Willebrand antigen

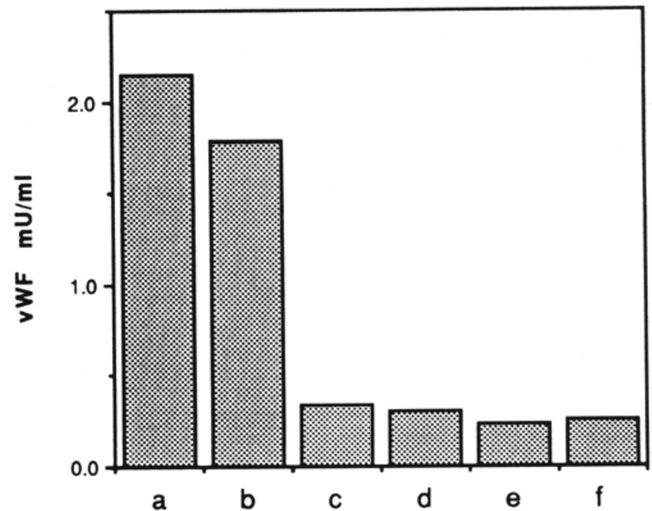

FIG. 3. Von Willebrand antigen is released from EA cells treated with calcium ionophore. Matched confluent EA cultures were treated with 10 μM A23187 (*a,b*), with 0.1% dimethyl sulfoxide as solvent controls (*c,d*), or mock treated (*e,f*). Concentration of von Willebrand antigen in the medium after 40 min was determined by the ELISA described in Materials and Methods.

released into the culture medium within 40 min in the presence of 10 μM A23187. There is 6 times more von Willebrand antigen released from the calcium ionophore treated cultures than from the cultures treated with solvent alone.

Multimeric forms of von Willebrand antigen, which are thought to be preferentially processed through WPBs (26) are produced by EA cells. Figure 4 demonstrates that an EA cell lysate contains von Willebrand antigen in multimers that are larger than most of those found in normal plasma, in addition to small multimeric forms. A 5-d conditioned culture medium sample from EA cells contains the small forms as well as some very large multimers that are just detectible in the unconcentrated sample represented in Fig. 4. Our EA samples are indistinguishable from our HUVE cell samples (data not shown) in terms of their von Willebrand factor multimer distribution.

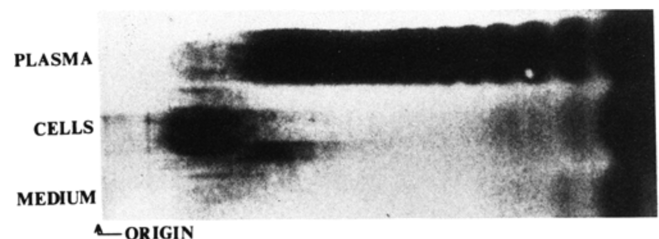

FIG. 4. EA cells contain large multimers of von Willebrand antigen as demonstrated by agarose electrophoresis and immunochemical staining. *Top lane* represents human plasma pooled from 10 healthy volunteers and diluted 1 to 10. *Middle lane* represents lysed EA cells at 27×10^6 cells/ml. *Bottom lane* represents culture medium after 5 d in contact with confluent EA cells at 0.27 ml/cm^2.

Discussion

The ultrastructural complexity of WPBs suggests that a number of molecular components could be involved. One or more proteins may be represented by the unit membrane, by the tubules, and by the less dense regions within and between the tubules. Von Willebrand factor was the first protein shown to be associated with WPBs (30), and GMP-140 (or PADGEM) has recently been shown by immunoelectron microscopy (20) to be another component. Other components may represent additional tissue-specific gene expression. Any cell that produces WPBs would have to coordinately express all of these genes. The demonstration of WPBs in EA cells significantly advances the characterization of this continuous cell line in terms of endothelium-specific properties.

The frequency of WPBs in EA cells, after more than 300 population doublings in culture and 3 successive clonal isolations, is as great as has been described for primary cultures of HUVE cells. Haudenschild (15) reported WPBs in 30 to 70% of sections from primary HUVE cells where they are considered particularly numerous. In the densest regions of EA cultures we found WPBs in 70% of en face sections, each of which represents only a small part of a cell. Postconfluent EA cultures have a swirling cobblestone morphology with areas of extra high cell density. The number of ultrastructurally identified WPBs per cell and the intensity of von Willebrand factor immunofluorescence is greatest in the regions where the cell density is greatest. In other regions of the same culture of these clonally derived EA cells, the prevalence of WPBs was considerably less. The frequency of WPBs also varies widely in endothelium from different anatomical sites and species (15).

Weibel-Palade bodies have been associated with large multimers of von Willebrand antigen because these large multimers are specifically depleted from A23187-treated HUVE cells in which the prevalence of WPBs (as visualized by immunofluorescence) is also decreased (26). It has been suggested that the function of WPBs is storage of these large von Willebrand antigen multimers. Therefore, we have demonstrated that A23187 treatment results in a decreased incidence of ultrastructurally recognizable WPBs in EA cells, that it causes rapid release of von Willebrand antigen from EA cells (Fig. 3), and that untreated cells contain large multimers of this antigen (Fig. 4). These data are consistent with suggestion that WPBs in EA cells serve the same function as those in HUVE cells. It should be noted that various laboratories observe different relative amounts of large and small von Willebrand antigen multimers in HUVE cell samples, as recently summarized by Tsai et al. (28). This may reflect lab-to-lab differences in culture parameters, sample handling, the epitope specificities of the antibodies used, or other parameters. What is relevant for this report is that the proportions of large and small von Willebrand antigen multimers in lysates and culture fluid from EA cells are the same as those in our HUVE cell samples.

Inasmuch as EA cells produce WPBs and replicate vigorously (more than 16-fold per week), these cells represent a convenient experimental model for further studies of the structure and function of this poorly understood organelle of the endothelium. A wide range of other endothelial cells functions has also been demonstrated in EA cells as detailed above. Relatively few of the more than 200 different cell types in mammals are represented by continuous cell lines that retain a wide spectrum of well-differentiated functions.

References

1. Ager, A.; Gordon, J. L.; Moncada, S., et al. Effects of isolation and culture on prostaglandin synthesis by porcine aortic endothelial and smooth muscle cells. J. Cell Physiol. 110:9–16; 1982.
2. Beretz, A.; Freyssinet, J. M.; Gauchy, J., et al. Stability of the thrombin-thrombomodulin complex on the surface of endothelial cells from human saphenous vein or from the cell line EA.hy926. Biochem. J. 259:35–40; 1989.
3. Bonfanti, R.; Furie, B. C.; Furie, B., et al. PADGEM (GMP-140) is a component of Weibel-Palade bodies of human endothelial cells. Blood 73:1109–1112; 1989.
4. Bradley, L. A.; Franco, E. L.; Reisner, H. M. Use of monoclonal antibodies in an enzyme immunoassay for factor VIII-related antigen. Clin. Chem. 30:87–90; 1989.
5. Bussolino, F.; Biffignandi, P.; Arese, P. Platelet-activating factor—a powerful lipid autacoid possibly involved in microangiopathy. Acta Haematol (Basel) 75:129–140; 1986.
6. Bussolino, F.; Wang, J. M.; Defilippi, P., et al. Induction of human endothelial cells migration and proliferation by granulocyte- and granulocyte-macrophage-colony stimulating factor. Nature 337:471–473; 1989.
7. Cramer, E. M.; Meyer, D.; le Menn, R., et al. Eccentric localization of von Willebrand factor in an internal structure of platelet alpha-granule resembling that of Weibel-Palade bodies. Blood 66:710–713; 1985.
8. DeBault, L. E.; Esmon, N. L.; Esmon, C. T., et al. Thrombomodulin expressed in human endothelial cell hybrid. Fed. Proc. 43:7183; 1984.
9. Del Vecchio, P. J.; Smith, J. R. Aging of endothelium in culture: decrease in angiotensin converting enzyme activity. Cell. Biol. Int. Rep. 6:379–384; 1982.
10. Edgell, C. J. S.; McDonald, C. C.; Graham, J. B. A permanent cell line expressing factor VIII related antigen established by hybridization. Proc. Natl. Acad. Sci. USA 80:3734–3737; 1983.
11. Emeis, J. J.; Edgell, C. J. S. Fibrinolytic properties of a human endothelial hybrid cell line (EA.hy926). Blood 71:1669–1675; 1988.
12. Ewenstein, B. M.; Warhol, M. J.; Handin, R. I., et al. Composition of the von Willebrand factor storage organelle (Weibel-Palade body) isolated from cultured human umbilical vein endothelial cells. J. Cell Biol. 104:1423–1433; 1987.
13. Fath, K. R.; Edgell, C. J. S.; Burridge, K. Distribution of distinct integrins in focal contacts is determined by the substratum composition. J. Cell Sci. 92:67–75; 1989.
14. Gimbrone, M. A. Culture of vascular endothelium. Prog. Hemostasis. Thromb. 3:1–28; 1976.
15. Haudenschild, C. C.; Cotran, R. S. Gimbrone, M. A., et al. Fine structure of vascular endothelium in culture. J. Ultrastruct. Res. 50:22–32; 1975.
16. Johnston, G. I.; Kurosky, A.; McEver, R. P. Structural and biosynthetic studies of the granule membrane protein, GMP-140, from human platelets and endothelial cells. J. Biol. Chem. 264:1816–1823; 1989.
17. Johnston, G. I.; Cook, R. G.; McEver, R. P. Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. Cell 56:1033–1044; 1989.
18. Lawrie, A. S.; Hoser, M. J.; Savidge, G. F. Phast assessment of VWF:Ag multimeric distribution. Thromb. Res. (in press); 1990.
19. Loesberg, C.; Gonsalves, M. D.; Zandbergen, J., et al. The effect of calcium on the secretion of factor VIII-related antigen by cultured human endothelial cells. Biochim. Biophys. Acta 763:160–168; 1983.
20. McEver, R. P.; Beckstead, J. H.; Moore, K. L., et al. GMP-140, a platelet α-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. J. Clin. Invest. 84:92–99; 1989.
21. Pearson, J. D.; Carleton, J. S.; Hutchings, A. Prostacyclin release stimulated by thrombin or bradykinin in porcine endothelial cells cultured from aorta and umbilical vein. Thromb. Res. 29:115–124; 1983.
22. Reinders, J. H.; de Groot, P. G.; Gonsalves, M. D., et al. Isolation of a

storage and secretory organelle containing von Willebrand protein from cultured human endothelial cells. Biochim. Biophys. Acta 804:361–369; 1984.

23. Reinders, J. H.; de Groot, P. G.; Sixma, J. J., et al. Storage and secretion of von Willebrand factor by endothelial cells. Haemostasis 18:246–261; 1988.
24. Rieber, A. J.; Edgell, C. J. S. Endothelium-specific gene expression. Thromb. Haemostasis 62:98; 1989.
25. Rosen, E. M.; Noveral, J. P.; Mueller, S. N., et al. Regulation of angiotensin I-converting enzyme activity in serially cultivated bovine endothelial cells. J. Cell Physiol. 122:30–38; 1985.
26. Sporn, L. A.; Marder, V. J.; Wagner, D. D. Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell 46:185–190; 1986.
27. Suggs, J. E.; Madden, M. C.; Friedman, M., et al. Prostacyclin expression by a continuous human cell line derived from vascular endothelium. Blood 68:825–829; 1986.
28. Tsai, H. M.; Nagel, R. L.; Hatcher, V. B., et al. Multimeric composition of endothelial cell-derived von Willebrand factor. Blood 73:2074–2076; 1989.
29. Van Oost, B. A.; Edgell, C. J. S.; Hay, C. W., et al. Isolation of human von Willebrand factor cDNA from the EA.hy926 cell line. Biochem. Cell Biol. 64:699–705; 1986.
30. Wagner, D. D.; Olmsted, J. B.; Marder, V. J. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. J. Cell Biol. 95:355–360; 1982.
31. Weibel, E. R.; Palade, G. E. New cytoplasmic components in arterial endothelia. J. Cell Biol. 23:101–112; 1964.
32. Zimmerman, T. S.; Batlle, F. J.; Ruggeri, Z. M., et al. Multimeric analysis of factor VIII/von Willebrand factor in the diagnosis of von Willebrand's disease. In: Nakamura, R. M.; Dito, W. R.; Tucker, E. S., eds. Clinical laboratory assays: new technology and future directions. New York: Masson Publishing USA, Inc.; 1983:249–264.