

Vesiculo-Vacuolar Organelles and the Regulation of Venule Permeability to Macromolecules By Vascular Permeability Factor, Histamine, and Serotonin

By Dian Feng, Janice A. Nagy, John Hipp, Harold F. Dvorak, and Ann M. Dvorak

From the Department of Pathology and the Orthopedics and Biomechanics Laboratory, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215

Summary

In contrast to normal microvessels, those that supply tumors are strikingly hyperpermeable to circulating macromolecules such as plasma proteins. This leakiness is largely attributable to a tumor-secreted cytokine, vascular permeability factor (VPF). Tracer studies have shown that macromolecules cross tumor vascular endothelium by way of a recently described cytoplasmic organelle, the vesiculo-vacuolar organelle or VVO (VVOs are grapelike clusters of interconnected, uncoated vesicles and vacuoles). However, equivalent VVOs are also present in the cytoplasm of normal venules that do not leak substantial amounts of plasma protein. To explain these findings, we hypothesized that VPF increased the permeability of tumor blood vessels by increasing VVO function and that the VVOs of normal venules were relatively impermeable in the absence of VPF stimulation. To test this hypothesis, VPF was injected intradermally in normal animals after intravenous injection of a soluble macromolecular tracer, ferritin, whose extravasation could be followed by electron microscopy. VPF caused normal venules to leak ferritin, and, as predicted by our hypothesis, ferritin extravasated by way of VVOs, just as in hyperpermeable tumor microvessels. Ultrathin (14-nm) serial electron microscopic sections and computer-aided three-dimensional reconstructions better defined VVO structure. VVOs occupied 16–18% of endothelial cytoplasm in normal venules. Individual VVOs were clusters of numerous (median, 124) interconnected vesicles and vacuoles that formed complex pathways across venular endothelium with multiple openings to both luminal and abluminal surfaces. Like VPF, histamine and serotonin also stimulated ferritin extravasation across venules by way of VVOs. Together, these data establish VVOs as the major pathway by which soluble plasma proteins exit venules in response to several mediators that increase venular hyperpermeability. These same mediators also increased the extravasation of colloidal carbon, but this large particulate nonphysiological tracer exited venules primarily through endothelial gaps.

Mammals and other vertebrates supply their tissues with nutrients and clear waste products by means of the “exchange vessels” of the microcirculation, primarily the capillaries and postcapillary venules (1–5). Normally, the vast majority of transport occurs across capillaries, thin vessels that are considerably more numerous than venules and that provide a much greater total surface area for molecular passage. Gases, hydrophobic species, and small hydrophilic molecules cross capillaries rapidly and without significant impedance; however, extravasation of circulating macromolecules is highly restricted and only small amounts of plasma proteins cross the endothelial barrier.

A very different situation prevails in tumors where microvessels are hyperpermeable to plasma proteins (6, 7). This hyperpermeability is largely attributable to the secretion by tumors of a cytokine, vascular permeability factor (VPF¹, also known as vascular endothelial growth factor or VEGF) that increases microvascular permeability to macromolecules with a potency some 50,000 times that of histamine. By using suitable tracers, macromolecules were found to extravasate across tumor vascular endothelium by way of a previously unrecognized cytoplasmic organelle, the vesiculo-vacuolar organelle or VVO (8). VVOs are grapelike clusters of uncoated vesicles and vacuoles interconnected by stomata that may be open or are closed by

This work was presented in part at the annual meeting of the American Society for Cell Biology in Washington, D.C. on Dec. 12, 1995. Portions of this work have appeared in abstract form (1995. *Mol. Biol. Cell.* 6:335a).

¹Abbreviations used in this paper: VPF, vascular permeability factor; VVO, vesiculo-vacuolar organelle.

thin diaphragms. VVOs structurally similar to those present in tumor microvessels have now also been identified in the postcapillary venules of several normal tissues (8).

Taken together, these findings suggested that tumor cell-secreted VPF rendered tumor microvascular endothelium hyperpermeable by activating VVO function. If so, VPF might be expected to activate the function of the VVOs that are present in normal venular endothelium. To test this hypothesis, we injected recombinant human VPF into the skin of normal animals of several species and determined the pathways by which a circulating plasma protein tracer, ferritin, crossed the vascular endothelium. In parallel experiments, vascular permeability was enhanced locally with histamine or serotonin in order to determine whether inflammatory mediators might also upregulate VVO function. Finally, colloidal carbon was used as a tracer to determine if circulating large particulates also exited venules by way of VVOs in response to VPF or inflammatory mediators.

Materials and Methods

Venular permeability was measured in young adult Hartley guinea pigs of either sex (Elm Hill, Chelmsford, MA), female A/J mice (The Jackson Laboratory, Bar Harbor, ME), or male Sprague-Dawley rats (Charles River, Wilmington, MA). Flank skin was shaved (guinea pigs flanks were also depilated) 1 d before experimentation. Animals were lightly anesthetized (guinea pigs with ketamine and xylazine, mice with tribromoethanol, and rats with pentobarbital) immediately before i.v. injection of ferritin (0.5–1 mg/g; Sigma Chemical Co., St. Louis, MO) or colloidal carbon (1.0 ml/250 g; Guenther Wagner, Hannover, Germany). Ferritin is a soluble anionic protein of ~11 nm diameter; individual ferritin molecules are readily visualized by conventional transmission electron microscopy. Normally present in plasma at low levels, ferritin is cleared slowly and serves as a useful marker for following bulk transport extravasation of plasma proteins (8). Colloidal carbon is a suspension of particles of mean diameter ~50 nm.

Immediately after i.v. tracer injection, 0.1 ml recombinant human VPF (5–100 ng, Peprotech, Rocky Hill, NJ), histamine, serotonin (both 1 µg, Sigma Chemical Co.), or HBSS was injected intradermally at different sites on flank or scrotal skin and animals were killed 10 s–30 min thereafter. In some experiments, skin injection sites were immersed in paraformaldehyde-glutaraldehyde, cut into small pieces, and fixed for 1–4 h at room temperature (8). Alternatively, fixation was accomplished by perfusing animals with paraformaldehyde-glutaraldehyde through the aorta. Rat cremaster muscle was harvested and fixed as described (9). After fixation, tissues were processed for electron microscopy (8).

A total of 2,190 blocks of tissue was studied by electron mi-

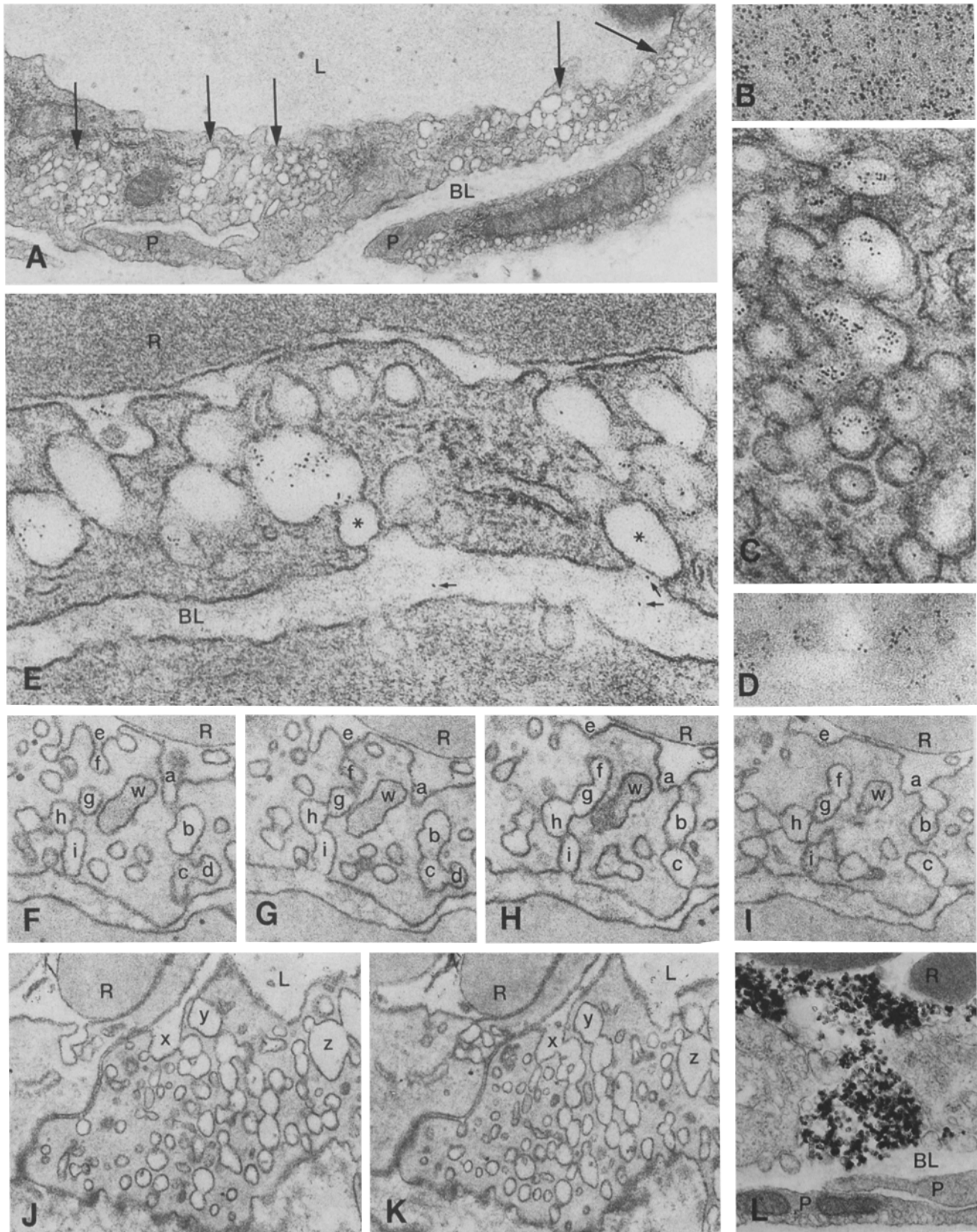
croscopy. Electron microscopic sections were cut at different thicknesses for different purposes: standard silver-gold (70-nm) sections for routine viewing; ultrathin (14-nm-thick) serial sections for tracing connections between adjacent VVO vesicles and vacuoles and for preparing computer-aided three-dimensional reconstructions; and serial 100-nm sections for determining the dimensions and composition of individual VVOs in venular endothelium. Data were analyzed with the Mann-Whitney or Kruskal-Wallis multiple comparison test, as appropriate.

Results and Discussion

VVOs were prominent in the largely continuous endothelium that lined venules of normal flank skin of mice (Fig. 1 *A*), rats, and guinea pigs; moreover, their appearance and frequency were identical with both methods of tissue fixation. Like other plasma proteins, circulating ferritin was largely retained within the microvessels of normal skin. However, ferritin extravasated extensively at sites injected with VPF (guinea pigs, mice, rats), histamine (guinea pigs), or serotonin (mice). Within seconds of intradermal injection of these mediators, and continuing for some minutes thereafter, increasing amounts of circulating ferritin entered VVO vesicles contiguous with the venular lumen and proceeded across the endothelium through a succession of interconnecting VVO vesicles and vacuoles to reach the vascular ablumen and underlying basal lamina (Fig. 1, *B–E*). Frequently, the stomata connecting adjacent VVO vesicles and vacuoles to one another and to the luminal and abluminal plasma membranes were open and did not seem to restrict ferritin passage (Fig. 1 *C*). Other stomata, however, were closed by thin diaphragms (Fig. 1 *E*); ferritin molecules sometimes accumulated in vesicles or vacuoles proximal to such diaphragms, suggesting that diaphragms served as a barrier that limited their further transcellular passage (Fig. 1 *E*). Interendothelial cell junctions remained intact (Fig. 1, *J* and *K*) and ferritin was never observed in junctions or free in endothelial cell cytoplasm.

To quantitate the passage of ferritin across venules under different circumstances, we counted individual ferritin molecules per square micrometer in representative, randomly selected electron micrographs of normal mouse flank skin or of mouse flank skin injected with VPF, serotonin, or HBSS. Ferritin concentrations were determined in venule plasma, in VVOs, and in the basal lamina immediately subjacent to VVOs (Table 1). 5 min after intradermal injection of VPF or serotonin, ferritin concentration in VVOs and in

Figure 1. Electron micrographs of venules from mouse or guinea pig skin (*A–K*) or from rat cremaster (*L*). As indicated, ferritin or colloidal carbon was injected intravenously, followed immediately by local injection of 12.5–100 ng VPF. Tissues were harvested 5 min later. (*A*) Venule in normal mouse skin illustrating five distinct VVOs (*arrows*) in the cytoplasm of a single endothelial cell. (*B–E*) VPF injection sites in guinea pig skin illustrating progressive extravasation of circulating ferritin from plasma across venular endothelium via VVOs. (*B*) Ferritin particles (*black dots*) in plasma. (*C*) A succession of ferritin-containing VVO vesicles and vacuoles at mid-level of endothelial cell cytoplasm. (*D*) Ferritin particles in subjacent extracellular matrix. (*E*) VVO with ferritin-containing vesicles and vacuoles. Ferritin is present in several vesicles that open to the vascular lumen or are separated from it by thin diaphragms. Two vesicles (***) in continuity with the abluminal plasma membrane do not contain ferritin but exhibit stomata closed by diaphragms. One starred vesicle (*left*) is also separated by a diaphragm from a more proximal (more luminal) vacuole that contains numerous ferritin particles; micrographs such as this suggest that stomatal diaphragms represent barriers that restrict the transcellular passage of macromolecules. Scattered ferritin particles are also present in the underlying basal lamina, several of which are indicated by arrows. (*F–I*) Four (of a set of 36) consecutive serial ultrathin (14 nm) sections illustrate two sequences of interconnecting VVO vesicles–vacuoles (*a–d*; *e–i*) that traverse venule endothelial cell cytoplasm from vascular lumen



to ablumen. (J, K) Two consecutive serial ultrathin sections of a single VVO. Three sequences of interconnecting vesicles-vacuoles are illustrated, the most luminal of which are designated *x*, *y*, and *z*; each sequence nearly forms a transendothelial channel in just two consecutive sections. Note closed interendothelial junction left of figure. (L) VPF-induced endothelial gap allows carbon extravasation from venule in rat cremaster muscle. *L*, lumen; *R*, red blood cell; *BL*, basal lamina; *P*, pericyte; and *W*, Weibel-Palade body. *A*, $\times 25,000$; *B-D*, $\times 145,000$; *E*, $\times 105,000$; *F-I*, $\times 60,000$; *J* and *K*, $\times 50,000$; and *L*, $\times 26,000$. (All magnifications are before 9.5% reduction.)

Table 1. Concentration of Ferritin in Venule Plasma, Venular Endothelial Cell VVOs, and Immediately Subjacent Subendothelial Space in Normal Mouse Skin Versus Skin Injected with VPF, Serotonin, or HBSS

Intradermal injection	Ferritin concentration (mean particles/ μm^2)			Percent plasma concentration	
	Plasma	VVOs	Subendothelial space	VVOs	Subendothelial space
VPF	710	167	140	23.5	19.7
Serotonin	670	121	129	18.1	19.3
HBSS	690	59	38	8.6	5.5
None	707	51	25	7.2	3.5

A/J mice were injected intravenously with ferritin and intradermally with VPF, serotonin, or HBSS as described in Materials and Methods. 5 min later, mice were killed by CO_2 narcosis and skin test sites were fixed and processed for electron microscopy. A total of 78 venule sections were analyzed in electron micrographs. Ferritin molecules were counted in plasma, in 365 separate VVOs, and in the basal lamina immediately subjacent to VVOs. The areas of the several compartments counted were measured simultaneously so as to determine ferritin concentration (number of ferritin molecules/ μm^2). Ferritin concentration in VVOs and subendothelial space at VPF and serotonin injection sites was significantly greater than in either control site ($p < 0.001$).

the subjacent basal lamina was substantially greater than that at control sites or at sites injected with HBSS. Thus, ferritin extravasates minimally from normal venules by way of VVOs and mediators such as VPF and serotonin greatly increase ferritin extravasation by this route.

VVOs are three-dimensional organelles and their structure could not be fully appreciated in individual electron microscopic sections. Moreover, standard 70-nm sections were too thick for reliably tracing the connections that linked individual vesicles and vacuoles to one another and to the plasma membrane. Therefore, we prepared serial ultrathin 14-nm sections of VPF-injected and control mouse skin. Study of 38 such sets of serial ultrathin sections, each comprised of 9–61 serial sections, revealed a network of interconnecting vesicles and vacuoles and established that VVOs formed continuous pathways across venular endothelium from lumen to ablumen (Fig. 1, F–K). Serial ultrathin sections also permitted computer-assisted three-dimensional reconstructions of individual VVOs from both VPF-injected (Fig. 2) and normal skin. These confirmed that the vesicles and vacuoles comprising VVOs interconnected to form serpentine pathways that traversed venular endothelium and opened to both the lumen and ablumen at multiple sites.

Morphometric measurements revealed that VVOs occupied a substantial portion (16–18%) of venular endothelial cell cytoplasm in both normal and VPF-injected mouse skin. Individual VVOs commonly extended through endothelial cell cytoplasm for distances of 1–2 μm . Based on 52 sets of serial 100-nm-thick sections (each set comprised of 9–115 sequential sections), single VVOs were found to be comprised of 79–362 individual vesicles or vacuoles (median, 124). Smaller VVO vesicles resembled capillary caveolae (8, 10–12). However, taken together, the vesicles and vacuoles comprising VVOs measured 108 ± 32 nm (mean \pm SD, range: 50–415 nm) in internal diameter and were therefore significantly larger and more heterogeneous than capillary caveolae whose diameters measured, on aver-

age, only 58 ± 9 nm (range: 38–141 nm, $p < 0.01$) (Fig. 3). Thus, the vesicles and vacuoles comprising VVOs differ from the caveolae of capillary endothelium in several important respects: significantly larger average size, greater size heterogeneity, and organization into a cohesive organelle (the VVO) that occupies nearly one fifth of venular endothelial cell cytoplasm and that links the vascular lumen with the ablumen. The large size and complexity of VVOs suggest that they are sessile structures and that their component vesicles and vacuoles are unlikely to shuttle back and forth across endothelial cytoplasm as has been proposed for capillary caveolae (10, 11). VVOs resemble the vesicular-vacuolar networks that have been described in amphibian capillary cytoplasm (13, 14), but, as shown here, differ in that VVOs traverse the entire thickness of endothelial cell cytoplasm, opening to both lumen and ablumen, and have a prominent role in macromolecule extravasation.

Earlier investigators, including ourselves, had reported that VPF and inflammatory mediators, such as histamine or serotonin, increased venular permeability by inducing the formation of interendothelial gaps (9, 15–18). However, in our present experiments with ferritin as tracer, gaps were exceedingly rare (<1 per 2,000 junctions studied). In attempting to explain this difference, we noted that, with one exception (19), studies demonstrating endothelial gaps had used colloidal particulates (carbon or Monastral blue) as tracers. To determine whether gap formation might depend on physical properties (e.g., size, particulate nature) of the tracer, we performed permeability studies with colloidal carbon as tracer instead of ferritin. In contrast to our results with ferritin, we found frequent venular endothelial gaps through which carbon extravasated at sites of VPF (Fig. 1 K) or histamine injection. Carbon particles were only rarely found in VVOs and were, on average, too large to pass through the stomata that interconnected VVO vesicles and vacuoles with one another and with the plasma membrane. These findings suggest that endothelial gap formation is related, at least in part, to properties of the tracer

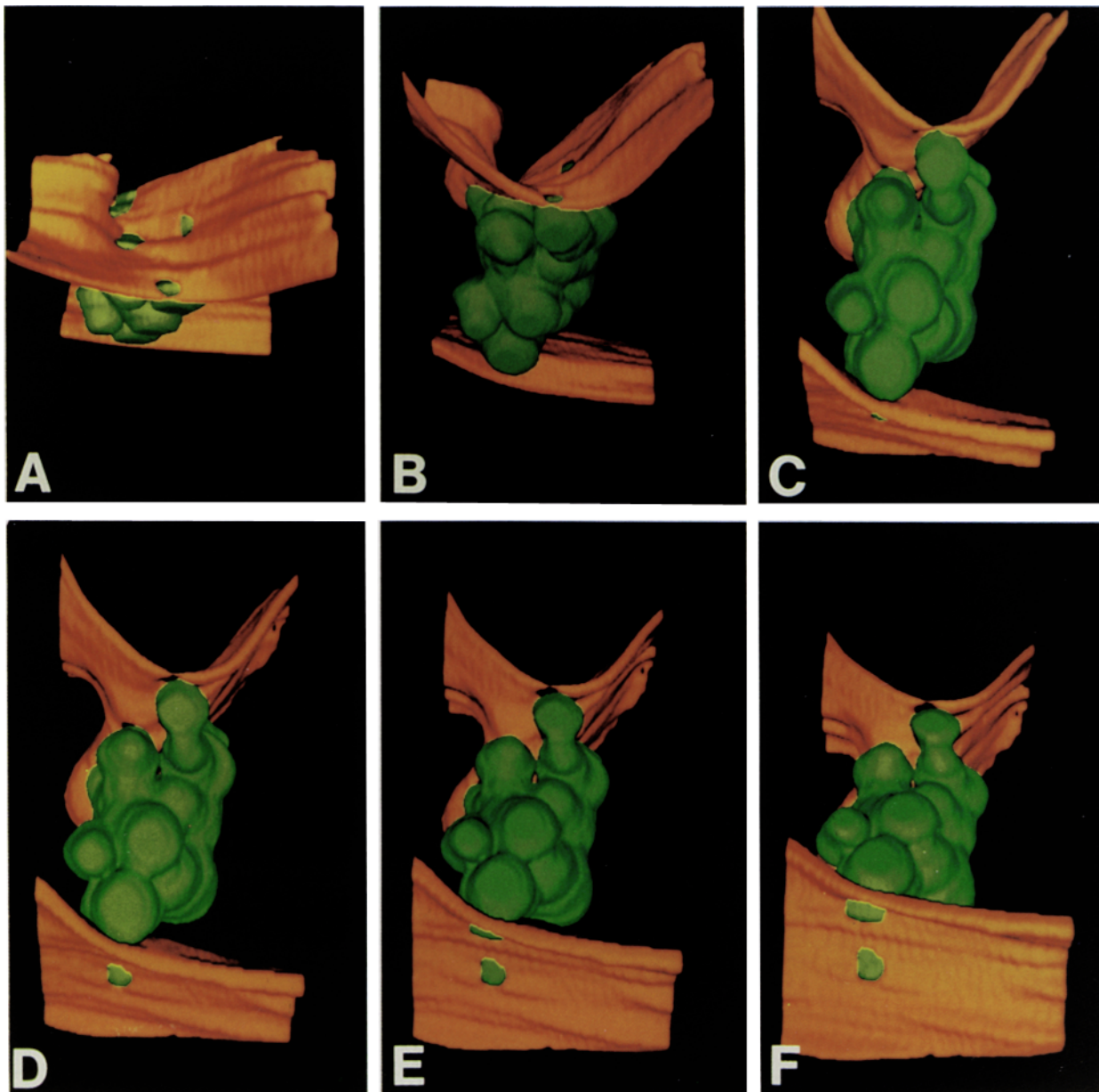
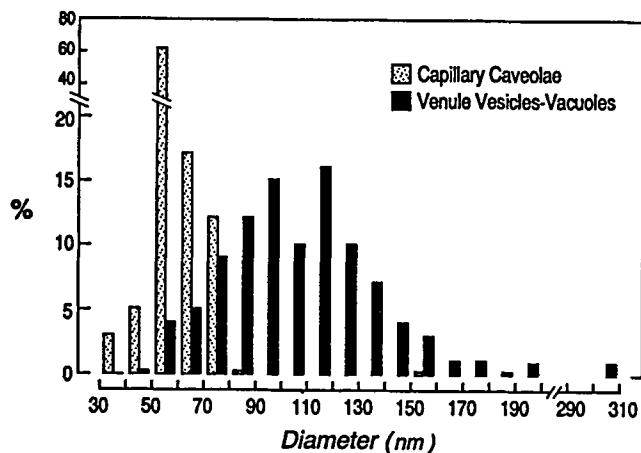


Figure 2. Computer-generated three-dimensional reconstruction of portion of a venular VVO from mouse skin injected 5 min earlier with 50 ng VPF. The interior volumes of VVO vesicles and vacuoles in successive electron micrographs were traced onto transparent overlays with reference marks to retain register. Tracings were digitized at a resolution of 5.9 nm/pixel. Since section thickness (15 nm) was greater than in-plane resolution, bicubic interpolation was performed between sections and convolutions were used to smooth surfaces. (A–F) Portion of a VVO (15 consecutive serial, ultrathin electron microscopy sections illustrating 25 individual vesicles–vacuoles) reconstructed with Advanced Visual Systems (Waltham, MA) software, here viewed in successive rotations around a horizontal axis at intervals of 30° (except 15°, C and D). There are two openings (E and F) to the vascular lumen and four to the abluminal surface (A).

itself; i.e., that particulate tracers such as colloidal carbon interact with endothelium to induce gap formation at test sites injected with VPF or inflammatory mediators.

In summary, we have identified VVOs as the primary anatomic pathway by which ferritin, a physiological protein useful for measuring bulk transport, exits normal venules in response to VPF, histamine, and serotonin. Presumably, other plasma proteins also exit venules by way of VVOs. Our data are consistent with the view that the hyperpermeability characteristic of tumor microvessels is at-

tributable to upregulation of VVO function by tumor-secreted VPF and with immunocytochemical evidence that has localized VPF to VVOs in tumor vessel endothelium (20). Remaining to be worked out are the molecular mechanisms by which VPF or inflammatory mediators interact with specific endothelial receptors to activate VVO function. The stomata that link individual VVO vesicles and vacuoles to one another and to the plasma membranes are often closed by thin diaphragms similar to those that close capillary caveolae (10, 11). We suggest that VPF and



other mediators that increase venule permeability provoke the opening of these diaphragms, whether physically or functionally, to allow *trans*-endothelial cell passage of macromolecules. Also remaining to be investigated are the mechanisms by which colloidal carbon and likely other particulates interact with venular endothelium, together with VPF or inflammatory mediators, to induce the formation of endothelial gaps.

Figure 3. Size distribution of vesicles and vacuoles comprising VVOs ($n = 1,657$) in venular endothelium compared with caveolae ($n = 1,225$) of capillary endothelium. Data from normal uninjected mouse skin and mouse skin injected 5 min earlier with VPF did not differ and were pooled. Venules were distinguished from capillaries according to Simionescu (11).

This work was supported by U.S. Public Health Service National Institutes of Health grants CA-50453 and AI-33372 and by salary support from the BIH Pathology Foundation, Inc.

Address correspondence to Dr. Harold F. Dvorak, Department of Pathology, Beth Israel Hospital, Boston, MA 02215.

Received for publication 28 November 1995 and in revised form 8 February 1996.

References

- Renkin, E.M. 1992. Cellular and intercellular transport pathways in exchange vessels. *Am. Rev. Respir. Dis.* 146:28-31.
- Michel, C.C. 1988. Capillary permeability and how it may change. *J. Physiol. (Lond.)*, 404:1-29.
- Clough, G. 1991. Relationship between microvascular permeability and ultrastructure. *Prog. Biophys. Mol. Biol.* 55:47-69.
- Curry, F.E. 1992. Modulation of venular microvessel permeability by calcium influx into endothelial cells. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:2456-2466.
- Rippe, B., and B. Haraldsson. 1994. Transport of macromolecules across microvascular walls: the two-pore theory. *Physiol. Rev.* 74:163-219.
- Dvorak, H.F., L.F. Brown, M. Detmar, and A.M. Dvorak. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.* 146:1029-1039.
- Jain, R.K. 1990. Vascular and interstitial barriers to delivery of therapeutic agents in tumors. *Cancer Metastasis Rev.* 9:253-266.
- Kohn, S., J.A. Nagy, H.F. Dvorak, and A.M. Dvorak. 1992. Pathways of macromolecular tracer transport across venules and small veins. Structural basis for the hyperpermeability of tumor blood vessels. *Lab. Invest.* 67:596-607.
- Joris, I., H.F. Cuenoud, G.V. Doern, J.M. Underwood, and G. Majno. 1990. Capillary leakage in inflammation. A study by vascular labeling. *Am. J. Pathol.* 137:1353-1363.
- Palade, G.E. 1988. The microvascular endothelium revisited. In *Endothelial Cell Biology in Health and Disease*. N. Simionescu and M. Simionescu, editors. Plenum Press, New York. 3-22.
- Simionescu, N. 1983. Cellular aspects of transcapillary exchange. *Physiol. Rev.* 63:1536-1579.
- Dvorak, A.M., S. Kohn, E.S. Morgan, P. Fox, J.A. Nagy, and H.F. Dvorak. 1995. The vesiculo-vacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. *J. Leukocyte Biol.* 59:100-115.
- Bundgaard, M. 1980. Transport pathways in capillaries—in search of pores. *Annu. Rev. Physiol.* 42:325-336.
- Frokjaer-Jensen, J. 1991. The endothelial vesicle system in cryofixed frog mesenteric capillaries analysed by ultrathin serial sectioning. *J. Electron. Microsc. Tech.* 19:291-304.
- Majno, G., and G.E. Palade. 1961. Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. *J. Biophys. Biochem. Cytol.* 11:571-605.
- Majno, G., S.M. Shea, and M. Leventhal. 1969. Endothelial contraction induced by histamine-type mediators: an electron microscopic study. *J. Cell Biol.* 42:647-672.
- Senger, D.R., S.J. Galli, A.M. Dvorak, C.A. Perruzzi, V.S. Harvey, and H.F. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science (Wash. DC)*. 219:983-985.
- Roberts, W., and G. Palade. 1995. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J. Cell Science* 108:2369-2379.
- Hulstrom, D., and E. Svensjo. 1979. Intravital and electron microscopic study of bradykinin-induced vascular permeability changes using FITC-dextran as a tracer. *J. Pathol.* 129:125-133.
- Qu-Hong, J.A. Nagy, D.R. Senger, H.F. Dvorak, and A.M. Dvorak. 1995. Ultrastructural localization of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) to the abluminal plasma membrane and vesiculovacuolar organelles of tumor microvascular endothelium. *J. Histochem. Cytochem.* 43:381-389.