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Vascular Permeability and Pathological Angiogenesis in Caveolin-1-Null Mice

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Caveolin-1, the signature protein of endothelial cell caveolae, has many important functions in vascular cells. Caveolae are thought to be the transcellular pathway by which plasma proteins cross normal capillary endothelium, but, unexpectedly, cav-1$^{-/-}$ mice, which lack caveolae, have increased permeability to plasma albumin. The acute increase in vascular permeability induced by agents such as vascular endothelial growth factor (VEGF)-A occurs through venules, not capillaries, and particularly through the vesiculo-vacuolar organelle (VVO), a unique structure composed of numerous interconnecting vesicles and vacuoles that together span the venular endothelium from lumen to ablumen. Furthermore, the hyperpermeable blood vessels found in pathological angiogenesis, mother vessels, are derived from venules. The present experiments made use of cav-1$^{-/-}$ mice to investigate the relationship between caveolae and VVOs and the roles of caveolin-1 in VVO structure in the acute vascular hyperpermeability induced by VEGF-A and in pathological angiogenesis and associated chronic vascular hyperpermeability. We found that VVOs expressed caveolin-1 variably but, in contrast to caveolae, were present in normal numbers and with apparently unaltered structure in cav-1$^{-/-}$ mice. Nonetheless, VEGF-A-induced hyperpermeability was strikingly reduced in cav-1$^{-/-}$ mice, as was pathological angiogenesis and associated chronic vascular hyperpermeability, whether induced by VEGF-A^{164} or by a tumor. Thus, caveolin-1 is not necessary for VVO structure but may have important roles in regulating VVO function in acute vascular hyperpermeability and angiogenesis. *(Am J Pathol 2009, 175:1768–1776; DOI: 10.2353/ajpath.2009.090171)*

Caveolae (also referred to as plasmalemmal vesicles) were described by Palade and Bruns in capillary endothelial cells as 50- to 100-nm diameter smooth membrane-bound vesicles.[1,2] Palade and Bruns proposed that caveolae shuttled across capillary endothelium from lumen to ablumen, carrying with them "cargoes" of plasma and in this manner provided the small amounts of plasma proteins that are required for maintaining tissue health. Later work demonstrated that caveolae could also form short chains of two to three linked vesicles that spanned the short distance across the capillary endothelium.[3] Together these studies implied that, whether shuttling or interconnected into short chains, capillary caveolae were de facto the elusive "large pores" that physiologists had postulated to account for plasma protein extravasation.[4,5]

Since their initial discovery, much has been learned about caveolae and their signature protein, caveolin.[6–10] Caveolin is thought to be necessary for caveolae formation and overexpression of caveolin can induce caveolae in cells that normally lack them.[11] Caveolin exists in three isoforms.[12–14] The first two isoforms, cav-1 and cav-2, are highly expressed in vascular endothelium, pericytes and smooth muscle, among other cell types, whereas cav-3 is confined to muscle.[15] Caveolae and caveolin have many functions besides plasma protein transport, including regulation of cholesterol homeostasis and sorting of signaling molecules such as endothelial nitric oxide synthase, heterotrimeric G proteins, and nonreceptor tyrosine kinases.[7,9,14,16,17]

Cav-1$^{-/-}$ mice have contributed much to our understanding of caveolin and caveolae. Cav-1$^{-/-}$ mice are viable and fertile but lack caveolae and exhibit various types of vascular dysfunction, including impaired nitric oxide and Ca^{2+} signaling.[12,18–22] However, there is controversy on some other points, such as whether tumor

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growth and angiogenesis are altered in cav-1$^{-/-}$ mice, and, if so, in what direction and by what mechanism.[22–29] Recently, cav-1$^{-/-}$ mice have been found to be systemically hyperpermeable to plasma albumin[25,30,31]; this finding was unexpected in that caveolae have been thought to be necessary for transporting plasma proteins across capillary endothelium under basal conditions.[1–3]

However, vascular permeability is not of a single type.[32] In contrast to the normal, low level basal vascular permeability (BVP) of normal tissues, two distinctly different types of increased vascular permeability are found in pathological conditions.[32] Vascular permeabilizing factors, such as vascular endothelial growth factor (VEGF)-A, histamine, and others, induce acute vascular hyperpermeability (AVH), a characteristic feature of acute inflammation. Chronic vascular hyperpermeability (CVH), on the other hand, is found in the pathological angiogenesis induced by tumors, healing wounds, and chronic inflammatory diseases; as its name implies, CVH persists for long periods of time—days to weeks and sometimes indefinitely. AVH and CVH differ from BVP not only in terms of the much greater amounts of plasma that extravasate but also with respect to the microvessels that leak. BVP takes place in capillaries.[2–4] In contrast, AVH takes place primarily in postcapillary venules[33–37] and is thought to involve an organelle, the vesiculo-vacuolar organelle (VVO), that is uniquely present in venular endothelial cells. VVOs are grapelike clusters of hundreds of uncoated, trilaminar unit membrane-bound, interconnecting vesicles and vacuoles that extend across the relatively tall cytoplasm of venular endothelium from lumen to ablumen. The relationship of VVOs to caveolae is uncertain.[36–40] Unlike caveolae, which are of relatively uniform size, the vesicles and vacuoles that comprise VVOs vary widely in size from caveolae-sized vesicles to those with a cross-sectional areas more than 10-fold greater.[37] They attach to each other and to the endothelial plasma membrane by stomata that are normally closed by thin diaphragms. In this respect, VVO stomata and diaphragms closely resemble the analogous structures by which caveolae attach to each other and to the luminal and abluminal plasma membranes of capillary endothelium.[36–38,41] On exposure to acute permeabilizing agents such as VEGF or histamine, the diaphragms interconnecting VVO vesicles and vacuoles open to provide a trans-endothelial cell pathway for plasma extravasation.[36,37,42] Others have reported leakage through a paracellular route, independent of VVOs.[43,44] In CVH, yet another type of blood vessel, the "mother" vessel, accounts for the bulk of vascular hyperpermeability.[45] Mother vessels are greatly enlarged, thin-walled, pericyte-poor sinusoids that derive from pre-existing normal venules after longer exposures to VEGF and other angiogenic stimuli.[46] VVOs participate in mother vessel formation and associated CVH.[45,47]

The experiments reported here made use of cav-1$^{-/-}$ mice to investigate the relationship between caveolae and VVOs and the role of caveolin-1 in VVO structure, in AVH and CVH, and in pathological angiogenesis. We report here that some, but not all, VVO vesicles and vacuoles express caveolin-1. Nonetheless, VVOs are present in normal numbers and with unaltered structure in cav-1$^{-/-}$ mice. Further, we find that AVH and CVH are strikingly reduced in cav-1$^{-/-}$ mice. Angiogenesis is also reduced in cav-1$^{-/-}$ mice, whether induced by an adenoviral vector expressing VEGF-A^{164} (Ad-VEGF-A^{164}) or by a tumor, the B16 melanoma.

Materials and Methods

Animals, Adenoviral Vector, and Tumors

Four- to 6-week-old female wild-type C57BL/6 and caveolin 1 knockout (cav-1$^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Angiogenesis was induced in flank skin either with (Ad-VEGF-A^{164})[46–48] or with the B16 melanoma.[49] All studies were performed under protocols approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Tissue Processing for Light Microscopic Immunohistochemistry

Animals were sacrificed by CO_2 narcosis. Flank skin was fixed in 4% paraformaldehyde and processed either for frozen or paraffin sections and immunohistochemical analysis, as described previously.[50] Two different rabbit polyclonal antibodies, one directed against N-terminal amino acids 1 to 97 (BD Biosciences, San Jose, CA) and the other against N-terminal amino acids 1 to 20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), were used to identify caveolin-1. Mean vascular density was calculated by counting CD31-positive structures with lumens in the five most highly vascularized fields at ×400 magnification. Statistical analysis was performed using an unpaired *t*-test.

Electron Microscopy

Tissues were fixed and processed for electron microscopy as described previously.[51] Morphometric analysis was performed on randomly selected electron micrographs of wild-type and cav-1$^{-/-}$ flank skin venules and capillaries for quantification of VVOs, vesicles, and caveolae.[35,37] Data were analyzed with the Kruskal-Wallis nonparametric analysis of variance test and with Dunn's multiple comparisons test.

Electron Microscopic Immuno-Nanogold Cytochemistry

Immuno-nanogold cytochemistry was performed as described previously.[50] Tissues were fixed for 4 hours at room temperature in 4% paraformaldehyde-0.02 mol/L PBS, pH 7.4, and were washed in 0.02 mol/L PBS, pH 7.4. before immersion in 30% sucrose in 0.02 mol/L PBS, pH 7.4, overnight at 4°C. Tissues were embedded in OCT compound (Miles, Elkhart, IN), snap-frozen, and stored in liquid nitrogen. The same two rabbit polyclonal antibodies used for light microscopic immunohistochemistry