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Cerebral capillary endothelial cells are covered by the VEGF-expressing foot processes of astrocytes

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ABSTRACT

Molecules that have crucial functions in both nervous and vascular systems have attracted keen attention recently, and the name "angioneurins" has been proposed. The most striking example of angioneurins is vascular endothelial growth factor A (VEGF), which was originally identified as a key regulator of angiogenesis and has only recently been found to have important functions in the nervous system. In this study, we compared VEGF expression in the vasculature in the brain with that in the aorta and the vasculature in the kidney in mice. In larger vessels containing smooth muscle cells, VEGF was expressed by smooth muscle cells covering the lining of endothelial cells, both in and outside the brain. In cerebral capillaries lacking smooth muscle cells, endothelial cells were closely covered by VEGF-expressing foot processes of astrocytes, whereas capillaries were surrounded by VEGF-expressing processes of podocytes in the renal glomeruli. We also found that cultured cerebral microvessel endothelial cells do not express VEGF, whereas cultured cortical astrocytes do express VEGF.

It has recently been suggested that neurons, glia, and microvessels (capillaries) are organized into "neurovascular units," which are involved in the regulation of cerebral blood flow [1]. Within the neurovascular unit, the abluminal surface of the cerebral microvessels is almost completely covered by the foot processes of astrocytes [13,15], and this peculiar anatomical feature, which is proposed to be called the "gliovascular unit" [1], has been supposed to be the structural basis for the induction and maintenance of the blood–brain barrier by astrocytes [12]. The mechanism, however, by which the cerebral microvessels are covered by the astrocytic foot processes, has remained entirely unknown. On the other hand, it has been suggested that blood vessels in the brain are not silent bystanders as originally considered but active regulators that play crucial roles in neural development and functions [26]. In this respect, molecules that have crucial functions in both nervous and vascular systems have attracted keen attention recently, and the term "angioneurins" has been proposed [26]. Angioneurins are involved, to name a few functions, in the regulation of angiogenesis, blood–brain barrier integrity, vascular perfusion, neuroprotection, neuroregeneration, and synaptic plasticity. The most striking example of angioneurins is vascular endothelial growth factor A (VEGF), which was originally identified as a key regulator of angiogenesis [23] and has only recently been found to have important functions in the nervous system [26].

To our knowledge, VEGF expression in the neurovascular unit has never been examined. In this study, we examined VEGF expression in the neurovascular unit in the mouse brain, and found that VEGF was expressed by the processes of astrocytes, but not by neurons or endothelial cells.

ICR mice were purchased from SLC (Hamamatsu, Shizuoka, Japan). All procedures were performed in accordance with the guidelines for Animal Experimentation at Gunma University Graduate School of Medicine and were approved by Gunma University Ethics Committee.

Mouse cerebral astrocytes were prepared from the cerebral hemispheres of newborn (postnatal day 0–3) mice, as previously described [22]. In brief, the meninges were removed, and the cortical tissue was mechanically dissociated in α-minimum essential medium supplemented with 10% fetal bovine serum, 0.6% glucose, and 200 μg/ml penicillin/streptomycin (all obtained from Life Technologies Corp., Carlsbad, California). Dissociated cells were seeded into 100 mm non-coated tissue culture dishes (BD Falcon, Franklin Lakes, New Jersey). After growth of astrocytes, the cells were passaged on poly-L-lysine-coated 12 well slide glasses

Abbreviations: VEGF, vascular endothelial growth factor A; AQP4, aquaporin-4; αSMA, α-smooth muscle actin; GFAP, glial fibrillary acidic protein; RT-PCR, reverse transcription-polymerase chain reaction; SMC, smooth muscle cell; MVEC, microvascular endothelial cell.

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(Matsunami Glass Ind., Ltd., Kishiwada, Osaka, Japan) and maintained in 5% CO_2/95% air at 37 °C. Primary cultures of mouse cerebral microvascular endothelial cells (MVECs) were prepared from 8-week-old mice, as previously described for preparation of MVECs from rat brains [25], suspended in culture medium (EBM-2; Lonza Walkersville, Inc., Walkersville, Maryland) containing 4 μg/ml puromycin, plated onto collagen type I-coated 60 mm dishes (BD Falcon), and maintained in 5% CO_2/95% air at 37 °C. Forty-eight hours later, puromycin was removed from the culture medium.

Mice were anesthetized with diethyl ether ($C_2H_5OC_2H_5$) and perfused transcardially with 0.1 M phosphate-buffered saline (pH 7.4) containing 4% paraformaldehyde. The perfused brains, kidneys, and aortas were excised, fixed in the same fixative overnight at 4 °C, and cryoprotected with 30% sucrose in 0.1 M phosphate-buffered saline. All samples were embedded in Tissue-Tek OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and frozen at −80 °C. Frozen sections (10-μm thickness) were cut on a cryostat (CM3050S; Leica Microsystems GmbH, Wetzlar, Germany), mounted on precoated glass slides (Matsunami Glass Ind., Ltd.), and kept at room temperature for 1 h to air dry. The sections were stored at −80 °C until staining.

Primary antibodies for immunostaining included anti-VEGF rabbit polyclonal (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, California), anti-VEGF rabbit polyclonal (1:100, IBL-America, Inc., Minneapolis, Minnesota), anti-aquaporin-4 (AQP4) rabbit polyclonal (1:500, [2]), anti-AQP4 goat polyclonal (1:500, Santa Cruz Biotechnology, Inc.), anti-CD31 rat monoclonal (1:50, BD Biosciences Pharmingen, San Diego, California), anti-α smooth muscle actin (αSMA) mouse monoclonal (1:800, Sigma–Aldrich Co., St. Louis, Missouri), and anti-glial fibrillary acidic protein (GFAP) mouse monoclonal (1:400, Millipore Corp., Billerica, Massachusetts) antibodies. The cells or the sections were fixed with 4% paraformaldehyde for 10 min, incubated with 2% bovine serum albumin–phosphate buffered saline containing 0.4% Triton X-100 for 30 min, and incubated with the primary antibodies overnight at 4 °C. After washing with phosphate buffered saline, the cells or the sections were incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488-conjugated donkey anti-rabbit or anti-goat immunoglobulin G (1:200, Life Technologies Corp.), and rhodamine-conjugated donkey anti-rabbit, anti-rat, or anti-mouse immunoglobulin G (1:100, Jackson Immuno Research Laboratories, Inc., West Grove, Pennsylvania) for 1 h at room temperature. TO-PRO-3 (2.5 μM, Life Technologies Corp.) was used for nuclear staining of the cells. The cells or sections were washed with phosphate buffered saline, mounted in Vectashield (Vector Laboratories, Inc., Burlingame, California), and observed using a confocal laser-scanning microscope (LSM510Meta; Carl Zeiss GmbH, Jena, Germany).

The presence of VEGF in cultured astrocytes or cerebral MVECs was examined by reverse transcription-polymerase chain reaction (RT-PCR). Total ribonucleic acid was extracted using Trizol reagent (Promega, Madison, Wisconsin). Complementary deoxyribonucleic acid synthesis used the SuperScript III DNA synthesis kit (Life Technologies Corp.). Determination of *VEGF* gene expression used two specific primers synthesized based on the mouse *VEGF* sequence. The sequences for the forward and reverse VEGF primers were 5′-TGG ATG TCT ACC AGC GAA GC-3′ and 5′-AC AAG GCT CAC AGT GAT TTT-3′, respectively. RT-PCR was performed by denaturation at 94 °C for 45 s, annealing at 54.2 °C for 45 s, and extension at 72 °C for 45 s.

We first examined VEGF expression in the vasculature of the mouse brain. As shown in Fig. 1A and B, the cells positive for CD31, a marker for endothelial cells [8], were surrounded by processes positive for AQP4, a marker for astrocytes [19], confirming that the cerebral capillaries are covered by the foot processes of astrocytes [16,18]. We did not observe VEGF expression in the cell bodies of astrocytes (Fig. 1A). The CD31-positive endothelial cells did not express VEGF, as shown in Fig. 1C and D. In the larger vessels containing smooth muscle cells (SMCs), VEGF was expressed by the SMCs (cells positive for αSMA, a marker for SMCs [24]), as shown in Fig. 2F. We then examined whether neurons, essential constituents of the neurovascular unit, express VEGF. As shown in Fig. 1E–G, the cells positive for NeuN, a nuclear marker for neurons, did not express VEGF. In contrast, AQP4-positive astrocytic processes expressed VEGF, as shown in Fig. 1E–G, confirming that the endothelial cells in the neurovascular unit are covered by the perivascular foot processes of astrocytes. As indicated by the arrowheads in Fig. 1F and G, the AQP4-positive processes of astrocytes were sometimes also located in close proximity to NeuN-positive neurons.

For comparison, we also examined VEGF expression in the vascular system outside the brain. In the aorta, VEGF was expressed by the αSMA-positive SMCs (Fig. 2A), but not by the CD31-positive endothelial cells (Fig. 2B). In the kidney, VEGF was expressed by the αSMA-positive SMCs in larger vessels (Fig. 2C) and smaller vessels (presumably afferent or efferent arterioles, Fig. 2D), but not by the CD31-positive endothelial cells (Fig. 2D and E). As shown in Fig. 2E, capillaries in the renal glomerulus were surrounded by VEGF-expressing processes (arrowheads).

We further examined expression of VEGF in the MVECs prepared from the mouse brain and cultured mouse cortical astrocytes. As shown in Fig. 3A and B, VEGF was expressed by the cultured AQP4-positive astrocytes. This finding was confirmed by the expression of VEGF by the cells positive for GFAP, another marker for astrocytes, as shown in Fig. 3C. In sharp contrast to *in vivo* expression, where VEGF was expressed only in the perivascular and perineuronal processes of astrocytes, VEGF expression was not polarized within the cultured astrocytes (Fig. 3A–C). VEGF was not expressed by the MVECs prepared from the mouse brain, as shown in Fig. 3D. RT-PCR analysis confirmed VEGF expression by cultured astrocytes, but not by cultured MVECs, as shown in Fig. 3E.

Although astrocyte-derived VEGF is reported to stabilize vessels in the developing retinal vasculatures [21], to our knowledge, our study was the first to examine VEGF expression in the neurovascular unit in the mouse brain. Our study revealed that capillary endothelial cells in the mouse brain do not express VEGF under normal circumstances. Although it had been believed that endothelial cells do not express VEGF, it was subsequently revealed that these cells express VEGF under hypoxic conditions [13]. We are planning to examine VEGF expression by cerebral capillary endothelial cells when the animals are subjected to hypoxic stress. Our study also revealed that the perivascular and perineuronal processes of astrocytes express VEGF. We did not observe VEGF expression in the cell bodies of astrocytes, whereas VEGF expression was not polarized in cultured astrocytes. Localized expression in astrocytic foot processes also occurs for AQP4 [19] and Kir4.1, a potassium channel subunit [14]. These molecules may use the same transport vesicles for localization in the astrocytic processes. Additionally, the polarized distribution of these molecules may be induced by capillary endothelial cells or neurons. Co-culturing astrocytes with MVECs or neurons may give a clue to this problem. Cultured astrocytes are reported to express VEGF and increase its expression under hypoxic conditions [10]. So we are also planning to examine VEGF expression by astrocytes when the animals are subjected to hypoxic stress. VEGF stimulates its own production in MVECs from bovine retina and human dermis, but not in aortic macrovascular endothelial cells [3]. Our study revealed that capillary endothelial cells in the gliovascular unit are surrounded by VEGF-expressing foot processes of astrocytes, so the possibility that these endothelial cells may be induced to express VEGF by the paracrine VEGF from astrocytes is apparently not the case. Astrocytes may increase