Cerebral microvascular endothelial cell tube formation: role of astrocytic epoxyeicosatrienoic acid release

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Munzenmaier, Diane H., and David R. Harder. Cerebral microvascular endothelial cell tube formation: role of astrocytic epoxyeicosatrienoic acid release. Am J Physiol Heart Circ Physiol 278: H1163–H1167, 2000.—Cerebral microvascular endothelial cells (CMVEC) form tubes when cocultured with astrocytes (AS). Therefore, it appears that AS may be important in mediating angiogenesis in the brain. We hypothesized that AS modulate CMVEC tube formation by releasing a soluble factor. Thymidine incorporation in cultured CMVEC increased 305% when incubated with 50% conditioned AS medium for 24 h (control: 52,755 ± 4,838 counts per minute (cpm) per well, conditioned 161,082 ± 12,099 cpm/well, n = 8). Because our laboratory has previously shown that AS can produce epoxyeicosatrienoic acids (EETs), which are known mitogens, we investigated whether release of EETs by AS is responsible for tube formation in the CMVEC-AS coculture. AS were seeded on Lab-Tek slides, CMVEC were seeded on the AS the next day, and cultures were allowed to progress for another 5 days with and without cytochrome P-450 epoxyenase blockade by 17-octadecenoic acid (17-ODYA). Tube formation in cocultures receiving 17-ODYA was significantly inhibited compared with control (93.8%). These data suggest that tube formation requires the release of EETs by AS.

METHODS

Cerebral angiogenesis has been shown to be stimulated in adult patients by various perturbations such as hypoxia (9, 15, 18, 24), ischemia (14), and even by physical activity and motor skill learning (12). Certain studies have shown this increased vascularity to have a therapeutic effect (14, 20), suggesting that it may be an adaptive response to a need for improved local tissue oxygenation. In a study of patients with Moya Moya disease, angiogenesis was induced by dissecting a scalp artery free yet leaving it attached to its circulation, removing the skull immediately beneath the artery and laying the artery directly onto the surface of the brain (20). Revascularization between the scalp artery and the cerebral cortical vasculature was stimulated when perfusion to the area became impaired. The results of the study indicated that the angiogenesis improved cortical hemodynamics in these patients and their clinical symptoms improved or stabilized. Another study by Krupinski and co-workers (14) reported that increased angiogenesis after stroke correlated with increased survival. Thus several studies suggest that not only can the phenomenon of hypoxia or stroke-induced angiogenesis occur in adults, but that it is a beneficial adaptation to a stressful stimulus.

Although little is known about what signals stroke-induced angiogenesis (hypoxia, ischemia, metabolite excess, etc.), it is understood that astrocytes (AS) are in some way essential to the process (16, 22, 25, 28). Laterra and Goldstein (16) developed a coculture model whereby cerebral endothelial cells (EC) form capillary-like tubes only when cultured with AS. The mechanism by which AS may contribute to this phenomenon is unknown. In the present study, we used a modification of this model to define a role for specific cytochrome P-450 arachidonic acid metabolites in the process of endothelial tube formation in AS-cerebral microvascular endothelial cell (CMVEC) coculture.

Our laboratory and others have shown that AS release arachidonic acid cytochrome P-450 epoxyenase products known as epoxyeicosatrienoic acids (EETs) (1, 2, 19). EETs have been shown to have many mitogenic and cell-growth-related actions (4, 5, 10, 11, 17, 23). We hypothesize that EETs released by AS act as a paracrine factor to stimulate EC to undergo the process of cerebral angiogenesis in vivo.

In the present study, we use a modification of Laterra and Goldstein’s coculture technique (16) as an in vitro cerebral angiogenesis model to test the hypothesis that astrocytic release of EETs stimulates CMVEC tube formation. Data from this study may help to elucidate the complex interactions between these cell types in vivo.

Cerebral microvessel isolation. Cerebral capillaries were isolated using a modification of a technique by Lamanna et al. (15). For each preparation, three 4- to 6-wk-old Sprague Dawley rats were anesthetized with pentobarbital sodium (65 mg/kg ip). The cervical spinal cord was resected, the cranium removed and the brain gently harvested. Cerebral cortical tissue was dissected out, minced into 1-mm cubes, and placed in a glass homogenizer containing 5 ml of ice-cold HEPES-buffered salt solution with 0.9% glucose. Brain tissue was homogenized using a Teflon pestle that had been machined to allow a 0.25-mm clearance on each side. Homogenate was centrifuged at 1,000 g for 10 min at 4°C. The pellet was then resuspended in a HEPES-buffered salt solution containing 15% dextran and centrifuged twice at 5,000 g for 20 min at
4°C. The pellet was resuspended in HEPES buffer containing glucose and loaded on a glass bead column. The column was washed with ~500 ml of cold buffer over a 90-min period. Capillaries adhering to the beads were released by sharply shaking the beads in buffer in a capped-tube centrifuge several times. The supernatant was decanted and centrifuged at 35,000 g for 20 min at 4°C.

CMVEC dissociation and culture. The microvessel pellet was resuspended in a sterile solution of collagenase (500 µg/ml) in DMEM (BioWhittaker, Walkersville, MD) + 10% fetal bovine serum (FBS, Sigma, St. Louis, MO). After 10 min of incubation at room temperature, the suspension was vortexed and centrifuged at 1,000 g. The pellet was resuspended in a small amount of complete DMEM and added to a T-25 flask that had been incubated for 30 min with complete medium. Cultures were grown using RPMI + 20% FBS, 1% penicillin-streptomycin, 0.1% gentamycin. Cells were incubated at 37°C in a 95%-5% mixture of atmospheric air and CO₂. Medium was changed daily for the first 3 days and every third day subsequently. For all experiments proposed here, we used subconfluent first passage CMVEC ~7 days after passage.

Astrocyte dissociation and culture. AS were cultured from hippocampi of 2- to 3-day-old Sprague Dawley rat brains under aseptic conditions. Briefly, brain tissue was dissected free of meninges, cut into small pieces and transferred to a sterile dish containing 20 U/ml papain (Worthington Biochemical, Freehold, NJ) and 0.2 mg/ml cysteine (Sigma) dissolved in Earle’s balanced salt solution (GIBCO BRL, Grand Island, NY). Tissue pieces were incubated at 37°C for 40 min with gentle agitation and then washed three times in complete medium containing DMEM (GIBCO BRL) with 10% FBS (Sigma) and 1% penicillin-streptomycin solution (Sigma). The tissue was then dissociated by triturating with a serological pipette. The cell suspension was diluted with complete medium and seeded into 75-cm² flasks. AS were incubated at 37°C in 95% O₂-5% CO₂. The medium was changed after 2 days and twice a week subsequently. For all experiments proposed here, we used subconfluent first passage cells ~5 days after passage.

AS/CMVEC coculture. The coculture assay used is a modification of a model developed by Laterre and Goldstein (16). Primary AS were grown in culture for 1 wk after isolation. AS were trypsinized, reconstituted in DMEM + 10% FBS, and plated at a density of 80,000 cells/well in 4-well Lab-Tek cover glass chamber slides (Nalge Nunc International, Naperville, IL) coated with fibronectin. The next day, medium was removed, and CMVEC were plated at a density of 80,000 cells/well in RPMI + 20% FBS + ascorbate (50 µg/ml). Twenty-four hours after EC seeding, DMSO vehicle or 17-octadecynoic (17-ODYA) was added to a final concentration of 10 µM. Five days after the addition of CMVEC, cocultures were incubated with dil-acyl LDL (10 µg/ml) to fluorescently label EC and the number of tubes formed in each 9 mm × 20 mm well were quantitated using a fluorescence inverted microscope imaging system (Nikon Diapath and I mage-1 software, Universal Imaging, West Chester, PA).

Tritiated thymidine incorporation assay. CMVEC were seeded at a density of 25,000 cells per well in 24-well plates. Forty-eight hours later, cells were treated with AS medium (DMEM + 10% FBS), EC medium (RPMI + 20% FBS), or 50% AS medium conditioned by confluent AS for 72 h and 50% EC medium. After 18 h incubation, [³H]thymidine (2 µCi/well, NEN) was added and allowed to incorporate for 6 h. EC were washed with PBS and treated with TCA (15%) for 30 min at 4°C. Wells were washed gently with water and allowed to dry in a hood. Precipitate was solubilized in 1 ml of 1 N NaOH for 20 min at 37°C. An equal amount of 1 M HCl was added to neutralize solution, and the contents of each well were transferred to a scintillation vial. Five milliliters of scintillation fluid (Ecoscint, National Diagnostics, Atlanta, GA) were added, and samples were counted for 5 min. Results are expressed as counts per minute per well.

Statistical analysis. Pooled data values are reported as means ± SE. Significant differences were evaluated using a Student’s t-test. The coculture tube formation was analyzed using a paired t-test. In all cases, P < 0.05 was considered statistically significant.

RESULTS

To determine the effect of soluble factors released from AS on CMVEC, thymidine incorporation was measured in CMVEC that were treated with 50% fresh EC medium and 50% AS medium that had been conditioned by AS for 72 h. Both AS and EC media were used as controls to discount the possible effect of differences in FBS concentration. EC treated with AS-conditioned medium showed a dramatic increase in thymidine incorporation after 18 h of stimulation (Fig. 1).

AS-induced EC tube formation was initiated by seeding first passage CMVEC on a 24-h culture of first passage AS. Tube formation was evident within 48 h and continued to progress through the first week (Fig. 2, A-C). AS-conditioned medium alone did not stimulate CMVEC tube formation (data not shown). In this study, tube formation was allowed to proceed for 5 days at which time the process had stabilized and number of tubes formed could be quantitated.

Cocultures were treated with 17-ODYA (10 µM) or DMSO vehicle beginning 24 h after CMVEC seeding through the end of the experiment. This allowed CMVEC attachment to the AS monolayer but was before any visible tube formation. Previous reports from our laboratory and others have shown that 10 µM 17-ODYA in culture is sufficient to block all cytchrome

Fig. 1. Tritiated thymidine incorporation in primary cerebral microvascular endothelial cells (CMVEC) after 18 h of incubation with astrocyte (AS)-conditioned medium. AS medium, AS growth medium (DMEM + 10% fetal bovine serum (FBS)); endothelial cell (EC) medium. EC growth medium (RPMI + 20% FBS); 50% EC medium. 50% AS medium cond., 1:1 EC medium and AS medium conditioned by primary AS for 72 h. *Statistically significant difference between conditioned medium treatment group and either unconditioned medium control (n = 8, P < 0.05).
P-450 epoxygenase activity (7, 29, 30). Figure 3 summarizes the raw data from nine paired experiments. Because of potential variability in primary cultures, experiments were always done in a paired fashion. Both cell types were split from the same culture and seeded into vehicle and treated wells and differences in tube formation between these paired wells were assessed. Although some variability existed between experiments, tube production was always less in coculture with epoxygenase blockade than in those treated with vehicle. The pooled data are summarized in Fig. 4.

DISCUSSION

Cerebral angiogenesis is an intricate process that differs greatly from the analogous process in non-neural tissues. A significant difference is the presence and involvement of AS in angiogenesis within the nervous system. AS are a complex cell type with a myriad of known functions. Juxtaposed between the neurons and the capillaries, they are in a strategically effective position to act as sensors of the microenvironment and to maintain homeostasis. Their foot processes surround the capillaries, helping the EC to form and maintain the blood-brain barrier (13, 27). This close association of AS with the EC that form the capillaries may also be important in angiogenesis. In fact, Schnitzer (22) made the observation that regions of the retina devoid of AS are also avascular. Furthermore, AS implanted in the brain parenchyma stimulate angiogenesis toward the implant (25). Thus it is clear that AS have a unique and necessary function in the process of cerebral angiogenesis.

In the present study, we utilized an in vitro technique that further demonstrates the functional interplay between AS and EC. CMVEC form a monolayer when grown in solo culture. When grown in the presence of AS-conditioned medium, thymidine incorporation is increased dramatically but CMVEC still form monolayers. CMVEC grown on an AS monolayer form capillary-like tubes, indicating that AS direct EC tube formation

Fig. 2. A: CMVEC after 3 days in coculture with primary cultured AS. CMVEC first cluster together and then begin to migrate out into long projections. B: after 5 days in coculture, CMVEC tubes have begun to form and become more defined. C: after 7 days, tube formation has peaked and tube morphology has stabilized.

Fig. 3. Paired-study quantitation of EC tube formation after 5 days in coculture after treatment with 17-octadecynoic acid (17-ODYA, 10 µM) or DMSO vehicle. Developed tubes or branches within each 9 mm × 20 mm chamber were manually counted.

Fig. 4. Pooled results of EC tube formation in AS-EC coculture after treatment with 17-ODYA (10 µM) or DMSO vehicle. *Statistically significant difference between treatment group and vehicle-treated control (n = 9, P < 0.05).
in some way. When EETs production was blocked by 17-ODYA, CMVEC tube formation was greatly inhibited. This suggests that EETs are involved in the process of tube formation.

Although we have focused our attention on the role of EETs as soluble mediators of EC tube formation, it is certainly possible that other factors such as vascular endothelial growth factor (VEGF) or epidermal growth factor (EGF) are also involved in the process of tube formation in this model and cerebral angiogenesis in vivo. VEGF is produced by astrocytomas and has been shown to have a key role in glioma angiogenesis. Also, EGF has been shown to stimulate EETs release in a cell line transfected with a $14,15$-epoxygenase (5). It is possible that VEGF and/or EGF are also involved in the process of tube formation in this model and cerebral angiogenesis in vivo though no evidence to that effect has been reported to date. In fact, it is likely that many mediators are involved in this complex and potentially critical process. However, because epoxygenase blockade effectively inhibited tube formation in this study, EETs apparently provide at least a permissive function in this process. Growth factors may act as controllers or initiators in vivo, stimulating EETs release in response to hypoxic conditions. This is an interesting area of research that warrants further study.

The cytochrome P-450 inhibitor used, 17-ODYA (17-octadecynoic acid), is a potent epoxygenase inhibitor ($IC_{50} = 100$ nm) that also inhibits cytochrome P-450 $\omega$-hydroxylase activity but has no affect on cyclooxygenase activity (7, 29, 30). The $\omega$-hydroxylase enzyme is primarily responsible for the production of 20-hydroxyeicosatetraenoic acid (20-HETE). This pathway is most prevalent in vascular smooth muscle, liver, and kidney (3, 6, 8, 26). In fact, neither AS nor EC have been shown to produce 20-HETE (4, 19). Thus this cross-reactivity of the inhibitor is not a cause for concern in this study. In addition, no study to date has shown any effect of 17-ODYA on the activity of non-cytochrome P-450 enzymes.

Because the inhibitor is added to the coculture, it can theoretically inhibit epoxygenase activity in both AS and CMVEC cell types simultaneously. Although EC from the vasculature of other tissues have been shown to produce EETs (21), cerebral EC have not been shown to have this capability. In addition, this study demonstrates that AS do release some factor that stimulates mitogenesis in cerebral EC. Thus we assume that EETs released by the AS are acting on the EC and that blockade of the epoxygenase enzyme is blocking the release of EETs from the AS.

Inhibition of endogenous epoxygenase activity was used rather than exogenous application of EETs for a variety of reasons. First, this study investigates a long-term process of tube formation that takes several days to complete. To stimulate tube formation with exogenously applied EETs, they would have to be continually supplemented over that period because of the short half-life of EETs. This would be extremely expensive and difficult to do in a controlled fashion. Second, although $14,15$-EET has been shown to be the most potent mitogen in some systems (4, 5) and to be the major epoxygenase metabolite in cultured AS (2), we do not yet know the relevant EETs regioisomers involved in this model. Lastly, we know from our conditioned media studies that more than EETs stimulation of EC alone is required for tube formation. Without physical contact between the EC and AS, it is unlikely that tube formation would occur.

This study demonstrates the necessary role of astrocytic EETs in EC tube formation as assessed by epoxygenase inhibition. However, release of the soluble factor alone is not sufficient because treatment with astrocyte-conditioned (cell-free) medium caused increased thymidine incorporation but not tube formation. Clearly, AS must serve yet another function. Further studies are ongoing to determine the role of physical interactions between the cell types in the process of tube formation and cerebral angiogenesis.

In summary, we have shown that a soluble factor released by cultured AS stimulates thymidine incorporation in CMVEC. Furthermore, when grown with AS, CMVEC form capillary-like tubes that are dependent on physical association of the two cell types. This tube formation is blocked by inhibition of EETs formation, suggesting that astrocytic release of EETs is a necessary step in this in vitro process and perhaps in angiogenesis as well.

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REFERENCES


3. Aoyama T, Hardwick J, Imaoka S, Funae Y, Gelbouf HV, and Gonzalez FJ. Clofibrate-induced rat hepatic IVA1 and IVA3 catalyze the $\omega$- and ($\omega$-1)-hydroxylation of fatty acids and the $\omega$-hydroxylation of prostaglandin E$_1$ and F$_2\alpha$. J Lipid Res 31: 1477–82, 1990.


