Endothelial Cell Culture From Human Cerebral Cavernous Malformations

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Background and Purpose—The cerebral cavernous malformation (CCM) is a common and frequently unrecognized cause of stroke and epilepsy. It consists of blood-filled caverns lined by endothelial cells (EC) and devoid of mature vessel wall structure. Cultured EC obtained from CCM may express phenotypic and genotypic alterations contributing to CCM pathogenesis. We report the first successful isolation and growth in vitro of primary EC lines from human CCM lesions.

Methods—We developed a procedure for the isolation and growth of EC from human CCM, confirmed their EC origin by a panel of molecular markers, and determined by immunocytochemistry the basic expression patterns of 6 transmembrane receptor protein kinases comparing brain, skin, and CCM primary EC lines grown identically.

Results—Several CCM EC lines were established from 2 patients after we treated the excised specimens with 0.3% trypsin/1% EDTA, selective cloning, and growth in MCDB107 containing 0.3 g/L heparin, 0.15 g/L endothelial cell growth supplement, and 15% FBS. The CCM EC showed contact inhibition and a rounded cobblestone appearance. The cells expressed CD31, CD105, von Willebrand factor, and binding sites for *Ulex europaeus* agglutinin, type 1 and acetylated LDL. They showed low levels of Flt-1, Flk-1, transforming growth factor (TGF)-β RI, and TGF-β RII expression but stained strongly with antibodies against Tie-1 and Tie-2.

Conclusions—Cultured CCM EC retained their endothelial phenotype. Brain, skin, and CCM EC lines did not significantly differ in their staining patterns with antibodies against Flt-1, Flk-1, TGF-β RI, TGF-β RII, Tie-1, and Tie-2. These cell lines will assist in defining molecular phenotype and genotype alterations in association with CCM. (*Stroke*. 1998;29:2426-2434.)

Key Words: angiogenesis • cavernous malformations • endothelium • growth factors

The cerebral cavernous malformation (CCM) is a common vascular lesion affecting ≈0.5% of the population. It is a frequently unrecognized cause of hemorrhagic stroke and other neurological symptoms. The CCM lesion is characterized by blood-filled caverns lined by a single layer of endothelial cells (EC). The cavern walls are devoid of mature vessel wall structure, elastin, and smooth muscle cells (SMC).

The CCM lesions are known to develop in the setting of genetic predisposition with multiple lesions inherited in an autosomal dominant mendelian mode, recently linked to >1 gene locus. The abnormal gene product(s) responsible for the disease has not been identified, and the molecular factors that mediate lesion genesis or hemorrhagic progression are not known. It is hypothesized that CCM may result from errors occurring during blood vessel formation or maintenance, both involving EC-mediated mechanisms. We speculate that cultured EC obtained from CCM lesions may express phenotypic and genotypic alterations contributing to CCM pathogenesis. Such cells can serve as a useful experimental model for studying the biology of CCM lesions in vitro and may yield molecular genetic information relevant to host or somatic mutations mediating lesion genesis. There have been to date no reports of successful isolation or maintenance of EC cultures from CCM lesions.

We report here for the first time the successful isolation and growth in vitro of primary EC lines from excised human CCM lesions. We compare cell morphology, growth patterns, and protein expression of a panel of EC-associated antigens and angiogenesis-related receptors in several primary EC lines from CCM lesions and from human skin and brain grown under identical conditions.

Methods

Patients and Tissue Specimens

Surgery was performed for traditional indications unrelated to this research project. There was no sex, age, or race bias in the selection of cases for surgical treatment. This study was approved by an institutional review committee, and all subjects provided informed consent.

The CCM were surgically excised by the senior author (I.A.A.). At the time of surgical biopsy, a 10×4×4-mm fragment of scalp dermis was also obtained along the line of the craniotomy incision for isolation of skin EC. Human brain EC were cultured from approxi-
mately 10-mm³ cortical pial fragments of excised temporal lobectomy specimens performed for intractable epilepsy by the Yale Epilepsy Surgery Program (courtesy of Professor D.D. Spencer, New Haven, Conn). The excised tissue specimen was divided with contiguous sections submitted for routine clinical neuropathologic examination. All cell cultures were handled anonymously from the time of harvesting. After its resection, the tissue specimen was processed immediately for cell culture.

EC Isolation

Specimens from a CCM lesion were rolled onto pieces of sterile gauze to remove as much adherent blood as possible, rinsed with fresh sterile Hanks’ balanced salt solution (HBSS) without calcium, magnesium chloride, magnesium sulfate, and sodium phosphate and transferred into a tissue culture dish (100 × 20 mm) (Becton Dickenson Labware) filled with 10 mL of 0.3% trypsin/1% EDTA, or collagenase, type II, Clostridium histolyticum (1 mg/mL) (Calbiochem). The tissue culture dish was placed at 37°C for 30 minutes, then trypsin activity was blocked with FBS (20% final concentration). To extrude the endothelial cells, the predigested tissue was extensively massaged with a metal spatula. The resulting suspension was triturated extensively with the use of a glass Pasteur pipette. The cell suspension was centrifuged at 2000 rpm/30g for 5 minutes at 20°C on a Beckman TJ-6 centrifuge. The cell pellet was washed twice, first with HBBS supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin, 0.5 μg/mL amphotericin B, and 20% FBS, and then with complete growth medium. The cells were finally suspended in fresh MCDB107 and transferred into a fibronectin precoated 100-mm tissue culture dish.

After selective passaging based on EC morphology, the isolated cell populations were plated on uncoated tissue culture polystyrene plastic vials or vials precoated with fibronectin or gelatin. After trials in 7 cases, we empirically identified the conditions that permitted the isolation of CCM EC from the 2 reported cases and that supported the growth of primary CCM EC lines in vitro. The CCM tissue sample from the first patient was treated with collagenase, and the tissue sample from the second patient was treated with trypsin/EDTA. The first growing cell colonies appeared 8 to 10 days after initial seeding. Seven cell colonies with cobblestone morphology were cloned from each clinical case, and 1 clone from each case was used in this study. CCM EC lines grew better in complete MCDB107 medium (see below) and on fibronectin precoated polystyrene surfaces.

EC from brain microvessels were isolated by a modification of the method of Vinters et al.2 Briefly, the cortical brain tissue was cleaned from the adherent blood, freed from the meninges, rinsed with fresh HBSS, chopped into 1-2 mm pieces, treated with collagenase/ dispase from Vibrio alginolyticum/Bacillus polymyxa (Boehringer Mannheim) (1 mg/mL) for 60 minutes at 37°C. The predigested tissue was triturated with a 10-mL pipette for 5 minutes. The tissue suspension was then filtered consecutively through a 183-μm and a 118-μm Nitex nylon screen, and the microvessels trapped by the latter were rinsed extensively with serum-free growth medium. The screen was placed upside down in a fibronectin precoated 100-mm tissue culture dish filled with complete growth medium, agitated quickly in the medium to free the attached microvessels, and then discarded. For this study, we used brain microvessels EC obtained from 2 patients. The first growing cell colonies of brain EC appeared 1 to 2 weeks after initial seeding. Only colonies with cobblestone appearance were cloned further.

Skin microvessels were isolated by adapting the technique of Karasek.3 Briefly, the dermal layer of the skin was separated from the epidermal layer by means of a surgical blade, cut into 2-mm pieces, and treated with 0.3% trypsin/1% EDTA for 30 minutes at 37°C. To extrude the microvessels, the partially digested skin pieces were extensively massaged by means of a small wooden spatula. The collected microvessels were washed extensively by centrifugation and finally resuspended in complete growth medium. After trials in 12 cases, we were able to isolate and grow EC lines from 5 adult skin EC specimens.

Cell Culture

All primary cell cultures were grown in complete MCDB107 medium. MCDB107 was prepared by supplementing MCDB105 medium (Sigma Chemical Co) with 0.015 g/L glycine (2 × 10⁻⁴ mol/L) and 0.15 g/L KCl (2 × 10⁻¹ mol/L).³ Complete MCDB107 medium contained in addition 0.3 g/L heparin, 0.15 g/L endothelial cell growth supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 15% heat-inactivated FBS. All primary EC cultures were grown in tissue culture polystyrene vials precoated with fibronectin (from bovine plasma, purchased from Sigma Chemical Co) at concentration 0.01 μg/mm². All primary cell cultures were grown at 37°C in humidified air saturated with 5% CO₂. The medium was exchanged initially after 7 days in culture and then twice weekly. EC colonies were distinguished from SMC colonies by their appearance. EC had cobblestone shapes and showed cell contact inhibition under phase-contrast microscopy. In contrast, SMC were spindle-shaped and grew in bundles in a characteristic “hills and valleys” pattern.6 Both types of colonies were expanded by trypsinization with the use of cloning cylinders (Bel-Art Products). The cell cultures were left to grow to near confluence and then split 1:2.

For this study, we established 2 primary EC lines from each source: normal brain, scalp skin, and CCM.

Cell Characterization

Antibodies

Monoclonal antibodies (MAB) against human von Willebrand factor (vWF) (clone F8/86, anti-vWF),¹ CD31 (platelet–endothelial cell adhesion molecule-1; PECAM-1) (clone IC70A, anti-CD31),¹² glial fibrillary acidic protein (GFAP) (clone 6F/2, anti-GFAP),¹ and α-smooth muscle actin (α-SMA) (clone 1A4, anti-α-SMA)¹⁴ were purchased from DAKO A/S, Glostrup, Denmark. MAB against human smooth muscle myosin (SMM) (clone hSM-V, anti-SMM)¹³ was from Sigma, St Louis, Mo. Dr M. Letarte (Toronto, Ontario, Canada) kindly provided a MAb against CD105 (endoglin) (clone SN66 hours, anti-CD105). Rabbit polyclonal serum against Ulex europaeus agglutinin, type 1 (anti–UEA-1) and U. europaeus agglutinin, type 1 (UEA-1) were purchased from DAKO. Affinity-purified and specificity-tested rabbit polyclonal antibodies against Flk-1 (C-1158), Flt-1 (C17), Tie-1 (C-18), Tie-2 (C-20), transforming growth factor (TGF)-β RI (V-22), and TGF-β RI (C-16) and their corresponding blocking peptides were obtained from Santa Cruz Biotechnology, Inc, Santa Cruz, Calif.

Immunocytochemistry

Two primary EC lines of each—normal brain, scalp skin, and CCM—were tested for antigen expression by immunocytochemistry. The tested cells, not exceeding passage number 4, were seeded at 5 to 10 × 10⁴ cells per chamber on fibronectin precoated (0.05 μg/mL) 4-chamber Labware (Nalge Nunc International) and allowed to recover overnight. For testing with MAB against vWF, CD31, GFAP, α-SMA, and SMM, the cells were fixed with acetone/methanol (2:3) for 10 minutes at −20°C. Endoglin (CD105) expression was determined on cells fixed with 3% paraformaldehyde/2% sucrose in PBS for 20 minutes at room temperature because of the detrimental effect of methanol on SN66 epitope (M. Letarte, PhD, written communication, 1997). All rabbit polyclonal antibodies were tested on methanol-fixed cells (10 minutes at −20°C). All primary antibodies were used at their optimal dilution, as determined on human umbilical vein endothelial cells (HUVEC) and isolated SMC. RPM11640 medium supplemented with 10% FBS served as a negative control to the anti-vWF, anti-CD31, anti-GFAP, and anti–α-SMA (all supplied as supernatants) and anti-SMM and anti-CD105 antibodies. UEAI receptor expression was determined on cells incubated first with UEAI-1 for 30 minutes at room temperature. The cells were washed and then incubated in parallel under identical conditions with the anti–UEA-1 antibody and anti–UEAI-1 that was preincubated with 0.2 μL/mL 2-fucose (Sigma Chemical), used as a lectin blocker, for 2 hours at room temperature. Corresponding blocking peptides were used as specificity controls for all other rabbit polyclonal antibodies, except for Flk-1. For neutralization,
each rabbit antibody, at its optimal dilution, was reacted with a 10-fold excess of peptide antigen in PBS for 2 hours at room temperature. The neutralized antibody was then allowed to react with the cells in parallel to the unblocked antibody. The primary MAb anti-vWF, anti-CD31, anti-GFAP, and anti–α-SMA were used in dilution 1:100, anti-SMM in 1:500, and anti-CD105 in 1:4000. The rabbit polyclonal sera against Flk-1, Flt-1, Tie-1, TGF-β RI, and TGF-β RII were used in dilution 1:200, while anti–UEA-1 and anti–Tie-2 sera were used in dilution 1:400. All primary antibodies were allowed to react with their respective antigens for 30 minutes at room temperature. Antibody reactivity was detected with a Vectastain Elite ABC kit, mouse IgG or rabbit IgG, followed by a DAB Substrate kit for peroxidase (all purchased form Vector Laboratories, Inc). Finally, the cells were counterstained with Gill’s hematoxylin, according to instructions of Vector Laboratories, mounted, and observed and photographed with a BH-2 Olympus research microscope.

**Acetylated LDL Uptake**

Cultured cell lines were tested for their ability to scavenge acetylated LDL.16 The cells were seeded at $5 \times 10^4$ cells per chamber in a fibronectin-precoated (0.05 µg/mm²) 4-chamber Laboratory-Tek Permanox chamber slide system (Nalge Nunc International) and allowed to recover overnight. Acetylated LDL labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL) (Biomedical Technologies, Inc) was added directly to the culture medium at a final concentration of 10 µg/mL, and the cells were incubated at 37°C for 4 hours. The cells were washed extensively with HBSS, fixed with 3% paraformaldehyde for 20 minutes at room temperature, and rinsed with distilled water. The gasket was removed, and the slide was mounted in Fluoromount-G medium (Southern Biotechnology Associates, Inc). Similarly labeled cultures of positive (HUVEC) and negative (SMC, fibroblasts) cell types were included as negative controls. Dil-Ac-LDL uptake was visualized on a BH-2 Olympus microscope that used standard rhodamine excitation equipped for epifluorescence.

**Results**

Under observation with a phase-contrast microscope, isolated CCM cells grew initially in separate colonies with round borders and cobblestone appearance. Those colonies contained mostly rounded cells that were larger than brain and skin EC (Figure 1, panels 1A and 1B) but also included some extended, irregular cells with wide cytoplasmic protrusions. CCM EC cells showed signs of contact inhibition when reaching confluence. Cultured CCM EC were larger than brain (Figure 1, panels 2A and 2B) and scalp skin EC (Figure 1, panels 3A and 3B) and more rounded in appearance. CCM cells cultured from case 1 appeared slightly larger than those grown from case 2. Subconfluent brain and dermal EC cultures formed islands bordered by crescent-shaped cells, while CCM cells grew separated from one another. CCM EC divided faster when maintained at higher densities and also before they reached their sixth passage. At lower cell densi-
ties as well as after the sixth passage, our CCM cultures consisted mostly of nonproliferating, degenerating, or dying cells. We were able, however, to keep some of our primary CCM EC cultures continuously for 6 months, at which time they consisted of mostly bizarre-looking, nonproliferating cells. When frozen and kept in liquid nitrogen (between their second and fourth passages), CCM EC grew well after thawing and expanding under our standard conditions. Such cells did not lose their EC phenotype after their cryopreservation (not shown) and could be used as an alternative to freshly isolated CCM cells.

All primary EC lines established from brain, skin, and CCM lesions were grown under identical conditions, and cells that did not exceed their fourth passage were screened for EC-associated molecular markers. Almost all isolated CCM EC expressed CD31 (PECAM-1) (Figure 2, panel 1C), were strongly stained with a MAb against CD105 (endoglin) (Figure 3, panel 1B), and carried binding sites for UEA-1 (Figure 3, panel 2B). Anti-CD31, anti-CD105, and anti-UEA-1 antibodies stained the cultured CCM cells uniformly, without apparent differences in intensity between the individual cells of the same line (Figures 2 and 3). Most cultured brain and skin cells stained with the anti-CD31 antibody (Figure 2, panels 2C and 3C), and we could not detect strong differences in staining intensity with that antibody between brain, skin, and CCM cells in vitro (Figure 2). Brain, skin, and CCM EC grown in vitro demonstrated structural heterogeneity when fixed and stained with an antibody against CD31 (Figure 2, panels 1C, 2C, and 3C), an antigen expressed on cell surface membrane of EC. The staining pattern showed that cultured CCM EC were of different shape and larger than their brain counterparts, which themselves outsized skin EC.

An anti-vWF antibody stained more than half of the cultured CCM cells. That staining was located both diffusely and in the perinuclear cytoplasm in distinct rod-shaped granules, although with varying intensity between the cells of the same line (Figure 2, panel 1B). More than half of the cultured brain cells and almost all skin EC stained with the anti-vWF antibody in the same granular pattern (Figure 2, panels 2C and 3C). We were unable, however, to determine the exact number of anti-vWF–positive cells among brain, skin, or CCM lines because of the varying staining intensity between the cells of the same line. CCM cell lines grown in vitro incorporated Dil-Ac-LDL as brain and skin EC did at the same conditions (Figure 2, panels 4A, 4B, and 4C). None of the studied CCM cell lines stained significantly with the MAb against α-SMA, SMM, or GFAP (not shown), which

Figure 2. Cultured EC isolated from CCM express 2 EC-associated antigens, vWF (1B) and CD31 (PECAM-1) (1C), similarly to cultured EC from brain (2B and 2C, respectively) and skin microvessels (3B and 3C, respectively), as determined by immunocytochemistry using a Vectastain Elite ABC kit (1A, 2A, and 3A, corresponding negative controls). All 3 types—cultured CCM EC (4A), EC isolated from brain (4B), and scalp skin microvessels (4C)—are capable of Dil-Ac-LDL uptake, as visualized by epifluorescence microscopy using rhodamine excitation. Bar=50 μm.
are markers for SMC (and pericytes) and astrocytes, respectively. Close to 10% of the cells of our brain lines and <5% of the cells of our skin lines stained with the antibodies against α-SMA and SMM (not shown). No cells from the primary brain and skin cell lines stained with the anti-GFAP antibody (not shown).

Primary EC cultures established from brain, skin, and CCM lesions all expressed low levels of vascular endothelial growth factor (VEGF) receptors, VEGF-R2 (Flk-1) and VEGF-R1 (Flt-1) (Figure 4, panels 3B and 4B, respectively). Similarly, they all expressed low levels of TGF-β receptors, types I and II (TGF-β RI and TGF-β RII) (not shown). In contrast, cultured CCM EC, as well as EC cultures from brain and skin blood vessels, expressed higher levels of both Tie-1 and Tie-2 receptors (Figure 4, panels 1B and 2B, respectively). Anti–Tie-1 and anti–Tie-2 staining patterns were almost identical, both antibodies strongly staining CCM EC in a characteristic fusiform pattern (Figure 4, panels 1B and 2B). That staining could be specifically abrogated by preincubating those antibodies with their corresponding blocking (immunizing) peptides (Figure 4, panels 1A and 2A, respectively). We observed the same staining pattern for brain and skin EC, as well (not shown). We found empirically, however, that MCDB107 medium containing 0.3 g/L heparin, 0.15 g/L endothelial cell growth supplement, and 15% heat-inactivated FBS supported the growth of those cells. Ham’s MCDB107 medium has been initially developed for the clonal growth of human diploid fibroblasts9 and later adapted for the maintenance of EC. After establishing the ideal growth conditions for the maintenance of CCM EC, we used identical conditions for the growth of the primary EC lines established from brain and scalp skin biopsies.

After 2 to 3 weeks in culture, most CCM cell colonies contained 2 morphologically different cell populations. One had a polygonal spindle-shaped appearance with a rapid growth rate, the other a rounded appearance with a lower growth rate. Both populations had a vesicular cytoplasm and a round nucleus with several prominent nucleoli, and both retained their EC phenotype (see below). A similar dual morphology was reported for EC isolated from human bone marrow.19 Vascular EC in vitro exhibit high morphological heterogeneity depending on the type, size, and origin of the vessel from which they had been isolated.20,21 Interestingly, the rounded CCM cells in culture also resembled in appearance the “giant cells” seen in oncogene transformed (immortalized) human EC cultures.22 In a mouse model, expression of the polyoma virus middle T oncogene was associated with a profound subversion of normal vascular development, which resulted in the formation of endothelial tumors termed hemangiomas, which represent cystic structures resembling CCM in humans.23

Discussion
Reports about the culturing of EC from human intracranial vascular malformations are scarce. Here we describe for the first time a simple protocol for the isolation and growth in vitro of EC from human CCM. This protocol involved a modification of the technique used by Rhoten et al17 for EC culture from brain arteriovenous malformations. CCM lesions consist of caverns filled with blood and thrombi at varying stages of organization. The excised tissue was therefore rinsed extensively with HBSS and rolled onto pieces of sterile gauze to remove as much adherent blood as possible. Different tissue processing protocols, enzymatic treatments, growth media, and support conditions were reported to support the growth of brain EC in vitro.18 Most of those conditions have been determined empirically. In our protocol, we first minced the CCM specimens and then treated them with 0.3% trypsin/1% EDTA or 1 mg/mL collagenase, type II for 30 minutes. We found that trypsin/EDTA treatment gave a better cell yield and viability (not shown). The small sizes of CCM specimens obtained from surgery did not permit us to conduct an extensive analysis of the growth requirements of primary CCM EC. We found empirically, however, that MCDB107 medium containing 0.3 g/L heparin, 0.15 g/L endothelial cell growth supplement, and 15% heat-inactivated FBS supported the growth of those cells. Ham’s MCDB107 medium has been initially developed for the clonal growth of human diploid fibroblasts9 and later adapted for the maintenance of EC. After establishing the ideal growth conditions for the maintenance of CCM EC, we used identical conditions for the growth of the primary EC lines established from brain and scalp skin biopsies.
We did not encounter the difficulties others have met in growing EC isolated from adult skin. After using the same original isolation technique, we did not encounter the need to supplement our standard MCDB107 medium with cAMP, adenyl cyclase activators, thymidine, hypoxanthine, conditioned tumor medium, or pooled human serum to grow the isolated skin EC. In addition, we did not observe any loss of epithelioid morphology or phenotypic changes in our primary skin EC cultures by using standard MCDB107, as reported by the same authors.

We found that primary EC lines established from CCM retained their endothelial phenotype and may serve as a reliable in vitro model of those lesions. Cultured CCM EC expressed several well-established endothelial molecular markers. While none of them is entirely specific for EC, the simultaneous expression of several such molecules by the same cell population is highly suggestive of an endothelial phenotype. Most of our cultured CCM cells expressed binding sites for UEA-1, as did our cultured brain and skin EC. Another protein, vWF (factor VIII–related antigen) is expressed at significant levels only in EC, in megakaryocytes, and in the human syncytiotrophoblast. We were able to localized vWF both diffusely in the perinuclear cytoplasm and in distinct rod-shaped granules of CCM EC, an expression pattern characteristic for that molecule in cultured human EC. Only approximately half of our CCM cultures stained for vWF, a lower proportion than that we found in the brain and skin EC cultures. While vWF remains one of the most specific EC markers, a great heterogeneity in its expression has been reported between EC isolated from large vessels as opposed to microvessels, microvessels from different sources, and fresh as opposed to late-passage cultures.

Most cultured CCM EC, as well as brain and skin EC, stained with anti-CD31 antibody. CD31 (PECAM-1) is a member of the immunoglobulin superfamily that is expressed on the surface of circulating platelets, monocytes, neutrophils, and selected T-cell subsets. It is a major
constituent of the EC intercellular junction, mediates a homophilic adhesion between those cells, and is involved in transmembrane signal transduction and integrin affinity modulation, but its real function in EC still remains obscure.\textsuperscript{32} EC of all vessels, including capillaries, veins, and arteries in adult and embryonic human tissues as early as 4 weeks of gestation, as well as EC in culture, express CD105 (endoglin).\textsuperscript{33} We found that most cultured CCM EC also expressed CD105. The phenotype of the primary CCM EC lines included in this study also corresponded well to the immunologic phenotype recently reported for endothelial cells, as established through a large panel of antibodies directed against cell surface antigens.\textsuperscript{34} The primary EC lines that we established from CCM lesions, brain, and scalp skin were also able to incorporate Dil-Ac-LDL, which is a function attributed to EC and macrophages.\textsuperscript{16}

We obtained final confirmation for the EC origin of our primary CCM cell lines by determining the expression of 4 other EC selective markers by those cells, namely, the VEGF receptors VEGF-R1 (Flt-1)\textsuperscript{35} and VEGF-R2 (Flk-1/KDR),\textsuperscript{36} and Tie-1 and Tie-2/Tek receptors.\textsuperscript{37} Most cells of our CCM, brain, and skin EC lines expressed all 4 of these receptors.

Cultured CCM EC expressed low levels of VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR), which did not differ significantly from those of brain and skin EC lines. VEGF-R2 has been implicated in the determination of a hematangioblast progenitor and then of EC.\textsuperscript{38} We were much more interested in determining the levels of expression of VEGF-R1 (Flt-1) because of its reported role in tube and functional vessel formation,\textsuperscript{39} which are clearly disrupted in CCM lesions built of caverns lined by EC but lack the structure of mature vessel elements. By using an immunocytochemistry technique, we were indeed able to confirm the expression of the 2 receptors. However, the detection of any subtle differences in the expression of Flt-1 between normal and CCM EC will require more sensitive quantitative techniques, such as flow cytometry (fluorescence-activated cell sorting analysis). This can also be used to determine precisely the differences in expression of CD105 (endoglin), Tie-1, Tie-2 (Tek), TGF-β RI, and TGF-β RII, none of which showed gross differences in staining intensity between CCM EC and brain and skin EC when tested by immunocytochemistry.

It was established recently that the EC putative receptor tyrosine kinases Tie-1 and Tie-2 (Tek) play important roles in angiogenesis and remodeling, 2 steps that are subsequent to the action of VEGF. Tie-1 is involved in the fluid exchange across capillaries and in hemodynamic stress resistance, while Tie-2 controls the capability of EC to recruit stromal cells to encase the endothelial tubes so as to stabilize the structure and function of blood vessels.\textsuperscript{40} Unlike VEGF, angiopoietin-1, the first identified ligand for Tie-2, is not a mitogen for EC, nor does it induce tube formation. Instead, it regulates the recruitment of pericytes and SMC precursors to the cell wall.\textsuperscript{41} An angiopoietin-1 relative, termed angiopoietin-2, was identified recently and shown to be a naturally occurring antagonist for angiopoietin-1 and Tie-2.\textsuperscript{42} They both contain 2 immunoglobulin-like domains, 3 epidermal growth factor–like domains, and 3 fibronectin III–like repeats in the extracellular region, followed by a transmembrane region and a short kinase insert sequence and C-terminal tail in the intracellular part.\textsuperscript{43}

Most cultured EC from brain, skin, and CCM stained strongly with the affinity-purified rabbit polyclonal antibodies against Tie-1 and Tie-2. That reactivity was specifically abrogated by preincubating the antibodies with their corresponding immunizing peptides. We did not observe significant differences in Tie-1 and Tie-2 expression between brain, skin, and CCM EC. Surprisingly, instead of reacting exclusively with cell surface membrane proteins, anti–Tie-1 and anti–Tie-2 antibodies stained single-cell preparations of cold methanol-fixed EC mostly in a fibrillary-like pattern. We are aware that such staining pattern may have been the result of the fixation procedure used. However, cell fixation and permeabilization with cold acetone and methanol was the procedure recommended by the manufacturer of the antibodies, and it is widely used and considered reliable.\textsuperscript{44} Such staining pattern may also suggest colocalization of Tie-1 and Tie-2 with the microtubular structures or intermediate filaments, most probably vimentin. Both antibodies were produced against short amino acid stretches at the carboxy (cytoplasmic) terminus of Tie-1 and Tie-2, and a significantly truncated version of the Tie-2 protein, an isoform completely lacking the extracellular domain but retaining its kinase insert, has been found.\textsuperscript{45} A similarly truncated form of the platelet-derived growth factor receptor that lacks much of the extracellular domain has also been found in mouse carcinoma and embryonic stem cells, and it may function independently of its ligand.\textsuperscript{46} A close association of an enzymatically active nonreceptor tyrosine kinase, c-src, with tubulin, the main building element of the microtubules, has been recently implied in cell substrate recognition.\textsuperscript{47} Several other nonreceptor protein-tyrosine kinases were implicated in controlling cellular responses to the engagement of cell surface integrins, including cell spreading and migration, survival, and proliferation.\textsuperscript{48,49} Truncated version(s) of Tie-1 and Tie-2 may bind similarly to the cytoskeleton and play a modulatory role in the signal transduction of human endothelial cells.

Our primary CCM cell lines did not contain mature SMC, a finding in accordance with the histopathologic features of the lesion.\textsuperscript{7} We found, however, some scattered cells in the skin and brain EC cultures, which stained for α-SMA and SMM and probably represented mature myocytes or pericytes. We tried to keep those cell types at low levels by selective cloning. We did not detect astrocyte contamination in any of the EC cultures, probably because of the distinct growth requirements of those cells in vitro. We are aware that the ability to isolate and maintain pure EC populations from excised CCM lesions, brain, and skin microvessels is paramount for future studies of receptor expression. The emphasis is on isolating pure populations of EC because selective conditions that support the exclusive growth of EC have not yet been identified. Several techniques that take advantage of certain endothelial-
specific cellular characteristics have been used for that purpose. It may be possible to increase the yield of EC from CCM by using an anti-CD31 antibody followed by fluorescence-activated cell sorting or immunomagnetic isolation.

Our primary CCM EC lines are currently being used for DNA analysis, including positional cloning and differential display, which will contribute to the ongoing work on identifying abnormal genotypes associated with this disease as well as other somatic mutations that may be present in lesional EC.4–6

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References

Editorial Comment

The investigators in the above article describe a method to grow the endothelial cells in culture from cerebral cavernous malformations surgically resected from 2 patients. In early passage (≤6) the cells retained several characteristics of endothelial cells, such as immunohistochemical evidence of CD31, von Willebrand factor, CD105, and binding sites for EEA-1 and Dil-Ac-LDL. There were no qualitative differences between these cells and endothelial cells obtained from skin microvessels in the same patients or from brain microvessels from other patients. The authors suggest that these cells can be used to determine genotypic and phenotypic differences between normal endothelial cells and those from patients with cavernous malformations, although they do not demonstrate such differences in this publication. I agree that the ability to grow cells from these malformations is a very important step that will be required to study the pathogenesis of cavernous malformations, particularly since there is no animal model for this disease.

Several factors need to be kept in mind. The endothelial cells are the cells that line the cavernous vascular spaces in cavernous malformations. There are usually no or only few smooth muscle cells in the walls of the blood vessels in the malformations. The fibrosis and hyalinization of the walls of the vessels suggests that other cells must be present to produce this reaction. Mutations of the long arm of chromosome 7 have been discovered in patients with familial cerebral cavernous malformations. Therefore, all the cells in the organs of these patients will contain the mutation. Studying the endothelial cells of the lesion may disclose some genetic alteration. The authors imply that the endothelial cell is the cell in which this genetic defect is of importance, although it is possible that it is related to some other cell in the malformation.

There are other possible limitations of this approach. There is a well-known tendency of cells to alter their phenotype from that present in vivo when they are cultured in vitro. There is always a concern that the cells that grow in culture are a select subpopulation of cells that do not represent the only or the dominant phenotype present in vivo. Another bias could be introduced if cells could be obtained from some cavernous malformations but not others. Endothelial cells are known to have different features depending on the artery size, sex, age, and embryological origin and in disease.

Recognizing these possible limitations, the ability to culture cells from cavernous malformations is a key first step in being able to study this disease.

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References
Endothelial Cell Culture From Human Cerebral Cavernous Malformations
Nikolay I. Baev and Issam A. Awad

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