Morphological Observation and In Vitro Angiogenesis Assay of Endothelial Cells Isolated From Human Cerebral Cavernous Malformations

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Background and Purpose—Little is known about the role of endothelial cells (ECs) in the pathogenesis of cerebral cavernous malformation because of the difficulties to obtain highly pure ECs. Thus, this study attempted to establish a reliable procedure to isolate and culture ECs from human cerebral cavernous malformation lesions. The biological features and the angiogenic potential of the cultured ECs were also investigated.

Methods—A modified protocol was developed to isolate and culture cerebral cavernous malformation endothelial cells (CECs) from surgically resected human specimens. The biological features of CECs were investigated by electron microscope, immunostaining, real-time polymerase chain reaction, fluorescence-activated cell sorter, and Western blotting. The tube formation by CECs was examined in an in vitro angiogenesis model with or without the addition of vascular endothelial growth factor.

Results—CECs from the specimens unaffected by the intraoperative bipolar coagulation were cultivated successfully with higher than 95% purity. Comparing to the ECs from control brain tissue, CECs presented primitive nucleus in ultrathin section, expressed higher levels of vascular endothelial growth factor receptor-1 and vascular endothelial growth factor receptor-2, and spontaneously formed tube structures in a 3-dimensional collagen matrix. The tube formation by CECs was significantly promoted by vascular endothelial growth factor treatment.

Conclusions—A modified protocol for the attainment of purified CECs and the first in vitro angiogenesis model of CECs were successfully established. We provided initial evidence that CECs had enhanced angiogenic potential and showed increased responsiveness to vascular endothelial growth factor.

Key Words: angiogenesis ■ cavernous malformations ■ endothelial cells

The cerebral cavernous malformation (CCM) affects 0.4% to 0.5% of the population and represents 10% to 20% of overall cerebral vascular abnormalities. The introduction of MRI allowed CCM to be diagnosed without the need for pathologic confirmation, thus improving clinical knowledge of these lesions. Although CCM does not exhibit the high-flow profile of arteriovenous malformation and is rarely associated with severe apoplectic hemorrhage, the repetitive hemorrhage of CCM is being increasingly recognized as a cause of seizures and focal neurological deficits.

Extensive work has been performed to explore the biological nature and pathogenesis of this vascular anomaly. These advances were mainly focused on either pathology or linkage analysis. Pathological studies using resected CCM specimens have shown that CCM was composed of immature proliferating vessel and exhibited brittle vascular morphology devoid of smooth muscle and elastin layers. Linkage analysis using peripheral blood monocytes from patients has resulted in the exciting discoveries of 3 specific gene loci (CCM1, CCM2, and CCM3) responsible for the pathogenesis of the familial CCM. However, we still cannot precisely explain the phenomenon of the de novo or progression of lesions. The underlying mechanisms of the pathogenesis of CCM have not been fully elucidated because of the lack of a reliable model system.

Endothelial cells (ECs) play pivotal roles in angiogenesis, which is crucial in the development and progression of various pathological processes. CCM is consisted of blood-filled or thrombus-filled caverns lined by a single layer of ECs, while its intercavernous spaces lack mature vessel wall elements. Therefore, it is possible that CCM endothelial cells (CECs), as the major component of the vessel wall, may play a central role in the pathogenesis of CCM characterized by abnormal angiogenesis. Thus, it is important to isolate...
CECs with high purity to serve as a model for further investigation of their role in CCM. Despite a previous report on the cultivation of CECs, a more reliable protocol is yet to be developed because of the contamination of non-EC components. In addition, it is of great interest to further characterize how the cultivated CECs undergo morphological changes and finally assemble into 3-dimensional abnormal networks, because the differentiation of ECs, ie, formation of a capillary-like lumen, is a prerequisite for angiogenesis. No previous study has been focused on this aspect of research.

In this study, we modified a previously reported protocol and successfully obtained CECs with better purity and yield from surgically resected CCM specimens. We further investigated, for the first time to our knowledge, the angiogenic potential of CECs using an in vitro angiogenesis model with 3-dimensional collagen matrix. The effect of vascular endothelial growth factor (VEGF) on the tube formation of CECs was also explored.

Materials and Methods

Patients and Tissue Specimens

From December 2001 to September 2005, surgical resection was performed on 70 patients with CCM in the Neurosurgery Department of Huashan hospital affiliated with Fudan University. Patient enrollment was based on surgical indications without sex, age, or race bias. There were 31 males and 39 females, aged from 4 to 42 years (average, 33.4 years old). They commonly presented with seizures, hemorrhages, and focal neurological deficits. Anticonvulsant medication was based on surgical indications without sex, age, or race bias. From December 2001 to September 2005, surgical resection was performed following the routine steps of collagenase digestion, trituration, filtering, and etc.

Isolation and Culture of CECs

After surgical resection, CCM specimens were immediately placed into ice-cold Hank's balanced salt solution (Life Technologies) and repeatedly rinsed with Hank's balanced salt solution to remove surrounding glotic tissues. The CCM vesicles were then carefully dissociated and the mixed ingredients (eg, adherent blood cells, thrombus, calcified tissues, etc.) were completely removed under a dissecting microscope (Figure 1). After rinsed with Hanks balanced salt solution 3 times again, the isolated vesicles were digested with 0.1% type II collagenase (Sigma) at 37°C for 30 minutes. The digestion reaction was then blocked with 20% fetal bovine serum and resuspended in fresh Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 20% fetal bovine serum, 25 U/mL heparin, 10 mmol/L HEPES, and 100 U/mL penicillin, 100 mg/L streptomycin, and 0.9 g/L L-glutamine. The cell suspension was then plated onto a 1% gelatin-precoated culture dish at 37°C in a humidified incubator (5% CO₂, 95% air) with the addition of 200 mg/L endothelial cell growth supplement (Sigma) and 10 mg/L epidermal growth factor (R&D Systems). Culture medium was initially changed after 5 to 7 days of static culture to remove the nonadherent cells and then twice weekly. For subculture, the cells were split at a ratio of 1:2 and cells from the second to fifth passage were used for subsequent studies. ECs from control brain microvessels were isolated and cultured as previously described. Briefly, the procedure was performed following the routine steps of collagenase digestion, trituration, filtering, and etc.

Immunocytochemistry

For immunofluorescence staining, the specific primary antibodies were rabbit anti-human factor VIII-related antigen (von Willebrand factor), IgG (1:200; Sigma), mouse monoclonal antibody of anti-CD34 (1:100; Santa Cruz), rabbit polyclonal antibody of anti-Flt-1 (1:100; Nordic Immunological Laboratory), and anti-Flk-1/KDR (1:100; Nordic Immunological Laboratory). For cellular contamination detection, the anti-α-smooth muscle actin (1:100; Santa Cruz) and anti-γ-glutamylcarboxylic acid protein (1:100; Santa Cruz) antibodies were used as primary antibodies. The observation was made under a fluorescent microscope (Olympus). Smooth muscle cells were used as negative controls.

Flow Cytometry

Briefly, cells were rinsed with phosphate-buffered saline, detached, and resuspended in phosphate-buffered saline containing 1% bovine serum albumin, and then were incubated with rabbit anti-human von Willebrand factor antibody (1:200; Sigma) or mouse anti-CD34 mAb (1:100; Santa Cruz) or isotype control mAb for 1 hour at 4°C. The cells were then washed with phosphate-buffered saline and incubated with 1:100 dilution of FITC-labeled goat anti-rabbit or mouse IgG (Molecular Probes) for 1 hour at 4°C. After a final wash, labeled cells were subjected to FACScan (BD Biosciences). Data were analyzed by CellQuest software (BD Biosciences).

Real-Time Polymerase Chain Reaction

Briefly, total RNA was isolated from ECs using Trizol Reagent (Invitrogen). Isolated RNA was reverse transcribed into first strand cDNA. The mRNA levels of VEGF receptor-1 (VEGFR-1) and VEGFR-2 were determined by real-time polymerase chain reac-

Figure 1. Pretreatment of CCM specimen. The typical CCM lesion composed of EC-lined caverns (arrow) filled with blood cells and thrombus (triangle) were observed under a microscope (HE staining ×200) (A). After careful dissociation and rinsing of CCM vesicles under a dissecting microscope, the mixtures inside caverns were completely removed and only the EC-lined walls of caverns were retained (arrows indicating the positive immunohistochemical staining for the EC-specific marker of CD34, ×200) (B).
tion (PCR). The primer sequences were designed as the following: VEGFR-1: sense, 5'-CAGGCCAGTTCTCCATT-3'; antisense, 5'-TTCCAGCTAGGCTGGTCTGA-3'; VEGFR-2: sense, 5'-CCAGCAAGCCACGGATCTGT-3'; antisense, 5'-TGGATGTCATCGGAGTGATATCC-3'. PCR was performed using SYBR green PCR master mix (Applied Biosystems). Amplification and detection were performed using an ABI Prism 7700 system (Applied Biosystems) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase was used as reference gene. The experiments were repeated 3 times.

Western Blot Analysis

Briefly, total cell lysates were electrophoresed on SDS-PAGE, transferred to nitrocellulose, and blotted with VEGFR-1 or VEGFR-2 mAb (1:500; Nordic Immunological Laboratory) followed by horseradish peroxidase conjugated anti-mouse IgG. After detection, the blots were stripped and re-blotted against glyceraldehyde-3-phosphate dehydrogenase (1:5000; Sigma) as a protein loading control. The blots were visualized by the ECL Western blotting kit (Amersham Pharmacia Biotech).

In Vitro Tube Formation Assay

Type I collagen from rat tail (Sigma) was dissolved, and collagen gel was prepared according to a previously described method. Both ECs from CCM and control brain tissue were seeded on the collagen gel and incubated for ~1 day in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. When cells became subconfluent monolayer, culture medium containing fetal bovine serum was removed and cells were rinsed with Hanks balanced salt solution twice. ECs were then treated with or without human recombinant VEGF at various concentrations (1, 5, 10, and 50 μg/L; Sigma) in serum-free Dulbecco's modified Eagle's medium for 2 hours. Medium was then removed and a second layer of collagen gel prepared as described previously was applied to the cell monolayer. Fresh Dulbecco’s modified Eagle’s medium containing 3% fetal bovine serum was then added to the collagen gel and cells were incubated at 37°C for 24 hours. The reorganization of the subconfluent monolayer ECs in 3-dimensional collagen matrix was monitored and photographed with a phase contrast microscope (Nikon). Tube formation of the ECs was identified by the appearance of intraluminal space inside the tube and quantified by measuring the length (mm) or area (mm²) of tube-like structures with an image analyzer (Leica QWinV3). Human umbilical vein endothelial cells purchased from Clonetics were used as another control to test the feasibility of our method.

Statistics

For quantitative real-time PCR, statistical analysis was made by using the Student t test. The differences were considered to be statistically significant when P<0.05. For tube formation, at least 4 EC samples each were selected from the CCM and control brain tissue. Each sample was divided into 2 groups, with or without VEGF. Two culture wells were used for each group and 4 different random fields of view were examined for each well. The results were shown as mean±standard deviation. Statistical analysis was made by using Scheffe test after a 1-way analysis of variance. The differences were considered to be statistically significant when P<0.01.

Figure 2. CEC morphorlogy. Cultured EC confluent monolayer with cobblestone appearance from human CCM (A) and control brain tissue (B). CEC morphology was approximately divided into 2 groups: spindle shape and round shape (arrow). Bar=50 μm.

Figure 3. CEC ultrastructure. A giant nucleus (N) with clear nucleolus (n) was centrally located in the CEC (A). Organelles such as mitochondrion (M) and rough reticulum (E) were also observed (A, B). Tight junctions between the adjacent cells (arrow) and pinocytotic vesicles under cell membrane (triangle) were found (B). In contrast, smaller nucleus without visible nucleolus was presented in the ECs from control brain tissue (C, D). Scale bars: A and C=1 μm; B and D=0.5 μm.
Results

Culture of CECs
The 70 CCM specimens were divided into 2 groups, 34 specimens unaffected and 36 specimens affected by the intraoperative bipolar coagulation as assessed by the neurosurgeons. A total of 25 CEC lines were successfully established from the group unaffected by the intraoperative coagulation and no CEC line was generated from the other group. In the primary culture, CEC monolayer reached confluence with signs of contact inhibition at 20.0±2.721 (n=25) days after seeding. Most CECs demonstrated a uniform cobblestone appearance and were larger than control brain ECs (Figure 2). Cell morphology could be divided into 2 groups. One group was bigger and in a short spindle shape, which grew faster and accounted for ≈60% of all cells. The other group was in a round shape and grew slowly, accounting for ≈40% of all cells. In the subculture, the average lifespan of each passage was 8.5±1.2 days (n=40) and growth deterioration usually occurred after 6 to 8 passages.

Ultrastructure Feature
In ultrathin sections, cultured CECs presented a primitive status with giant nucleus centrally located and bounded by a nuclear envelope consisting of 2 parallel membranes. The heterochromatin was distributed below the nuclear membranes and the nucleolus was clearly observed. There are plenty of organelles such as mitochondrion, rough reticulum, ribosome, etc, in the cytoplasm. Although no Weibel-Palade body was seen, another 2 characteristic ultrastructure features of ECs, the pinocytotic vesicles and tight junctions were found under the cell membrane and between the adjacent cells, respectively. In contrast, smaller nucleus with no visible nucleolus was presented in the ECs from brain tissue (Figure 3).

Immunostaining, Real-Time PCR, and Western Blot
More than 95% CECs and 90% ECs from brain tissue were intensively stained with von Willebrand factor and CD34 as

![Immunostaining and quantitative analysis of ECs. Both ECs from CCM and control brain tissue were intensively stained with von Willebrand factor and CD34 (A). Bar=50 μm. Fluorescence-activated cell sorter analysis of cultured ECs showed positive staining of von Willebrand factor and CD34 (B, green; specific antibody; blue, isotype control mAb). CECs showed moderate staining of VEGFR-1 and VEGFR-2, which were rarely detected in ECs from control brain tissue (C). Expression of VEGFR-1 and VEGFR-2 in both cultured ECs was measured by Western blot (D) and the mRNA expression levels of VEGFR-1 and VEGFR-2 were measured by real-time PCR (E). *P<0.05. Each bar represents 3 independent experiments and the error bar depicts standard deviation.](http://stroke.ahajournals.org/)

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measured by immunostaining and fluorescence-activated cell sorter (Figure 4A and 4B). No detectable smooth muscle cells or astrocyte contamination was identified by immunostaining for α-smooth muscle actin or glial fibrillary acidic protein, respectively (data not shown). Almost all isolated CECs displayed mild to moderate staining for VEGFR-1(Flt-1) and VEGFR-2(Flk-1), which were rarely expressed in ECs from brain tissue (Figure 4C). Furthermore, Western blotting (Figure 4D) and real-time PCR (Figure 4E) also demonstrated that VEGFR-1 and VEGFR-2 expression levels were higher in CECs than that in ECs from brain tissue. \((P<0.05)\).

In Vitro Angiogenesis Assay
The subconfluent monolayer of CEC established on the surface of the collagen gels underwent a dramatic reorganization when covered with a second collagen layer. In VEGF-free culture, apparent branching networks with obvious lumens were quickly found at the first 24 hours of incubation (Figure 5). When VEGF was added to the CEC monolayer, marked increase in the formation of these capillary-like structures was observed (Figure 5). The total length or area of tubular structures induced by VEGF was significantly increased when compared with the VEGF-free culture \((P<0.01; \text{Figure 6A and 6B})\). In contrast, only anastomosing cell cords without visible lumens were detected in the control groups of ECs from brain tissue and human umbilical vein ECs (Figure 5) in the absence of VEGF. For human umbilical vein ECs and ECs from brain tissue, the obvious tubular structure formations could be only induced when VEGF was added (Figure 5), which were still significantly less than that of CECs \((P<0.01; \text{Figure 6A, 6B})\). Furthermore, the tube formation inducing effects of VEGF on CECs occurred in a dose-dependent way as shown in Figure 6C.

Discussion
A reliable culture method is needed to establish a model system to study the pathogenesis of CCM. Despite a previous report of culturing ECs from CCM specimens by routine EC culture method, successful cultivation of CECs is rare. Because repetitive hemorrhages of lesions always result in the mixture of blood and thrombus filling the caverns,\(^{16}\) thus limiting the isolation of ECs from CCM specimens. In this study, therefore, we modified procedures used by Baev and Awad in CECs culture\(^{12}\) and successfully isolated and cultured highly pure CECs from 25 patients. In addition, we, for the first time to our knowledge, established an in vitro angiogenesis model allowing the further characterization of these lines.

Twenty-five CEC lines from 34 specimens unaffected by the burning of intraoperative bipolar coagulator were cultivated successfully. Therefore, the strict selection of suitable specimen is the first important step toward a successful culture. Then, the mixtures inside caverns must be effectively removed by the careful dissociation and rinsing of the CCM.

Figure 5. Collagen gel-induced in vitro tube formation of ECs. ECs from CCM and control brain tissue as well as human umbilical vein endothelial cells were sandwiched between 2 layers of type I collagen in the absence or presence of 10 μg/L VEGF. Only a few anastomosing cell cords (arrow) without visible lumens were detected in human umbilical vein ECs and ECs from control brain tissue, while the apparent branching networks with obvious lumens (triangle) quickly formed in CECs. The addition of VEGF significantly increased the formation of capillary-like structures, especially for CECs. Bar=100 μm.
vesicles under the dissecting microscopy. The content of ECs in the specimen could be dramatically elevated after this second key step. In addition, prolonging the initial culture medium change from 1 to 4 hours to 5 to 7 days may also allow more CECs to attach, thus increasing EC content. With these modifications, 95% purity of CECs is achieved in our culture systems as determined by fluorescence-activated cell sorter, almost a 90% improvement from the previous reported procedure. In addition to the positive staining for von Willebrand factor and CD34, which are specific EC markers, the uniform cobblestone appearance, the contact inhibition, and the existence of tight junctions and pinocytotic vesicles of these cells also suggest the EC origin of cultured CECs.

It is of note that we have also observed 2 distinct cell types in our culture system, similar to that reported by Baev and Awad. The exact functions of these 2 types of cells are yet to be elucidated. Cultured CECs presented a primitive status with giant nucleus and abundant organelles in ultrathin sections. Furthermore, almost all of them displayed mild to moderate expression of Flt-1 and Flk-1, which are abundantly expressed during embryonic development but are absent in most normal adult vascular beds, including the cerebral vasculature, except under certain physiological and pathological conditions. These observations suggest that CECs may be a kind of primitive-state EC and may have altered angiogenesis potential when comparing to normal ECs. CCM may develop as a result of irregular organization of CECs, for which the detailed process still needs to be clarified. Recently, Konya et al assessed the angiogenic potential of tissue specimens from arteriovenous malformations. However, their model cannot completely exclude the effects of some mingled components inside the cerebral vascular malformation specimen, such as blood cells, fibroblasts, and surrounded gliotic brain tissues, which may also have significant angiogenic potentials. Therefore, the establishment of an in vitro angiogenesis model with purified CECs may allow further objective studies of the angiogenic activities of CCM. Indeed, in our study, capillary-like tubular structures appeared soon after CECs were cultured in the 3-dimensional collagen gels even without the addition of VEGF. This spontaneous tube-formation ability is more apparent in CECs than in ECs from control brain tissue. Such phenomenon has not been previously reported in studies regarding ECs from other origins, such as human foreskin microvascular ECs, bovine aortic ECS, rat cerebral capillary ECs, immortomouse brain ECs. These studies have actually implied that the in vitro capillary-like tubular structures cannot be induced in the absence of exogenous angiogenic factors, such as VEGF, basic-fibroblast growth factor, epoxyeicosatrienoic acid, etc. In our preliminary studies on the proliferation and migration CECs, we discovered that CECs had significantly increased proliferation and migration potential, especially for the latter when compared with that of the control brain ECs. It is well-known that proliferation and migration of ECs are involved in the formation of tube structures. Thus, the spontaneous tube formation property of CECs in the absence of VEGF suggests that CECs have increased angiogenic or differentiation potentials than that of ECs from other origins.
In addition, previous immunohistochemical studies have also revealed that the interaction of VEGF and its tyrosine kinase receptors is closely related to the genesis and subsequent biological behavior of CCM. In our model system, the formation of significant capillary networking structures in the presence of VEGF demonstrates that CECs are sensitive to VEGF, possibly because of the increased expression of Flt-1 and Flk-1 on these cells. Signaling through these receptors may influence the angiogenic activity of CECs. In our preliminary studies, VEGF treatment has triggered significantly higher activation of AKT and ERK, two major downstream signal molecules associated with angiogenesis. In CECs than that in normal brain ECs. Studies are ongoing to further explore signaling transduction pathways in this model.

Taken together, the successful culture of ECs from excised CCM specimens and the establishment of a 3-dimensional CEC culture model provided us an ideal in vitro model to further explore the pathogenesis of CCM. Further studies are ongoing to clarify the molecular mechanisms mediating abnormal vessel development and maintenance in CCM.

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**Disclosures**

None.

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