CCM2 Expression Parallels That of CCM1

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Background and Purpose—Mutations in CCM2 (MGC4607 or malcavernin) cause familial cerebral cavernous malformation (CCM), an autosomal dominant neurovascular disease. Both the function of this molecule and the pathogenesis of the disease remain elusive.

Methods—We analyzed the mRNA expression of Ccm1 and Ccm2 in the embryonic and postnatal mouse brain by in situ hybridization. Subsequently, we generated CCM2-specific polyclonal antibodies and tested their specificity using transient transfection experiments in various cell lines. We then investigated CCM2 protein expression in cerebral and extracerebral tissues by Western blot analysis as well as immunohistochemistry and compared these results with CCM1 (KRIT1) protein expression.

Results—In situ analysis shows similar temporal and spatial expression patterns for Ccm1 and Ccm2, although Ccm1 expression appears more widespread. Immunohistochemical analysis shows that CCM2 is expressed in various human organs, most noticeably in the arterial vascular endothelium. As is the case with CCM1, CCM2 is not expressed in other vascular wall elements such as smooth muscle cells or the venous circulation. Within cerebral tissue, it is also expressed in pyramidal neurons, astrocytes, and their foot processes. In extracerebral tissues, CCM2 is present in various epithelial cells necessary for blood–organ barrier formation.

Conclusions—CCM1 and CCM2 have similar expression patterns during development and postnatally thereafter. Given the fact that the disease phenotypes caused by mutations in either gene are clinically and pathologically indistinguishable, the significant overlap in expression pattern supports the hypothesis that both molecules are involved in the same pathway important for central nervous system vascular development. (Stroke. 2006;37:518-523.)

Key Word: cavernous malformations

Cerebral cavernous malformation (CCM) is a neurovascular disease characterized by raspberry-like angiomas comprised of abnormally enlarged vascular channels; lesions can be either sporadic or familial. Familial cases are attributable to mutations in 1 of 3 loci: CCM1 on 7q21.2, CCM2 on 7p15-p13, or CCM3 on 3q25.2-q27. All 3 genes have been identified: KRIT1 or Krev1/Rap1A Interaction Trapped 1 (CCM1), MGC4607 or malcavernin (CCM2), and PDCD10 or the Programmed Cell Death 10 gene (CCM3). Despite the identification of these 3 genes, CCM pathogenesis remains largely elusive.

Based on the fact that both sporadic and familial CCM lesions are clinically and pathologically indistinct, we previously hypothesized that all 3 genes are likely to be involved in the same biochemical pathway. To test this hypothesis and to better understand the function of the CCM2 protein, we analyzed the expression patterns of Ccm1 and Ccm2 mRNA in the embryonic and postnatal mouse brain. We also generated polyclonal antibodies against CCM2 and studied the expression pattern of the CCM2 protein in adult human tissue, which we then compared with that of the CCM1 protein KRIT1. Here we report that CCM2 expression pattern closely resembles that of CCM1 both temporally and spatially.

Materials and Methods

In Situ Hybridization

Mouse brains were fixed by intracardiac perfusion with 4% paraformaldehyde according to institutional animal care and use committee guidelines and sectioned at 36 μm. In situ hybridization was performed as described previously with minor modifications. RNA probes complementary to mouse Ccm1 (IMAGE clone 5390736) and Ccm2 (IMAGE clone 4952288) were prepared and labeled with digoxigenin-11-UTP.

In Vitro Cultures

Human umbilical vein endothelial cells (HUVECs) were cultured in media containing 199 serum supplemented with 20% FBS, 200 mmol/mL L-glutamine, 1% Pen/Strep (Invitrogen), and endothelial cell growth supplement. Cos7 cells were grown in low-glucose DMEM (GIBCO/BRL) with 10% FBS and 1% antibiotic-antimycotic. Cells harvested for protein for Western blot analysis were grown to confluence.
CCM2 Antibody Characterization

A CCM2-specific antibody was synthesized and affinity purified by Zymed Laboratories Inc. The antibody corresponds to the C-terminal peptide sequence for open reading frame 22 on Chromosome 7, amino acids 410 to 423: DDRSAPSEGDEWDR. Antibody specificity was tested by transfecting Cos7 cells, which have a low level of endogenous CCM2 expression, with an expression vector encoding a CCM2–GFP fusion protein. The full-length CCM2 cDNA was cloned into pcDNA-DEST53 (Invitrogen) and transfected into Cos7 cells using Lipofectamine (Invitrogen). Nontransfected cells served as control. Protein extracts prepared from these cells were fractionated by SDS-PAGE and analyzed by Western blot with the CCM2 antibody. For peptide competition assays, the CCM2 antibody was competed with a 2-fold molar excess of the immunizing peptide before primary incubation.

Analysis of CCM2 Expression in Mouse Tissue

Various organs from CD-1 mouse were homogenized, and 15 μg of the resulting protein lysates were analyzed by Western blot.
Immunohistochemical Analysis

Sections of human organ tissue were obtained from the Department of Pathology at Yale University School of Medicine (HIC 7680). Tissues were fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned to 0.5 mm thickness. They were then incubated in a 60°C oven followed by Xylene treatments and rehydrated by short incubations through a 100% to 70% ethanol gradient. To optimize CCM2 antigen exposure, slides were placed in 10 mmol/mL citric acid solution and microwaved. Tissue sections were incubated with the CCM2 antibody customized from Zymed using standard immunohistochemical protocols. Antibody staining was visualized using diaminobenzidine substrate from Vector Laboratories, Inc., followed by counterstaining with hematoxylin to detect nuclei. Tissue sections incubated in preimmune serum served as negative control.

Results

Expression of Ccm1 and Ccm2 mRNA in Mouse Brain

We analyzed the expression of Ccm1 and Ccm2 mRNA in mouse brain, both at embryonic and postnatal stages. Overall, the pattern of expression of these 2 molecules shows considerable overlap, with Ccm1 expression being generally higher than Ccm2, particularly at embryonic stages.

Embryonic Day 16.5

Both Ccm1 and Ccm2 are expressed in the ventricular zone, where glial cells are generated at this stage, as well as the developing cerebral cortex, striatum, hippocampal formation, and in various thalamic and hypothalamic nuclei (with the differentiating intermediate thalamic and hypothalamic neuroepithelium showing intense Ccm2 expression; Figure 1). Ccm1 is expressed in the oculomotor nucleus, and both Ccm1 and Ccm2 are expressed in the developing cerebellum, whereas Ccm2 is not at this stage.

Postnatal Day 8

Both Ccm1 and Ccm2 are expressed in the glomerular and mitral cell layers of the olfactory bulb; the motor, somatosensory, and piriform areas of the cortex (all layers), the induseum griseum, the caudate putamen, the hippocampus, and dentate gyri, many thalamic and hypothalamic nuclei, the inferior colliculus, the cerebellum (external granular and Purkinje cell layers), and the medulla (Figure 2).

Postnatal Days 14 and 21

Both genes continue to be expressed in the cortex (with Ccm2 expression higher in the deep layers); Ccm2 is particularly strong in the piriform cortex. Expression persists in the hippocampus, dentate gyrus, thalamus, and hypothalamus. In the cerebellum, Purkinje cells express both Ccm1 and Ccm2, whereas the granule cells appear to gradually downregulate both genes (supplemental Figure, available online at http://stroke.ahajournals.org).

Figure 3. Western blots of Cos7 cell extracts stained with CCM2 antibody (A and B). Cells were transfected with an expression vector encoding a full-length CCM2-GFP fusion protein (+). Untransfected cells served as controls (−). A, In transfected cells, the CCM2 antibody detects an ~82-kDa protein (expected size for the fusion protein). No staining is observed in untransfected controls. B, Peptide competition eliminates staining, demonstrating the specificity of this antibody for the CCM2 protein. C, Multitissue Western blot reveals CCM2 protein expression in the brain, heart, lung, and kidney.

Figure 4. CCM2 stains the vascular endothelium of various human organ tissues. A, In the heart, CCM2 localizes to endothelial cells of the arteries (black arrows) but not veins (blue arrow). B and C, Vasa vasorum of the heart and the aorta shows endothelial expression (arrows). D and E, Higher magnification of the myocardium confirms arterial expression of CCM2. F and G, The alveoli of the lungs express CCM2 in capillary endothelium. H, Western blot of protein extracts from HUVECs and human aortic endothelial cells (HAECs) give a band of ~50 kDa corresponding to the endogenous human CCM2 protein.
Characterization of the CCM2 Antibody
Antibodies were raised against a 14-aa peptide at the C terminus of the CCM2 protein and affinity purified as described in Materials and Methods. Antibody specificity was determined by comparison of protein lysates from Cos7 cells transfected with a GFP-tagged CCM2 construct with those from untransfected control Cos7 cells. Western blot analysis indicated that the antibody recognized a protein of the expected size of ~84 kDa (human CCM2 clone ~49 kDa plus GFP protein ~35 kDa) in extracts of the transfected Cos7 cells (Figure 3A). Specificity was confirmed by a 2-fold molar excess peptide competition with the immunizing peptide (Figure 3B).

CCM2 Expression in Mouse Tissue
To investigate CCM2 protein expression in different tissues, lysates of various mouse organs were analyzed by Western blot. CCM2 was detected as an ~50-kDa isoform in all tissues examined including brain, heart, lung, and kidney. The antibody also recognized an ~43 kDa isoform in the lung, indicating tissue-specific splice variants of the CCM2 protein (Figure 3C).

CCM2 Is Expressed in Arterial Endothelial Cells
The expression pattern of CCM2 was determined by immunohistochemical staining of human tissue. Our results indicated that CCM2 was expressed in the arterial endothelium of the vasculature of all organs analyzed but was absent from the smooth muscle cells, as observed in the heart (Figure 4A through 4E), including the coronary arteries and vasa vasorum (Figure 4B and 4C). The same pattern was also observed in the lungs (Figure 4F and 4G).

To confirm endothelial cell expression of CCM2 at the mRNA and protein levels, both RT-PCR and Western blot analyses were performed on 2 distinct human endothelial cell lines from umbilical vein and aortic endothelium (HUVECs and human aortic endothelial cells, respectively). RT-PCR analysis confirmed the presence of CCM2 mRNA (data not shown), whereas Western blot analysis confirmed expression of the CCM2 protein in both cell lines (Figure 4H).

Consistent with expression in other tissues, CCM2 was present in the endothelium of cerebral capillaries and arteries (Figure 5A). High magnification of these cerebral arteries clearly shows CCM2 expression, whereas other vascular elements, such as smooth muscle cells, do not (Figure 5B and 5C).

CCM2 Expression in Extracerebral Tissues
In addition to the arterial endothelium, CCM2 staining was observed in the myocardial cells (Figure 6A), as well as certain epithelia, such as bile duct epithelium (Figure 6B), reticular cell epithelium that sheaths the blood–thymus barrier (Figure 6C and 6D), and the white pulp node epithelium that marks the closed circulation in the spleen (Figure 6E and 6F). In the lung bronchioles, the columnar epithelium and epithelial cilia expressed CCM2 (Figure 6G and 6H). In the kidney, on the other hand, CCM2 was observed in the endothelium of interconnected glomerular capillaries and the inner visceral layer of the Bowman’s capsule of the glomerulus (Figure 6I). The proximal tubules of the kidney, identifiable by their closed lumens, showed CCM2 expression, whereas the distal tubules did not (Figure 6I).

CCM2 Expression in Cerebral Cortex
A cross-section of the cerebral cortex at low magnification showed that CCM2 was expressed in various cell types (Figure 5). In addition to endothelial cells (Figure 5A through 5E), the astrocytes and astrocytic foot processes that surround these cells were observed to be CCM2 positive, a pattern closely resembling that of CCM111 (Figure 6D and 6E). Pyramidal neurons of the cerebral cortex also express CCM2 (Figure 6F and 6G). The CCM2 staining was mainly cytoplasmic.

Discussion
CCM is the most common vascular malformation that affects the central nervous system (CNS). Mutations in 3 genes (KRIT1, MGC4607 [Malcavernin], and PDCD10) have been found to cause familial cases of CCM. Given that familial as well as sporadic CCM lesions are clinically and pathologically indistinct, we hypothesized previously that all 3 genes
Figure 6. CCM2 localizes to various epithelia in the extracerebral organs. A, CCM2 is present in cardiac myocytes (arrowheads) and arterial endothelium (black arrows) but not venous endothelium (white arrow) of the heart. B, CCM2 is expressed in bile duct epithelium of the liver (black arrow) and hepatic artery endothelium (data not shown) but not hepatic vein endothelium or smooth muscle cells (white arrow). C, CCM2 is expressed in the vasculature of the thymus; arterial endothelium expresses CCM2 (black arrows), whereas thymic vein has no CCM2 protein expression (white arrow). D, Reticular cells of the thymic epithelium stain for CCM2 (black arrows). E, Connective tissue protein expression (white arrow). D, Reticular cells of the thymic epithelium express CCM2 (black arrows). F, Central artery of the lymphatic nodule in the white pulp region expresses CCM2 (black arrow), as does the white pulp region itself. Smooth muscle lining the nodule does not stain for CCM2 (white arrow) nor do the endothelia of the corresponding venous regions (data not shown). G, Low magnification of the bronchial tissue shows that CCM2 localizes to the columnar epithelium and the active cilium (black arrow). H, Higher magnification of the lung shows expression in the columnar epithelium (black arrow). I, Capillary endothelium and the podocyte processes in the kidney glomerull stain for CCM2 (arrowheads) as does the epithelia of the proximal convoluted tubules (white arrow).

are likely to be involved in the same molecular genetic pathway important in the cross-talk between the neural and glial elements (neurons and astrocytes) and the endothelium of the CNS. This could possibly explain why CCM lesions are CNS specific. The evidence presented in this study showing the similar temporal and spatial expression patterns of CCM1 and CCM2 further supports this hypothesis.

The CCM1 gene was identified using yeast 2-hybrid analysis with Krev1/Rap1A as bait. The gene encodes the KRIT1 protein, which contains a N terminus NPXY motif now known to bind integrin cytoplasmic domain associated protein-1α (ICAP-1α) through the phosphotyrosine binding domain (PBD). ICAP-1α also binds the cytoplasmic tail of β1-integrin through association of the NPXY motif and the PBD domain, thereby implicating integrin signaling in CCM pathophysiology. In addition, KRIT1 has been shown to associate with microtubules, specifically β-tubulin. These studies suggest that through integrin signaling, KRIT1 may mediate bidirectional signaling between the extracellular matrix and the cellular cytoskeleton. KRIT1 is expressed in arterial and microvascular endothelium along with neurons and astrocytes in the CNS. These data are consistent with the phenotype of CCM1/Krit1 knockout mice that show arterial pathology, with homozygous mutant embryos dying at an early embryonic age from closure of the dorsal aorta caused by increased endothelial cell proliferation.

The CCM2 gene, recently identified as MGC4607 or malcavernin, encodes a protein containing a putative PBD. CCM2 protein sequence analysis does not reveal any other conserved region, nor does it offer implications on the function of the molecule. In this study, we show that CCM2 is expressed in a variety of tissues. CCM2, like CCM1, is expressed by the endothelial cells and, more specifically, by the arterial and microvascular endothelium. In the brain, CCM2 protein is also expressed in pyramidal cells and astrocytes, once again mimicking CCM1. CCM2 expression is observed in astrocytes with foot processes terminating on cerebral blood vessels. These foot processes are responsible for forming the glial membrane that encapsulates the vessel, thus establishing the blood–brain barrier. Interestingly, CCM lesions lack astrocytic foot processes and tight adherence junctions between endothelial cells. These lesions appear as immature vessels with possibly proliferating, nonadherent endothelium that is rich in fibronectin and poor in laminin.

Although little is known with regard to the molecular function of CCM2, its mouse homolog, OSM (Osmosensing Scaffold for MEKK3), provides some insight into its function and its relationship with the CCM1 molecule KRIT1. OSM is involved in mechanosensing and osmosensing and has a PBD and mediates p38MAPK signaling by directly interacting with MEKK3, Rac, and actin. ICAP-1α, a binding partner of KRIT1, is known to inhibit Rac signaling; thus, Rac might be a potential point of interaction between CCM1 and CCM2 molecules. Further studies are under way to investigate this hypothesis.

Our results suggest that CCM molecules might indeed signal together in a novel pathway, most likely important in CNS angiogenesis. This is further supported by the recent observation that CCM1 and CCM2 interact in a complex. The types of
cells, namely neural and endothelial cells, that express both CCM1 and CCM2 are especially intriguing. A recent study demonstrated that mice lacking αv integrin develop cerebral hemorrhage. This phenotype was dependent on the astrocytic expression of αv integrin because mice devoid of αv integrin in the endothelium were normal.22 By analogy, and based on the expression data presented in this article, CCM signaling might also function to establish the communication between neural and endothelial cells of the neurovasculature. Supporting this hypothesis, both KRIT1 and CCM2 are expressed in microstructures important in CNS angiogenesis, and, as we have shown here, CCM2 has a pattern of arterial endothelium expression paralleling that of KRIT1. Consistent with this observation, the homozygous Ccm1−/− knockout mice show arterial pathology.17 The aforementioned hypothesis will be further tested with the study of the CCM2 knockout mouse, which will likely implicate CCM2 protein in normal angiogenesis and arterial morphogenesis as well. It is interesting to note that CCM3 is caused by mutations in PDCD10/FAR-15, a gene involved in the induction of programmed cell death, another process integral to arterial morphogenesis. Previous research has already implicated this gene in cerebrovascular development or remodeling.8 Although little is yet known with regard to this gene in particular, there is evidence that apoptosis in smooth muscle cells is mediated by β1-integrin signaling pathway,23 which is known to interact with KRIT1 and CCM2. Future study of integrin involvement and the p38 pathway may help elucidate the molecular pathology of CCM.

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