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Cerebral cavernous malformation is a vascular disease associated with activated RhoA signaling

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Abstract

Cerebral cavernous malformation (CCM) involves the homozygous inactivating mutations of one of three genes, *ccm1*, -2, or -3 resulting in hyperpermeable blood vessels in the brain. The CCM1, -2, and -3 proteins form a complex to organize the signaling networks controlling endothelial cell physiology including actin dynamics, tube formation, and adherens junctions. The common biochemical defect with the loss of CCM1, -2, or -3 is increased RhoA activity leading to the activation of Rho-associated coiled coil-forming kinase (ROCK). Inhibition of the ROCK rescues CCM endothelial cell dysfunction, suggesting that the inhibition of RhoA-ROCK signaling may be a therapeutic strategy to prevent or arrest the progression of the CCM lesions.

Keywords

actin dynamics; CCM; cerebral cavernous malformation; RhoA; ROCK

Introduction

Cerebral cavernous malformation (CCM) is a genetic disease where loss of CCM1, -2, or -3 is associated with dilated, hyperpermeable blood vessels primarily in the brain (Robinson et al., 1991). The CCM lesions are less frequently found in the spinal cord, retina, liver, and skin. Many patients with CCM experience symptoms; other patients develop neurological deficits including seizures and stroke from the extravasation of blood due to an increased vascular permeability or hemorrhage. The treatments for CCM include surgical resection or radiation therapy, both are associated with substantial risks; therefore, the current standard of care is observation until the symptoms necessitate intervention (Batra et al., 2009). The goal of an intense research effort by several laboratories is to develop a pharmacological treatment to arrest and/or reverse the vascular hyperpermeability of the CCM lesions.

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The CCM lesions develop upon the homozygous mutation of *ccm1*, -2, or -3 in endothelial cells. (Laberge-le Couteulx et al., 1999; Liquori et al., 2003; Guclu et al., 2005). Germline homozygous loss of a *ccm* gene is embryonic lethal, and it is estimated that 1 in 200 people in the general population harbor a mutation in one allele of the three *ccm* genes. The frequency may be as high as 1 in 70 in Hispanics due to a founder mutation (Günel et al., 1996). Although a speculation at this time, the high frequency of heterozygous carriers in the general population suggests there may be a selective advantage to have one mutant allele of a *ccm* gene. The mRNA for the CCM2 protein is highest in macrophages, suggesting that a loss of one allele of a *ccm* gene could, for example, influence the innate immune response. Currently, no evidence for such a selective advantage has been discovered. The homozygous mutation of the *ccm* genes in endothelial cells from patient lesion samples, but not in surrounding normal brain tissue, has given rise to the hypothesis of CCM as a disease defined by the loss of heterozygosity, similar to the 'two-hit' hypothesis for neoplastic cancer progression (Knudson, 1971; Pagenstecher et al., 2009). One report suggests that the loss of the *ccm3* gene expression by conditional deletion in glial cells may induce CCM disease (Louvi et al., 2011). Familial CCM, in which a patient has inherited one mutant *ccm* allele and the subsequent loss of heterozygosity appears to be responsible for more than 2/3 of the patients harboring lesions (Labauge et al., 1998). The familial CCM patients often present between infancy and the third decade of life. The non-familial, sporadic CCM patients often do not present with lesions and neurological deficits until later in life, presumably because of the longer time duration involved in the accumulating mutations in both alleles of a *ccm* gene in the brain endothelial cells. Genetic testing suggests that some patients thought initially to be sporadic are most likely familial (Labauge et al., 1998). The ability to rapidly sequence the *ccm* genes using the next generation deep sequencing methods should provide a more comprehensive understanding of the *ccm* mutations.

CCM1, CCM2, and CCM3

Clinically, the inactivating mutation of both alleles in any one of the three *ccm* genes, referred to as *ccm1*, -2, and -3, gives rise to phenotypically similar lesions, indicating that the three proteins are genetically in the same pathway (Faurobert and Albiges-Rizo, 2010). Consistent with this hypothesis, Hilder et al. were able to demonstrate through multidimensional protein identification technology (MudPIT) that the three CCM proteins coimmunoprecipitate in a complex from cell lysates and can be shown to form a CCM1-CCM2-CCM3 protein complex *in vitro* (Hilder et al., 2007). A major conclusion from the MudPIT analysis was that the CCM proteins were involved in regulating the cytoskeleton. This hypothesis has now been proven using different endothelial cell models of CCM including RNAi studies in human and mouse endothelial cells, CCM1, -2, and -3 inhibitory morpholinos in zebrafish, and targeted *ccm* gene knockouts in mice (Boulday et al., 2009; Kleaveland et al., 2009; Whitehead et al., 2009; Zheng et al., 2010). Cumulatively, it is clear that a major function for CCM1, -2, and -3 proteins in endothelial cells is the regulation of cytoskeletal dynamics and the control of polarity, migration, and adherens junction stability (Glading et al., 2007; Borikova et al., 2010; Lampugnani et al., 2010; Stockton et al., 2010). It is also evident that the subcellular location of CCM1, -2, and -3 is dynamic, and the three proteins are not always found in a ternary complex, indicating that they have functions independent of each other in addition to their function as a complex.

A feature of the three CCM proteins is that none has a defined catalytic activity, and their primary function appears to be the scaffolding of protein complexes for the control of specific endothelial cell functions including the regulation of actin dynamics (Figure 1). A patient mutation in the CCM2 phosphotyrosine-binding (PTB) domain (L198R) that disrupts the binding of target proteins including CCM1 is consistent with *in vitro* data describing the PTB domain interactions of CCM2 being required for the normal maintenance of endothelial

cell physiology (Denier et al., 2004; Zawistowski et al., 2005). Many CCM protein mutations found in the lesions are at splice junctions, suggesting that the protein may not be expressed because of non-sense-mediated mRNA decay (Stahl et al., 2008).

Of the three CCM proteins, CCM1 has the largest number of defined binding partners, including several that regulate the endothelial cell adherens junctions and vascular permeability (Figure 1) (Zawistowski et al., 2005; Glading et al., 2007). CCM3 has recently been shown by several groups to bind the STE20-like Mst4/STK25 serine/threonine kinases, which can phosphorylate the ezrin-radixin-moesin (ERM) cytoskeleton-associated proteins that function to link the actin filaments to the plasma membrane (Zheng et al., 2010; Ceccarelli et al., 2011; Fidalgo et al., 2012). CCM2 binds CCM3 outside of the PTB domain in what has been named the Karet domain (Zawistowski et al., 2005; Hilder et al., 2007; Li et al., 2010; Costa et al., 2012). CCM2 also binds the E3 ubiquitin ligase SMAD ubiquitin regulatory factor (Smurf1) (Croise et al., 2009). Given that the loss of CCM1, -2, or -3 protein results in similar pathological lesions, the common dysregulated biochemical pathway in endothelial cells is increased expression and activation of the small GTPase RhoA (Whitehead et al., 2009; Borikova et al., 2010; Stockton et al., 2010; Zheng et al., 2010). This dysregulated RhoA expression is consistent with the initial proteomic studies of Hilder et al. predicting that the CCM protein complex binds proteins that are involved in the regulation of the cytoskeleton (Hilder et al., 2007). The question becomes how do CCM1, -2, and -3 integrate their individual scaffolding functions to regulate the dynamic RhoA-mediated control of the endothelial cell actin cytoskeleton, and is RhoA signaling an important target to arrest CCM pathology?

RhoA GTPase

The initial evidence for dysregulated RhoA signaling in CCM pathology came from experiments where RNAi-mediated loss of CCM1 or -2 protein expression led to an increased stress fiber formation (Glading et al., 2007; Whitehead et al., 2009; Stockton et al., 2010). Later studies also showed that a loss of CCM3 resulted in an increased stress fiber formation; however, one study challenged this finding and proposed that CCM3 loss did not increase stress fiber formation (Zheng et al., 2010; Chan et al., 2011). It is well chronicled in many cell types, including endothelial cells, that activated RhoA stimulates stress fiber formation (Ridley and Hall, 1992). Like other GTPases, RhoA is active when GTP is bound and inactive when GDP is bound to the guanine nucleotide-binding site of the GTPase (Figure 1). Specific guanine nucleotide exchange factors (GEFs) activate GTPases, such as RhoA, by stimulating the exchange of GDP for GTP, and GTPase-activating proteins (GAPs) inactivate the GTPase by promoting the intrinsic GTPase activity. The Rho GTPases also bind the guanine nucleotide dissociation inhibitors (GDIs), which block GDP dissociation, thereby keeping the GTPase in an inactive state (Jaffe and Hall, 2005).

Measurement of activated RhoA (GTP bound) levels in CCM1, -2, or -3-deficient endothelial cell lysates demonstrated an increased active RhoA compared to the wild-type control endothelial cells (Whitehead et al., 2009; Stockton et al., 2010; Zheng et al., 2010). This finding is consistent with the increased formation of stress fibers in the endothelial cells lacking CCM1, -2, or -3. Two primary RhoA effectors cooperate to control actin polymerization and stress fiber formation: Rho-associated coiled coil-forming kinase (ROCK) and mammalian homolog of *Drosophila* diaphanous (mDia) (Watanabe et al., 1999). mDia is a formin protein that catalyzes the formation of long straight actin filaments (Figure 2A). The ROCK, in contrast, is a serine-threonine kinase whose substrates include myosin light chain (MLC), MLC phosphatase, and LIM kinase (LIMK) (Riento and Ridley, 2003) (Figure 2B). The phosphorylation of MLC phosphatase inhibits its catalytic activity, and the resulting increase in the phosphorylated MLC increases myosin cross-linking with

actin, which results in actomyosin contractility. LIMK is activated when phosphorylated by the ROCK. LIMK-catalyzed phosphorylation of cofilin inhibits the actin depolymerizing and severing activity of cofilin. The treatment of cells with a ROCK inhibitor, such as Fasudil or Y-27632, results in the disruption of actin filaments leaving diffuse, disrupted actin filaments in the cell and abolishes increased stress fibers due to the activation of RhoA.

The increased RhoA activity in CCM1, -2, or -3-deficient endothelial cells results in the increased phosphorylation of the MLC, consistent with the increased stress fiber formation. Functionally, CCM1, -2, or -3-deficient endothelial cells display loss of migration, invasiveness, ability to form three-dimensional tubes, and form a stable permeability barrier as a monolayer (Whitehead et al., 2009; Borikova et al., 2010; Stockton et al., 2010). Each of these functions was rescued by the treatment of the cells with the ROCK inhibitor or RNAi knockdown of the ROCK, consistent with the activated RhoA and increased actin-based stress fibers driving the CCM phenotype (Whitehead et al., 2009; Borikova et al., 2010; Stockton et al., 2010). Thus, the CCM proteins must be regulating RhoA activity.

The emerging role of proteasomal degradation for control of RhoA signaling

In addition to the widely appreciated role of specific GEFs, GAPs, and GDIs in the regulation of RhoA activity, it is now realized that ubiquitin-mediated proteasomal degradation of RhoA is important for the control of localized RhoA signaling and the control of RhoA protein levels (Ding et al., 2011). Ubiquitination resulting in proteasomal degradation of RhoA is catalyzed by two different E3 ubiquitin ligases: Smurf1 and Cullin3. Smurf1 and Cullin3 are members of the HECT domain and the RING/U-box families of the E3 ubiquitin ligases, respectively.

Smurf1 has a C2-WW-HECT domain architecture with the E3 ligase activity encoded in the HECT domain. It is a member of the NEDD4 subfamily of HECT domain E3 ligases. The C2 domain targets Smurf1 to the membranes, and the WW domain generally binds the substrate proteins for ubiquitination by the HECT domain. Among its target substrates, Smurf1 ubiquitinates RhoA, leading to its degradation and, therefore, regulates cell shape, polarity, cell-cell contact, and motility (Wang et al., 2003). Smurf1 is thought to inhibit RhoA signaling through targeted degradation of active RhoA at the sites of active membrane protrusion during the dynamic regulation of cell polarity and migration that involves an integrated response of additional GTPases Rac1 and Cdc42 and the PAR6-PKC ζ polarity complex (Wang et al., 2003). This localized RhoA degradation prevents reactivation of the targeted RhoA protein by a Rho GEF, which inhibits RhoA signaling in the cellular locations having active Smurf1. Interestingly, the overall levels of RhoA do not generally change significantly when Smurf1 is either overexpressed or knocked down using RNAi in the fibroblasts or cells of epithelial origin such as HEK293 cells. This finding has been used to support the notion that Smurf1 mediates the degradation of the localized active RhoA at sites of active membrane protrusion at the leading edge of migrating cells and not a generalized degradation of RhoA (Wang et al., 2003). The Smurf1 knockdown phenotype has not been defined in the vascular endothelial cells.

Cullin3 is a member of the Cullin-RING-Ligases (CRLs) that have been shown to target RhoA for ubiquitination and proteasomal degradation (Chen et al., 2009). RhoA was identified indirectly as a target for Cullin3 by the phenotype observed in the cells where Cullin3 had been knocked down by RNAi (Chen et al., 2009). The Cullin3 knockdown cells display a remarkable network of actin stress fibers, inhibited migration, and altered cell morphology. Unlike the Smurf1 knockdown cells, the Cullin3 knockdown cells had a markedly increased RhoA expression due to the loss of Cullin3-mediated RhoA degradation.

The expression of other GTPases was not affected by the Cullin3 knockdown, indicating that RhoA is a selective GTPase for ubiquitination by Cullin3.

RhoA activation, expression, and stress fiber formation in CCM

A hallmark of RNAi-mediated knockdown of CCM1, -2, or -3 in endothelial cells in culture is increased stress fiber formation due to the aberrant RhoA activity (Glading et al., 2007; Whitehead et al., 2009; Stockton et al., 2010; Zheng et al., 2010). This increased RhoA activity has also been seen with the RNAi knockdown of STK25, a serine-threonine protein kinase that binds CCM3 (Zheng et al., 2010). The knockdown of ezrin and moesin, members of the ERM cytoskeleton-associated proteins that are phosphorylation substrates for STK25, also activated RhoA (Zheng et al., 2010). This finding suggests that membrane-localized actin-associated complexes regulated by STK25 and the ERM proteins control RhoA activation. Studies using a well-characterized Förster Resonance Energy Transfer (FRET) biosensor for RhoA showed that the loss of CCM1, -2, or -3 did, indeed, result in an increase in RhoA activation in the endothelial cells (Borikova et al., 2010). The knockdown of CCM1 gave a pronounced activation of RhoA, but the loss of any of the three CCM proteins increased RhoA activity both at the sites near the plasma membrane and in the cell body.

Cröse et al. demonstrated that CCM2 bound Smurf1 through a CCM2 PTB domain-Smurf1 HECT domain interaction that localized Smurf1 at the plasma membrane (Cröse et al., 2009). In these studies, it was shown that the CCM2 interaction with Smurf1 regulated RhoA degradation. Interestingly, CCM2 was first cloned as a scaffold-like protein that was originally named *Osmosensing Scaffold for MEKK3* (abbreviated OSM) (Uhlík et al., 2003). In this study, OSM was highly expressed in the primary macrophages and localized to the sites of newly polymerized actin in the membrane ruffles. This localization is consistent with a targeted role of CCM2/OSM in controlling RhoA function.

The Smurf1 knockdown in other cell types has indicated that there is a redistribution of F-actin to cortical actin and not a general increase in stress fiber formation throughout the cell body (Wang et al., 2003). Smurf1 is proposed to be localized to the membrane protrusions at the leading edge of the migrating cells and membrane ruffles, which is similar to the localization of CCM2 in the macrophages, where RhoA is ubiquitinated and degraded. Several laboratories have reported the knockdown of CCM1, -2, or -3 that resulted in stress fiber formation in human and mouse endothelial cells, suggesting that mechanisms, in addition to the dysregulated Smurf1 ubiquitination of RhoA, might contribute to the CCM phenotype if Smurf1 has similar regulatory functions in the cells of endothelial and epithelial origin (Whitehead et al., 2009; Stockton et al., 2010; Zheng et al., 2010).

Our studies using the stable shRNA knockdown of CCM1, -2, or -3 have demonstrated an increase in the RhoA protein levels in addition to the increased RhoA activity (Borikova et al., 2010). This experiment has been done in multiple endothelial cells of human and mouse origin. Other laboratories studying the phenotype of CCM knockdown in endothelial cells have generally used siRNA strategies with a significantly shorter time between the introduction of the siRNAs and analysis (~5–7 days) vs. the drug selection after lentiviral infection for the expression of shRNAs to stably knockdown the CCM proteins (~14 days). In general, the siRNA strategies have not given a significant increase in the RhoA expression that we have observed with the stable shRNA knockdown. The reason for this discrepancy is unclear because increased stress fibers are apparent in endothelial cells for more than one study where siRNAs were used for CCM protein knockdown (Whitehead et al., 2009; Stockton et al., 2010; Zheng et al., 2010). This increase in stress fibers was not found in one study using the CCM3 siRNA knockdown in HUVECs, even though a loss of tube formation was observed (Chan et al., 2011). The CCM1, -2, and -3 shRNA knockdown

phenotype, characterized by an activated RhoA, increased RhoA expression, and stress fiber formation, does not appear to be an off target effect of the shRNAs. Multiple shRNAs for targeting CCM1, -2, or -3 give similar phenotypes, and the loss of migration, invasion, and tube formation are reversed with the inhibition of the ROCK, demonstrating that the phenotype is driven by RhoA activation (Borikova et al., 2010). The difficulty in comparing the siRNA and shRNA studies is the turnover of two proteins, CCM1, -2, or -3, and RhoA is potentially being altered. The turnover of these proteins appears to be relatively slow, making the timing of the assays of RhoA activation and protein expression after knockdown critical. If both Smurf1 and Cullin3 are involved in the CCM phenotype, then the influence of their dysregulation may have different temporal as well as spatial consequences on RhoA function.

Our current understanding of the roles of Smurf1 and Cullin3 in regulating RhoA ubiquitination and degradation, in cell types other than vascular endothelial cells, suggests the possibility of distinct regulatory functions for the two E3 ligases in endothelial cells that could be dysregulated in CCM. Smurf1 has been shown to be involved in cell polarity and directed migration that is required for normal endothelial cell physiology. Cullin3 regulates RhoA protein levels, ROCK-dependent formation of stress fibers, and cell migration. The loss of Cullin3 expression using RNAi was sufficient to increase the basal activation of RhoA sufficiently to induce stress fibers (Chen et al., 2009). Functionally, CCM2 binds Smurf1, but it is presently unclear if the CCM proteins interact with the Cullin3 protein complex and regulate Cullin3-dependent RhoA degradation.

Mechanistically, the loss of CCM1, -2, or -3 could result in an increased RhoA GEF or decreased RhoA GAP activity. The functional regulation of RhoA by GDIs could also be deregulated. Each loss of the CCM protein-dependent change could result in increased basal RhoA activity and stress fiber formation. It is difficult to understand how the RhoA protein is increased with no change in transcription without decreased degradation of RhoA. Studies with the RhoA biosensor readily measured the spatial localization of the activated RhoA in cells (Borikova et al., 2010). The activated RhoA was located not just at the cell edge and membrane protrusions, but pronounced activity was also seen in the cell body. The increased RhoA activity was very pronounced in the cell body and the nucleus of the CCM1 knockdown endothelial cells. It will be of interest to determine what the effect of Smurf1 and Cullin3 knockdown has on the spatiotemporal activity of RhoA. Clearly, more studies are required to define how the CCM proteins regulate RhoA activity and if the control of RhoA degradation is a major mechanism for the CCM control of RhoA-dependent endothelial cell physiology.

ROCK inhibition and RhoA geranylgeranylation as therapeutic interventions for CCM

Inhibition of the ROCK is able to rescue *in vitro* and *in vivo* the CCM phenotypes suggesting that the ROCK small molecule inhibitors could provide a pharmacological intervention for CCM (Whitehead et al., 2009; Borikova et al., 2010; Stockton et al., 2010). The ROCK inhibition or RNAi knockdown of ROCK2 rescues the endothelial cell actin cytoskeletal dynamics, *in vitro* tube formation, and permeability barrier function (Whitehead et al., 2009; Borikova et al., 2010; Stockton et al., 2010). *In vivo*, the ROCK inhibition decreased the vascular leak stimulated by LPS in CCM1- and CCM2-deficient mice (Stockton et al., 2010). Two structurally distinct ROCK inhibitors, H-1152 and Fasudil, rescue *in vitro* and *in vivo* the CCM phenotypes, with the RNAi knockdown of the ROCK rescuing *in vitro* the CCM1, -2, or -3-deficit phenotypes. Simvastatin inhibits HMG-CoA reductase and interferes with the production of geranylgeranyl pyrophosphate, which is required for RhoA lipidation, targeting RhoA to the membrane where it functions to control

the actin cytoskeleton (Takemoto and Liao, 2001). Simvastatin, by inhibiting the geranylgeranylation of RhoA, is capable of rescuing the CCM phenotype (Whitehead et al., 2009). Cumulatively, the findings indicate that the activated RhoA seen with the loss of CCM1, -2, or -3 is responsible for the CCM phenotype and that inhibition of RhoA signaling or the ROCK activity can prevent and/or reverse specific endothelial cell defects seen in CCM. Simvastatin is an FDA-approved drug for lowering cholesterol, and Fasudil has been used in Japan for cerebral vasospasm after subarachnoid hemorrhage since 1995 and displays positive outcomes with a few adverse effects (Suzuki et al., 2007). Other small molecule ROCK inhibitors in early stage development for the treatment of cardiovascular disease have been well-tolerated in mice, suggesting that suppression of RhoA geranylgeranylation and/or ROCK inhibition provides viable molecular targets to prevent or treat the CCM lesions (Surma et al., 2011). Thus, despite our lack of complete understanding of how the loss of CCM1, -2, or -3 protein expression alters RhoA activation and RhoA protein levels, it is probable that a therapeutic strategy for patients can evolve from the discovery of the dysregulated RhoA signaling as a primary pathway responsible for the CCM endothelial cell pathophysiology.

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Figure 1.

Defined structural domains and interacting proteins for CCM1, -2, and -3. Interactions were defined using multiple techniques, including coimmunoprecipitation coupled with mass spectroscopy and/or immunoblotting and yeast 2-hybrid screens. The CCM1 four-point-one, ezrin, radixin, moesin (FERM) domain binds multiple proteins. The NPAY, NPLF, and NPYF sequences in CCM1 bind to the PTB domains. CCM1 also encodes a nuclear localization (NLS) sequence. CCM2 encodes a PTB domain and Karet domain. CCM3 has a FRAP-ATM-TRAP (FAT) domain that binds MST4, STK24, and STK25 serine/threonine protein kinases.

Figure 2.

CCM proteins regulate RhoA activation and signaling.

(A) The CCM proteins form a ternary complex and negatively regulate RhoA activity and protein levels. CCM2 associates with the E3 ubiquitin ligase Smurf1, which targets active RhoA for degradation. RhoA is activated by GEF-mediated GDP to GTP exchange after ligand binding to a GPCR or RTK. Activated RhoA activates both mDia and ROCK. mDia stimulates actin polymerization. The ROCK phosphorylates and inhibits the MLC phosphatase (MLCPase) and directly phosphorylates the MLC and LIMK. LIMK phosphorylates the actin severing protein cofilin, leading to a decreased activity. This process leads to the stabilization and cross-linking of actin and myosin. (B) The RhoA-bound ROCK phosphorylates multiple substrates that affect global actin cytoskeletal dynamics, which in the context of CCM leads to stress fiber formation and junctional protein destabilization. Current pharmacological approaches to treat CCM based on this model are through the inhibition of RhoA geranylgeranylation with statins or through ROCK inhibition to restore proper endothelial cytoskeletal dynamics.