Apoptotic Functions of PDCD10/CCM3, the Gene Mutated in Cerebral Cavernous Malformation 3

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Background and Purpose—Mutations in the Programmed Cell Death 10 (PDCD10) gene cause autosomal dominant familial cerebral cavernous malformations (CCM3). To date, little is known about the function of this gene and its role in disease pathogenesis.

Methods—We examined the effects of overexpression of wild-type and 2 human disease-causing variants of PDCD10 on cell death using 3 different methods (TUNEL and MTT assays and caspase-3 activation). We analyzed expression of CCM3, activated caspase-3, and p38 in endothelial cell lines using the serum deprivation model of apoptosis induction. Finally, we assayed the effects of siRNA-mediated inhibition of endogenous PDCD10 expression on cell death in endothelial cell cultures.

Results—Overexpression of wild-type CCM3, but not disease-linked mutant forms, induced apoptosis as confirmed by TUNEL and increased levels of activated caspase-3. Serum starvation of endothelial cells, an inducer of apoptosis, led to increased expression of CCM3 and activation of p38 and ultimately activated caspase-3. siRNA-mediated inhibition of CCM3 expression resulted in decreased levels of p38 and activated caspase-3, and decreased cell death.

Conclusions—CCM3 is both necessary and sufficient to induce apoptosis in vitro in well-defined cell culture systems. Even though it is currently unclear whether this effect on apoptosis is direct or indirect through modulation of cell cycle, these results led to the novel hypothesis that CCM lesions may form as a consequence of aberrant apoptosis, potentially altering the balance between the endothelium and neural cells within the neurovascular unit. (Stroke. 2009;40:1474-1481.)

Key Words: cerebral cavernous malformations ■ CCM3 ■ PDCD10 ■ apoptosis ■ p38 ■ caspase-3

Cerebral cavernous malformations (CCM) are vascular lesions affecting almost exclusively the central nervous system (CNS) and the retina. Grossly, they are a collection of enlarged sinusoidal vascular channels almost resembling a raspberry. Ultrastructurally, these channels are lined by a single layer of endothelium, lack normal vessel wall elements such as smooth muscle and are devoid of intervening normal CNS parenchyma. These lesions can occur sporadically, or as a familial form attributable to mutations in 3 different genes: CCM1, CCM2 (malcavernin) and Programmed Cell Death 10 (PDCD10); alternative name: CCM3. Overall, mutations in these 3 genes account for approximately 96% of familial cases. Despite the identification and characterization of these CCM genes, several questions regarding this complex disorder remain unanswered. These include a sophisticated understanding of the function of the CCM genes and how mutations lead to formation of lesions that almost exclusively affect the CNS and are focal in nature. It has been suggested that the CCM lesions may be the consequence of hemorrhage-induced proliferation, a process by which abnormal vascular beds cause reactive angiogenesis with new vessel formation and coalescence, but mechanistic insight into lesion formation is lacking.

Recent molecular biological studies on the CCM1 and 2 proteins shed some light into their functions. CCM1 was shown to bind to microtubules in vitro and to interact individually with Krev-1/Rap1a, ICAP1a (integrin cytoplasmic domain associated protein 1 alpha), β-catenin, and CCM2 in various in vitro systems. CCM2 binds CCM1 and MEKK3 in a ternary complex and acts as a scaffolding protein signaling through p38 after extracellular stimulation. More recently, CCM3 was shown to be a part of this complex in vitro. Despite this recent progress, little is known about the biological functions of the CCM3 protein. It was first identified through a screen of genes expressed during the induction of apoptosis in a premyeloid cell line. Recent evidence suggests

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that CCM3 binds to and is phosphorylated by serine/threonine kinase 25 (STK25) and is dephosphorylated by binding to the phosphatase domain of Fas-associated phosphatase-1.27

Based on these limited data, we sought to investigate the biological function of CCM3 and specifically focused on its potential involvement in apoptotic pathways. We modulated CCM3 expression using cell culture based assays in vitro and examined its effects on cell death. Our results indicate that CCM3 has a proapoptotic function in the cell culture models tested and led to the novel hypothesis that CCM lesions potentially form as a result of aberrant apoptosis in the vasculature within the neurovascular unit.

Methods

Cell Culture

HeLa cells were plated in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. Primary human umbilical vein endothelial cells (HUVECs) were obtained from the Vascular Biology and Transplantation Program at Yale University (New Haven, Conn). HUVECs were grown in M199 medium with 20% fetal bovine serum (FBS) and 1% endothelial cell growth supplement (ECGS), on gelatin-coated (0.1%) plates (J.T. Baker Inc). Cells used in this study were obtained between passages 3 and 6. For serum starvation assays, HUVECs were seeded on 0.1% gelatin coated culture slides and after 48 hours of attachment in standard media, cells were washed in 0.2% FBS medium, followed by an additional 24-hour incubation in 0.2% FBS.

Expression Vector Construction

A PCR-amplified human PDCD10/CCM3 (hCCM3) cDNA fragment was subcloned into XhoI and HindIII sites of the pEGFP-C3 vector (Clontech) or a pRFP-C3 vector containing monomeric red fluorescent protein, resulting in a C-terminal GFP- or RFP-tagged CCM3, respectively. Both expression constructs were indistinguishable in terms of transfection efficiency and cytotoxicity in HeLa cells (data not shown). Expression plasmids carrying two human disease-associated with caspase-3 activation. Caspase-3 has previously been identified as an effector caspase in several apoptotic pathways.25 We analyzed the effects of CCM3 overexpression in cell culture by transfecting HeLa cells with the expression plasmid pEGFP-C3-hCCM3 (referred as GFP-CCM3 henceforth). GFP-CCM3 (wild-type) overexpression resulted in increased levels of apoptosis compared to vector alone (Figure 1). A significantly larger proportion of apoptotic cells with fragmented nuclei was observed in the population of GFP-positive (and, therefore, CCM3 expressing) cells transfected with GFP-CCM3 compared to vector alone (Figure 1). A significantly larger proportion of apoptotic cells with fragmented nuclei was observed in the population of GFP-positive (and, therefore, CCM3 expressing) cells transfected with GFP-CCM3 compared to vector alone (Figure 1).

Apoptosis Assays

Cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice and the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction mixture (In Situ Cell Death Detection Kit, TMR-red, Roche) was applied for 1 hour at 37°C. Cell counts were performed at 10 different fields in 3 separate transfection experiments.

Cell Viability Assays (MTT)

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay. Briefly, 72 hours after transfection, MTT was added to each well at a final concentration of 0.5 mg/mL for 4 hours; the cells were subsequently lysed in dimethyl sulfoxide (DMSO) and the amount of MTT formazan was quantified by determining the absorbance at 550 nm, using a microplate reader. Cell viability is expressed as a percentage of the control value (GFP plasmid transfection group).

PDCD10 siRNA Inhibition

HUVECs were seeded on 6-well plates (5 × 10⁴ cells per well). After attachment overnight, cells were washed in PBS, and transfected with 3 sets of PDCD10 Stealth select siRNAs (25 bp; Unigene ID: Hs.478150), following the manufacturer’s protocol (Invitrogen Corporation). As control siRNA, stealth RNAi negative control (Invitrogen) was used. After 72 hours of incubation, HUVECs were washed and exposed to low serum (0.2% FBS). At different time points, HUVECs were harvested for Western blotting. The membranes were incubated with the following antibodies: antiphospho-p38 (Cell Signaling Technology), anti-CCM3,29 or anti-GAPDH (Santa Cruz).

To quantify cell death occurring in the cultures, terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) analysis was performed as above. The percentage of apoptotic cells was calculated from the positively labeled cells in each field (400×) from at least 5 fields for each group.

Statistical Analyses

Data are presented as means±SE of absolute values or percent of control and were analyzed using either Student t test (Figures 1 and 2) or 1-way ANOVA (Figures 3 to 5). We initially performed a global ANOVA test to check for significance and when proven, proceeded with pair-wise comparisons to examine which groups were significantly different from the baseline. Bonferroni correction was applied to correct for multiple testing unless specified otherwise and P<0.05 was considered significant. The global ANOVA probability values and Bonferroni corrected probability values for pair-wise ANOVA tests are reported.

Results

CCM3 Induces Apoptosis in HeLa Cells

We analyzed the effects of CCM3 overexpression in cell culture by transfecting HeLa cells with the expression plasmid pEGFP-C3-hCCM3 (referred as GFP-CCM3 henceforth). GFP-CCM3 (wild-type) overexpression resulted in increased levels of apoptosis compared to vector alone (Figure 1). A significantly larger proportion of apoptotic cells with fragmented nuclei was observed in the population of GFP-positive (and, therefore, CCM3 expressing) cells transfected with GFP-CCM3 compared to those control transfections with empty vector (Figure 1b). Protein expression of GFP and GFP-CCM3 was confirmed by immunoblotting with anti-GFP (Figure 1c).

We then investigated whether CCM3-mediated cell death is associated with caspase-3 activation. Caspase-3 has previously
been established as a terminal executioner of the apoptotic cell death pathway. Activated caspase-3 was detected in apoptotic cells with fragmented/condensed nuclei expressing CCM3 (Figure 2). These data indicate that CCM3 overexpression in HeLa cells results in caspase-3 activation and cell death.

**Disease-Causing Mutated CCM3 Fails to Induce Apoptosis**

We had previously reported 4 novel disease-causing *PDCD10/CCM3* mutations in CCM families. We introduced 2 of these point mutations (c.283C>T [M1], c.192delA [M2]) into the GFP-CCM3 expression vector. Both mutations lead to production of truncated proteins (Figure 3a). We tested the effects of overexpression of mutated forms of CCM3 in HeLa cells. As expected, overexpression of wild-type CCM3 resulted in increased cleaved caspase-3 levels, whereas overexpression of either mutant form of CCM3 (c.283C>T, c.192delA) had no effect on cleaved caspase-3 expression (Figure 3b). In addition, overexpression of either CCM3 mutant resulted in reduced cell loss by the MTT assay compared to wild type CCM3 (Figure 3c), further suggesting that mutant forms of CCM3 fail to induce apoptosis.

**Serum-Deprivation of Endothelial Cells Results in Increased CCM3 Levels**

Expanding on a previously established assay for the induction of apoptosis in endothelial cells, we cultured human umbilical vein endothelial cells (HUVECs) in low-serum media. Serum-deprived HUVECs demonstrated an increase in cleaved caspase-3 protein levels by Western blotting after 3 hours as expected (Figure 4a). Interestingly, CCM3 expression increased on serum-deprivation starting at approximately 30 minutes, before the increase in cleaved caspase-3 levels (Figure 4b). After serum starvation, reincubation of cells in normal media (20% FBS) allows for cell recovery and restores (reduces) CCM3 expression to normal levels (Figure 4c). To establish the mechanism of this increase in CCM3 expression, we incubated the cells with different concentra-
In summary, serum deprivation of endothelial cells results in increases in CCM3 and phosphorylated p38 protein levels followed by caspase-3 activation.

**siRNA Inhibition of CCM3 Expression Reduces Endothelial Cell Apoptosis During Serum Deprivation**

Using the same serum-starvation model, we then reduced CCM3 protein levels with siRNAs as described in Methods. HUVECs treated with *CCM3* siRNA demonstrated decreased levels of cell death, as shown by the TUNEL assay (Figure 5a and 5b). We then evaluated whether the reduction of cell death correlated with reduction of caspase-3 activation. Serum-starved HUVECs also demonstrated decreased activated caspase-3 levels after treatment with *CCM3* siRNA (Figure 5c). This decrease in CCM3 and activated caspase-3 levels correlates with decreased p38 activation after *CCM3* siRNA transfection compared to cells treated with siRNA controls (stealth RNAi negative control; Figure 5d).

**Discussion**

Because CCM lesions in all 3 inherited forms of the disease are clinically and pathologically nearly identical, we had hypothesized over 10 years ago that genes causing familial cavernous malformations CCM1, 2, and 3 (KRIT1, CCM2, and *PDCD10*, respectively) all participate in a common or parallel pathways, and thus disruption of signaling would lead to similar disease manifestations. To test this hypothesis, we previously investigated the expression patterns of CCM1, 2, and 3 and showed that these proteins spatially and temporally show similar expression patterns. These proteins are specifically expressed throughout the endothelium in many tissues and organs and within the neurovascular unit of the CNS that consists of astrocytes, neurons, and vascular endothelium. Consistent with this observation, recent data from yeast and mammalian systems demonstrate that CCM1, 2, and 3 physically interact, confirming findings in zebrafish which demonstrate that CCM1 and 2 act in a common pathway with *ccm1* and *ccm2* mutants displaying comparable vascular defects. Similarly, in vivo studies in the mouse based on *Ccm1* and 2 knockout models have revealed their nonredundant, vital roles in angiogenesis but failed to reveal any breakthrough insights into their function.

On the other hand, multiple levels of in vitro evidence suggest that the CCM genes might play a role in a variety of biological processes linking extracellular signals, including adhesion (through β1 integrin or β-catenin interactions) or stress (including osmo- or mechano-stress) to various biological responses, including changes in morphology and proliferation. In this article, we present data that link CCM signaling to apoptotic pathways.

Indeed, in vitro work has shown that the CCM1 protein, KRIT1, binds to microtubules in a folded state. Both Rap1a and ICAP1α can disrupt the association of KRIT1 with microtubules localizing it to endothelial cell–cell junctions. Interestingly, only ICAP1α appears to be able to unfold KRIT1. After localization to the cell membrane through its FERM domain, KRIT1 binds to β-catenin and plays a role in stabilizing adherens junctions and regulates...
endothelial permeability. Through its NPXY domains, KRIT1 also binds CCM2, potentially recruiting CCM2 to these junctions. CCM2 has been identified as a human paralog of the mouse osmosensing scaffold protein for MEKK3 (OSM), which, after binding with CCM1 and potentially this complex, might participate in the p38 mitogen-activated protein kinase (p38 MAPK) stress signaling pathway. More recent evidence suggests that CCM3, at least in certain biological states, might be part of this complex. Furthermore, on activation of this complex, CCM1 shuttles from the cytoplasm and the cell membrane into the nucleus and therefore might interact with other nuclear molecules, including transcription factors, which might influence basic cellular functions such as proliferation and apoptosis. Consistent with this hypothesis is the observation in C elegans, in which kri-1, a CCM1 ortholog, interacts with DAF-16, a transcription factor, such that mutations in kri-1 lead to an increase in life span.

Based on these observations, we investigated the biological functions of CCM3 using overexpression or siRNA-mediated inhibition of PDCD10/CCM3 expression. Previously published studies showed CCM3 to be a part of a cohort of proteins upregulated on growth factor deprivation and apoptosis induction in the TF-1 premyleloid cell line as well as in a fibroblast cell line. Based on these data which suggested that CCM3 might play a role in apoptosis signaling, we initially focused our attention at understanding the capacity of CCM3 to induce apoptosis in the absence of exogenous proapoptotic stimuli. Overexpression of CCM3 in HeLa cells was in fact sufficient to induce increased levels of cell death as evidenced by both nuclei morphology as well as caspase-3 activation, a well established common end pathway protein in apoptosis. This increase in apoptosis and activated caspase-3 levels was abrogated when cells were transfected with wild-type CCM3 increases activated caspase-3 expression levels, whereas transfection with either mutated CCM3 construct has no effect on cleaved caspase-3 expression. *P<0.05 compared with untransfected group (n=3). c, Transfections with mutated CCM3 fail to elicit significant cell loss in comparison with wild-type CCM3. Cell viability was measured using the MTT assay (see Methods) and is graphed as a percentage of the control culture value (GFP vector alone transfection group) as quantified by determining the absorbance of cell cultures at 550 nm. *P<0.05 vs GFP plasmid transfection group (n=3).

Figure 3. Mutant CCM3 fails to induce cell death in HeLa cells. Transient transfections in HeLa cells with the following expression plasmids: N=untransfected; V=vector alone; CCM3= wild-type GFP-CCM3 construct; M1=GFP-CCM3 construct harboring the 283C>T mutation; M2=GFP-CCM3 construct harboring the 192delA mutation. a, Western blotting for anti-GFP demonstrates transfection efficiency. HeLa cells were transiently transfected, cell lysates were prepared and immunoblotted with an antibody against GFP. The anti-CCM3 antibody is downstream to the mutated sequences and thus fails to recognize the truncated protein. b, Transfection with wild-type CCM3 increases activated caspase-3 expression levels, whereas transfection with either mutated CCM3 construct has no effect on cleaved caspase-3 expression. *P<0.05 compared with untransfected group (n=3). c, Transfections with mutated CCM3 fail to elicit significant cell loss in comparison with wild-type CCM3. Cell viability was measured using the MTT assay (see Methods) and is graphed as a percentage of the control culture value (GFP vector alone transfection group) as quantified by determining the absorbance of cell cultures at 550 nm. *P<0.05 vs GFP plasmid transfection group (n=3).
affect both proliferation and apoptosis in a cell type-dependent manner, potentially explaining observed effects of CCM3 on these biological processes. Further work will be needed to answer these questions. Our results, however, lead to the novel hypothesis of attenuated apoptosis affecting endothelial cells within the neurovascular unit as a molecular mechanism underlying cavernous malformations. If proven to be true in vivo, this might lead to a mechanistic understanding of the role of apoptosis and cell cycle control in CCM pathophysiology, potentially having significant implications in designing new therapies.

Conclusions
CCM3 is both necessary and sufficient to induce apoptosis in well-defined cell culture systems. These results led to a novel hypothesis that CCM lesions may form because of aberrant apoptosis, potentially altering the balance between the endothelium and neural cells within the neurovascular unit. Even though it is currently unclear whether this effect on apoptosis is direct or indirect through modulation of cell cycle, the current data provide a testable hypothesis about CCM lesion development and has potentially important implications for a mechanistic understanding of CCM pathophysiology.

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**Figure 5.** siRNA inhibition of CCM3 expression in endothelial cells results in decreased cell death, p38, and cleaved caspase-3 expression. a, Serum-deprived HUVECs treated with CCM3 siRNA show decreased cell death. Representative images (20× original magnification) at the 3-hour time point are shown for untransfected HUVECs (no siRNA group, left panel) or those transfected either with control siRNA (middle panel) or CCM3 siRNA (right panel). All groups were subjected to serum deprivation (see Methods) and cell death was analyzed at various time points by TUNEL assay. TUNEL-positive cells (green) are scarce in the sample treated with CCM3 siRNA (right panel) compared to those untreated (left panel) or treated with control siRNA (middle panel). b, The percentage of apoptotic cells was calculated from TUNEL-positive cells in each field (400×) and in at least 5 fields for each group (global ANOVA P<10⁻³ in both control groups across all time points and within the 3-hour group). *Corrected P<0.05 compared with the equivalent groups before serum deprivation (0 hour; n=3). c, Serum-deprived HUVECs show decreased cleaved caspase-3 levels after treatment with CCM3 siRNA (global ANOVA P<10⁻³ in both control groups across 2 time points). HUVECs were transfected with control siRNA or CCM3 siRNA, then were cultured under serum deprivation conditions (0.2% FBS). Cells lysates were prepared at various time points and cleaved caspase-3 levels were analyzed using Western blotting. Serum deprivation results in increase of cleaved caspase-3 in both the no siRNA and control siRNA treated groups, but not in the CCM3 siRNA-treated group. *Corrected P<0.05 compared with the equivalent groups before serum deprivation (0 hour; n=3). d, HUVECs treated with CCM3 siRNA demonstrate decreased p38 activation (global ANOVA P<10⁻³ in p-p38/p38 and CCM3/GSDPH groups across 2 time points). Similar to the procedure in c, serum deprivation results in increase of p38 activation and CCM3 protein levels in the no siRNA- and control siRNA-treated groups, but not in the CCM3 siRNA-treated group. *Corrected P<0.05 compared with the equivalent groups at each time point (n=3).

**Disclosures**

None.

**References**

gene (CCM1) to a 4-cm interval of chromosome 7q contained in a well-defined yac contig. **Genome Res.** 1995;5:368–380.


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