Upregulation of transmembrane endothelial junction proteins in human cerebral cavernous malformations

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Object. Cerebral cavernous malformations (CCMs) are among the most prevalent cerebrovascular malformations, and endothelial cells seem to play a major role in the disease. However, the underlying mechanisms, including endothelial intercellular communication, have not yet been fully elucidated. In this article, the authors focus on the endothelial junction proteins CD31, VE-cadherin, and occludin as important factors for functional cell-cell contacts known as vascular adhesion molecules and adherence and tight junctions.

Methods. Thirteen human CCM specimens and 6 control tissue specimens were cryopreserved and examined for the presence of VE-cadherin, occludin, and CD31 by immunofluorescence staining. Protein quantification was performed by triplicate measurements using western blot analysis.

Results. Immunofluorescent analyses of the CCM sections revealed a discontinuous pattern of dilated microvessels and capillaries as well as increased expression of occludin, VE-cadherin, and CD31 in the intima and in the enclosed parenchymal tissue compared with controls. Protein quantification confirmed these findings by showing upregulation of the levels of these proteins up to 2–6 times.

Conclusions. A protocol enabling the molecular and morphological examination of the intercellular contact proteins in human CCM was validated. The abnormal and discontinuous pattern in these endothelial cell–contact proteins compared with control tissue explains the loose intercellular junctions that are considered to be one of the causes of CCM-associated bleeding or transendothelial oozing of erythrocytes. Despite the small number of specimens, this study demonstrates for the first time a quantitative analysis of endothelial junction proteins in human CCM.

(DOI: 10.3171/2010.6.FOCUS10125)

Key Words • cerebral cavernous malformation • cell contacts • adherence junction • tight junction • occludin • VE-cadherin

Cerebral cavernous malformations constitute 5%–10% of all cerebrovascular malformations with a prevalence of approximately 0.5%. The condition affects cerebral blood vessels, resulting in dilation as well as thinning of the vessel wall.¹,¹⁷ Depending on the location of the affected vessel, this often leads to bleeding, causing seizures, the main clinical sign of CCMs, or other neurological deteriorations.⁴,¹⁸ Currently, 2 types of CCMs can be distinguished: a familial form, which follows an autosomal dominant pattern of inheritance, and a sporadic form.¹⁰ The familial form is caused by loss-of-function mutations in 3 known genes:⁶,⁷,¹³,²⁰ KRIT1 (also known as CCM1), CCM2 (also known as malcalvernin or osm¹⁴), and PDCD10 (also known as CCM3). However, in many CCM patients such gene mutations are absent, which indicates additional pathogenic mechanisms. The recent findings of PTEN promotor methylation²⁸ or disease-modulating factors such as the HEG transmembrane receptor¹² and the RhoA GTPase²⁵ support this hypothesis.

In vitro cell studies have suggested that endothelial cells play a significant role in CCM¹⁵,²⁵ and that contacts to neighbor cells, such as neuroglia or neurons, and interendothelial cell contacts may be altered.⁹,¹⁵ Zhao et al.²⁷ showed an impairment of intercellular barrier function of endothelial cells, while Clatterbuck et al.² detected gaps in endothelial tight junction formation in CCM. These authors and others conclude that the absence of intercellular barrier components renders vessels more susceptible to hemorrhage.¹¹,¹⁹,²³,²⁶ Looking at the intercellular junctions in more detail, occludin, as an integral plasma-membrane

Abbreviations used in this paper: CCM = cerebral cavernous malformation; DAPI = 4,6′-diamino-2-phenylindole-dihydrochloride; DVA = developmental venous anomaly; PBS = phosphate-buffered saline; SDS = sodium dodecyl sulfate.

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protein, is found specifically at tight junctions. This protein, as well VE-cadherin, is specifically expressed in endothelial adherence junctions and plays a critical role in endothelial cell contacts. Intercellular cohesion and organization as well as lining and sealing are important features of these protein complexes. Their alterations lead to leaky cell contacts followed by permeability and paracellular outflow with oozing of erythrocytes. To analyze expression of these intracellular proteins within the pathological CCM tissue, we focused on the protein expression of occludin, VE-cadherin, and CD31 and the quantification of possible alteration of these proteins in human CCM tissue compared with control tissue.

**Methods**

**Patients and Tissue Specimens**

Surgery was performed for indications unrelated to this research project, and there was no sex, age, or race bias in the selection of patients. After ethics review board approval (No E-69/2008) and patient informed consent were obtained, 13 specimens were obtained from 13 patients (Table 1) undergoing neurosurgical resection of CCMs in accordance with institutional guidelines at the University Hospital Zurich. (All specimens were obtained by the same surgeon [H.B.]) Specimens of nontumor tissue obtained from patients undergoing tumor resection and of tissue obtained from patients undergoing epilepsy surgery were used as controls. Control tissue was treated in the same manner as CCM tissue. The median age of the 6 female and 7 male patients was 32 years (range 16–51 years). After resection, the tissue specimens were transferred immediately to the laboratory (Fig. 1) and divided into sections for protein extraction by snap-freezing in liquid nitrogen and for cryosection by embedding in optimal cutting temperature medium and frozen over dry ice. In addition, parts of the tissue were used for routine clinical neuropathological examination to verify that the specimens met the criteria for the diagnosis of CCM.

**Immunofluorescence Analysis**

For immunofluorescence analyses, tissue cryosections (12-µm thick) were mounted on uncoated slides and stored at −80°C. For analysis, slides were blocked with 0.1 M glycine in PBS for 5 minutes and permeabilized in 0.2% Triton X-100/PBS for 10 minutes. After blocking with 5% goat serum in 1% BSA/PBS for 30 minutes, primary antibodies against CD31 (Clon JC70A, monoclonal mouse antihuman antibody, DakoCytomation), occludin (polycyclonal rabbit anti–human antibody, Invitrogen Corp.), and VE-cadherin (polycyclonal rabbit anti–human antibody, Cell Signaling Technology Inc.) were added and incubated overnight at 4°C. After 3 washing steps with PBS (each for 5 minutes), secondary antibodies (Cy3-conjugated goat anti–mouse IgG, bivalent fragment antigen binding [F[ab′]2) fragments, as well as Cy5-conjugated goat anti–rabbit antibodies, both from Jackson Immuno Research) were added for 45 minutes. Thereafter the specimens were washed again 3 times with PBS and mounted in Lisbeth medium (0.1 M Tris-HCl (pH 9.5)/glycerol (3:7) containing 50 mg/ml n-propyl gallate. Qualitative analysis was performed by means of conventional fluorescence microscopy (Leica DM 6000B). Image processing was performed on a PC workstation using

**TABLE 1: Summary of patient and lesion characteristics***

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Clinical Presentation</th>
<th>mRS score (adm/30-day FU)</th>
<th>Radiol Findings</th>
<th>MRI Type†</th>
<th>Location</th>
<th>Side</th>
<th>Diam. (mm)</th>
<th>MRI Type, Multiple Lesions</th>
<th>MRI Type, Unoperated Lesions</th>
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<td>RH</td>
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* Adm = admission; FU = follow-up; N = no; NH-FND = nonhemorrhagic focal neurological deficit; mRS = modified Rankin Scale; NRH = no recent hemorrhage; Radiol = Radiological; RH = recent hemorrhage; SH = symptomatic hemorrhage; SR = symptomatic relative; Y = yes.
† The MRI type classification was used as previously described.‡ The letters before the virgule indicate whether or not a DVA was present; the letters after the virgule indicated whether a DVA was directly associated with the surgically treated CCM lesion.
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PhotoShop (Adobe Systems) and Image J 1.42q, and images were processed uniformly.

Polyacrylamide Gel Electrophoresis and Immunoblotting

The CCM and control samples were homogenized by slam freezing, resuspended in SDS sample buffer (3.7 M urea, 134.6 mM Tris–HCl, pH 6.8, 5.4% SDS, 2.3% NP-40, 4.45% β-mercaptoethanol, 4% glycerol, and 6 mg/100 ml of bromophenol blue) followed by boiling for 1 minute. Samples were run on 8% polyacrylamide gels together with All Blue (Bio-Rad Laboratories, Inc.) molecular weight marker. Equal amounts of protein were loaded for the different tissue extracts as judged by Coomassie blue staining from the previous gel. Blotting was carried out overnight onto nitrocellulose Amersham Hybond-C Extra (GE Healthcare). The same primary antibodies as those used for immunofluorescence (against CD31, occludin, and VE-cadherin) and an antibody against all actin isoforms (polyclonal rabbit anti–human antibody [Sigma-Aldrich]) were used for protein loading control. These primary antibodies, as well as horseradish peroxidase-conjugated anti–rabbit IgGs (Calbiochem), which were used as secondary antibodies, were diluted in low-salt buffer supplemented with 4% nonfat milk powder. Antibody incubation was set for 1 hour and was followed by repeated washing with low-salt buffer. Chemiluminescence reaction was performed with SuperSignal (Pierce) and visualized on Fuji Medical x-ray films (Fujifilm).

Statistical Analysis

Statistical data analysis was performed with Microsoft Excel and SPSS Statistics software. To assess whether 2 independent samples of observations came from the same distribution, a nonparametric test was used (Wilcoxon–Mann-Whitney test). Probability values less than 0.05 were considered significant.

Results

Occludin, VE-Cadherin, and CD31

Occludin, VE-cadherin, and CD31 were detected in all human cryosection specimens, with a preferred localization in the intima of the vascular structures. In the controls, these proteins were almost exclusively present in the small vessels and capillaries and were undetectable in the surrounding brain tissue (Figs. 2 and 3), whereas immunofluorescent analyses revealed expression of occludin, VE-cadherin, and CD31 beyond the vascular intima in the CCM specimens. The localization of these proteins was mainly detected in small vessels including the capillary beds in a dispersed pattern. In some parts of the CCM tissue samples, occludin and/or VE-cadherin were arranged in clusters with a high concentration, in other parts primarily CD31 was present. Furthermore, in addition to the intima of the vessels, the surrounding tissue layers next to these clusters also stained positive for these proteins.

Cell-Contact Protein Levels

Expression analysis of the proteins of interest was performed by means of Western blot, and all 3 proteins could be analyzed by triplicate measurements in all CCM and control samples (Fig. 4). Statistical analysis revealed a significantly higher expression of CD31 (p = 0.001), occludin (p = 0.044), and VE-cadherin (p = 0.028) in the CCM samples than in the control counterparts. The range of expression varied within the individual CCM samples (Fig. 4). Analyzing clinicopathological correlations, no significant results regarding the localization of the CCM lesion (supratentorial vs infratentorial, p = 0.25–0.56), associated DVA (p = 0.086–0.31) or number of CCM lesions (1 vs >1, p = 0.39–0.56) could be obtained. Comparing the active lesions with inactive CCM lesions showed differences (although not statistically significant) in the measured protein levels (Fig. 4). Active state was defined according to Al-Shahi Salman et al. 2 as a CCM lesion with an associated acute hemorrhage before surgery, detected by MR imaging/CT scan and confirmed during surgery and by pathological examination. The analyses in active CCM lesions revealed enhanced levels of CD31 and occludin and decreased levels of VE-cadherin compared with the inactive counterparts.

Discussion

Endothelial cells are thought to be the governing cell type involved in CCM, but little is known about intra- and intercellular pathogenetic mechanisms, especially in humans. 25,27 Although CCM patients are more likely symptomatic in lesions associated with acute hemorrhagic complications, detailed large human clinicopathological as well as experimental studies regarding these underlying molecular mechanisms of the disease are currently.
missing. Interestingly, it seems that CCM-associated bleeding is a multifactorial event at the capillary level caused by alteration of endothelial cell or blood-brain barrier components leading to leakage of red blood cells through these lesions into the surrounding brain tissue.\textsuperscript{13} In vitro studies of endothelial CCM cells showed that mutations of the known CCM genes induce dysfunction of endothelial cells by changes in the cascade of intracellular tubular proteins.\textsuperscript{10,20} While the exact cascades of involved intracellular factors and proteins are not completely understood,\textsuperscript{12,25} involvement of intercellular proteins, such as VE-cadherin, has been recently implicated in this process.\textsuperscript{10,13} Indeed, a growing body of evidence supports the notion of the alteration of intercellular junctions between neurons, glia, pericytes, and endothelial cells.\textsuperscript{5,11,19,23,27}

In this study, we quantified for the first time in human CCM tissue the transmembrane cell-contact proteins occludin and VE-cadherin (representing main proteins in tight and adherence junctions) and the vascular adhesion molecule CD31. We found a significant overexpression of these proteins in CCM tissue compared with control tissue, with slight variations within the patient samples (Fig. 4). These differences suggest that patient-specific or other unknown factors may influence the expression rate. The molecular CCM protein complex model of Hilder et al.,\textsuperscript{10} which interacts closely with cytoskeletal proteins and modulates interendothelial cell junctions, may explain these changes in protein regulation.

Based on these individual protein profiles, protein expression levels in the specimens obtained from CCM patients were associated to the corresponding clinical phenotype to provide further details within the different patient subgroups. Although patients with infratentorial CCMs are more likely to have neurological symptoms than those with supratentorial CCM, we did not find any correlation between differences in protein levels in CCM tissue and CCM location. The distinctive clinical symptoms found in association with infratentorial CCMs are rather related to the limitation of the infratentorial space and the presence of important and sensitive neuronal tracts and centers within the brainstem, representing a challenge for neurosurgical treatment of these lesions.\textsuperscript{3,8,21}

**Fig. 2.** Immunostaining of CCM (A–F) and control tissue (G–I) for occludin. A: Overlay of occludin (green), DAPI (red), and phalloidin (blue). B: Single-channel image, DAPI. C: Single-channel image, occludin. D: Negative control without primary antibodies; overlay of occludin (green), DAPI (red), and phalloidin (blue). E: Single-channel image, DAPI. F: Single-channel image, occludin. G: Control tissue, overlay of occludin (green), DAPI (red), and phalloidin (blue). H: Single-channel image, DAPI. I: Single-channel image, occludin. Arrows indicate localization of occludin in the inner layer of the vessel.
The differences in the protein levels in CCM lesions found by Western blot analysis with respect to the presence of acute associated hemorrhages were highly interesting, although they did not reach statistical significance. However, a precise reason for the higher mean rates of CD31 and occludin and the lower mean rate of VE-cadherin in active CCM lesions remains unclear. These findings therefore need to be further explored in studies involving larger patient groups. This also applies to CCM-associated DVAs and the number of CCM lesions in single patients, since in this study no significant results were found regarding the relationship between the levels of the proteins of interest and DVAs (their presence or absence) or number of CCMs (multiple or single locations).

The immunofluorescence analyses of the CCM tissues showed staining for VE-cadherin, occludin, and CD31 in the intima and the surrounding brain tissue. In some specimens an arrangement in clusters was found; these clusters seem to originate in the intima and expand to the surrounding brain tissue. However, the vessels in the controls specimens were only slightly stained and did not show any cluster arrangements or spread to the sur-

![Immunostaining of CCM (A–G) and control tissue (H) for CD31 and VE-cadherin.](image)

**Fig. 3.** Immunostaining of CCM (A–G) and control tissue (H) for CD31 and VE-cadherin. **A:** Overlay of CD31 (green), DAPI (red), and phalloidin (blue). **B:** Single-channel image, DAPI. **C:** Single-channel image, CD31. **D:** VE-cadherin (green), DAPI (red), and phalloidin (blue). **E:** Single-channel image, DAPI. **F:** Single-channel image, VE-cadherin. **G:** Negative control without primary antibodies, overlay of CD31 (green), VE-cadherin (blue), and DAPI (red). **H:** Control tissue, overlay of CD31 (green), VE-cadherin (blue), and DAPI (red). Arrows indicate localization of CD31 and VE-cadherin in the inner layer of the vessel.
rounding brain tissue. These immunohistochemical findings confirm the results of the increased protein levels revealed by Western blot and are suggestive of compensatory production due to nonfunctional cell contacts.

The weakness of this study can be found in the sample size, which may lead to an underpowered value of the clinicopathological correlation. However, this study does quantitatively identify alterations in endothelial junction protein expression in human CCMs for the first time. Future studies will be designed to pursue functional assays in vitro using tissue samples from a larger number of patients.

Conclusions

Based on the presented results we conclude that 1) the abnormal and discontinuous pattern in the investigated endothelial cell-contact proteins compared with control tissue explains the CCM-associated bleeding or oozing of erythrocytes; 2) endothelial cells appear to compensate for the loose cell contacts in the pathological situation by production of new proteins to strengthen their contacts; 3) the alterations of VE-cadherin, occludin, and CD31 in these CCM patients are not locally based or associated with multiplicity of lesions or the presence of DVAs. A difference in the expression level of the proteins of interest in the CCM lesions associated with acute hemorrhage compared with lesions without recent hemorrhage might be possible, but needs to be validated by means of functional assays and studies involving a larger number of patients.

Disclosure

This work was financially supported partly by the Swiss National Science Foundation and the Swiss Center of Integrative Human Physiology.

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Burkhardt, Schmidt, Bertalanfy, Hoerstrup. Acquisition of data: Burkhardt, Schoenauer, Agarkova. Analysis and interpretation of data: Burkhardt, Schmidt, Schoenauer, Brokopp, Bozinov, Hoerstrup. Drafting the article: Burkhardt, Schmidt, Schoenauer, Brokopp, Bozinov. Critically revising the article: Schmidt, Brokopp, Agarkova, Bozinov, Bertalanfy, Hoerstrup. Reviewed final version of the manuscript and approved it for submission: Burkhardt, Bertalanfy, Hoerstrup.

Acknowledgments

The authors thank Ines Lohse (Department of Radio-Oncology, University Hospital Zurich) for her support with respect to the control tissue and the Institute of Biostatistics at the University of Zurich for assistance with the statistic analyses.

References

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Accepted June 23, 2010.

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