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Contribution of endothelial-to-mesenchymal transition to the pathogenesis of human cerebral and orbital cavernous malformations

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Conflict of Interest

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Abstract

Background: The analysis of gene-targeted mouse mutants has demonstrated that endothelial-to-mesenchymal transition (EndMT) is crucial to the onset and progression of cerebral cavernous malformations (CMs). It has also been shown that Notch and ephrin/Eph signaling are involved in EndMT. However, their roles in the pathogenesis of human intracranial CMs remain unclear.

Objective: The aim of our study was to elucidate the contribution of EndMT, the Notch pathway and ephrin-B2/EphB4 signaling to the pathogenesis of human intracranial CMs.

Methods: Eight human intracranial CMs (five cerebral and three orbital CMs) were immunohistochemically investigated.

Results: CD31 (an endothelial marker) and EndMT markers, such as α -smooth muscle actin (a mesenchymal marker) and CD44 (a mesenchymal stem cell marker), were expressed in the endothelial layer of vascular sinusoids in all cases, suggesting that endothelial cells (ECs) have acquired mesenchymal and stem-cell-like characteristics and undergone EndMT in all cerebral and orbital CMs. EndMT was observed in about 70% and 35% of ECs in cerebral and orbital CMs, respectively. In all cases, Notch3 was expressed in the endothelial layer, indicating that ECs of vascular sinusoids have acquired mesenchymal features. In all cases, both ephrin-B2 and EphB4 were detected in the endothelial layer, suggesting that ECs of vascular sinusoids are immature or malformed cells and have both arterial and venous characteristics.

Conclusion: EndMT plays a critical role in the pathogenesis of human cerebral and orbital CMs. Modulating EndMT is expected to be a new therapeutic strategy for cerebral and orbital CMs.

Key Words

cavernous malformation, endothelial-to-mesenchymal transition (EndMT), ephrin-B2/EphB4, Notch signaling

Running title

EndMT in human cavernous malformations

Abbreviations

α -SMA, α -smooth muscle actin; **AVMs**, arteriovenous malformations; **CMs**, cavernous malformations; **ECs**, endothelial cells; **EndMT**, endothelial-to-mesenchymal transition; **HRP**, horseradish peroxidase; **ICD**, intracellular domain; **SCs**, stromal cells; **SMCs**, smooth muscle cells

Introduction

Endothelial-to-mesenchymal transition (EndMT) is a process in which endothelial cells (ECs) lose their cell-type-specific characteristics and acquire mesenchymal and stem-cell-like features.^{1,2} EndMT has been described in various pathologies, including diabetic nephropathy, cardiac fibrosis, systemic fibrosis and malignant tumors.¹⁻³ Recently, the analysis of gene-targeted mouse mutants has described that EndMT contributes to the onset and progression of cerebral cavernous malformations (CMs).² However, the role of EndMT in the pathogenesis

of human cerebral CMs remains unclear.

Cerebral CMs are slow-flow vascular malformations with a prevalence of 0.4–0.6% in the general population.^{4,7} Cerebral CMs can occur in a sporadic or autosomal dominant inherited form.^{8,9} It has been widely accepted that cerebral CMs are caused by loss-of-function mutations in the *CCM1* (*KRIT1*), *CCM2* (*OSM*), and *CCM3* (*PDCD10*) genes.¹⁰⁻¹³ Histologically, they are characterized as single or clusters of enlarged capillary-like channels with a single layer of flattened ECs. Importantly, they lack intervening brain parenchyma and the components of organized mature vessels such as smooth muscle cells (SMCs), pericytes, astrocytic foot processes and intact endothelial cell–cell junctions.^{11,14,15} It has been demonstrated that cerebral and orbital CMs belong to different pathological entities.¹⁶⁻¹⁸ Conversely, it has also been described that they belong to an identical pathological entity because they show considerable overlap in histology: dilated vascular spaces lined by a single layer of flattened ECs, thrombosis, chronic hemorrhage, and non-infiltrative lesion.¹⁹ Orbital CMs are also slow-flow vascular malformations just like cerebral CMs. Histologically, they are finely encapsulated masses containing a complex network of dilated vascular channels with a single layer of flattened ECs surrounded by abundant fibrous stroma.^{16,17,20} It is controversial whether cerebral and orbital CMs belong to an identical pathological entity or not, and the role of EndMT in the development of human orbital CMs also remains unclear.

Various studies have demonstrated a critical role of Notch signaling in EndMT,^{1-3,21} and it has been suggested that Notch signaling regulates ephrin-B2 during EndMT.¹ Notch signaling is an evolutionarily conserved pathway that is essential for cell-fate determination and vascular

development.²²⁻²⁴ Notch is a transmembrane protein and activated by its ligands. Owing to activation, the intracellular domain (ICD) of Notch is released and transferred into the nucleus, where the ICD forms a complex with the DNA-binding protein RBP-J. This complex induces expression of *Hes* and *Hesr/Hey* genes.²⁵⁻²⁷ Ephrin ligands and Eph receptors are involved in many important biologic processes.²⁸ Above all, signaling between ephrin-B2 and its tyrosine kinase receptor EphB4 (ephrin-B2/EphB4 signaling) is known to play a crucial role in arteriovenous differentiation.^{29,30} Mouse embryos lacking ephrin-B2 or EphB4 exhibit fatal defects of conduit vessels formed during early vascular development.^{29,30} However, in human cerebral and orbital CMs, the role of Notch signaling and ephrin-B2/EphB4 signaling in EndMT remains unknown.

The aims of this study were to elucidate the contribution of EndMT, the Notch pathway and ephrin-B2/EphB4 signaling to the pathogenesis of human cerebral and orbital CMs and to make a comparison between human cerebral and orbital CMs.

Methods

Patients

This study was performed under the guidelines provided by the ethics committee of Kyoto University Graduate School of Medicine. Eight patients underwent removal of intracranial CMs (five cerebral CMs and three orbital CMs) for four years at Kyoto University Hospital. The mean age was 36.8 years (range 10 to 65 years). In cerebral CMs, four patients presented with intracerebral hemorrhage and one with seizure. Four patients had multiple CMs. There was no

family history of cerebral and/or orbital CMs. The clinical features of the patients are summarized in Table 1.

Specimen Preparation and Immunohistochemical Studies

Cerebral and orbital CMs were obtained during surgical procedures. We also studied normal vessels in 3 control specimens obtained during surgery for epilepsy, which were involved in epileptogenic focus, but histologically normal. Positive control tissues were obtained from surgical specimens of hemangioblastomas and arteriovenous malformations (AVMs). We got informed consent from all patients for the research use of removed tissue. All specimens were fixed in 10% formalin overnight, and then embedded in paraffin.

For immunohistochemical staining, 6- μ m-thick tissue sections were cut from paraffin blocks. These sections were prepared for staining with horseradish peroxidase (HRP) system. Anti-CD31 (1:20; SANTA CRUZ), α -smooth muscle actin (α -SMA) (1:100; DAKO), CD44 (1:200; SANTA CRUZ), Notch1 (1:200; SANTA CRUZ), Notch3 (1:200; SANTA CRUZ), Hesr2/Hey2 (1:20; CHEMICON), ephrin-B2 (1:200; SANTA CRUZ) and EphB4 (1:20; SANTA CRUZ) antibodies were reacted at 4°C overnight. Mouse IgG (DAKO) and rabbit immunoglobulin fraction (DAKO) were used instead of primary antibodies as negative controls. For detection of antibody binding, EnVision[®]+ System-HRP (DAKO) and LSAB[®] 2 System-HRP (DAKO) were used. All sections were counterstained with Gill's hematoxylin to cell nuclei. Negative and positive controls are shown in Supplemental Figure, which are consistent with previous reports.^{31,32} Sections were examined using a whole slide scanner (ScanScope[®] XT scanner (Aperio, Vista,

CA, USA)).

Data Analysis

In each CM specimen, we avoided the portion affected by bleeding, and analyzed mainly the central area of the lesion. For quantitative studies, slides were scanned through a 20× objective lens. In every case, at least 2 sections (mean 2.75, range 2–7) were analyzed. In each section, we randomly selected 5 areas (500µm×800µm), and counted positively stained ECs. In normal brain specimens, ECs of arterioles and venules were also manually counted and analyzed. The immunoreactivity was quantitatively evaluated by two authors (S.T. and M.H.) in a blind manner according to ratios of positive cells (–, 0%; +–, 0–20%; +, 20–50%; ++, 50–75%; +++, 75–100%) in the endothelial layers of vascular sinusoids. Data are presented as mean ± standard error (SE) in parenthesis. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). The Mann-Whitney U test was used to test for all quantitative data. A p-value less than 0.05 was considered statistically significant.

Results

1. EndMT occurs in human cerebral and orbital CMs

To investigate the contribution of EndMT to the pathogenesis of human cerebral and orbital CMs, we first examined the expression of CD31 (an endothelial cell marker) and EndMT markers, such as α-SMA (a mesenchymal marker) and CD44 (a mesenchymal stem-cell marker) (Figure 1 and 2). Results of immunohistochemical analysis of ECs are summarized in Table 2.

In normal human brain vessels, CD31 was strongly expressed in the endothelial layer, whereas neither α -SMA nor CD44 could be detected (Figure 1M–R). There was no significant difference in immunohistological findings of all three control specimens. In human cerebral CMs, CD31, α -SMA and CD44 were strongly expressed in the endothelial layer of vascular sinusoids in all cases, suggesting that EndMT has occurred in all cerebral CMs (Figure 1A–C and 1G–I). In staining for EndMT markers, positive and negative cells were mixed in the endothelial layer of all vascular sinusoids (mosaic-like staining pattern) (Figure 1B and C). α -SMA and CD44 were positive in 61.6–75.4 % and 71.6–74.2 % of ECs, respectively (Table 2). These results suggest that EndMT was observed in about 70% of ECs in cerebral CMs. Similarly, in human orbital CMs, CD31, α -SMA and CD44 were strongly expressed in the endothelial layer of vascular sinusoids in all cases, suggesting that EndMT has occurred in all orbital CMs (Figure 2A–C and 2G–I). In staining for EndMT markers, mosaic-like staining pattern was also observed (Figure 2B and C). α -SMA and CD44 were positive in 32.9–41.3 % and 33.1–41.0 % of ECs, respectively (Table 2). These results suggest that EndMT was observed in about 35% of ECs in orbital CMs. EndMT occurred in ECs of cerebral CMs significantly more frequently than in orbital CMs, although it was observed in all cerebral and orbital CMs (Figure 3).

2. Notch signaling is involved in human cerebral and orbital CMs

It has been described that *Notch1* and *Notch3* are highly expressed in the endocardium during EndMT leading to foramen ovale closure in mice, and that activation of *Notch3* alone induces EndMT in cultured ECs.²¹ Silencing of *CCM1* in cultured ECs significantly diminishes the Notch

target gene *Hesr2/Hey2* mRNA levels.³³ Therefore, we examined the expression of Notch1, Notch3 and their effector *Hesr2/Hey2* (Supplemental Figure, Figure 4A–C). Results of immunohistochemical analysis of ECs are summarized in Table 3. In both cerebral and orbital CMs, Notch1 was expressed in the endothelial layer, whereas its effector *Hesr2/Hey2* was scarcely detected (Figure 4A and C). Notch3 was also expressed in the endothelial layer (Figure 4B). In each staining, mosaic-like staining pattern was observed. These results are consistent with previous findings in gene-targeted mouse mutants, and indicate that Notch signaling is involved in the formation of human cerebral and orbital CMs.

3. ECs are immature or malformed cells and have both arterial and venous characteristics in human cerebral and orbital CMs

It has been described that ephrin-B2 marks arterial ECs at early stages of vascular development, whereas EphB4 marks venous ECs.³⁰ However, in adult human, neither ephrin-B2 nor EphB4 is expressed in normal vessels.^{32,34} It has also been demonstrated that Notch signaling regulates ephrin-B2 during EndMT.¹ Therefore, we examined the expression of ephrin-B2 and EphB4 (Supplemental Figure, Figure 4 D and E). Results of immunohistochemical analysis of ECs are summarized in Table 4. Both ephrin-B2 and EphB4 were detected not only in the endothelial layer but also in the subendothelial layer of all cases (Figure 4D and E), whereas neither ephrin-B2 nor EphB4 was detected in the endothelial layer of normal brain vessel (Table 4). In each staining, mosaic-like staining pattern was observed. These results suggest that ECs are immature or malformed cells and have both arterial and venous characteristics in human cerebral

and orbital CMs.

Discussion

Although a recent investigation has demonstrated that EndMT contributes to the onset and progression of cerebral CMs in mice,² the role of EndMT has not been investigated in human cerebral CMs to date. Actually, in this investigation, EndMT markers were not examined in human specimens.² α -SMA is one of the earliest proteins expressed during differentiation of SMCs and found in a variety of mesoderm-derived cells including myofibroblasts.¹⁸ CD44 has been widely used as a marker of mesenchymal stem cells, which differentiate into various cell types including myofibroblasts, pericytes, and SMCs. It is well known that α -SMA and CD44 are not expressed in the endothelium of normal vessels. However, in our study, CD31, α -SMA and CD44 were expressed in the endothelium of vascular sinusoids in all human cerebral CMs, suggesting that ECs have acquired mesenchymal and stem-cell-like characteristics and undergone EndMT. To our knowledge, this is the first report showing that human cerebral CMs are formed by ECs undergoing EndMT.

While the pathogenesis of cerebral CMs has been well investigated, the pathogenesis of orbital CMs remains unclear to date. Although it has been described that the majority of vessel walls are stained positively for α -SMA in orbital CMs,^{20,35} it has not been examined whether ECs are positive for α -SMA or not. In our investigation, in human orbital CMs, α -SMA and CD44, EndMT markers, were expressed not only in the subendothelial layer but also in the endothelial layer, suggesting that EndMT also contributes to the pathogenesis of human orbital CMs.

It has been reported that Notch signaling is associated with the formation of cerebral CMs in the analysis of gene-targeted mouse mutants and cultured cells.^{2,33,36-38} Endothelial-specific *Hes*-mutant embryos exhibit vascular malformations in mice.³⁶ Freshly isolated brain ECs from *CCM1* loss-of-function mice show a significantly lower expression of *Hesr/Hey* genes.² Silencing of *CCM1* in cultured ECs significantly diminishes *Hesr2/Hey2* mRNA levels.³³ Silencing of *CCM3* in cultured ECs also down-regulates *Hesr2/Hey2* mRNA levels.³⁸ Although the Notch pathway acts downstream of *CCM* genes, the expression of Notch1 is preserved in *CCM1*-silenced cultured cells, *CCM3*-silenced cultured cells, freshly isolated brain ECs from *CCM1* loss-of-function mice and *CCM3*-silenced ECs derived from human cerebral CMs.^{2,33,38} In our study, in the endothelium of human cerebral and orbital CMs, Notch1 was expressed, whereas *Hesr2/Hey2* was scarcely detected. These results are consistent with the previous findings in the analysis of gene-targeted mouse mutants. It is well known that *Notch3* is expressed in vascular SMCs, but not in ECs. Recently, it has been described that Notch1 and Notch3 are highly expressed in the endocardium during EndMT leading to foramen ovale closure, and that activation of *Notch3* alone induces EndMT in cultured ECs.²¹ In our study, Notch3 was expressed in the endothelium, indicating that activated *Notch3* has induced EndMT in cerebral and orbital CMs. These results suggest that Notch signaling has a role in the formation of human cerebral and orbital CMs.

Although the important functions of ephrin-B2 and EphB4 in development of arteries and veins have been demonstrated in knockout mice, their roles in adult mice and human have not been established. Several studies have demonstrated that EphB4 is also expressed on arterial ECs at

low levels.^{39,40} The analysis of human normal tissue has demonstrated that ephrin-B2 is a marker of arterial ECs and EphB4 is expressed in arteries and veins.⁴⁰ In contrast, neither *ephrin-B2* nor *EphB4* mRNAs is detected in normal human brain vessels and superficial temporal arteries.^{32,34} In human subfascial congenital venous malformations, both ephrin-B2 and EphB4 were expressed in the endothelium.⁴⁰ In human arteriovenous malformations, both ephrin-B2 and EphB4 were expressed in the endothelium of immature vessels.³² It has been demonstrated that Notch signaling regulates ephrin-B2 during EndMT.¹ In our study, both ephrin-B2 and EphB4 were detected in the endothelial layer of cerebral CMs and orbital CMs, whereas neither ephrin-B2 nor EphB4 was detected in the endothelial layer of normal brain vessels. These results indicate that ECs have undergone EndMT and that immature or malformed ECs have both arterial and venous characteristics in human cerebral and orbital CMs. To date, these endothelial characteristics of human cerebral and orbital CMs have not been reported.

Limitation

Limitations of our study are that this research is descriptive and not mechanistic, and that only immunohistochemistry was used to confirm our postulation.

Conclusion

Our data suggest that EndMT plays a critical role in the pathogenesis of human cerebral and orbital CMs. Notch signaling is involved in the formation of human cerebral and orbital CMs, and the endothelium of them is composed of immature or malformed ECs that have undergone

EndMT and have both arterial and venous characteristics. Modulating EndMT is expected to be a new therapeutic strategy for cerebral and orbital CMs.

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Figure Legend

Figure 1.

Immunohistochemistry for endothelial and EndMT markers in human cerebral CMs.

A, CD31, an endothelial marker, was expressed in the endothelium of vascular sinusoids of cerebral CMs. **B**, **C**, α -SMA and CD44, EndMT markers, were expressed in the endothelium of cerebral CMs. **D–F**, Negative controls show no significant staining. **G–I**, Photomicrographs showing ECs that have undergone EndMT in cerebral CMs (serial sections). The endothelial layer is indicated by two dotted lines. CD31, α -SMA and CD44 are expressed in the ECs of vascular sinusoids. **J–L**, Photomicrographs showing ECs that haven't undergone EndMT in cerebral CMs (serial sections). The endothelial layer is indicated by two dotted lines. Neither α -SMA nor CD44 is expressed in the ECs, while CD31 is expressed. **M–R**, Photomicrographs showing ECs of normal brain vessels (arterioles and venules). CD31 is expressed in ECs, whereas neither α -SMA nor CD44 is detected. Bar = 100 μ m (**A–F**), 15 μ m (**G–L**) and 25 μ m (**M–R**). **α -SMA**, α -smooth muscle actin; **EndMT**, endothelial-to-mesenchymal transition

Figure 2.

Immunohistochemistry for endothelial and EndMT markers in human orbital CMs.

A, CD31, an endothelial marker, was expressed in the endothelium of vascular sinusoids of

orbital CMs. **B, C**, α -SMA and CD44, EndMT markers, were expressed in the endothelium of orbital CMs. **D–F**, Negative controls show no significant staining. **G–I**: Photomicrographs showing ECs that have undergone EndMT in orbital CMs (serial sections). The endothelial layer is indicated by two dotted lines. CD31, α -SMA and CD44 are expressed in the ECs of vascular sinusoids. **J–L**: Photomicrographs showing ECs that haven't undergone EndMT in orbital CMs (serial sections). The endothelial layer is indicated by two dotted lines. Neither α -SMA nor CD44 is expressed in the ECs, while CD31 is expressed. Bar = 100 μ m (A–F) and 15 μ m (G–L). **α -SMA**, α -smooth muscle actin; **EndMT**, endothelial-to-mesenchymal transition

Figure 3.

Quantitative analysis of EndMT markers in human cerebral and orbital CMs. The ratios of α -SMA⁺ and CD44⁺ cells to the total ECs in vascular sinusoids with SE are shown (Mann-Whitney U test, * P <0.05). In every case, at least 2 sections were analyzed. In each section, we randomly selected 5 areas (500 μ m $\times$ 800 μ m), and counted positively stained ECs. **α -SMA**, α -smooth muscle actin; **CMs**, cavernous malformations

Figure 4.

Analysis of Notch and ephrin/Eph pathways in human cerebral CMs. **A**, Notch1 was expressed in the endothelium of vascular sinusoids. **B**, Notch3 was also expressed in the endothelium. **C**, Hesr2/Hey2 was scarcely detected. **D**, Ephrin-B2 was expressed in the endothelium. **E**, EphB4 was also detected. Bar = 50 μ m (A–C) and 50 μ m (D and E).

Supplemental Figure

Negative and positive controls for immunohistochemical analysis of Notch and ephrin/Eph pathways. **A, B, E, F**, Stromal cells (SCs) in hemangioblastomas. **C, D**, Microvessels in hemangioblastomas. **G, H**, Arterial endothelia of AVMs. **I, J**, Venous endothelia of AVMs. **A, C, E, G, I**, Negative controls show no significant staining. **B**, Notch1 is detected in SCs. **D**, Notch3 is expressed in ECs of microvessels. **F**, Hesr2/Hey2 is detected in SCs. **H**, Ephrin-B2 is expressed in ECs of arteries in AVMs. **J**, EphB4 is detected in ECs of veins in AVMs. Bar = 15 μ m (A–J).

Figure 1

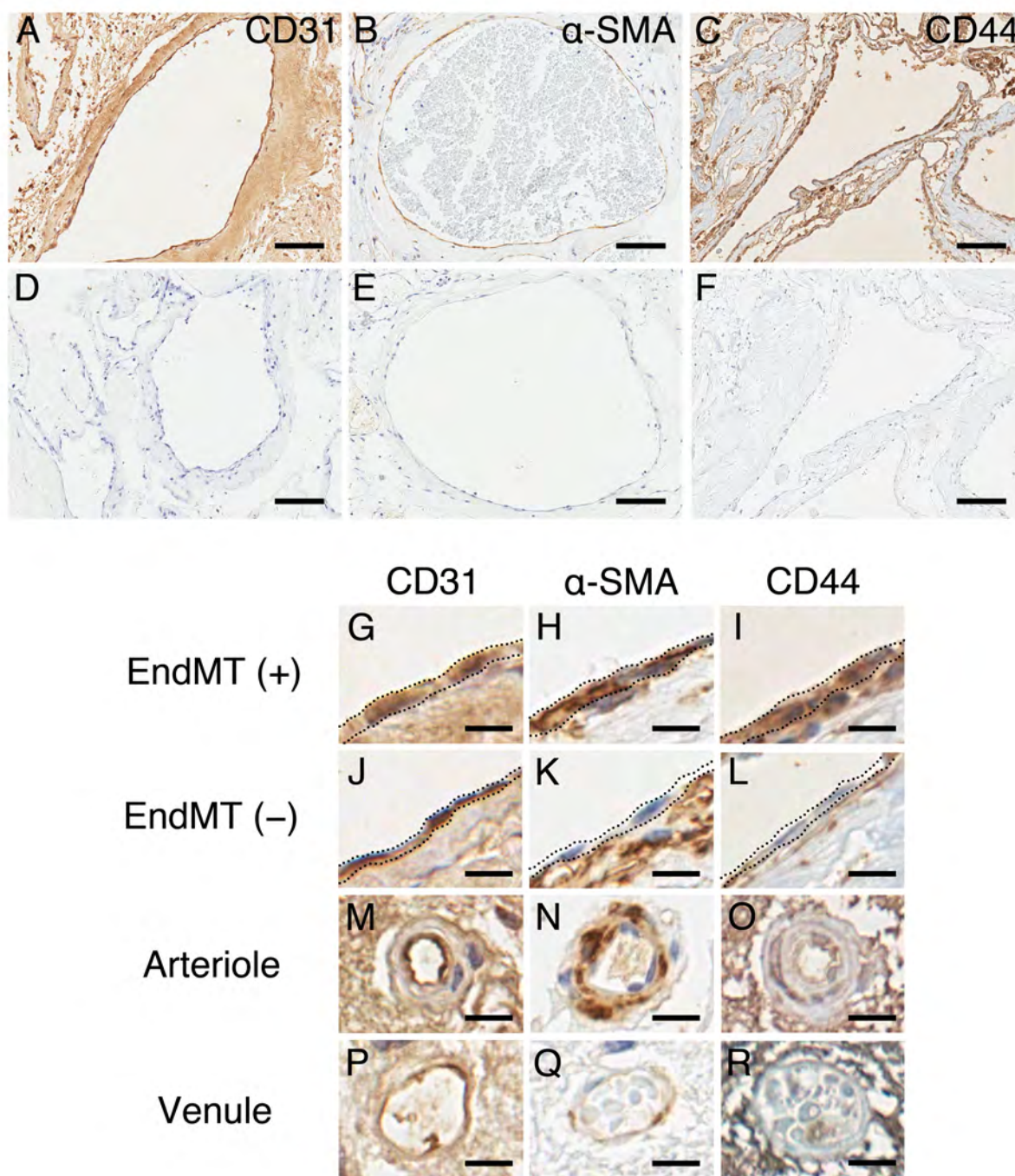


Figure 2

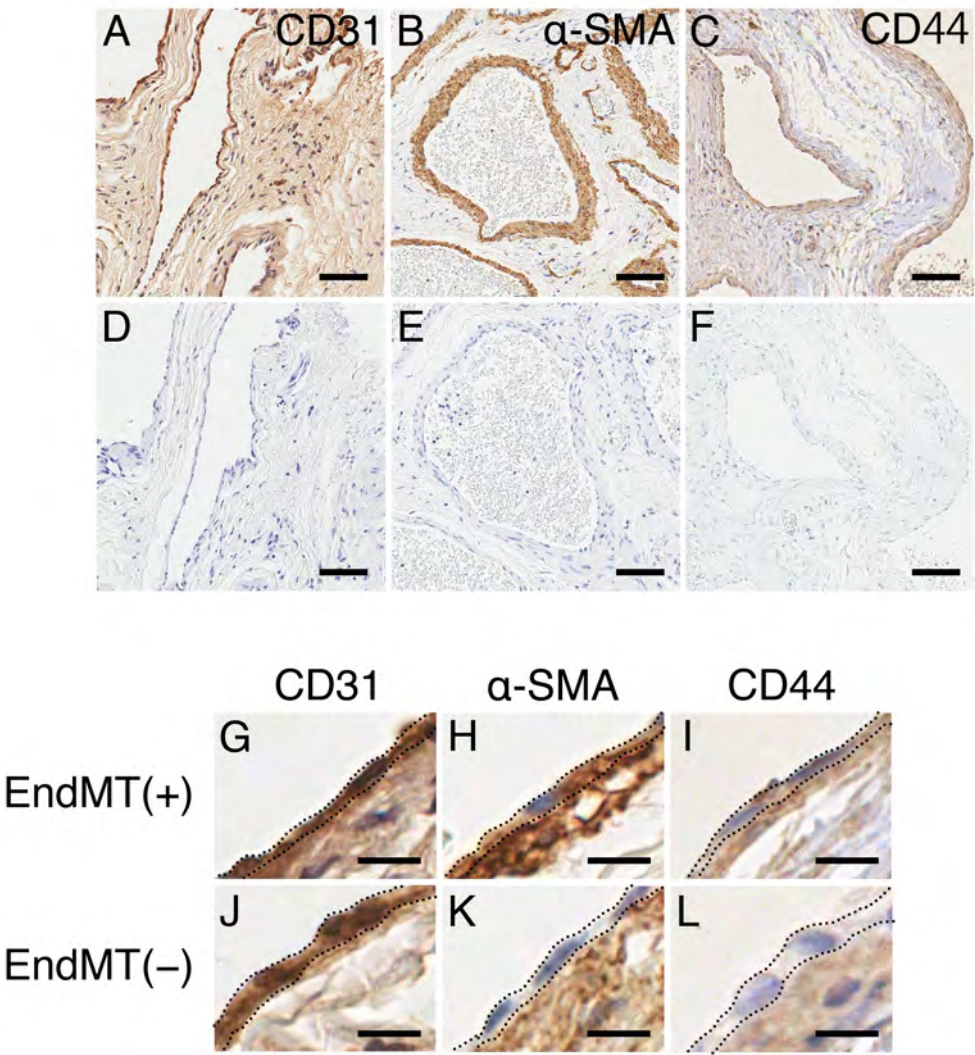


Figure 3

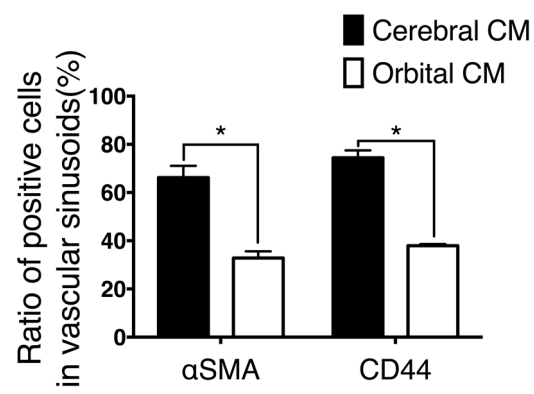
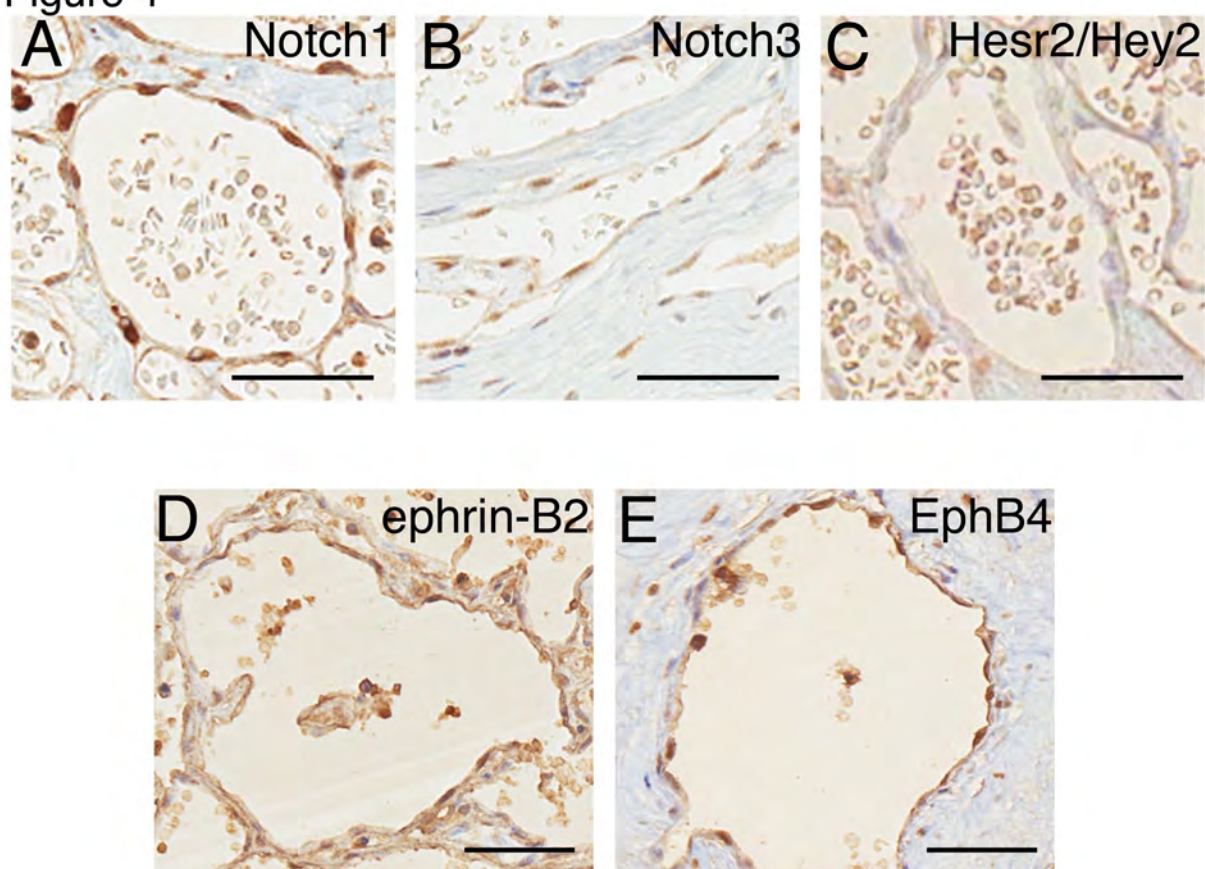


Figure 4



Supplemental figure

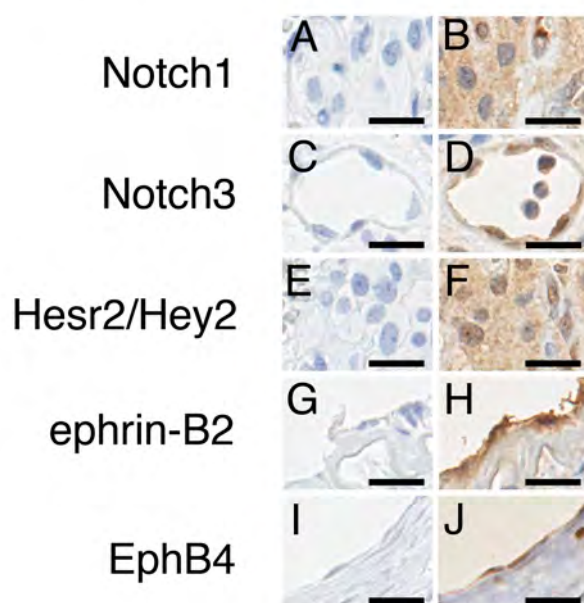


TABLE 1. Summary of Patients with Cavernous Malformations

Case No.	Age (yr), Sex	CM type	Lesion	Family History	Onset
1	29, F	Cerebral	Multiple	No	Hemorrhage
2	29, F	Cerebral	Multiple	No	Hemorrhage
3	22, F	Cerebral	Multiple	No	Seizure
4	10, M	Cerebral	Multiple	No	Hemorrhage
5	26, M	Cerebral	Single	No	Hemorrhage
6	54, F	Orbital	Single	No	Visual loss
7	59, M	Orbital	Single	No	Visual loss
8	65, F	Orbital	Single	No	Diplopia

CM, cavernous malformation

TABLE 2. Immunohistochemical Analysis of EndMT Markers in Endothelial Cells

Case No.	CD31		α -SMA		CD44	
1	+++	(100%)	++	(67.9 $\pm$ 3.1%)	++	(74.2 $\pm$ 3.3%)
2	+++	(100%)	++	(69.4 $\pm$ 2.7%)	++	(71.6 $\pm$ 2.7%)
3	+++	(100%)	++	(66.9 $\pm$ 1.9%)	++	(73.9 $\pm$ 4.6%)
4	+++	(100%)	+++	(75.4 $\pm$ 5.0%)	++	(73.6 $\pm$ 3.4%)
5	+++	(100%)	++	(61.6 $\pm$ 5.1%)	++	(71.7 $\pm$ 2.2%)
6	+++	(100%)	+	(33.2 $\pm$ 2.6%)	+	(39.5 $\pm$ 4.5%)
7	+++	(100%)	+	(41.3 $\pm$ 5.8%)	+	(41.0 $\pm$ 3.6%)
8	+++	(100%)	+	(32.9 $\pm$ 1.8%)	+	(33.3 $\pm$ 1.5%)
Arteriole	+++	(100%)	–	(0%)	–	(0%)
Venule	+++	(100%)	–	(0%)	–	(0%)

SMA, smooth muscle actin

Positively stained endothelial cells were counted and analyzed. The immunoreactivity was quantitatively evaluated according to ratios of positive cells (–, 0%; +–, 0–20%; +, 20–50%; ++, 50–75%; +++, 75–100%) in the endothelial layers of vascular sinusoids. Data are presented as means with standard errors in parentheses.

TABLE 3. Immunohistochemical Analysis of Notch Pathway in Endothelial Cells

Case No.	Notch1		Notch3		Hesr2/Hey2	
1	+++	(83.1±11.5%)	++	(53.1±3.6%)	+-	(9.1±2.9%)
2	+++	(94.0±2.9%)	+++	(83.7±2.7%)	+-	(12.9±2.4%)
3	+++	(91.7±2.6%)	+	(44.1±5.4%)	+-	(16.5±2.8%)
4	+++	(94.1±2.4%)	++	(57.8±7.8%)	+-	(12.9±5.8%)
5	+++	(95.7±2.5%)	+	(37.2±3.1%)	+-	(19.1±2.5%)
6	+++	(95.8±1.1%)	+	(24.3±2.8%)	+-	(16.1±2.5%)
7	+++	(94.3±1.9%)	+-	(18.9±2.4%)	+-	(10.8±1.3%)
8	+++	(93.4±2.8%)	+	(30.0±1.9%)	+-	(16.9±2.3%)
Arteriole	+	(66.7±16.7%)	-	(0%)	++	(50.0±9.6%)
Venule	+-	(16.7±16.7%)	-	(0%)	+	(22.2±11.1%)

Positively stained endothelial cells were counted and analyzed. The immunoreactivity was quantitatively evaluated according to ratios of positive cells (-, 0%; +-, 0–20%; +, 20–50%; ++, 50–75%; +++, 75–100%) in the endothelial layers of vascular sinusoids. Data are presented as means with standard errors in parentheses.

TABLE 4. Immunohistochemical Analysis of Ephrin/Eph Signaling in Endothelial Cells

Case No.	ephrin-B2		EphB4	
1	+	(32.0±4.1%)	+++	(98.8±1.2%)
2	+	(41.2±3.8%)	+++	(98.0±0.8%)
3	+	(27.1±3.4%)	+++	(96.9±1.3%)
4	+	(32.7±3.4%)	+++	(96.1±2.4%)
5	+	(29.8±1.6%)	+++	(98.6±0.9%)
6	+	(27.7±2.9%)	+++	(76.7±2.1%)
7	+	(27.8±3.0%)	++	(61.9±6.2%)
8	+	(20.3±1.3%)	+++	(76.9±1.6%)
Arteriole	–	(0%)	–	(0%)
Venule	–	(0%)	–	(0%)

Positively stained endothelial cells were counted and analyzed. The immunoreactivity was quantitatively evaluated according to ratios of positive cells (–, 0%; +–, 0–20%; +, 20–50%; ++, 50–75%; +++, 75–100%) in the endothelial layers of vascular sinusoids. Data are presented as means with standard errors in parentheses.