Analysis of Endoglin Expression in Normal Brain Tissue and in Cerebral Arteriovenous Malformations

Shunji Matsubara, MD; Annie Bourdeau, PhD; Karel G. terBrugge, MD; Christopher Wallace, MD; Michelle Letarte, PhD

Background and Purpose—A high incidence of arteriovenous malformations (AVMs) is associated with hereditary hemorrhagic telangiectasia type 1. Endoglin, the gene mutated in this disorder, is expressed at reduced levels on blood vessels of these patients. Since endoglin is a component of the transforming growth factor-β receptor complex critical for vascular development and homeostasis, we determined its expression in sporadic cerebral AVMs and in normal brain vessels.

Methods—Twenty cerebral AVMs and 10 normal brain samples were analyzed for endoglin, platelet endothelial cell adhesion molecule 1 (PECAM-1), α-smooth muscle cell actin, vimentin, and desmin by immunohistochemistry.

Results—In normal brain, endoglin was found not only on the endothelium of all vessels but also on the adventitial layer of arteries and arterioles. In cerebral AVMs, the numerous vessels present expressed endoglin on both endothelium and adventitia. Arterialized veins, identified by lack of elastin and uneven thickness of smooth muscle cells, revealed endoglin-positive mesenchymal cells in the adventitia and perivascular connective tissue. These cells were fibroblasts since they expressed vimentin but not actin and/or desmin.

Conclusions—This is the first report of endoglin expression on adventitia of normal brain arteries and on arterialized veins in cerebral AVMs. Increasing numbers of endoglin-positive endothelial and adventitial cells were seen in sporadic cerebral AVMs, but endoglin density was normal. Thus, it is not involved in the generation of these lesions. However, the presence of endoglin on fibroblasts in the perivascular stroma suggests an active role for this protein in vascular remodeling in response to increased blood flow and shear stress. (Stroke. 2000;31:2653-2660.)

Key Words: cerebral arteriovenous malformations ■ immunohistochemistry ■ pathology ■ transforming growth factors

The arteriovenous malformation (AVM) is the most dangerous of cerebral vascular malformations and is defined as a direct communication between 1 or more arteries of the cerebral parenchyma and 1 or more draining veins, without an intervening capillary bed. Direct shunting of blood at arterial pressures causes dilation and tortuosity, particularly in the involved veins. Focal depositions of layers of elastic tissue can be observed, as well as proliferation of fibroblasts and increased cellularity in the wall, leading to focal thrombosis, necrosis, and hemorrhage. The involved arteries also show changes, such as duplication or destruction of the elastica, fibrosis of the media, and focal thinning of the wall. Although it is generally accepted that many AVMs are developmental anomalies, it is likely that a combination of congenital predisposition and extrinsic factors lead to their generation. The vast majority of cases are sporadic, in which no familial association is observed. No specific gene mutations have been reported for these AVMs.

Endoglin (CD105) is a component of the receptor complex for transforming growth factor (TGF)-β1 and -β3. It binds ligand via its association with the TGF-β receptor II, a ser/thr kinase that transmits signal by phosphorylating the ser/thr kinase receptor I. Endoglin is a homodimeric integral membrane glycoprotein of 180 kDa, predominantly expressed on vascular endothelium. It is recognized as an endothelial cell antigen (CD105) and is expressed on capillaries, veins, and arteries. Endoglin is the gene mutated in hereditary hemorrhagic telangiectasia (HHT) type 1, a disease associated with AVMs and characterized by haploinsufficiency. The mutant proteins, if expressed at all, are only detected as transient intracellular species, resulting in half levels of endoglin in endothelial cells. All vessels of HHT1 patients, in situ, also show reduced levels of endoglin. Vessels within pulmonary and cerebral AVMs still expressed detectable levels of endoglin, similar to those found in unaffected vessels, indicating that HHT1 AVMs are not associated with a further loss of endoglin.
Current data suggest that HHT1 families have a much higher prevalence of pulmonary AVMs than HHT2 families. The gene mutated in HHT2 is a type I receptor of the TGF-β superfamily, activin-like kinase 1 (ALK-1). Cerebral AVMs often cluster in families with a higher prevalence of pulmonary AVMs and are frequent in HHT1 families. However, whereas 70% of all cases of pulmonary AVMs are associated with HHT, only 5% to 10% of cerebral AVMs are found in HHT patients. This implies that reduced endoglin levels are more often associated with pulmonary than cerebral AVM and suggests a more complex etiology for cerebral AVMs.

A critical role for endoglin in vascular development has been confirmed by the observations that endoglin null mice die at embryonic day 10 to 10.5. Vascular channels of the primitive yolk sac cannot form proper vessels, embryonic vessels are dilated, and hemorrhages are seen in both yolk sac cavity and embryo. We have also generated endoglin heterozygous mice, confirming that a single copy of this gene confers susceptibility to the disease. However, only certain strains of mice manifested clinical signs of HHT, suggesting that additional genes are contributing to the heterogeneity and severity of the disease, such as the generation of AVMs.

In the present study we analyzed the expression of endoglin, a modulator of TGF-β effects, in normal brain vessels and in sporadic AVMs occurring in patients without a family history of HHT. Since TGF-β plays a major role in vascular remodeling, we determined whether endoglin distribution and level of expression were altered in the abnormal vessels found in sporadic AVMs. The localization of endoglin was compared with that of platelet endothelial cell adhesion molecule 1 (PECAM-1) (CD31), an endothelial cell–specific antigen. Vimentin staining revealed the mesenchymal cells, while α-smooth muscle cell (SMC) actin and desmin staining identified both SMCs in the media and myofibroblasts in the perivascular connective tissue.

Subjects and Methods

Characteristics of Patients and Clinical Samples
Twenty cerebral AVM pathological specimens, obtained by surgical resection between 1996 and 1998, were selected for analysis on the basis of prior evaluation of their morphology on sections stained with hematoxylin and eosin. The patients ranged in age from 26 to 67 years (Table 1). Initial signs of disease were intracranial hemorrhage in 9 cases, seizures in 10, and headache in 1. None of these patients showed clinical manifestations of HHT. Before surgery, 6 patients underwent embolization treatment with n-butyl cyanoacrylate mixed with lipidol and tantal powder; none underwent preoperative radiation therapy (Table 1). As for control samples, 10 normal brain specimens showing intracranial vessels were used: 5 from temporal lobectomy, 4 from autopsy, and 1 from tissue adjacent to an AVM. All samples were prepared in the pathology laboratory. All research protocols were reviewed by departmental or hospital committees of the University Health Network and the Hospital for Sick Children.

Antibodies
Monoclonal antibody (MAb) SN6h to endoglin (CD105) was an ascites obtained through the Sixth Leukocyte International Workshop and used at an 8000-fold dilution for immunostaining. MAb JC70A to the endothelial marker PECAM-1 (CD31), an endothelial cell–specific antigen. Vimentin staining revealed the mesenchymal cells, while α-smooth muscle cell (SMC) actin and desmin staining identified both SMCs in the media and myofibroblasts in the perivascular connective tissue.
hybridoma supernatant (Dako) used at a 16-fold dilution. Ascites fluid and hybridoma culture supernatant derived from the P3X63-Ag8 myeloma cell line were used as negative controls in the experiments and at dilutions corresponding to those of the specific antibodies.

Immunohistochemical Staining

All specimens were fixed in 10% formalin or 3% paraformaldehyde, and paraffin-embedded tissue sections (6 μm) from all specimens were stained with hematoxylin and eosin and/or hematoxylin phloxine saffron and elastic martius-scarlet-blue. Immunohistochemical staining was performed with the use of the alkaline phosphatase detection system, essentially as described previously. Blocking with either 5% normal goat serum or 3% bovine serum albumin was done before incubation with optimal concentration of primary antibodies for 2 hours at 4°C and for 1 hour with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) F(ab’)_2 (diluted 400-fold; Jackson Immunolabs, BioCan). In other experiments the LSAB^1 Dako amplification system was used. In that case, slides were incubated for 15 minutes at 23°C with biotinylated anti-immunoglobulin reagent followed by streptavidin-conjugated alkaline phosphatase. Most specimens were stained at least twice and in different experiments that included normal brain control sections. Images were acquired from the stained tissue sections with an Olympus BX50 microscope linked to a charge-coupled device video camera using Image Pro Software Analysis (Carsen Medical Scientific) and digitization on a Power Macintosh G3 computer. Staining intensity was measured from images converted to black and white and using the software NIH Image 1.61/fat for Power Macintosh (http://rsb.info.nih.gov/nih-image/) and Image Pro-Plus. Multiple measurements were taken for endoglin, PECAM-1, and control ascites on endothelial and adventitial layers of all vessels and on fibroblasts in the perivascular space using the free line tool set at a defined thickness. The average gray value of the selected pixels, ranging from 0 to 256 shades of gray, was recorded for these markers in each of 5 cases of normal brain and 7 cases of cerebral AVMs that were quantified in detail. A mean±SD value is estimated for each case for endoglin and PECAM-1 on endothelial and mesenchymal cells (which include adventitial and perivascular fibro-
blistas for AVMs). SMC actin and vimentin were also quantified in a similar manner.

Results

Expression of Endoglin in Endothelium and Adventitia of Normal Leptomeningeal Arteries and Intracerebral Arterioles

Normal brain samples were examined for endoglin expression, focusing on extracerebral leptomeningeal and intracerebral vessels. Figure 1A illustrates 2 leptomeningeal arteries showing α-SMC actin expression in their media and 2 veins recognized by a single layer of SMCs. PECAM-1 was restricted to the endothelial layer of both arteries and veins (Figure 1B). Endoglin, as expected from its distribution in several human tissues, was found on the endothelium of these vessels (Figure 1C). However, its expression on the adventitia of the leptomeningeal arteries (Figure 1C) represents the first report of endoglin association with the outer fibroblastic layer of the vessel wall. Figure 1D confirms staining specificity since no reactivity was observed with the control nonimmune ascites. This pattern of endoglin distribution on both endothelial and adventitial layers of leptomeningeal arteries was observed in the 9 samples of normal brain in which such vessels were present.

Figure 1E illustrates a normal intracerebral arteriole in the white matter, showing α-SMC actin reactivity in its narrow media. Endoglin was observed on both endothelial and adventitial layers of this arteriole (Figure 1F), while PECAM-1 was restricted to endothelium (not shown). A venule in the same section revealed a single layer of cells staining for actin (Figure 1G) and the endothelial layer staining for endoglin (Figure 1H). In the 10 cases of normal brain studied, intracerebral arterioles showed adventitial and endothelial endoglin expression. Levels of endoglin and PECAM-1 were relatively similar between normal arteries and veins in the 5 cases of normal brain that were analyzed quantitatively (Table 2). A relative ratio of endoglin to PECAM-1 close to 1 was observed for both arteries and veins, in agreement with our previous report. We also demonstrate that levels of endoglin on adventitia of normal arteries are similar to those found on the endothelial layer (Table 2).

Expression of Endoglin on Endothelial and Mesenchymal Cells in Sporadic Cerebral AVMs

Figure 2A shows a typical case (case 2) of sporadic cerebral AVM with its collection of abnormal vessels and an intervening parenchyma. A large artery can be distinguished by the elastica lamina, absent from the other vessels, presumed to be veins. This is further substantiated by the presence of α-SMC actin on all the vessels, most of which show an abnormal convoluted wall with a variable but definite thickness of SMCs (Figure 2B). PECAM-1 was found on the luminal side of the vessels and was completely absent from media and adventitia (Figure 2C). PECAM-1 is also on platelets in the thrombus of some vessels.

Endoglin was found on the endothelium of all vessels (Figure 2D), giving a luminal pattern of staining very similar to that of PECAM-1. It was also seen on the adventitia, but not in the media of the vessels or in glial tissues (Figure 2D). The arterialized veins appear to be forming adventitial layers, in addition to acquiring SMCs in the media. These fibroblast-like endoglin-positive adventitial cells were seen in arteries as well as in arterialized veins in all 20 cases of sporadic cerebral AVMs analyzed.

Figure 3A illustrates another case (case 6) of cerebral AVM in which the process of vein arterialization is highly apparent, with all vessels in the section showing uneven thickness of α-SMC actin-positive cells. PECAM-1 (Figure 3B) and endoglin (Figure 3C) were present on the endothelium of the vessels. Endoglin was also seen on the adventitial side of the arterialized veins and in multiple layers at the junction of adjacent vessels (Figure 3D). Eight cases (2, 3, 6, 7, 8, 10, 11, and 20) of sporadic cerebral AVMs showed these endoglin expressing fibroblast-like cells in the connective tissue of the nidus. Staining of endothelial, adventitial, and stromal cells was observed with a high dilution of the SN6h MAb (1:8000) and was specific since the control ascites, at the same dilution, showed no reactivity.

To establish the type of cells staining for endoglin in adventitia and perivascular space, 11 of 20 sporadic AVM cases were examined simultaneously for endoglin, PECAM-1, vimentin, desmin, and α-SMC actin expression. Figure 4A illustrates endothelial, adventitial, and perivascular endoglin staining in case 8. Vimentin was present at high levels not only on endothelial cells and SMCs but also on fibroblast-like cells in adventitia and perivascular area (Figure 4B). Desmin, associated with myocyte differentiation, was observed on the transverse layer of SMCs in the media (Figure 4C), which were also reactive with antibodies to actin and vimentin but not to endoglin. A few desmin-positive cells were seen in the perivascular stroma, which also stained for actin and represent myofi-

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**TABLE 2. Analysis of Endoglin Levels in Cerebral Vessels**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Endothelium</th>
<th>Adventitia</th>
<th>Endoglin Ratio Adventitia/Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endoglin Level, Pixels</td>
<td>PECAM-1 Level, Pixels</td>
<td>E/P</td>
</tr>
<tr>
<td>Normal brain</td>
<td>Arteries and veins</td>
<td>78±17</td>
<td>80±17</td>
</tr>
<tr>
<td></td>
<td>Arteries</td>
<td>72±18</td>
<td>79±18</td>
</tr>
<tr>
<td></td>
<td>Veins</td>
<td>87±16</td>
<td>80±16</td>
</tr>
<tr>
<td>Cerebral AVMs</td>
<td>Arteries and veins</td>
<td>71±24</td>
<td>79±24</td>
</tr>
</tbody>
</table>

E/P indicates endoglin level/PECAM-1 level at the surface of endothelial cells, estimated as described in Subjects and Methods.
broblasts (Figures 4C and 4D). Thus, the majority of endoglin-positive cells present in the perivascular connective tissue of cerebral AVMs are vimentin-positive, desmin-negative, and α-SMC actin-negative fibroblasts. It was not possible to determine whether the few myofibroblasts present in the connective tissue of this AVM were endoglin-positive.

All 20 sporadic cerebral AVM cases studied showed endothelial and adventitial endoglin staining, while 8 cases showed endoglin-positive fibroblasts, with 0% to 15% of myofibroblasts present in the perivascular connective tissue. In the 7 cases that were quantified, the level of endoglin was found to be similar in endothelial and adventitial cells (71±24 versus 79±24, respectively) (Table 2). The level of endoglin on mesenchymal cells of the perivascular connective tissue was very similar to that of the adventitial layer, so that results shown for AVMs in Table 2 represent the mean of all mesenchymal cells in a given section. When the levels of endoglin and PECAM-1 were compared between cerebral AVMs and normal samples, no significant difference was seen. Although AVMs are highly vascularized and contain multiple abnormal vessels, their density of endoglin and PECAM-1 is unchanged (Table 2). Thus, the level of endoglin in sporadic AVMs is normal.

One case of cerebral AVM (case 8), resected 2 months after embolization, contained an unusual number of small vessels. Figure 5 reveals that a large-vessel endothelium had a high density of both endoglin (Figure 5A; 129±4) and PECAM-1 (Figure 5B; 167±8) as well as vimentin (Figure 5C; 151±15,
versus 60 in other cases). This endothelial layer appears to be budding and shedding, as suggested by the staining pattern of these 3 markers. Multiple small vessels, best identified by PECAM-1 staining (Figure 5B; 77±16), were also present within media and perivascular connective tissue. The perivascular space also contained multiple layers of fibroblasts staining for vimentin and endoglin, with densities of 110±30 and 71±13, respectively. Very few α-SMC actin–positive myofibroblasts were seen in this sample (Figure 5D). This case suggests that proliferation of endothelium in large vessels and generation of multiple small vessels might occur soon after embolization as part of the remodeling process in the vascular lesion.

Discussion

Our studies demonstrate that endoglin, a known endothelial cell marker, is present on endothelium of normal brain vessels of all types. The endoglin/PECAM-1 ratio estimated on normal brain vessel endothelium was close to 1, confirming that these 2 antigens are expressed at similar levels, as reported previously. The expression of endoglin on the adventitial layer of normal brain arteries and leptomeningeal and intracerebral arterioles represents a novel finding. PECAM-1 is absent from the adventitial cells, while vimentin is present, confirming their mesenchymal origin. The density of endoglin on adventitial cells of normal brain arteries was similar to that observed on endothelial cells (Table 2).

The analysis of sporadic cerebral AVMs revealed normal levels of endoglin on the endothelium of the abnormal veins and arteries (Table 2), indicating that sporadic AVMs are not associated with reduced expression of endoglin. In contrast, endoglin levels were reduced by 50% on the endothelium of

Figure 4. Characterization of the endoglin-positive cells in the connective tissue of a parietal left cerebral AVM (case 8). A, Endoglin is expressed on fibroblast-like cells (open arrowheads) in the perivascular space and on vascular endothelium (open arrow). B, Vimentin is found on endothelium (open arrow), SMCs of the media (arrowheads), and fibroblasts within the connective tissue stroma (open arrowheads). C, Desmin is present on the transverse SMC fibers of the media (arrowhead) and on very few cells within the stroma. D, α-SMC actin is expressed at high levels on all SMCs of the media but more diffusely on the transverse fibers (arrowhead). A subset of cells in the stroma, presumably myofibroblasts, shows α-SMC actin staining (open arrowhead). Bar=200 μm.

Figure 5. High endothelial content in a cerebral AVM (parietal left, case 8). A budding endothelial layer shows a high intensity of endoglin (A), PECAM-1 (B), and vimentin (C) (open arrows) and is outlined by the underlying α-SMC actin layer (D) (arrow). Multiple small vessels are revealed by PECAM-1 staining in media and perivascular space (B) (arrowheads). Mesenchymal cells in the perivascular connective tissue are stained for endoglin (A) and vimentin (C) (open arrowheads). Bar=200 μm.
AVMs of patients with HHT1. However, it is important to state that unaffected vessels in these patients also express 50% endoglin in situ, in agreement with the haploinsufficiency model proposed for the disorder. This reduced expression of endoglin on vessels of HHT1 patients might predispose to cerebral AVM formation, which often occurs in newborns and children. However, our recent observations that only certain strains of mice develop HHT suggest the presence of additional so-called modifier genes that would amplify the effects of reduced endoglin expression, and (A. Bourdeau, PhD, et al, unpublished data, 2000). Such genes could belong to the TGF-β pathway or be implicated in vascular development and/or homeostasis. The human counterparts of some of these modifier genes are likely associated with the generation of cerebral vascular malformations seen in approximately 10% of HHT families. One can speculate that these additional genes might also contribute to the generation of the so-called sporadic AVMs.

Endoglin was expressed on the adventitial layer of both arteries and arterialized veins and on mesenchymal cells in the perivascular space within the lesions. The density of endoglin on these cells was similar to that observed on endothelial cells and was not significantly different from that found in adventitia of normal brain arteries. Considering that cerebral AVMs are highly enriched in blood vessels relative to the adjacent brain parenchyma, more endoglin/surface area is present in sporadic AVMs than in normal brain (Table 2). The expression of endoglin on adventitial and perivascular fibroblasts of sporadic AVMs suggests an active role in remodeling of the lesion rather than in their generation.

Endoglin was first described on stromal cells of connective tissue in murine uterus, ovary, and gastrointestinal system, which are highly vascularized and subject to frequent remodeling. We also reported massive upregulation of endoglin at 4 to 9 weeks of gestation on cushion tissue mesenchymal cells that give rise to valves and septa in human embryos. In murine embryos, we detected endoglin on endocardial cells transforming into mesenchymal cells and subsequently proliferating and migrating to form the cushion tissue. This process does not occur in endoglin null mice, impairing heart development and leading to death of the embryos at mid-gestation. Cushion tissue formation is known to be regulated by TGF-β1 and/or -β3 and appears to require endoglin, capable of modulating responses to these 2 isoforms.

We now suggest that fibroblasts of the connective tissue of AVMs are implicated in vascular remodeling, mediated by TGF-β and endoglin. Paradoxically, it has been reported that vessels of AVMs have reduced levels of TGF-β2/β3 and TGF-β receptor II, relative to normal brain vessels; the fibrous wall of the vessels was said to be unreactive for TGF-β and receptors. We have recently demonstrated that overexpression of endoglin in murine fibroblasts led to reduced production of extracellular matrix proteins, such as fibronectin and plasminogen activator inhibitor 1, decreased migration, and changes in cellular morphology, in the absence of exogenous TGF-β1 stimuli. Thus, there might be a TGF-β-independent function of endoglin. This is supported by observations that endoglin is capable of binding other growth factors of the TGF-β superfamily such as activin, bone morphogenetic protein (BMP)-7, and BMP-2 by association with their respective ligand binding receptors. Subsequent studies should clarify whether TGF-β1/β3 or any other growth factors of the superfamily are present in AVMs and being modulated by endoglin.

AVMs consist of relatively mature vessels in an activated state, as suggested by the presence of angiogenic factors. Vascular endothelial growth factor was upregulated in cerebral AVMs and surrounding vasculature. It was found on endothelial and subendothelial layers and in intervascular and perivascular tissues, suggesting a diffuse activation of angiogenesis throughout the vascular malformations. Basic fibroblast growth factor was expressed, albeit at lower levels than vascular endothelial growth factor, and was localized to the media of vessels, around individual myocytes and fibroblasts. The endothelial specific angiopoietic receptor tyrosine kinase Tie-1, also implicated in angiogenesis, was found at high levels in the abnormal arteries and veins of the AVM nidus and in the capillaries bordering the lesion.

The analysis of AVM sections suggests that the angiogenic process is also associated with proliferation and migration of adventitial cells. This was particularly obvious where adventitial layers of adjacent vessels merge and vimentin- and endoglin-positive fibroblasts invade the perivascular space. Very few myofibroblasts were present in the connective tissue stroma, detectable in 8 of 20 cases of AVMs studied. This is in agreement with a previous study reporting conversion of fibroblasts into myofibroblasts only after stereotaxic radiosurgery of AVMs. Radiation induced the proliferation of fibroblasts and their transformation into myofibroblasts, whose contractile activity could contribute to shrinking and eventual occlusion of the AVM. The stromal spindle-shaped and proliferating cells were closely related to the adventitial layer of the vessels but did express α-SMC actin, desmin, and vimentin. It is of interest to determine whether radiation-induced myofibroblasts express endoglin.

In the present study we have demonstrated that endoglin is expressed at similar levels in endothelial and adventitial layers of normal brain arteries and arterioles and on endothelium and adventitium of arteries and arterialized veins of sporadic cerebral AVMs. Contrary to those derived from HHT1 patients, these AVMs express normal levels of endoglin on their endothelium. However, since the reduction in endoglin in AVMs associated with HHT1 is similar to that seen in unaffected vessels of these patients, we must conclude that endoglin reduction is not responsible for the generation of cerebral AVMs. Overall endoglin expression is in fact higher in sporadic cerebral AVMs than in normal brain parenchyma because of the large number of vessels present in these lesions. Furthermore, in 40% of cases, endoglin and vimentin-positive fibroblasts were found in the perivascular stroma. Our data suggest that endoglin present in the adventitial layer of normal brain arteries and in arterialized veins of sporadic AVMs is implicated in the response of vessels to sustained arterial blood flow. Endoglin, via its capacity to potentially regulate responses to several members of the TGF-β superfamily, must be functionally implicated in this intense remodeling process.

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References

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