Cerebral Vascular Abnormalities in a Murine Model of Hereditary Hemorrhagic Telangiectasia

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Background and Purpose—Hereditary hemorrhagic telangiectasia type 1 (HHT1) is an autosomal dominant vascular dysplasia caused by mutations in the endoglin gene and characterized by dilated vessels and arteriovenous malformations (AVMs). To understand the etiology of this disorder, we evaluated the cerebral vasculature of endoglin heterozygous (Eng+/−) mice, which represent the only animal model of HHT1.

Methods—The cerebral vasculature of Eng+/− and Eng+/+ mice from C57BL/6 (B6) and 129/Ola (129) strains with a differential susceptibility to HHT1 was studied with corrosion casting. Casts were observed by scanning electron microscopy to detect malformations and evaluate arterial diameters and orientation of endothelial nuclei. Measurements were taken to assess relative constriction at arteriolar branching points and downstream relative dilatation.

Results—Three of 10 Eng+/− mice demonstrated abnormal vascular findings including AVMs, while none of 15 Eng+/+ mice did. The incidence of relative constriction at arteriolar branching points was significantly less in both Eng+/− groups than in their Eng+/+ counterparts. The occurrence of relative dilatation was significantly greater in B6-Eng+/− than in B6-Eng+/+ mice. Endothelial nuclei were significantly rounder and deviated more from the direction of blood flow in Eng+/− than in Eng+/+ mice.

Conclusions—Eng+/− mice showed significant structural alterations in cerebral blood vessels, indicating that the level of endoglin on endothelium is critical for maintenance of normal vasculature. Since endoglin haploinsufficiency is associated with HHT1, such changes in arteriolar structures might occur in HHT1 patients and predispose them to AVMs and their sequelae. (Stroke. 2003;34:783-789.)

Key Words: arterioles ■ endothelium ■ microscopy, electron ■ transforming growth factors ■ vascular malformations

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder affecting 1 of 8000 people and associated with epistaxis and telangiectases. Major complications are due to pulmonary, hepatic, and cerebral arteriovenous malformations (AVMs). Cerebral AVMs are observed predominantly in newborns and children and can often be fatal.

Two genes are responsible for HHT: endoglin mutated in HHT type 1 (HHT1) and activin receptor-like kinase 1 mutated in HHT type 2 (HHT2). A higher prevalence of pulmonary and cerebral AVMs is observed in HHT1 families, suggesting that an endoglin mutation may be a predisposing factor. Levels of endoglin protein are reduced by half in umbilical vein endothelial cells from newborns harboring an endoglin mutation and in activated monocyes from patients with HHT1 because mutant proteins are unstable and nonfunctional. A 50% reduction in endoglin was also estimated on presumed normal arteries, veins, and capillaries from lung and brain of a newborn who died subsequent to rupture of a cerebral AVM. These data suggest that all blood vessels in HHT1 patients express reduced endoglin and support haploinsufficiency as the disease model.

Endoglin is a component of the transforming growth factor-β (TGF-β) receptor complex, preferentially expressed on vascular endothelium. Mice lacking endoglin or other TGF-β receptors die at mid-gestation of cardiovascular defects. Mice heterozygous at the endoglin locus (Eng+/−) mature to adulthood and can develop HHT. Immunostaining of sections from several murine tissues revealed reduced endoglin levels on arteries, veins, and capillaries, reflecting overall expression of a single allele. In mice and humans, age of onset and extent of organ involvement are heterogeneous. Modifier genes likely contribute to HHT, as suggested...
by the 129 strain being more susceptible than the B6 strain.\textsuperscript{14,17}

We investigated the ultrastructure of cerebral vessels in B6- and 129-Eng\textsuperscript{+/+} and Eng\textsuperscript{+/−} mice by analyzing vascular corrosion cast images obtained by scanning electron microscopy. Our aim was to identify potential vascular abnormalities in Eng\textsuperscript{+/−} mice and test HHT susceptible and nonsusceptible strains.

**Materials and Methods**

**Mice**

Eng\textsuperscript{+/−} mice were generated by homologous recombination with the use of embryonic stem cells of 129/Ola (129) origin and a construct containing the Endoglin (ENG) gene including the promoter and the location where exon 1 was replaced by LacZ and Neo genes.\textsuperscript{14} Male chimeras were mated with wild-type (WT) 129 mice (Harlan, Bicester, UK), giving rise to inbred progeny, homozygous at all alleles except endoglin. Male chimeras were successively backcrossed with C57BL/6 (B6) females (Taconic Farms, Germantown, NY), yielding mice of N5-N6 generations, with 85% to 97% probability of being homozygous for B6 alleles at any locus. All protocols were approved by the Animal Care Committee of the Hospital for Sick Children.

**Western Blot Analysis**

Eng\textsuperscript{+/−} and Eng\textsuperscript{+/+} mice were perfused through the left ventricle with PBS. Brains were homogenized in 0.05 mol/L Tris-HCl (pH 7.4) with 1% Triton X-100 and protease inhibitors. Extracts were pre-cleared 3 times with Protein A Sepharose CL-4B and Protein G Sepharose Fast Flow (Amersham-Pharmacia Biotech AB), and aliquots (40 μg protein) were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions, transferred onto nitrocellulose, and probed with rat monoclonal antibody JC7/18 (1:200; Pharmingen) conjugated goat anti-rat IgG (1:2000; ICN Biochemical), essentially as described before.\textsuperscript{10} Specific bands were visualized by chemiluminescence and quantified by densitometry.

**Vascular Corrosion Casts**

Previous methods\textsuperscript{18,19} were adapted. Mice were anesthetized with sodium pentobarbital (50 mg/kg IP), and laparotomy/thoracotomy was performed. Mice were manually perfused with 20 mL of heparinized (20 U/mL) PBS (37°C) into the left ventricle (right atrium was cut for drainage) followed by 10 mL of Batson’s No. 17 plastic (Polysciences) at a rate of 1 mL/min. This resin is suitable for quantifying luminal diameters and endothelial nuclei images.\textsuperscript{20,21} The lack of resin nasal outflow and good filling of the eye’s choroid vessels were indicative of no hyperperfusion or hypoperfusion.\textsuperscript{18} After polymerization (24 hours at 23°C), the whole brain was removed and digested in 20% KOH for 24 to 72 hours, with intermittent water rinses. The remaining vascular cast was air-dried, mounted onto a stub with colloidal silver paste, sputter-coated with gold, and examined by scanning electron microscopy (Hitachi S570) at 15 keV.

**Image Analysis**

The replica of the vascular luminal space was analyzed qualitatively and quantitatively. For qualitative analysis, vascular casts were examined blindly for 45 to 60 minutes; the extent of perfusion was determined, and vascular abnormalities were examined.

For quantitative analysis, vessels were chosen from well-perfused areas of superficial gray matter irrespective of hemisphere or lobe. Photomicrographs were taken of the proximal middle cerebral artery (MCA) and 10 randomly chosen arterioles (10 to 60 μm diameter) located near the cerebral cortex surface, along with their parent arteries. Diameters of the proximal MCA and parent arteries served as references for gross vascular changes. Diameters at the origin (at branching point), proximal (10 μm downstream), and distal (narrowest point within 10 to 50 μm of origin) positions were measured for 10 randomly selected arterioles. For parent arteries and their arterioles, 10 measurements were taken per mouse. The origin/distal and
proximal/distal ratios were calculated; origin/distal <1 represented a relative constriction at the branching point, while proximal/distal >1 indicated a relative downstream dilatation.

The impressions of 50 nuclei located neither at the edge of the image nor near a branching point were analyzed from a minimum of 5 randomly chosen straight arteriolar segments in each mouse. Surface area, length, width, width/length ratio, and deviation (absolute angle between long axis and blood flow direction) were determined for the selected nuclei. A total of 50 measurements were collected per mouse for each parameter with the use of the Image Processing Toolkit 3.0 in Adobe Photoshop (Adobe Systems Incorporated).

All vessels were confirmed as arterial if they originated from parent arteries with regularly spaced elongated nuclei oriented along the direction of flow.\textsuperscript{18} Veins have less organized and rounder nuclei.\textsuperscript{18,22} Digitized photomicrographs were taken if both ends of the vessels of interest were at near 0° tilt angle, thus minimizing depth of focus distortion. Wilcoxon rank statistics compared variables measured once in each animal. Generalized estimating equations were used to analyze the data and incorporate the repeated measures (vessel diameter and nuclear geometry) on each animal with the GENMOD procedure in SAS version 8.01 (SAS Institute). \( P < 0.05 \) was considered significant. Data are presented as box-and-whisker plots.

**Results**

**Reduced Endoglin Expression in Cerebral Vasculature of Eng\textsuperscript{+/−} Mice**

To ascertain endoglin level in Eng\textsuperscript{+/−} mice, Western blot analysis was performed on whole brain lysates. Endoglin was resolved as a homodimer of 180 kDa (Figure 1A), and its expression was reduced to 59.3±5.5% of control values in Eng\textsuperscript{+/−} mice (Figure 1B; \( P < 0.05 \)), as expected for mice with a single allele.

**Cerebrovascular Malformations in Eng\textsuperscript{+/−} Mice**

Vascular casts from WT B6, N5/N6 B6-Eng\textsuperscript{+/−} and -Eng\textsuperscript{+/−}, and WT 129 and 129-Eng\textsuperscript{+/−} mice were analyzed by scanning electron microscopy. Major intracranial arteries were readily identifiable, and arterial branching could be followed to the capillary bed, which showed abundant capillary anastomoses (Figure 1C). Veins were not generally perfused; postcapillary venules were identified by increased diameter when exiting the capillary bed. Intracranial vessel diameters ranged from 200 \( \mu \text{m} \) for major arteries to 8 \( \mu \text{m} \) for precapillary arterioles, while capillaries were between 3 and 7 \( \mu \text{m} \).

No apparent gross abnormalities were observed in the majority of mice. However, abnormal findings were observed in 3 of 10 Eng\textsuperscript{+/−} mice. Two arteriovenous connections were found in an N5 B6-Eng\textsuperscript{+/−} mouse that had no external signs of HHT but showed some dilated vessels in lung, liver, and intestine before perfusion. One of the connections appears normal since the vessels were nontortuous and showed direct branching of a metarteriole from a parent artery to a venule without capillary anastomoses (Figure 2A). The second connection represents a micro-AVM with a nidus of 50 \( \mu \text{m} \) in diameter with identifiable feeding arteriole and draining venule (Figure 2B). A 129-Eng\textsuperscript{+/−} mouse, which had an ear...
telangiectasia and dilated vessels in lung, liver, stomach, and intestine, demonstrated a remarkable AVM displaying a mass of intertwined vessels and several perinidal aneurysms in adjacent irregularly sized capillaries (Figure 2C). Another N5 B6-Eng−/− mouse, which had no external or internal sign of disease, revealed a severe dilatation of the proximal portion of an arteriole to a diameter greater than that of the parent artery (Figure 2D). None of the B6- or 129-Eng+/− mice displayed cerebrovascular abnormalities.

Quantitative Differences in Arteriolar Structure Between Eng−/− and Eng+/− Mice

Figure 3 illustrates the caliber and shape of arterioles in the vascular casts. Arterioles without relative constriction or dilatation (Figure 3A), with a relative constriction at the branching point (origin/distal ratio <1; Figure 3B), with a relative dilatation 10 μm downstream (proximal/distal ratio >1; Figure 3C), and with both a relative constriction and dilatation (Figure 4D) are shown.

The proximal MCA diameters ranged from 132 to 163 μm, and their distribution showed no significant differences between groups (Figure 4A), supporting the validity of our analysis. In all mice, the parent vessels of randomly selected arterioles were also of similar sizes (20 to 80 μm), with no significant differences between groups (Figure 4B). The absolute diameters at origin, proximal, and distal positions of selected arterioles were also not different between groups (data not shown). Although examples of the various arterioles shown in Figure 3 were found in all groups of mice, their distribution was variable between groups. Relative constriction at the origin was significantly less prevalent in Eng−/− groups than in their respective Eng+/− controls, as shown by a higher origin/distal ratio (Figure 4C). The 129 strain had significantly less such relative constrictions than the B6 strain (Figure 4C). Relative dilatation at the proximal portion of arterioles was significantly greater in B6-Eng−/− mice than in B6-Eng+/− mice, as indicated by a higher proximal/distal ratio (Figure 4D). While not significant, a similar trend was noted in the 129 strain.

Preliminary observations suggested changes in arteriolar endothelial nuclei patterns in Eng−/− mice. Imprints of endothelial nuclei were clearly visualized on arterial casts down to the precapillary level. Figure 5 demonstrates representative arterioles and their endothelial nuclei for each mouse group. Endothelial nuclei of WT B6 arterioles (Figure 5A), N6 B6-Eng−/− arterioles (Figure 5B), and WT 129 arterioles (Figure 5D) were elongated and oriented in the direction of flow. However, endothelial nuclei of N6 B6-Eng−/− (Figure 5C) and 129-Eng+/− (Figure 5E) arterioles were rounder and smaller in area than their respective controls.

B6-Eng−/− nuclei were significantly smaller in surface area than B6-Eng+/− nuclei, while no difference in surface area was observed in the 129 strain (Figure 6A). Nuclei were wider in 129-Eng+/− arterioles than in WT 129 (P<0.001;
significant difference between all groups. C, distribution of the diameters of parent arteries of randomly selected arterioles was also not distinct between all groups. C, proximal MCA diameters was similar in all groups of mice: WT B6, B6-Eng−/−, WT 129 and 129-Eng−/−. B, The distribution of the diameters of parent arteries of randomly selected arterioles was also not distinct between all groups. C, Origin/distal (O/D) diameter ratio was significantly higher in the Eng−/− groups than in the Eng+/+ controls (P<0.001 for B6 and P<0.05 for 129) and in WT 129 versus B6 (tP<0.001). D, Proxi-

mal/distal (P/D) diameter ratio was significantly greater in B6-Eng−/− than in B6-Eng+/+ mice (P<0.05). Data are present-

ed as box-and-whisker plots with cross (+) indicating the mean, central line, the median, boxes the 25th and 75th percentiles, and whiskers minimum and maximum values.

median = 6.1 versus 5.3 μm) and shorter in B6-Eng+/+ than in B6-Eng−/− arterioles (P<0.001; median = 12.5 versus 15.1 μm). The width/length ratios were significantly higher in Eng−/− groups than in Eng+/+ groups (Figure 6B). Deviation of the long axis of nuclei from the direction of flow was greater in Eng−/− than in Eng+/+ arterioles (Figure 6C). The maximum values of the deviation distribution were particularly noticeable in the Eng−/− groups, and there was also a lesser but significant difference between B6-Eng+/+ and WT B6 mice.

Discussion

With the use of scanning electron microscopy of vascular casts, structural abnormalities were identified in the cerebral vasculature of Eng−/− mice. Malformations, reminiscent of AVMs seen in the brain of HHT1 patients,2–5 were found in 30% of Eng−/− mice, but none were found in control mice. These data suggest that mice with a single copy of endoglin might be predisposed to AVM development. Additionally, a reduction in the occurrence of relative constriction at arterio-

lar branching points and a greater propensity for relative downstream dilatation were observed in Eng−/− mice. Endo-

thelial nuclei were rounder and less aligned with the direction of flow, further indicating an abnormal vasculature in Eng−/− mice.

One of the arteriovenous connections seen in an Eng+/−
mouse (Figure 2A) resembled normal arteriovenous anasto-

moses described in canine and human brain.23 Such structures are rare in cerebral vessels but have been observed in other tissues.24,25 The other 2 arteriovenous connections were more like disease-associated AVMs.26,27 Small aneurysms seen adjacent to 1 AVM (Figure 2C) might be due to resin extravasation; however, they were not detected anywhere else and infer focal vessel fragility.

Cerebrovascular abnormalities were observed in 30% of Eng−/− mice, while none were found in control mice. In humans with HHT, the incidence of cerebral AVMs has been estimated at 12% to 23%.27,28 However, cerebral and pulmonary AVMs are higher in HHT1 families.5,8,9 Analysis of a larger number of Eng−/− mice is needed to determine whether incidence of AVMs is similar in mice and humans with a single functional endoglin copy.

Quantitative analysis of cerebral vascular casts revealed that proximal MCA and parent arteries of randomly selected arterioles were of similar size in Eng−/− and Eng+/+ mice, as were the absolute arteriolar diameters measured at origin, proximal, and distal positions. However, arterioles of Eng−/− mice showed less relative constriction at the branching point and more proximal relative dilatation than Eng+/+ arterioles. These morphological observations are indicative of focal points of fragility and perhaps impaired vascular integrity but do not shed light on the physiological and molecular parameters affected. Hemodynamic and biochemical studies are under way to elucidate possible mechanisms that lead from reduced endothelial endoglin expression to altered vascular integrity.

Our previous data indicated that the 129 strain was more susceptible to clinical manifestations of HHT.14,17 The present study did not reveal a higher incidence of cerebrovascular abnormalities in these mice, with the exception of the arteriolar origin/distal ratio being significantly greater in the 129 than the B6 strain, indicating less relative constriction at the branching point. Previous studies reported reduction and
truncation of peripheral vessels in liver and lung and portal shunting in 70% of 129 mice.29,30 These data suggest that genetic components may contribute to susceptibility of the 129 strain to vascular abnormalities. In this report, the effects of endoglin haploinsufficiency were much stronger than the strain differences.

Figure 5. Altered endothelial nuclear patterns in Eng+/− cerebral arterioles. Five representative nuclei imprints are outlined for emphasis. A, WT B6; B, N6 B6-Eng+/−; C, N6 B6-Eng−/−; D, WT 129; E, 129-Eng−/−. Endothelial nuclei in Eng+/− mice are rounder and less aligned with the long axis of the arteriole than those in Eng−/− mice. Bars=25 μm.

We analyzed endothelial cell nuclei rather than cell shape because nuclei were more readily visible in arterioles. The larger cerebral and basilar arteries revealed cell borders and depressions representing nuclei, whereas arteries <125 μm in diameter only showed nuclear imprints. Shape and orientation of endothelial cells and their nuclei were reported to change in vitro in response to shear stress or flow patterns.31–35 In vivo studies have shown that at major artery branching points, where both turbulent flow and jet flow are present, endothelial cells are spindle-shaped with disrupted borders or cobblestone-shaped and disoriented with respect to the direction of flow.36 Endothelial nuclei impressions located near a branch that could be affected by complicated local flow conditions were therefore excluded from the study. In Eng+/− mice, endothelial nuclei were rounder and had a more irregular orientation, indicative of local reduction in shear stress or turbulent flow and consistent with increased incidence of relative dilatation and abnormal arteriolar structure.

We observed that endoglin heterozygosity can result in morphological abnormalities such as AVMs, focal decreases in relative arteriolar constriction at branching points, and focal increases in downstream relative dilatation. It remains to be determined whether these localized changes can lead to the generation of AVMs and be present in HHT patients.

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