Mouse Model for Hereditary Hemorrhagic Telangiectasia Has a Generalized Vascular Abnormality

Evelyn Torsney, PhD; Richard Charlton, PhD; Austin G. Diamond, PhD; John Burn, MD; James V. Soames, PhD, Helen M. Arthur, PhD

Background—Mutations in endoglin or activin like kinase-1, both involved in the endothelial transforming growth factor-β signaling pathway, cause the autosomal dominant bleeding disorder hereditary hemorrhagic telangiectasia. We and others have reported mouse models for this disease that share the characteristic phenotype of dilated vessels and sporadic hemorrhage. The reasons for the variable phenotype in hereditary hemorrhagic telangiectasia are not understood.

Methods and Results—After a detailed immunohistochemical analysis of 129/Ola mice, which are heterozygous for a targeted deletion in the endoglin gene, we observed intrinsic abnormalities in the vascular walls throughout the cutaneous vasculature. Postcapillary venules were dilated, and up to 70% of the vascular wall had no smooth muscle cells. The supporting layers of collagens and elastin were irregular, with thin areas, adding to the fragility of these vessels. A variable hemorrhagic phenotype was observed in which local bleeding is associated not only with fragile vessels but also with regions of inflammation.

Conclusions—These findings have relevance to our understanding of the molecular basis of vascular integrity in a wide range of diseases. (Circulation. 2003;107:1653-1657.)

Key Words: vasculature ■ angiogenesis ■ hemorrhage ■ inflammation

Endoglin, an auxiliary receptor for transforming growth factor (TGF)-β, is required for angiogenesis during development.1–3 It is expressed primarily in endothelial cells and is upregulated during endothelial cell activation, inflammation, and angiogenesis.4–6 Endoglin associates with type II receptors of the TGF-β family in the presence of ligand and with the type I signaling receptors activin-like kinase-1 and activin-like kinase-5, even in the absence of exogenous ligand.7–9 Mutations in endoglin cause hereditary hemorrhagic telangiectasia type I (HHT1), an autosomal dominant disease of variable severity, characterized by cutaneous telangiectases, increasingly severe nose bleeds, and gastrointestinal hemorrhage.10 In addition, major arteriovenous malformations (AVMs) involving direct connections between artery and vein may occur in lung, liver, or brain and may cause severe morbidity and mortality.11,12 Mutations in activin-like kinase-1 can also produce the HHT phenotype,13 and in one family, a possible third locus for this disease is still to be mapped.14 Pulmonary AVMs affect 33% of HHT1 patients but seem to be less common in HHT2.15

Immunohistochemistry of 1 pulmonary and 1 cerebral AVM showed no detectable loss of endoglin protein in the vascular endothelium compared with the rest of the vasculature. This fails to support local loss of heterozygosity at the endoglin locus as the cause of the AVM but leaves open the question of how they develop.16

To investigate the primary causes of HHT and the role of endoglin in blood vessel formation, we and others independently derived a mouse that was heterozygous for a targeted deletion of the endoglin gene and that exhibited localized dilated weak-walled blood vessels similar to those of HHT patients.1,3 Mice homozygous for the targeted deletion of the endoglin gene exhibited severe defects in angiogenesis and heart development, resulting in midgestation lethality.1–3 The angiogenic defects appeared to be a result of delayed smooth muscle development around the neovasculature.2

We proposed that reduced levels of circulating TGF-β1 in the 129/Ola background might explain why symptoms of HHT developed in this strain but not in the NIH or C57Bl/6 strains,3 which was subsequently shown in mice carrying a different endoglin mutation.17 However, in this study, only five 129/Ola animals carrying the endoglin mutation were autopsied for histology, and their analysis focused primarily on the progeny of intercrosses between the 129/Ola and C57Bl/6 strains. In an F1 generation, all mice are heterozygous at every locus (for any allele that varies between these 2 strains), and in the F2 generation, alleles segregate in a random manner, raising particular difficulties when 1 of the

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parent strains (129/Ola) has an unusual vasculature tree compared with other strains. In this study, the first to explore in detail the general microscopic structure of the distal vascular tree in HHT, we focus exclusively on 129/Ola mice, all of which are genetically identical except for the targeted endoglin mutation. We report that in addition to earlier reports on localized dilation and vascular abnormalities in these mice, there are several additional intrinsic abnormalities in the organization of the whole peripheral vasculature in HHT. These observations are relevant to our general understanding of angiogenesis and the maintenance of vascular integrity.

Methods

Animals

Mice (bred in-house) were maintained according to procedures permitted under UK Home Office license and are heterozygous for a targeted deletion of the endoglin gene. This mutation was generated in 129/Ola ES cells, and crossing to this line maintains the mice in a pure 129/Ola background. Males were kept in separate cages both for the well-being of their cagemates and to avoid any confusion with bleeding caused by fighting wounds. Health screening showed that the colony was free of bacterial and viral infection. Detailed records were kept for >2 years, and postmortem examinations were performed on a total of 128 mice (60 wild-type and 68 eng<sup>+</sup>−<sup>−</sup>) during this period.

Immunohistochemistry

Both bleeding and asymptomatic tissues were examined. Care was taken to fix tissue rapidly in buffered 10% formalin to maximize preservation of morphology. When necessary, PBS/0.8 mol/L EDTA was used to decalcify bone tissue. Tissues were dehydrated and processed in paraffin, and 5-μm sections were cut. For general histology, tissue sections were stained with hematoxylin and eosin. Immunohistochemistry was performed using primary antibodies for von Willebrand factor and α-smooth muscle actin (DAKO) to detect vascular endothelial cells and vascular smooth muscle cells, respectively. Primary antibodies were visualized with the Histomouse kit (Zymed), which detects mouse primary antibodies on mouse tissue without background because of endogenous Ig and uses a 3-aminophenyl-9-ethylcarbazole substrate to give a red-brown reaction product. By use of these stains, capillaries were counted in ×20 fields of view, and numbers were compared by a Student’s 2-tailed t test. For postcapillary venules, the regions lacking smooth muscle were quantified as a percentage of total vessel diameter by use of KS300 quantification software (Imaging Associates). Elastin and collagens were identified by the elastin–van Gieson histochemical method, which stains elastin blue/black, collagens red, and muscle yellow. Mast cells were stained with toluidine blue and counted in a ×20 field of view. All tissue sections were examined with the investigator blinded to genotype.

Results

Significant mortality and morbidity were associated with the endoglin mutation in the 129/Ola background (see Table 1). One quarter of the eng<sup>+</sup>−<sup>−</sup> mice either died spontaneously, without any obvious external symptoms, or showed significant morbidity and were euthanized. Postmortem examination revealed that death was caused by internal hemorrhage or multiorgan vascular dilatation causing heart failure or, in one third of cases, by an unknown cause. Externally visible hemorrhage was noted in 44% of the eng<sup>+</sup>−<sup>−</sup> mice, with bleeding from the uterus, gut, ear, nose, or eye. The earliest onset of bleeding was at age 12 weeks, and the average age at onset was 36 weeks. In addition, blood was observed regularly in cages housing 6 of the 31 eng<sup>+</sup>−<sup>−</sup> males, but was

### Table 1. Summary of HHT Phenotypes of the eng<sup>+</sup>−<sup>−</sup> Mouse

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild-Type (95% CI)</th>
<th>eng&lt;sup&gt;+&lt;/sup&gt;−&lt;sup&gt;−&lt;/sup&gt; (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major AVMs</td>
<td>0% (0–6%)</td>
<td>6% (1–14%)</td>
</tr>
<tr>
<td></td>
<td>(subdermal; uterine; liver)</td>
<td></td>
</tr>
<tr>
<td>Spontaneous death or moribund and euthanized</td>
<td>3.3% (0.4–11%)</td>
<td>25% (15–37%)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>1.6% (0–9%)</td>
<td>44% (32–57%)</td>
</tr>
<tr>
<td></td>
<td>(uterus)</td>
<td>(ear 6; eye 8; gut 4; uterus 6; nose 6)</td>
</tr>
<tr>
<td>Average No. of litters per female</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Average No. of surviving pups per litter</td>
<td>3.5</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Sample sizes are 60 wild-type and 68 eng<sup>+</sup>−<sup>−</sup>.

### Table 2. Detailed Histological and Immunohistochemical Comparison of General Dermal Vascular Architecture in eng<sup>+</sup>−<sup>−</sup> and Wild-Type Mice

<table>
<thead>
<tr>
<th>Vascular Phenotype (Ear/Eye/Abdominal Skin)</th>
<th>Wild-Type (n=10)</th>
<th>eng&lt;sup&gt;+&lt;/sup&gt;−&lt;sup&gt;−&lt;/sup&gt; (n=12)</th>
<th>Level of Statistical Significance, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilated venules in papillary dermis</td>
<td>5/10</td>
<td>9/12</td>
<td>NS</td>
</tr>
<tr>
<td>Average numbers of capillaries across the dermis in a ×20 field of view</td>
<td>20.1±1.8*</td>
<td>30.6±1.76*</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Presence of dilated vessels in reticular dermis and subcutaneous tissue</td>
<td>2/10</td>
<td>12/12</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Animals with irregular or thin-walled vessels in reticular dermis and subcutaneous tissue</td>
<td>0/10</td>
<td>12/12</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Smooth muscle actin staining around individual postcapillary venules as proportion of vessel perimeter</td>
<td>0.72±0.1*</td>
<td>0.31±0.1*</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Average numbers of mast cells across the dermis in a ×20 field of view</td>
<td>14.8±1.8*</td>
<td>24.4±2.4*</td>
<td>&lt;0.005§</td>
</tr>
<tr>
<td>Average proportion of mast cells that were degranulated</td>
<td>0.21±0.04*</td>
<td>0.75±0.1*</td>
<td>&lt;0.001§</td>
</tr>
</tbody>
</table>

<sup>*</sup>Standard error. Statistical tests: †2×2 χ2; ‡Student’s 2-tailed t test.
attributed to nose bleeds only after histological examination of nasal tissues.

The endoglin mutation had a severe effect on female fecundity, which is already poor in the 129/Ola strain (Table 1). Wild-type females had 2 or 3 litters and an average of 3 to 4 pups per litter. In contrast, the eng<sup>−/−</sup> female mice had fewer litters during their reproductive life (between 1 and 2 litters per female), and only 2 animals from a total of 35 litters survived beyond 24 hours after birth. Because this may have been a result of defects in the uterine vascular architecture, we compared uterine histology in 4 wild-type and 6 eng<sup>−/−</sup> female mice and found an increased frequency of thick-walled vessels, microvascular dilatation, and hemorrhage in half of the eng<sup>−/−</sup> females (not shown). These abnormalities may have contributed to reduced fertility.

Detailed histological and immunohistochemical analyses of both unaffected and hemorrhagic areas of skin from the abdomen, ear, and eyelids from 22 animals are summarized in Table 2. Dilated vessels were observed in all 129/Ola animals irrespective of the endoglin mutation. This was not unexpected, because the 129/Ola strain has an abnormal vasculature, including dilated vessels, reduced branching, and an increased shunt frequency.<sup>18,20</sup> Dilated vessels were seen in the delicate ear vasculature in both wild-type and eng<sup>−/−</sup> mice and were not recorded as telangiectases. However, the severity of dilation of capillaries and postcapillary venules in the skin of endoglin mice was far greater than in wild-type mice. The vessel walls were uneven because of
variation in the numbers of perivascular smooth muscle cells, numbers of collagen fibers, and integrity of elastin (Figure 1, B, D, F, and G). In the postcapillary venules, vascular wall thickness was irregular across the diameter of a vessel, and there were significant regions completely lacking smooth muscle cells (Figure 1, F and G; Table 2). In contrast, wild-type vessels had regular, even walls of smooth muscle cells, collagens, and elastin (Figure 1, A, C, and E). We also noted that the smooth muscle cells and the collagen fibers are more loosely organized in the eng−/− mice than in the wild-type animals (Figure 1B). This was also reflected in a whole-mount view in which vessels showed irregular dilated and constricted regions along their length (Figure 1J). Irregular vessel walls were also seen in the vasculature of the lung of a human HHT patient with a mutation in the endoglin gene (Figure 1, K and L). In addition, the eng−/− mice showed a 50% increase in the number of capillaries in the papillary dermis compared with wild-type mice (Table 2).

Four major AVMs were found in the eng−/− mice: 2 subcutaneous, 1 uterine, and 1 hepatic. These had varied structures, but had all lost the distinctive vascular structure defining arteries and veins. In addition, immunohistochemistry revealed that there were extremely abnormal regions within the AVMs with no endothelial cells, smooth muscle cells, or elastin fibers (Figure 2, A–F).

When bleeding lesions developed on the ears, secondary inflammation followed rapidly, with bleeding persisting for periods of up to 16 weeks. In the eyelid, 129/Ola mice are susceptible to ulcerative blepharitis, which developed at a similar frequency (5%) in both wild-type and susceptible to ulcerative blepharitis, which developed at a similar frequency (5%) in both wild-type and eng−/− mice.3 In contrast, inflammation followed rapidly, with bleeding persisting for periods of up to 16 weeks. In the eyelid, 129/Ola mice are susceptible to ulcerative blepharitis, which developed at a similar frequency (5%) in both wild-type and susceptible to ulcerative blepharitis, which developed at a similar frequency (5%) in both wild-type and eng−/− mice.3 However, inflammation of the eyelids invariably led to bleeding in the eng−/− but not the wild-type animals. Again, bleeding persisted for up to 20 weeks. The pathology of bleeding lesions was complex because of secondary events associated with inflammation, such as leukocyte recruitment, vascular dilation, and angiogenesis in granulation tissue. A histological and immunohistochemical analysis revealed that bleeding vessels were dilated and unusually close to the epidermal surface (Figure 2G). In some cases, these vessels had few smooth muscle cells (Figure 2I). In contrast, inflammation in wild-type animals was associated with a proliferation of small vessels with endothelial cells expressing von Willebrand factor protein and surrounded by smooth muscle cells (Figure 2, J and K). Blood was found in the nose of 6 eng−/− males and appeared to be hemorrhaging from dilated vessels near the vomeronasal organ (Figure 2L).

**Discussion**

We have found that 129/Ola mice, heterozygous for a targeted deletion in the endoglin gene, have a widespread abnormality of the cutaneous vasculature. The postcapillary venules throughout the cutaneous vasculature have irregular layers of smooth muscle cells, elastin, and collagen, which result in vulnerable vessels with areas of extremely thin walls. Furthermore, we have identified inflammation as a potential precipitant of vascular bleeding, which may also be important in the pathobiology of lesions in human HHT patients.

Epistaxis, a classic feature of HHT disease with almost 100% penetrance in human HHT, was seen in only 9% of eng−/− mice. The frequency of animals with nasal bleeding may be reduced because the temperature-controlled and disease-free conditions in which the animals were kept minimize any environmental influences that may trigger bleeding. We found major AVMs in 6% of eng−/− mice, which, although less frequent than the 30% of human HHT1 patients, showed a similar breakdown of normal vascular structure. However, because they occur at such a low frequency, the possibility remains that they are spontaneous hemangiomatous. The low fecundity of the eng−/− mice has not been seen in human HHT patients and may be manifest because of the already low fecundity of the 129/Ola strain.

The eng−/− mice showed an increased frequency of dilated vessels in the skin, and in occasional eng−/− animals, dilatation led to an extreme phenotype, as reported previously.3 The increased numbers of perivascular degranulated mast cells seen in the eng−/− mice may contribute to vasodilation, because they release vasodilatory factors such as histamine, prostaglandins, and nitric oxide.23,24

We were surprised to note that there was a 50% increase in the number of capillaries in the eng−/− mice compared with wild-type animals. This has not been reported as a feature of human HHT; rather, there are reduced capillaries within an AVM.25 However, this was a general feature (not one associated with AVMs) and may be caused by secondary vascular remodeling to reduce capillary blood pressure when there are dilated arterioles.

Normally, the close proximity of endothelial and smooth muscle cells activates TGF-β1, which promotes differentiation and inhibits proliferation of smooth muscle cells.26,27 However, in the eng−/− mice, as in HHT1 patients, endoglin levels may fall below a critical level required to promote TGF-β1 signaling. It is possible that in this situation, TGF-β1 signaling is no longer effective and vascular smooth muscle cells proliferate or dedifferentiate. The irregular smooth muscle wall was mirrored by irregular layers of collagen fibers in the eng−/− mice. Collagens are normally secreted by smooth muscle cells in response to TGF-β1.28 The thin-walled regions of the vessels also have reduced elastin, which is normally synthesized during development by the smooth muscle cells as tropoelastin.29 Because elastin mRNA is stabilized by TGF-β1 signaling,30 this would also be explained by a local region of reduced TGF-β1 signaling in endothelial cells. In addition, the occurrence of the HHT phenotype only in the 129/Ola background is also consistent with low circulating TGF-β1 levels in this strain.17

In the eng−/− mouse, inflammation in the ears and eyelids almost invariably caused bleeding from thin-walled dilated vessels that were unusually close to the epidermis. Angiogenesis associated with inflammation results in vessels that are larger and weaker-walled than normal in the eng−/− mice.31 In addition, this occurs unusually close to the epidermis, where vessels are normally small, so that inflammation enhances susceptibility to trauma and hemorrhage. For many HHT patients, persistent hemorrhage from mucosal surfaces lining the mouth, nose, and gut, where blood vessels are normally close to the surface and there is no protective layer of keratinocytes, is the most clinically significant symptom of the disease.
The vascular abnormalities of the eng\textsuperscript{+/−} mice mirror a number of vascular defects seen in human HHT disease. Irregular vessel walls have been reported in vascular lesions of the lung, lip, and skin in HHT patients\textsuperscript{25,31} and were observed in bleeding nasal telangiectases from 5 HHT patients (obtained through the kind cooperation of Dr C. Mummery and Dr K. Westermann) in whom an inflammatory infiltrate was also observed around the abnormal vessels (not shown). This feature was first observed during development of telangiectases in the skin.\textsuperscript{25}

Our data suggest that in HHT, there is an intrinsic reduction in vessel strength, but a second event is necessary to rupture the vessel. The requirement for a second event may explain incomplete penetrance of the disease in both mice and humans. The variation in disease severity has been ascribed to modifier genes in human patients, and a possible link to circulating TGF-β levels has been proposed.\textsuperscript{3,17} It is clear from this study that all mice heterozygous for the endoglin mutation in the 129/Ola background had abnormal vessels but that not all animals developed hemorrhagic symptoms. The use of mice with an isogenic background excludes variation at other gene loci as the sole explanation. Instead, hemorrhage was triggered by other secondary events, such as physical trauma or inflammation. If inflammatory events also trigger bleeding in human HHT disease, then anti-inflammatory treatments (those that are not antithrombotic) may provide protection against vascular damage and hemorrhage.

The demonstration that haploinsufficiency for endoglin can cause a generalized vascular abnormality in the mouse has relevance to our understanding of the molecular basis of new vessel formation and emphasizes the importance of endoglin in this process.

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8. Mummery and Dr K. Westermann) in whom an inflammatory infiltrate was also observed around the abnormal vessels (not shown). This feature was first observed during development of telangiectases in the skin.\textsuperscript{25}
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