Endoglin Deficiency in Bone Marrow is Sufficient to Cause Cerebrovascular Dysplasia in the Adult Mouse After Vascular Endothelial Growth Factor Stimulation

Eun-Jung Choi, PhD; Espen J. Walker, PhD; Vincent Degos, MD, PhD; Kristine Jun, BS; Robert Kuo, BS; John Pile-Spellman, MD; Hua Su, MD; William L. Young, MD

Background and Purpose—Bone marrow–derived cells (BMDCs) home to vascular endothelial growth factor (VEGF)–induced brain angiogenic foci, and VEGF induces cerebrovascular dysplasia in adult endoglin heterozygous (Eng+/−) mice. We hypothesized that Eng−/− BMDCs cause cerebrovascular dysplasia in the adult mouse after VEGF stimulation.

Methods—BM transplantation was performed using adult wild-type (WT) and Eng−/− mice as donors/recipients. An adeno-associated viral vector expressing VEGF was injected into the basal ganglia 4 weeks after transplantation. Vascular density, dysplasia index (vessels >15 μm/100 vessels), and BMDCs in the angiogenic foci were analyzed.

Results—The dysplasia index of WT/Eng+/− BM mice was higher than WT/WT BM mice (P<0.001) and was similar to Eng−/−/Eng+/− BM mice (P=0.2). Dysplasia in Eng+/− mice was partially rescued by WT BM (P<0.001). WT/WT BM and WT/Eng−/− BM mice had similar numbers of BMDCs in the angiogenic foci (P=0.4), most of which were CD68+ Eng+/− monocytes/macrophages expressed less matrix metalloproteinase-9 and Notch1.

Conclusions—Endoglin-deficient BMDCs are sufficient for VEGF to induce vascular dysplasia in the adult mouse brain. Our data support a previously unrecognized role of BM in the development of cerebrovascular malformations. (Stroke. 2013;44:XXX-XXX.)

Key Words: adult mouse □ arteriovenous malformation □ brain angiogenesis

Mutations in human endoglin (ENG) cause hereditary hemorrhagic telangiectasia 1. Telangiectases and arteriovenous malformations have been viewed as a disorder of the extant endothelium.1 Vascular endothelial growth factor (VEGF)–induced cerebrovascular dysplasia in adult Eng heterozygous (Eng+/−) mice; the majority of bone marrow–derived cells (BMDCs) in the angiogenic foci were monocytes/macrophages (Mø), which contribute to vascular repair and angiogenesis.2 We hypothesized that Eng deficiency in BMDCs causes cerebrovascular abnormalities in mice after VEGF stimulation.

Methods

After institutional approval, the design and groups are listed in Figure I and Figure II in the online-only Data Supplement and methods described in the online-only Data Supplement were used.

Results

Adeno-associated viral vector expressing VEGF induced brain angiogenesis in all groups and caused abnormal cerebrovascular morphology in mice with Eng−/− BM (Figure 1A). Vascular densities (mean±SD) were as follows: 820±153 (wild type [WT]/WT BM), 720±150 (Eng−/−/WT BM), 653±120 (WT/Eng−/− BM), and 674±76 vessels/mm2 (Eng−/−/Eng−/− BM). Mice carrying Eng−/− somatic or BM cells showed a trend toward lower vascular density compared with WT/WT BM mice (P=0.06; Figure 1B). WT/Eng−/− BM mice had >5-fold greater dysplasia index than WT/WT BM mice (1.7±0.3 versus 0.3±0.3; P<0.001; Figure 1C), comparable with the dysplasia index of Eng+/−/Eng+/− BM mice (1.9±0.4; P=0.2). Transplantation of WT BM to Eng−/− mice partially rescued dysplasia (P<0.001; Figure 1C).

Using enhanced green fluorescent protein–expressing donors, we found that WT/WT BM and WT/Eng−/− BM mice had similar BMDC counts in the angiogenic foci (400±125 versus 339±112/mm2; P=0.4; Figure SIIIA and SIIIB in the online-only Data Supplement). The majority of BMDCs was CD68+ (WT/WT BM: 67%±8 versus WT/Eng−/− BM: 64%±10; P=0.6; Figure 2A and 2C; Figure SIIID in the online-only Data Supplement). Approximately 7% of BMDCs in both groups were CD31+ endothelial cells (ECs Figure 2B and 2D).

BM-derived Mø from WT and Eng−/− mice were cultured and treated with 4 doses of VEGF (0, 10, 50, and 100 ng/mL)
for 18 hours. Compared with WT, Eng expression was 50% lower in Eng+/− Mø (Figure SIV in the online-only Data Supplement). The presence of both Vegfr1/Flt1 and Vegfr2/FklKdr indicates that Mø can be stimulated by VEGF (Figure SIVB and SIVC in the online-only Data Supplement).

Discussion

This is the first demonstration that Eng haploinsufficiency in BMDCs was sufficient to cause cerebrovascular dysplasia in the adult mouse after angiogenic stimulation. The abnormal angiogenic response was associated with altered expression of angiogenesis-related genes in Mø. These findings are consistent with previous work in Eng+/− myocardial infarction mice showing that transfection of normal, but not hereditary hemorrhagic telangiectasia 1, human mononuclear cells rescued the defect.4

Transforming growth factor-β, VEGF, and Notch pathways act either synergistically with or antagonistically against each other during angiogenesis in a context-dependent manner.5 Notch signaling in Mø plays a critical role in angiogenesis and repair. Abrogation of monocytic Notch1 adversely affected repair after myocardial injury.6 Conditional deletion of Mø Notch1 caused abnormal anastomosis between angiogenic sprouts.7 Further study is needed to examine whether reduced Notch1 signaling in Eng+/− Mø contributes to a dysplastic phenotype.

VEGF dose-dependent effect on Mø depends on culture conditions. Chemotactic response of human Mø to VEGF peaked at 12 ng/mL and decreased after 40 ng/mL with 2-hour incubation.8 We found that 50 ng/mL of VEGF upregulated metalloproteinase-9 in murine Mø, whereas neither 10 nor 100 ng/mL had any effect. Possible explanations are as follows: (1) human cells respond to VEGF differently from mouse cells; and (2) various VEGF doses differentially trigger various signaling pathways to regulate diverse monocytic functions. Notch1 is induced by VEGF in arterial ECs.5 However, its expression in mouse Mø was not affected by VEGF (10–100 ng/mL) in our study, possibly because only a subpopulation of Mø expresses Notch1 during angiogenesis.7

Growth factors and cytokines produced by BMDCs can affect local angiogenesis via systemic signaling. We showed that the mobilization of metalloproteinase-9–deficient BMDCs into circulation in response to VEGF was reduced, which resulted in less BMDC homing and brain angiogenesis.9 VEGF may affect Mø polarization by effects on Notch signaling.10 Further studies should address the indirect/systemic effects of Eng deficiency on the BMDC function and the effect of VEGF on Mø polarization.
Eng deficiency in endothelial precursors may also play a role and deserves further study. In tumors, very few endothelial precursors are capable of triggering the angiogenic switch. Furthermore, only a small number of homozygously Eng-deleted ECs (≈1%) was sufficient to induce macroscopic cerebrovascular dysplasia after VEGF stimulation.
In summary, ≥1 subpopulations of Eng<sup>−/−</sup> BMDCs are sufficient to induce an abnormal vascular response to brain angiogenic stimulation. Highly relevant to hereditary hemorrhagic telangiectasia 1, it may be possible to envision development of a rescue strategy using BM transplantation therapy. The role of BMDCs in sporadic brain arteriovenous malformations needs further study, because M<sub>0</sub> are associated with lesion<sup>13</sup> and endothelial precursors incorporate into the abnormal vascular structures.<sup>14</sup> Consideration should also be given to the role of ENG in other cerebrovascular diseases, such as stroke.

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We thank Jeffrey Nelson for statistical consultation, Voltaire Gungab for manuscript preparation, and University of California, San Francisco brain arteriovenous malformation project members (http://avm.ucsf.edu/faculty_staff/) for support.

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Disclosures
None.

References
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Supplemental Methods

Bone Marrow Transplantation

BM transplantation was performed as previously described. Briefly, BM cells were collected from the tibia and femurs of 8 to 10-week-old male donor mice by flushing and aspiration with PBS containing 1% fetal bovine serum (FBS). Then, cells were centrifuged at 1,200 rpm for 10 minutes and resuspended in PBS at a concentration of 1×10⁷/ml. Two hundred µl of cell suspension (2×10⁶ cells) were immediately injected into lethally irradiated (9.7 Gy, GC3000 Irradiator, MDS-Nordion) recipient mice via the tail vein. To facilitate detection of BMDCs in the angiogenic foci, WT and Eng−/− mice expressing enhanced green fluorescent protein (EGFP) were used as donors in the BM homing study.

AAV-VEGF Stereotactic Injection

AAV-VEGF viral vector was described previously. CMV promoter was used to drive human VEGF₁₆₅ CDNA expression in the vector. The vector was packaged in an AAV serotype 1 capsid. This viral vector mediates VEGF expression in brain endothelial cells, neurons, and astrocytes. AAV-VEGF was injected into the brain 4 weeks after BM transplantation. After induction of anesthesia by isoflurane inhalation, mice were placed in a stereotactic frame with a holder (David Kopf Instruments), and a burr hole was drilled in the pericranium, 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. Two µl viral suspension containing 2×10⁹ genome copies (gcs) of AAV-VEGF were stereotactically injected into the right basal ganglia, 3 mm under the cortex, at a rate of 0.2 µl per minute using a Hamilton syringe. The needle was withdrawn after 10 min and the wound was closed with a suture.

Vascular Density and Dysplasia Index

Six weeks later, vascular density (the mean vessels obtained from six images) and dysplasia index (number of vessels >15 µm in diameter per 100 vessels) were assessed on two lectin-stained (1:200; Vector Laboratories) coronal sections per animal, 0.5 mm rostral and 0.5 mm caudal to the virus injection site 20 µm in thickness, by three blinded investigators using NIH Image 1.63 software. Images for quantification were taken from three areas (to the right and left of and below the injection site) of each section under the 20X microscopic objective lens.

Immunohistochemistry

Mice were anesthetized with isoflurane inhalation and perfused with 4% paraformaldehyde (PFA). Brain samples were collected, frozen in dry ice, and sectioned at 20 µm in thickness (CM1900 Cryostat, Leica). Two coronal sections per mouse, 0.5 mm rostral and 0.5 mm caudal to the virus injection site, were chosen and stained with primary antibodies against CD68 (1:200; AbD Serotec) and CD31 (1:50, Abcam). Expression was subsequently detected by fluorescent secondary antibodies Alexa Fluor 594 goat anti-rat IgG (1:500, Invitrogen) for CD68 and goat anti-rabbit IgG (1:500, Invitrogen) for CD31. Images for quantification were taken as detailed above.

Monocyte/macrophage Culture
BM was harvested from tibias and femurs of 8-week-old WT and Eng<sup>+/−</sup> mice. BMDCs were grown in the medium containing mouse macrophage-colony stimulating factor (M-CSF; 7.5 ng/ml, Akron Biotech) and 10% FBS for 7 days. The monocyte/macrophage-enriched culture was serum-starved in the macrophage serum-free medium (Invitrogen) for 6 hours. Then, the culture was treated with various concentrations of VEGF (0, 10, 50, and 100 ng/ml, R & D Systems) diluted in the macrophage serum-free medium for 18 hours.

**Quantitative Real-time qRT-PCR**

Total RNAs were isolated from WT and Eng<sup>+/−</sup> monocytes/macrophages using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized by reverse transcription using SuperScript III First-Strand Synthesis System kit (Invitrogen). TaqMan Gene Expression Assays (Applied Biosystems) was used for qPCR to quantify relative expression of each gene using Mx3000P QPCR System (Agilent Technologies). Predesigned qPCR primers are listed in Supplemental Table S1.

**Confocal Microscopy**

Confocal images were taken with a Spectral Confocal Microscope (Nikon) using three laser lines (405, 488, and 561 nm). Z-stacks were rendered into a three-dimensional (3D) image using the NIS-Elements AR 3.0 software (Nikon).

**Statistical Analysis**

Data are shown as mean ± SD. Two-way analysis of variance (ANOVA) was used to determine a statistical significance among groups, followed by pair-wise multiple comparisons using the post-hoc Bonferroni test. Student’s t-test was performed when two groups were compared. A p value of <0.05 was considered statistically significant.
### Supplemental Table S1. TaqMan Gene Expression Assays primers used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Taqman Assay ID</th>
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<tr>
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<tr>
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<td>Notch gene homolog 1 (Drosophila)</td>
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<tr>
<td>Gapdh</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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Supplemental Figure S1. Experimental design and groups for angiogenesis and vascular dysplasia analyses in the adult mouse brain.

(A) Experimental design for Figure 1. (B) Experimental groups for Figure 1.
Supplemental Figure S2. Experimental design and groups for BMDC homing assay.

(A) Experimental design for Figure 2 and Supplemental Figure S3. (B) Experimental groups for Figure 2 and Supplemental Figure S3.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BM Recipient</th>
<th>BM Donor</th>
<th>VEGF</th>
<th>n</th>
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<tbody>
<tr>
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<td>WT</td>
<td>GFP⁺ WT</td>
<td>+</td>
<td>6</td>
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<tr>
<td>WT + Eng BM</td>
<td>WT</td>
<td>GFP⁺ Eng⁺⁻</td>
<td>+</td>
<td>6</td>
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Supplemental Figure S3. WT and Eng^{+/−} BMDCs had equivalent homing ability and most of BMDCs in the angiogenic foci were CD68^{+} monocytes/macrophages. 

(A) Representative images of GFP^{+} WT and Eng^{+/−} BMDCs in the brain angiogenic foci. (B) Bar graph shows quantification of recruited GFP^{+} BMDCs. Data: mean±SD. n=6 per group. (C) A representative confocal image shows co-localization of GFP/CD68/DAPI. Scale bar is 10 µm.
Supplemental Figure S4. $Eng^{+/+}$ monocytes/macrophages expressed a half level of $Eng$ compared to WT and both $Vegfr1/Flt1$ and $Vegfr2/Flk1/Kdr$.

(A) Basal $Eng$ expression. Quantification of (B) $Vegfr1/Flt1$ and (C) $Vegfr2/Flk1/Kdr$ expression. Expression levels are relative to that of WT-untreated cells. Data: mean±SD from three independent experiments (n=3 per group).
Supplemental References


