Age-Dependent Lethality in Novel Transgenic Mouse Models of Central Nervous System Arteriovenous Malformations

Ian Milton; Dan Ouyang, MD; Caitlin J. Allen; Nathan E. Yanasak, PhD; James R. Gossage, MD; Cargill H. Alleyne, Jr, MD; Tsugio Seki, MD, PhD

Background and Purpose—The lack of an appropriate animal model has been a limitation in studying hemorrhage from arteriovenous malformations (AVMs) in the central nervous system.

Methods—Novel mouse central nervous system AVM models were generated by conditionally deleting the activin receptor-like kinase (Alk1; Acvrl1) gene with the SM22-Cre transgene. All mice developed AVMs in their brain and/or spinal cord, and >80% of them showed a paralysis or lethality phenotype due to internal hemorrhages during the first 10 to 15 weeks of life. The mice that survived this early lethal period, however, showed significantly reduced lethality rates even though they carried multiple AVMs.

Results—The age-dependent change in hemorrhage rates allowed us to identify molecular factors uniquely upregulated in the rupture-prone AVM lesions.

Conclusions—Upregulation of angiopoietin 2 and a few inflammatory genes were identified in the hemorrhage-prone lesions, which may be comparable with human pathology. These models will be an exceptional tool to study pathophysiology of AVM hemorrhage. (Stroke. 2012;43:1432-1435.)

Key Words: activin receptor-like kinase 1 ■ arteriovenous malformation ■ hereditary hemorrhagic telangiectasia ■ intracranial hemorrhage ■ stroke

Hemorrhage from arteriovenous malformations (AVMs) in the central nervous system (CNS) may cause severe neurological deficit or death. Limited research in the development of therapeutic medications for AVMs has been due, in a large part, to an absence of an appropriate animal model. In humans, a pathological ALK1 mutation causes hereditary hemorrhagic telangiectasia (HHT), a disease characterized by AVM formation in the CNS and other organs. Although AVM mouse models have been generated by conventional and conditional Alk1 deletions, all models caused severe vascular malformations in diverse organs and inevitable lethal hemorrhages.1-4 Recently, a mouse brain AVM model was developed by a viral vectormediated method using the Alk1 conditional deletion mice; however, this model does not cause hemorrhage or neurological deficit.5

We report novel CNS AVM mouse models exhibiting hemorrhage, paralysis, and partial lethality. All of these mice developed AVMs in their brains, and a majority of them died or were paralyzed due to internal hemorrhages before reaching 10 weeks of age. However, a subset of mice survived much longer despite carrying multiple AVMs. In addition, we identified variegated expression of angiopoietin 2 (Agpt2) and a few inflammation-related genes in the rupture-prone AVM walls.

Methods
Detailed Supplemental Methods are available in the online-only Supplement (http://stroke.ahajournals.org).

Animals and Blue Latex Injection
All mouse procedures carried out were reviewed and approved by the Georgia Health Sciences University Institutional Animal Care and Use Committee. Floxed-Alk1 conditional deletion mice Alk1(flox/flox),3,4 Alk1 deletion mice Alk1(+/-),2 and transgenic Tg(SM22-Cre) deleter mice (Jackson Laboratory, Bar Harbor, Maine)6 were intercrossed to generate SM22Cre-del models. For identification of Cre-recombined cells, R26R Cre-reporter mice (Jackson Laboratory)7 were crossed with the previously mentioned mouse lines. Littermate control mice were used for all experiments. Blue latex solution was systemically injected through the cardiac left ventricle, and tissues were imaged as previously described.3,4

Relative Quantitative Polymerase Chain Reaction
Total RNAs from the most AVM-prone segment of the cerebral hemisphere, roughly right striatum and basal ganglia regions, were isolated from 4 groups of animals (n=6 each): lethal period (LP) Flox-SM22Cre-del mice (4–8 weeks of age), LP littermate control mice, stable period (SP) SM22Cre-del mice (29–36 weeks of age), and SP litterate control mice. TaqMan Gene Expression Assays (Applied Biosystems) were used for the relative quantification of transcripts, and results are shown as fold difference compared with the SP control.

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Histological Analyses

Localizations of brain AVMs were identified by MR angiography, and tissues were collected without any cardiac perfusion. Colorimetric immunohistochemical staining was performed to observe unpredictable AVM histology and localization of specific protein on a single section. The antibodies used in immunohistochemical staining are listed in the Online Supplement. Single and double immunohistochemical staining was performed with commercially available kits with and without hematoxylin counter staining, respectively. X-gal and immunohistochemical double staining on frozen tissue samples was performed as described.\(^2\)

Statistical Analyses

Proportions of genotypes in offspring were tested with the \(\chi^2\) test. The log rank significant test was performed for Kaplan-Meier survival curves. The \(\Delta\Delta C_t\) values from quantitative polymerase chain reaction assays were analyzed with 1-way analysis of variance followed by a post hoc test.

Results

Two closely related CNS AVM mouse models were generated by conditionally deleting the \(\text{Alk1}\) gene using \(\text{Tg(SM22-Cre)}\) deleter mice. In the first \(\text{Alk1}^{\text{flox/flox}};\text{Tg(SM22-Cre)}\) model, both copies of \(\text{Alk1}\) were deleted by Cre recombinase expressed from the \(\text{SM22-Cre}\) transgene (Flox-SM22Cre-del mice hereafter). In the second \(\text{Alk1}^{\text{flox/}\text{flox}};\text{Tg(SM22-Cre)}\) model, 1 of the 2 copies of the \(\text{Alk1}\) gene was constitutively deleted in all cells, mimicking human HHT and the second \(\text{Alk1}\) copy was deleted by the \(\text{SM22-Cre}\) transgene (HHT \(\text{SM22Cre-del}\) model hereafter). Of note, although the \(\text{SM22-Cre}\) transgene was initially reported to induce Cre recombination in smooth muscle cells and cardiac muscles in embryos,\(^6\) a wide-range and inconsistent Cre activation was observed in adults (Supplemental Figures I and II). Furthermore, an involvement of both \(\text{Alk1}\)-null and \(\text{Alk1}\)-intact cells in AVM walls was identified (Supplemental Figure II).

Both SM22Cre-del models caused partial lethality before 2 weeks of age (Supplemental Tables I and II). Intriguingly, the difference in the ratio of surviving pups at 2 weeks of age (Flox-SM22Cre-del pups: 47% versus HHT \(\text{SM22Cre-del}\): 15%, \(\chi^2\): \(P<0.01\)) was the only notable difference between the 2 SM22Cre-del models, and their gross phenotypes after 2 weeks of age were virtually indistinguishable. Therefore, the following analyses were performed mainly using the Flox-SM22Cre-del mice unless otherwise specified.

Survival curves of the 2 SM22Cre-del models after 2 weeks of age were essentially identical (log rank significance: \(P=0.31\)), and a majority of mice suffered from spontaneous death, hindlimb paralysis, or whole body paralysis during the next 8 to 13 weeks (Figure 1A–B). There was no significant difference between female and male survival curves in either model (both \(P>0.3\), data not shown).

End points were hindlimb paralysis, whole body paralysis, and spontaneous death. Note that plots for control genotypes overlap at the top. The numbers of SM22Cre-del mice at risk (number at risk [NR]) are indicated in the parentheses on the X axis. C–D, Intracranial hemorrhage (\#) in Flox-SM22Cre-del (C) and HHT+SM22Cre-del (D) mice with whole body paralysis at 2 to 3 weeks of age are observed through skull (left panels) and absorption of brain tissue is seen after skull removal (right panels). Blue latex injection through cardiac left ventricle highlights some of the vascular structures.

Figure 1. Survival curve and intracranial hemorrhages. A–B, Survival curves of SM22Cre-del models from 2 weeks of age.
CNS hemorrhages were identified in the paralyzed SM22Cre-del mice. Hindlimb-paralyzed mice showed spinal cord hemorrhage in all cases, with some of them showing AVM histology (Supplemental Figure III). Severe intracranial hemorrhage was found in many of the completely paralyzed animals (Figure 1C–D). Because the blue latex solution injected through the cardiac left ventricle cannot enter microcapillaries due to its particle size,4,5 only arterial vessels should have been filled with latex. However, cerebral veins of these animals were filled with blue latex, indicating presence of AVMs. Overall, these findings suggest that Alk1-deletion by the SM22-Cre transgene induced malformed vessels in the CNS, which caused internal hemorrhages and hence the early partial lethality.

Interestingly, the survival of the mice was considerably improved when the Flox-SM22Cre-del and HHT+SM22Cre-del mice surpassed 10 and 15 weeks of age, respectively (Figure 1). The mice that survived this early LP (±10 weeks of age) continued to show partial lethality, however, at much reduced rates during the SP (±16 weeks of age). To elucidate the cause of this change, we considered 2 alternative hypotheses. The first is that a small population of mice does not develop AVMs, and these non-AVM-bearing mice live much longer. The second is that most mice develop AVMs but the AVM walls stabilize once they reach SP age and become protected from lethal hemorrhages. The first hypothesis implies few or no AVMs in SP mice, whereas the second suggests presence of (multiple) AVMs in SP mice.

The cardiac blue latex injection was used to determine whether SP mice carry AVMs. All brain tissues in 15 SP mice (18–102 weeks of age) were found with various numbers, morphologies, and sizes of malformed vessels (Figure 2; Supplemental Figure IV). A number of malformed vessels showed direct connections between arteries and veins (AVMs), but there were also many tortuous vessels (TVs) that did not show an apparent connection to veins. In addition, many of the AVMs/TVs were accompanied by adjacent brown pigments, the hemosiderin deposits formed due to prior hemorrhages. Intriguingly, sometimes relatively large AVMs were free of neighboring hemosiderin (Figure 2B), whereas some of the smallest TVs were accompanied by hemosiderin (Figure 2D), indicating that any malformed vessels could cause hemorrhage. On a side note, we found malformed vessels in intestines of 4 SP animals (data not shown). In contrast, no AVMs were found in 22 littermate control mice except some TVs mostly in the cerebellum region. These findings were confirmed by scoring the severities of AVMs/TVs using a newly developed grading system (Supplemental Table III). The AVM/TV scores clearly showed that all SP SM22Cre-del mice carried a number of AVMs/TVs and most mice experienced hemorrhage (Supplemental Figure V).

Because only the vasculature in LP SM22Cre-del mice was prone to bleed compared with the SP SM22Cre-del and LP/SP control mice, the LP SM22Cre-del brain may have a unique molecular environment that enhances the chances of hemorrhage from their vascular walls. Transcript levels of 31 candidate genes that may contribute to such an environment were examined by quantitative polymerase chain reaction using the brain tissues from these 4 groups regardless of AVM presence (Supplemental Figure VI). Interestingly, Agpt2 was the only gene significantly upregulated in LP SM22Cre-del brains compared with the 3 other groups (all P<0.01). In addition, interleukin-1β and tumor necrosis factor α were significantly upregulated in LP SM22Cre-del brains compared with LP control brains (P<0.05).

Of significance, proteins of some of these factors were induced in the AVM walls in a variegated fashion. Localization of AVMs in LP brain (5–6 weeks of age) were identified by MR angiography, and their histological sections were prepared (Supplemental Figure VII, Supplemental Movie I). The nuclei of AVM wall cells were occasionally stained with cell proliferation markers (Supplemental Figure VIII). Like in other Alk1-deletion models,3–5 smooth muscle cell coverage of AVMs was inconsistent and variegated (Figure 3; Supplemental Figure VII). Similarly, Agpt2 and interleukin-1β expression was found in a variegated pattern in AVM walls. In addition, variegated expression of cyclooxygenase 2 (COX2; PTGS2), a downstream molecule in inflammatory pathway, was also observed. Although smooth muscle cell coverage and the expression of these proteins were both variegated, Agpt2 and COX2 expression were actually often higher in the endothelial
of Agpt2 and inflammatory factors in AVM lesions were consistent with human AVM tissues, confirming the validity of these models.\(^{10,11}\) Although the limited availability of SP mice restricted the extent of this initial analysis, more comprehensive investigation is currently underway.

In addition, these mice will be an excellent model of human hemorrhagic CNS AVM. Because the LP mice are hemorrhage-prone, they can be used as a drug treatment model for hemorrhagic AVM. In addition, because human AVMs are stable and seldom bleed, the stable AVMs in SP mice may best replicate the human CNS AVM condition. We will be able to induce various pathophysiological insults on SP mice that cause vascular inflammation and/or injury, including hypertension, diabetes, and physical trauma, to test if these stimuli induce AVM hemorrhage.

**Acknowledgments**

Alk1\(^{flox/flox}\) and Alk1\(^{-/-}\) mice were provided by Dr S. Paul Oh (University of Florida). We thank Dr Hua Su and Dr William L. Young (both University of California San Francisco) for critical comments on the article. We thank the Georgia Health Sciences University Georgia Research Pathology Services and Histology Core Laboratory for histology sample preparation services.

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**Disclosures**

None.

**References**


**Figure 3.** Immunohistochemical (IHC) staining of lethal period (LP) brains. A, Anti-smooth muscle α actin (SMA) IHC staining visualizes inconsistent AVM wall coverage by SMCs. B–D, Variegated Agpt2 (B), IL-1β (C), and COX2 (D) expression in AVM walls. See a side-by-side comparison against control brains in Supplemental Figure VII. Filled and open arrowheads indicate IHC-stained and IHC-negative AVM wall segments, respectively. Panels are in the same magnification and the scale bar is equal to 50 μm. AVM indicates arteriovenous malformation; Agpt2, angiopoietin 2; IL-1, interleukin-1; COX2, cyclooxygenase 2.
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Supplemental Methods, Figures, Figure Legends, Tables, References and Legends for Video File for:

Age-Dependent Lethality in Novel Transgenic Mouse Models of Central Nervous System Arteriovenous Malformations

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1. Detailed Methods

Animals

All mouse procedures carried out were reviewed and approved by the Georgia Health Sciences University (GHSU) Institutional Animal Care and Use Committee. Floxed-Alk1 conditional deletion mice Alk1(flox/flox) (also designated as Alk1(2f/2f)), 1, 2 lacZ-knock-in Alk1 deletion mice Alk1(+/-), 3 and transgenic Tg(SM22-Cre) deleter mice (Jackson Laboratory, stock # 004746, allele symbol: Tg(Tagln-cre)1Her) 4 were intercrossed to generate SM22Cre-del and littermate control mice in a specific pathogen free animal facility. For identification of Cre-recombined cells, R26R Cre-reporter mice (Jackson Laboratory, stock # 003474, allele symbol: Gt(ROSA)26Sortm1Sor) 5 were crossed with the above mouse lines. The Alk1(+/flox) and Alk1(+/-) mice were developed originally in 129Sv genetic background, however backcrossed to C57BL/6 genetic background for several generations before intercrossed. The Tg(SM22-Cre) were generated in B6SJLF2 background and maintained by sister x brother cross in the Jackson Laboratory. R26R mice were backcrossed to C57BL6/J background for more than 10 generations in the Jackson Laboratory prior to the purchase. Therefore, the SM22Cre-del mice used in the experiments had mixed genetic background of the above genetically modified mouse lines. All experiments were carried out with littermate control mice to regulate genetic background effect. The offspring was genotyped at 2 weeks of age by polymerase chain reaction (PCR) using tail DNAs. Genotyping with a primer set specific for the Alk1 null allele (3F and 6R primers reported by Park et al) 1 showed a presence of Cre-recombined Alk1-null allele in SM22Cre-del tails, confirming a successful recombination of Alk1-flox allele by the SM22-Cre transgene (data not shown).

Paralyzed animals were euthanized promptly since they often showed difficulty in feeding. Animals found dead underwent necropsy, and most necropsy specimens appeared to show signs of significant hemorrhage in multiple internal cavities in partially decomposed bodies (data not shown). However, it is unclear whether it is postmortem artifact or an actual cause of death since comparable hemorrhagic lesions were identified in a few of the similarly decomposing littermate control mouse bodies.

Blue latex injection, whole mount imaging, and AVM grading

Blue latex solution (Connecticut Valley Biological Supply Company) was systemically injected through the left ventricle as previously described. 1, 2 Briefly, 2 mL of the blue latex solution was diluted with 1 mL of 0.9% NaCl or phosphate buffered saline (PBS) solution. A mouse was anesthetized with intra peritoneal (i.p.) injection of tribromoethanol (Avertin), and its chest cavity was exposed by thoracotomy. Following heparin perfusion through cardiac left ventricle, the diluted blue latex was perfused through the left ventricle to complete the vascular casting. The inferior vena cava (IVC) was cut during the heparin perfusion for bleeding.

Subsequent to an examination of arteriovenous malformation (AVM) incidence in abdominal cavity under microscopy, brain and spinal column tissues were collected, fixed with formalin, and dehydrated with methanol series. 1 The tissues were then cleared with 100% methyl salicylate (Sigma-Aldrich) and photographed. Since the latex cannot enter microcapillaries due to its particle size, AVM search could not be performed in some of the major organs, including the lungs and liver.

Relative quantitative PCR

Total RNAs from the most AVM-prone segment of the cerebral hemisphere, roughly right striatum and basal ganglia regions, were isolated using the NucleoSpin RNA II kit (Clontech Laboratories) from four groups of animals (n = 6 each): lethal period (LP) SM22Cre-del mice (4 – 8w of age), LP littermate control mice, stable period (SP) SM22Cre-del mice (29 – 36w of age), and SP littermate control mice. Based on the previous vascular malformation-related studies, 6-18 31 genes associated with angiogenesis, extracellular matrix degradation, inflammation, vascular malformation, and Alk1 signaling pathways were selected for quantification. Relative quantification of transcripts with comparative ∆∆Ct method was performed by following the manufacturer’s recommended protocols using the Applied
Biosystems 7500 Fast Real-Time PCR Systems and the following pre-designed TaqMan Gene Expression Assays (Applied Biosystems): Agpt1, gene symbol: Angpt1, gene name: angiopoietin 1, Assay ID: Mm01129232_m1; Agpt2, Mm00545822_m1; Tie-1, Mm0433287_m1; VEGF-A, Mm00437306_m1; VEGF-B, Mm00660047_m1; HIF-1α, Hif1α, hypoxia inducible factor 1, alpha subunit, Mm00468869_m1; MMP-2, Mm00439505_m1; MMP-9, Mm00441825_m1; TIMP-1, Timp1, tissue inhibitor of metalloproteinase 1, Mm00441818_m1; TIMP-2, Timp2, tissue inhibitor of metalloproteinase 2, Mm00441825_m1; TIMP-3, Timp3, tissue inhibitor of metalloproteinase 3, Mm00441826_m1; TIMP-4, Timp4, tissue inhibitor of metalloproteinase 4, Mm00446568_m1; IL-6, Il6, interleukin 6, Mm00434228_m1; IL-1β, Il1b, interleukin 1 beta, Mm00434228_m1; TNF-α, Tnf, tumor necrosis factor, Mm99999068_m1; COX-2, Ptgs2, prostaglandin-endoperoxide synthase 2, Mm00478374_m1; Krit-1, Krit1, KRIT1 ankyrin repeat containing, Mm00459502_m1; CCM2, Ccm2, cerebral cavernous malformation 2 homolog (human), Mm00524581_m1; Pdcd10, Pdcd10, programmed cell death 10, Mm00468256_m1; Smad4, Smad4, MAD homolog 4 (Drosophila), Mm03023996_m1; Tmem100, Tmem100, transmembrane protein 100, Mm00435245_m1; BMP-9, Gdf2, growth differentiation factor 2, Mm00444619_m1; Dll-4, Dll4, delta-like 4 (Drosophila), Mm00444619_m1; Notch-1, Notch1, Notch gene homolog 1 (Drosophila), Mm00435245_m1; Notch-4, Notch4, Notch gene homolog 4 (Drosophila), Mm00440525_m1.

The RNA sample loading was normalized using the Ct value of 18S RNA assay (Catalog #: 4319413E, Applied Biosystems) and then fold difference compared to the SP control sample was calculated (ΔΔCt method).

Whole mount tissue X-gal staining

The cells underwent Cre recombination were visualized by crossing Tg(SM22-Cre) Cre-deleter line with R26R(+/lacZ) Cre-reporter line, where Cre recombination causes reporter lacZ gene expression. Whole mount X-gal staining was performed as previously described. Briefly, fresh tissue samples were fixed in fixation solution consisting of 1% formaldehyde, 0.2% glutaraldehyde, 2mM MgCl₂, 5mM ethylene glycol tetraacetic acid (EGTA), and 0.02% NP-40 in PBS for 10 min with gentle rocking. After rinsing with PBS twice, the tissue samples were then incubated in X-gal staining solution consisting of 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, and 0.75 mg/mL X-gal, in PBS overnight at 37°C with gentle rocking. On the following day, the samples were rinsed with PBS twice and photographed.

Magnetic resonance angiography

Magnetic resonance (MR) imaging was performed using a Bruker 7T 20cm BioSpec MR spectrometer with a standard transmit/receive volume coil (35mm i.d.) (Bruker Instruments). Mice were anesthetized with isoflurane inhalation, positioned on a cradle to maintain a constant body temperature (37.8°C), and secured using medical tape. Electrocardiogram and respiratory signals were monitored. MR angiography images were collected to locate AVMs using a flow-compensated, axial 2D time-of-flight sequence (TE/TR = 4/17msec; FOV = 19 x 19mm²; Acq Matrix = 384 x 384; 80 slices; 0.2mm slice thickness; 12 averages). Maximum intensity projections (MIP) of the MR angiography image volumes were constructed using the NIH ImageJ program.

Histology, immunohistochemistry, and X-gal staining on frozen sections

The following antibodies were used for immunohistochemical (IHC) staining: anti-smooth muscle α actin (SMA, dilution 1:800, catalog # A2547, Sigma-Aldrich), anti-angiopoietin 2 (Agpt2, 1:300,
Ab65835, Abcam), anti-interleukin-1β (IL-1β, 1:800, NBP1-03300, Novus Biologicals), anti-platelet endothelial cell adhesion molecule (PECAM, 1:200, 550274, BD Pharmingen), anti-cyclooxygenase 2 (Cox2, 1:200, 160126, Cayman Chemical), anti-minichromosome maintenance complex component 7 (MCM7 also known as CDC47, 1:500, MS862P1, Lab Vision), and anti-proliferating cell nuclear antigen (PCNA, 1:6000, Ab29, Abcam).

Tissue samples for histological analyses were collected from Flox-SM22Cre-del and littermate control mice (n = 4 mice each, 5 – 6w of age) without any cardiac perfusion, followed by paraformaldehyde-fixation and paraffin-embedding, to preserve the fragile AVM tissue histology. Colorimetric IHC staining was performed since it enables us to observe unpredictable, irregular AVM histology and localization of specific protein on a single section. IHC staining was performed with VECTASTAIN Elite ABC Kit or Vector M.O.M. Peroxidase Kit along with ImmPACT DAB Peroxidase Substrate (Vector Laboratories), followed by hematoxylin counter staining. Antigen retrieval was performed for the following antibodies using Declere Pretreatment Solution and an electric pressure cooker (Cell Marque Corporation) by following the manufacturer’s recommended protocol: Agpt2, IL-1β, Cox2, MCM7, and PCNA.

For double IHC staining, the sample slides were treated with hydrogen peroxide and Avidin/Biotin Blocking (Vector Laboratories) solutions after the completion of the first IHC to eliminate the cross-reaction with the second IHC. Colorimetric reaction of the second IHC was performed using ImmPACT VIP Substrate. As a negative control, some of the sections were stained without the primary antibody for either the first or second (SMA) IHC. No counter staining was performed on these sections.

X-gal staining on frozen tissue samples was performed as described. Briefly, tissue samples were sequentially treated with paraformaldehyde (PFA) and sucrose solutions, embedded in OCT compound, sectioned, and X-gal stained overnight by following the published standard protocol. X-gal stained sections were then either cover-slipped without counter-staining; counter-stained with nuclear fast red (Vector Laboratories) followed by cover-slipping; or IHC-stained without subsequent counter-staining, as described. In order to detect the slightest X-gal staining, the samples cover-slipped without counter-staining were carefully examined under microscopy for very weak X-gal signals, which generally appear as a tiny blue dot. Any signal detected were then matched and identified on the next serially-sectioned IHC-stained sample to determine if it colocalized with IHC signals.

**Statistical and survival analyses**

Statistical and survival analyses were performed using SigmaPlot 11.0 software (Systat Software Inc.). Proportions of genotypes in offspring were tested with chi-square test. The logrank significant test was performed for Kaplan-Meier survival curves. Delta-delta-Ct values from Taqman quantitative PCR (qPCR) assays were analyzed with one-way analysis of variance (ANOVA) followed by the Holm-Sidak all pairwise multiple comparison test when the distribution of delta-delta-Ct values passed the Shapiro-Wilk normality test. For the genes failed to pass the normality test (Agpt1, VEGF-A, TIMP4, PDGF-B, and Cox2), Kruskal-Wallis one-way ANOVA on ranks test was used followed by the Tukey all pairwise multiple comparison test.
2. Supplemental Tables

**Supplemental Table I:** Number of surviving pups per genotype at 2 weeks of age in Flox-SM22Cre-del model. Mating of Alk1(flox/flox) and Alk1(+/flox);Tg(SM22-Cre) mice was expected to produce the listed four genotypes at an equal ratio. Note that the number of available Flox-SM22Cre-del pups at 2 weeks of age was significantly lower than the average of the control genotypes (47.0% of the average, 44 vs 93.7, chi-square: p<0.01), indicating an early lethal phenotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># of available pups at 2 weeks of age</th>
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<tbody>
<tr>
<td>Alk1(+/flox)</td>
<td>96</td>
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<tr>
<td>Alk1(flox/flox)</td>
<td>88</td>
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<tr>
<td>Alk1(+/flox);Tg(SM22-Cre)</td>
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<tr>
<td>Alk1(flox/flox);Tg(SM22-Cre) (Flox-SM22Cre-del)</td>
<td>44</td>
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</table>

**Supplemental Table II:** Number of surviving pups per genotype in HHT+SM22Cre-deletion model. Mating of Alk1(flox/flox) and Alk1(+/-);Tg(SM22-Cre) mice was expected to produce the listed four genotypes at an equal ratio. Note that HHT+SM22Cre-del pups were available at a significantly lower rate at birth (39.8%, 15 vs 37.7, chi-square: p<0.01) and at 2w of age (15.0%, 25 vs 166.7, p<0.01), indicating an early lethal phenotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># of available pups at newborn</th>
<th>at 2 weeks of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alk1(+/flox)</td>
<td>49</td>
<td>159</td>
</tr>
<tr>
<td>Alk1(flox/-)</td>
<td>37</td>
<td>(average 37.7)</td>
</tr>
<tr>
<td>Alk1(+/flox);Tg(SM22-Cre)</td>
<td>27</td>
<td>158</td>
</tr>
<tr>
<td>Alk1(flox/-);Tg(SM22-Cre) (HHT+SM22Cre-del)</td>
<td>15</td>
<td>25</td>
</tr>
</tbody>
</table>

*HHT: hereditary hemorrhagic telangiectasia

*The survival at 2w of age was significantly lower in HHT+SM22Cre-del mice compared to Flox-SM22Cre-del mice (15% vs 47%, chi-square: p<0.01). This difference may be caused by more frequent and early generation of Alk1-null cells in HHT+SM22Cre-del mice since one of their Alk1 alleles was constitutively deleted. Alternatively, it may be due to the Alk1 haploinsufficiency in the cells surrounding Alk1-null cells, which may have contributed to weakening of AVM walls and exaggerated the rupture rate in early ages.
**Supplemental Table III:** Graded features and scores in mouse AVM grading system. A number of AVMs/TVs, irregularity in diameter, and size of the biggest AVM/TV were independently scored using the blue latex images and added up to have a score from 0 to 6, where score 0 indicates no AVM/TV. Since hemorrhage is a critical indicator of severity, we scored AVMs/TVs with and without adjacent hemosiderin deposition separately (AVM/TV/+H and AVM/TV/-H, respectively). Furthermore, the brain and spinal cord were scored separately, resulting in obtaining the following four scores from each mouse: Brain AVM/TV/+H (+hemosiderin), Brain AVM/TV/-H, Spinal Cord AVM/TV/+H, and Spinal Cord AVM/TV/-H.

<table>
<thead>
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<th>Graded feature</th>
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| **Size:** Size of the biggest AVM/TV  
(Size of mouse brain: 10 – 15 mm in length) |                |
| Small: <0.5 mm        | 0              |
| Medium: 0.5 – 1.5 mm  | 1              |
| Large: > 1.5 mm       | 2              |
| **DI:** Diameter irregularity in any of the AVM/TV |                |
| Negative              | 0              |
| Positive              | 1              |
| **Number of AVM/TV**  |                |
| No lesion             | 0              |
| 1 or 2                | 1              |
| 3 – 5                 | 2              |
| >5                    | 3              |

*Score range: 0 – 6*

*AVM: arteriovenous malformation, TV: tortuous vessels, DI: diameter irregularity*
3. Supplemental Figures and Figure Legends

Supplemental Figure I: Diverse Cre recombination by Tg(SM22-Cre) transgene. The Cre recombination reporter mouse line R26R(+/lacZ) was crossed with Tg(SM22-Cre) reporter mice to reveal the cells undergone Cre recombination. The recombined cells are marked by blue color after X-gal staining. Organs from the compound transgenic mouse R26R(+/lacZ):Tg(SM22-Cre) (left panels) and its littermate control mouse R26R(+/lacZ) (right panels) at 3w of age show that Cre recombination occurred in a subset of arterial (a), venous (v), and intestinal wall smooth muscle cells (SMCs). In addition, many cells in the spleen, thymus (T), heart (H), lungs (L), and aorta (Ao) are recombined. Note that SMCs in large arteries, including aorta, were all recombined by SM22-Cre transgene, however these vessels did not cause AVM in SM22Cre-del models (data not shown). Each set of left and right panels is shown in the same magnification, and the scale bars in right panels are equal to 1mm.
Supplemental Figure II: Presence of both Alk1-intact and Alk1-null cells in AVM walls. a-c) Histological sections of control mouse brain carrying Tg(SM22-Cre) and R26R reporter transgene were
X-gal stained followed by immunohistochemical (IHC) staining with anti-smooth muscle α actin (SMA) antibody. Blue staining indicates the cells which have undergone Cre recombination, and brown staining denotes SMCs. Colors of arrowheads indicate staining pattern of the vessel walls, and color legends for arrowheads are shown in the box at the bottom. d-f) Cre recombined non-vascular cells in brain parenchyma. Double staining of R26R-X-gal and anti-platelet endothelial cell adhesion molecule (PECAM) IHC shows focal accumulations of Cre-recombined cells in brain parenchyma with a low magnification view (d). Most of these cells were not vascular cells since they do not colocalize with endothelial cell (EC)-specific PECAM IHC staining (e and f). g-l) Variegated Cre recombination in AVM walls in SP mice (21 – 51w of age). R26R-X-gal and SMA-IHC double staining reveals variegated distribution of Cre-recombined and non-Cre-recombined cells in AVM walls, and the proportions of these cell populations significantly differed from lesion to lesion. This indicates that the established AVM walls consisted of a mixture of Alk1-null and Alk1-intact cells, and their ratios varied greatly from lesion to lesion. Hemosiderin (H) deposition can be found in some of the lesions, which did not show any particular association with Cre recombination and SMC coverage statuses of adjacent AVM walls (l). m-p) Double staining of R26R-X-gal and anti-PECAM IHC showed EC lining in AVM walls (m and n). In addition, a few Cre recombined ECs are found in venous (o) and AVM (p) walls, although it is exceptionally rare. The scale bars in panels (a – c) and (e – p) are equal to 50μm and that in (d) is 1mm.
Supplemental Figure III: Spinal cord hemorrhage and AVM histology in a HHT+SM22Cre-del mouse at 3w of age. *ad* After removal of back skin, spinal cord hemorrhage is observed in a HHT+SM22Cre-del mouse with hindlimb paralysis as dark red discoloration in the spinal column (a) compared to a littermate control (b). *c&d* Histological sections of spinal cords in (a) and (b), respectively. A section 2–3mm anterior to the discoloration lesion shows multiple irregular shaped vascular openings (*) in (c), a typical AVM histology. A section at the comparable level of the spinal cord in a littermate control mouse shows normal histology (d).
Supplemental Figure IV: Multiple arteriovenous malformation and tortuous vessels (AVMs/TVs) in stable period (SP) mouse brains. Vasculature in SM22Cre-del (a, c, e, and g) and their littermate control (b, d, f, and h) mice was visualized by blue latex injection. The brains are sagittally sectioned at the midline, and images of both halves after clearing are taken from the inside cut surface. Locations of some of the AVMs/TV's are indicated by open arrowheads. A boxed area in (e) indicates the image shown in Figure 2C in the main manuscript. Ages of the mice are indicated in each panel. All panels are in the same magnification, and the scale bar in (h) is equal to 1mm.
Supplemental Figure V: Plots of AVM score in stable period (SP) mice against age. 

a&b) Brain AVM/TV/+H (+hemosiderin) and AVM/TV/-H (no hemosiderin) scores of 15 SM22Cre-del mice (solid circle) and 22 littermate controls (open circle) were determined using the mouse AVM grading system outlined in Supplemental Table III and are plotted against age. Solid and dashed lines indicate trend lines for SM22Cre-del and control mice, respectively. Note that all 15 SM22Cre-del mice had multiple AVMs/TVs in the brain and showed consistently high scores for both AVM/TV/+H and AVM/TV/-H throughout SP and no trends of increase, suggesting little or no increase in severity of AVM during SP. The brain AVM/TV/-H score in 22 control littermates showed positive values due to the cerebellar TVs.

c&d) Spinal cord AVM/TV/+H and AVM/TV/-H scores of the same set of animals were determined using the same grading system and are plotted. Note that spinal AVMs/TVs were found in 10 animals, however, most of these AVMs/TVs were not accompanied by hemosiderin deposition. This may be due to prior removals of paralyzed animals during lethal period (LP) since spinal hemorrhage generally caused hindlimb paralysis.
Supplemental Figure VI: Quantitative PCR (qPCR) analysis of SM22Cre-del mouse brain tissues (n=6 per group). Two statistical analysis results are shown. The first is ANOVA assay using all four groups and the results are shown in black brackets and asterisks (**) for p<0.01, * for p<0.05. The second is Student t-test using the two LP groups and the results are shown in red brackets and asterisks. Note that the Taqman Assay for Alk1 gene amplifies and detects the transcript at exons 10 – 11 region, whereas the Alk1-flox recombination/deletion occurs at exons 4 – 6. Therefore, the Alk1 assay is collectively detecting both recombined and non-recombined transcripts. Hence, the increase in total Alk1 transcript may be caused by compensatory upregulation of Alk1-null transcript evoked by ALK1 protein deficiency. Endoglin (Eng) upregulation may also be induced by the ALK1 protein absence since these two molecules are both involved in HHT pathogenesis in human and belong to the same TGF-β superfamily signaling pathways.
Supplemental Figure VII: Angiopoietin 2 and inflammatory protein expression in lethal period (LP) mouse brains. *a&b* A maximum intensity projection view of MR angiographic images taken from one of
the SM22Cre-del mouse brain (a), and its H&E stained section (b). Yellow arrowheads indicate locations of AVM. See rotating 3D reconstruction of (a) in Supplemental Movie I. c-f) SMCs in AVM walls (c and d), an arteriole in corresponding control brain parenchyma (e), and basilar artery in control brain (f) are visualized by IHC staining against SMA. Since figures in the main manuscript is limited to 4 panels, the image shown in Figure 3A is duplicated in the panel (c) to aid a comparison between AVM lesion and normal brain in a littermate control mouse. Similarly, the images in Figure 3B, 3C, and 3D are duplicated in the panels (g),(k), and (o), respectively. g-j) Variegated Agpt2 expression in AVM walls (g and h) and its absence in control brains (i and j). k-n) IL-1β expression in AVM walls (variegated) and adjacent brain parenchyma (ubiquitous) (k and l) as well as its absence in control brains (m and n). o-r) Variegated Cox2 expression in AVM walls (o and p), its absence in a control arteriole (q), and its EC-dominant expression in basilar arteries in a control brain (r). Filled arrowheads in panels (c-r) indicate IHC-stained AVM/vascular wall segments. Open arrowheads indicate IHC-negative AVM/vascular wall segments. Panels (c-r) are in the same magnification and the scale bar in (r) is equal to 50 μm.
Supplemental Figure VIII: Immunohistochemical (IHC) staining with antibodies against cell proliferation markers. a-d) In order to identify proliferating cells in lethal period (LP) AVM walls, anti-minichromosome maintenance complex component 7 (MCM7 also known as CDC47) antibody was used in IHC staining. Although expression of MCM7 protein in the nuclei of proliferating basal keratinocytes in wild-type mouse is easily detected (d), positive nuclei are only occasionally identified in AVM walls in a variegated fashion (a&b) and rarely in blood vessels in the littermate control brain (c). Filled and open arrowheads indicate IHC-stained and IHC-negative nuclei in AVM walls, respectively. e-h) IHC with antibody against another cell proliferation marker, proliferating cell nuclear antigen (PCNA), showed essentially the same results. Panels are in the same magnification and the scale bar is equal to 50 µm.
Supplemental Figure IX: Endothelial-dominant expression of Agpt2 and Cox2. 

*a and b* Serial sections of AVM walls were immunohistochemically stained with SMA antibody only (a, purple color) and double-stained with Agpt2 (brown) and SMA (purple) antibodies (b). Agpt2 stained cells are not necessarily colocalized with SMCs (marked by SMA). Positive staining in the cells lining the most inner layer of AVM suggests Agpt2 expression in endothelial cells (ECs). 

*c and d* Serial sections of AVM walls stained with Agpt2 alone (c) and double Agpt2+SMA (d) antibodies. Similar to (a and b), Agpt2 expression is dominant in the EC layer. 

*e and f* Serial sections of AVM walls stained with SMA alone (e) and double Cox2+SMA (f) antibodies. Similar to Agpt2, Cox2 is expressed dominantly in ECs, and its expression is not associated with SMC coverage. 

*g and h* Serial sections of AVM walls stained with Cox2 alone (g) and double Cox2+SMA (h) antibodies. Similarly, Cox2 expression seems to be predominant in the EC layer. All panels are shown in the same magnification, and the scale bar is equal to 50μm.
4. Supplemental References


5. Legend for Video File

Supplemental Movie I: Magnetic resonance (MR) 3D angiography of SM22Cre-del mouse head. The movie shows that AVMs are located in both brain hemispheres and both drain to surface veins.
中枢神経系動静脈奇形の新しいトランスジェニックマウスモデルにおける年齢依存性死亡率

Age-Dependent Lethality in Novel Transgenic Mouse Models of Central Nervous System Arteriovenous Malformations

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Abstract

背景および目的：適切な動物モデルの欠如は中枢神経系の動静脈奇形（AVM）による出血の研究の妨げとなっていた。

方法：SM22-Cre 導入遺伝子を用いてアクチビン受容体様キナーゼ（Alk1;Acvrl1）遺伝子を条件的に欠失させた、新しいマウス中枢神経系 AVM モデルを作製した。すべてのマウスで脳および/または脊髄に AVM が生じ、80%を超えるマウスで生後 10～15 週の間に脳内出血による麻痺または致死的表現型が認められた。この早期の致死期間を生き延びたマウスでは、複数の AVM がある場合でも死亡率が有意に低かった。

結果：年齢に依存した出血率の変化から、易破裂性 AVM 病変において独自に増加している分子的因子を同定することが可能であった。

結論：アンジオポエチン 2 およびいくつかの炎症性遺伝子の発現増加が易出血性病変において認められたが、これはヒトの病態と同等と考えられる。これらのモデルは、AVM 出血の病態生理を研究するための特別なモデルとなるであろう。

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図2
安定期（SP）の脳における AVM。A：生後 43 週における脳の内側切断面、正中位での矢状断面には、透明化（洗浄）の前（左側）および後（右側）の両方に、青色のラテックスで満たされた血管とヘモシデリンの沈着（赤色の矢頭）が認められる。洗浄によって、動静脉管構造、AVM、およびすべての深さでのヘモシデリンを観察が可能となる。B：脳内のヘモシデリンが認められない AVM/TV。C～D：脳内のヘモシデリン沈着（赤色の矢頭）を伴う AVM/TV。B～D は同じ倍率であり、スケールバー＝1 mm。AVM：動静脈奇形、TV：蛇行血管。

図3
致死期間（LP）の脳の免疫組織化学（IHC）染色。A：抗平滑筋αアクチシン（SMA）IHC 染色によって、SMC が AVM 壁を不均一に覆っているのが観察化されている。B～D：AVM 壁に発現した Agpt2（B）、IL-1β（C）および COX2（D）。補助の図 VII の対照脳と並べた比較を参照。黒色および白色的矢頭は、それぞれ IHC 陽性および IHC 陰性の AVM 壁断面を示す。パネルは同じ倍率、スケールバー＝50 μm。AVM：動静脈奇形、Agpt2：アンジオポエチン 2、IL-1β：インターロイキン 1α、SMC：平滑筋細胞、COX2：シクロオキシゲナーゼ 2。