ONLINE SUPPLEMENT

Potential influences of gut microbiota on the formation of intracranial aneurysm

Fumiaki Shikata, Kenji Shimada, Hiroki Sato, Taichi Ikedo, Atsushi Kuwabara, Hajime Furukawa, Masaaki Korai, Masakazu Kotoda, Kimihiko Yokosuka, Hiroshi Makino, Emma A. Ziegler, Daisuke Kudo, Michael T. Lawton, Tomoki Hashimoto

Department of Neurosurgery and Neurobiology, Barrow Aneurysm and AVM Research Center, Barrow Neurological Institute, Phoenix, AZ

Short title: Gut microbiota and intracranial aneurysm.

Corresponding author
Tomoki Hashimoto, MD
Department of Neurosurgery and Neurobiology, Barrow Aneurysm and AVM Research Center, Barrow Neurological Institute, 350 W. Thomas Road, Phoenix, AZ, 85013
Email: tomoki.hashimoto@barrowneuro.org
PHONE: 602-406-3487
FAX: 602-406-4172

Materials and Methods

Mouse model of intracranial aneurysms

Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. We used 10- to 12-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine). We combined induced systemic hypertension (deoxycorticosterone acetate-salt hypertension) and a single injection of elastase (35 milli-units; mU) into the cerebrospinal fluid at the right basal cistern as previously described.[1] This dose of elastase was chosen so that we can achieve 80-90% of the incidence of aneurysm formation, based on our previous studies.[1-8] We used the same lot of elastase for injections of all study groups. We performed a series of test injections for every 10 -15 mice to ensure the correct needle placement as previously described.[9] To induce systemic hypertension, we used deoxycorticosterone acetate(DOCA)-salt hypotension as previously described.[3, 10] DOCA-salt hypertension is a classical method for inducing hypertension that has been successfully used in various species.[3, 10] DOCA-salt hypertension requires left nephrectomy followed by implantation of DOCA pellet one week later; 1% sodium chloride drinking water was started on the same day as the DOCA pellet implantation as previously described.[3, 10] Angiotensin II was not used, as it is not part of the DOCA-salt hypertension.

To detect aneurysmal rupture, two blinded observers performed neurological examination daily as previously described.[1] Neurological symptoms were scored as follows: 0: normal function; 1: reduced eating or drinking activity demonstrated by a weight loss >2 g of body weight (≈10% weight loss) >24 hours; 2: flexion of the torso and forelimbs on lifting the whole animal by the tail; 3: circling to 1 side with a normal posture at rest; 4: leaning to 1 side at rest; and 5: no spontaneous activity. Mice were euthanized when they developed neurological symptoms (score, 1-5). Because our previous studies using this model showed that aneurysmal rupture occurs within 3 weeks of aneurysm induction, asymptomatic mice were euthanized 21 days after aneurysm induction as previously described.[2, 4, 5, 7, 11] The brain samples were perfused with phosphate-buffered saline, followed by a gelatin-containing blue dye to visualize cerebral arteries. Aneurysms were defined as a localized outward bulging of the vascular wall, whose diameter was greater than the parent artery diameter.

Two blinded observers assessed aneurysm formation and subarachnoid hemorrhage.

Elastase activity in the CSF

Elastase activity was assayed using SucAla3-pNA (S4760, Sigma) as the substrate. To prepare the triplicated sets of the standard curve, the substrate solution was mixed with the buffer that was made from Trizma base (T1503, Sigma) in ultrapure water. Before the addition of the substrate, the buffer pH was adjusted to 8.0 at 25 degrees with 1 M HCl. Then, the series of elastase standard and the CSF samples from mice were added to the mixed reagents on a 96 well plate. Immediately the plate was read the absorbance at 410 nm using a spectrophotometer for 5 minutes. From the record, we obtained the standard curves and the delta slopes (A_{410}/minute) respectively. The delta slopes plotted on the vertical axis, and the units of elastase plotted on the horizontal axis. As a result, we obtained the delta-delta slope (slope/unit). Finally, the elastase activity in the CSF was calculated using the standard curve.

Detection of bacterial DNA in cerebral arteries in this model.

We assessed the potential bacterial transmigration into the cerebral arteries by examining the presence of bacterial DNA in our model. We utilized the PCR analysis of bacterial 16s rRNA as previously described.[12-14] We tested 3 ruptured aneurysms, 3 unruptured aneurysms, and 3 cerebral arteries from mice that underwent aneurysm induction. We also tested the same samples for mammalian DNA.

Results

3-weeks treatment with antibiotics did not affect the elastase activity in the CSF.

In our previous study, we have assessed the dynamics of elastase activity in the CSF.[1] When 35 mU of elastase was injected, the elastase activity was under the detection level at 30 minutes and 6 hours. When 350 mU was used, we were able to detect the elastase activity at 30 minutes. However, 6 hours after elastase injection, the elastase activity was not detectable, showing the extremely short half-life of elastase in the CSF.[1]

We have repeated a similar study using a much more sensitive method to detect elastase activity. The elastase activity was still under the detection level at 1 hour and 3 hours after the injection of 35 mU of elastase, consistent with our previous study[1]. As a next step, we measured the elastase activity after an injection of elastase at 175 mU, a dose 5 times higher than what was used to induce aneurysms. We were able to detect the elastase activity at 1 hour. However, the elastase activity was not detectable at 3 hours even with this high dose. These findings, again, confirmed the extremely short half-life of elastase. Elastase in this model appears to be important for the initiation of aneurysm formation, but it probably does not play a significant role in the development of aneurysmal rupture that occurs at much later time points.

Then, we measured the elastase activity in the CSF after three weeks of treatment with antibiotics or vehicle. 10 mice received the antibiotics treatment, and another 10 mice received the vehicle treatment (Supplemental Table S1). After 3-weeks of the treatment, mice received 175 mU of elastase.

There was no difference in the elastase activity at 1 hour after elastase injection between antibiotics-treated group and vehicle-treated group (0.0081 U/mL ± 0.0105 vs. 0.0084 U/ml ± 0.0088; P = 0.84, Mann-Whitney test. n = 6 for each group). Then, in the remaining 4 mice that received antibiotics and 4 mice that received the vehicle for three weeks, we measured the elastase activity in the CSF 3 hours after elastase injection. Elastase activity in the CSF was under the detection levels in both groups at 3 hours (n = 4 for each group). Additionally, we measured the elastase activity in the CSF after 24 hours after the injection of 175 mU of elastase. We were not able to detect the elastase activity either group.

Therefore, it is unlikely that the antibiotics treatment affected the formation of aneurysms by changing the elastase activity in the CSF.

Detection of bacterial DNA in our model

As shown in the supplemental figure S1, bacterial DNA was not detectable in ruptured aneurysms, unruptured aneurysms, or cerebral arteries from our model, while mammalian-specific DNA was readily detected in these tissues.

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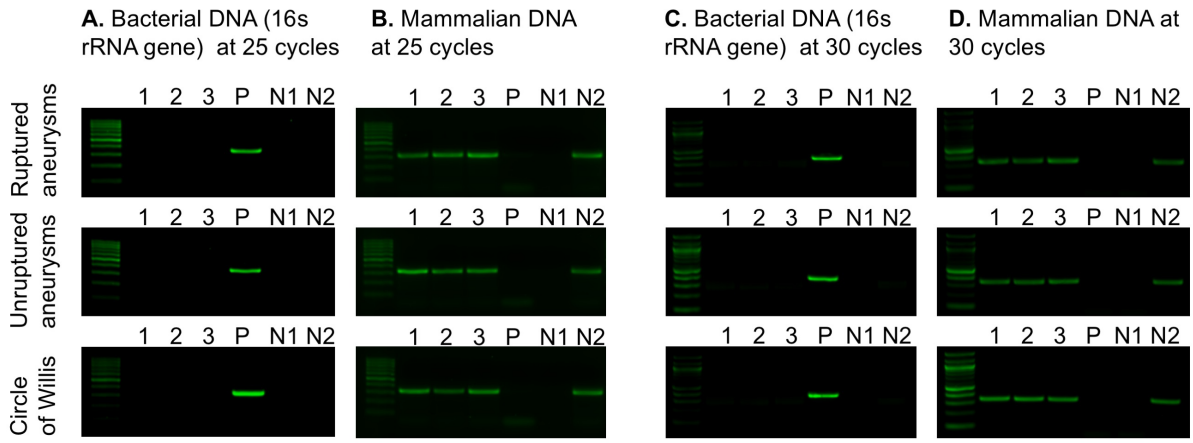
Supplemental Table S1

Time	Vehicle	Antibiotics	p value
1 hour after injection	0.0084 ± 0.0088	0.0081 ± 0.0105	0.84
3 hours after injection	0	0	N/A

Supplemental Table S1. Elastase activity (unit / ml) in the cerebrospinal fluid (CSF) after 3 weeks of the treatment with antibiotics and vehicle. Elastase activity was measured at 1 and 3 hours after elastase injection.

Supplemental Figure S1.



Supplemental Figure S1. A-D. Representative amplifications of 16S rRNA gene and mammalian DNA.

A. At 25 cycles, bacterial DNA (16S rRNA gene) was not detected in ruptured aneurysms, unruptured aneurysms, or Circle of Willis. B. At 25 cycles, mammalian DNA was detected in ruptured aneurysms, unruptured aneurysms, and Circle of Willis. C. At 30 cycles, bacterial DNA (16S rRNA gene) was not detected in ruptured aneurysms, unruptured aneurysms, and Circle of Willis. D. At 30 cycles, mammalian DNA was detected in ruptured aneurysms, unruptured aneurysms, and Circle of Willis. 1-3: Sample tissues P: Cultured bacteria for positive control. N1: DNA without primers (negative control). N2: Brain tissues (negative control). rRNA: ribosomal RNA.