SUPPLEMENTAL MATERIAL

Roles of Nicotine in the Development of Intracranial Aneurysm Rupture

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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 9-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 (17.5mU) into the cerebrospinal fluid at the right basal cistern as previously described.[1] 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] 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. Using the tail cuff method, systolic blood pressure was measured in the mice before the treatment and two and three weeks after the elastase injection as we previously described.{Kanematsu, 2011 #855}{Makino, 2012 #991}

Mice

We obtained α7nAChRflox/flox ($\alpha7^{f/f}$) mice[5], α7nAChR knockout (α7nAChR KO) mice[6], mice expressing Cre recombinase under the control of the myeloid–specific lysosome M (LysM) promoter (LysMCre[7]), the transgelin (smooth muscle protein 22-alpha) promoter (SM22Cre[8]), and the endothelial-specific receptor tyrosine kinase (Tie2) promoter (Tie2Cre[9]) from Jackson Laboratory (Bar Harbor, Maine). We used LysMCre, SM22Cre and Tie2Cre negative $\alpha7^{f/f}$ mice as control and LysMCre, SM22Cre, and Tie2Cre positive $\alpha7^{f/f}$ mice as myeloid-, smooth muscle cell-, and endothelial cell-specific α7nAChR knockout mice.

Real-time PCR analysis

For the real-time polymerase chain reaction (RT-PCR) analysis, the total RNA was collected at 6 days after aneurysm induction from the mice that were dedicated to the mRNA analysis. We assessed mRNA expression of vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor 2, transforming growth factor-beta (TGF-β), hepatocyte growth factor, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, matrix metalloproteinase-9 (MMP-9), and nuclear factor Kappa B. The total RNA was extracted using the RNeasy Mini Kit (Qiagen, CA). The total RNAs were

transcribed to cDNA using QuantiTect reverse transcription kit (Qiagen). The mRNA expression levels were determined using SYBR Green technology (Applied Biosystems, CA). Quantitative values were obtained from the threshold cycle value (CT), and the data were analyzed by the $2^{-\Delta\Delta CT}$ method.[2, 4, 10] The transcript amount of glyceraldehyde-3-phosphate dehydrogenase was quantified as an internal RNA control.

Immunohistochemistry

For analysis of macrophage infiltration, we collected the brain tissue samples from mice treated with nicotine at 6 days after aneurysm induction (n=6 from each group). These mice were dedicated for the immunohistochemical analyses. The brain tissues were frozen in optimal cutting temperature compound and were sectioned near the middle cerebral artery. Immunohistochemistry with antibodies for macrophage (CD68; Bio Rad, Hercules, CA) was performed to the brain tissue sections.

Drug treatment

Nicotine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline (0.9% sodium chloride), and administrated subcutaneously thorough Alzet osmotic minipumps (model 2004 (28 days), Durect Corporation, Cupertino, CA). The concentration of nicotine was adjusted for animal weight and the mini pump flow rate, resulting in 5 mg/kg/day.[11, 12] α7nAChR agonist (AR-R17779, Abcam, Cambridge, MA) and α7nAChR antagonist (Methyllycaconitine, Abcam,) were dissolved in 5%DMSO, and saline (0.9% sodium chloride), respectively. α7nAChR agonist (5mg/kg) and α7nAChR antagonist (5mg/kg) were injected intraperitoneally at a total volume of 1ml/100g body weight once a day.[13-15] Vehicle control groups received 0.9% sodium chloride and/or 5% DMSO. All drugs or vehicle were administered from one week before elastase injection, and the treatments were continued for 4 weeks.

Statistical Analysis

We used the Fisher's exact test (GraphPad Prism 6) to analyze the incidence of aneurysms (number of mice with any aneurysms [ruptured or unruptured]/total number of mice) and rupture rate (number of mice with ruptured aneurysms/number of mice with any aneurysms). Mice that did not show aneurysm formation were excluded from the calculation of the rupture rate. As an exploratory analysis, the survival analysis was performed using the log-rank test. Levels of mRNA and blood pressure were analyzed by two-way ANOVA, followed by Turkey-Kramer post hoc test. Statistical significance was accepted at $P < 0.05$. Quantitative results were expressed as mean ± SD.

Results

There was no difference in systolic blood pressure among all comparison groups(Supplemental Table I). As an exploratory analysis, we analyzed the location and size of aneurysms (Supplemental Table II). There was no significant difference in the location or size between all comparison groups.

Supplementary Table I. Systolic blood pressure

Blood pressure	1 week before aneurysm induction	1 week after aneurysm induction	2 weeks after aneurysm induction	3 weeks after aneurysm induction
VC	107.2 ± 10.7	132.9 ± 10.8*	138.5 ± 8.8*	137.2 ± 8.5*
Nicotine	107.5 ± 11.6 (NS)	134.0 ± 13.7* (NS)	135.0 ± 14.2* (NS)	137.1 ± 11.7* (NS)
VC	102.5 ± 11.5	126.3 ± 17.9*	146 ± 11.4*	135.8 ± 9.8*
α7nAChR agonist	106.6 ± 10.5 (NS)	121.5 ± 17.2* (NS)	138.5 ± 11.1* (NS)	139.4 ± 10.9* (NS)
Nicotine	101.5 ± 7.9	135.7 ± 9.1*	135.5 ± 8.3*	134.8 ± 5.6*
Nicotine + α7nAChR antagonist	99.1 ± 7.6 (NS)	131.0 ± 6.7* (NS)	136.2 ± 9.2* (NS)	132.4 ± 9.4* (NS)
α7nAChR antagonist	101.0 ± 10.7 (NS)	135.7 ± 12.0* (NS)	139.9 ± 10.1* (NS)	137.2 ± 10.8* (NS)
Wild-type + VC	104.8 ± 11.6	132.7 ± 14.7*	146.5 ± 11.4*	135.6 ± 8.5*
Wild-type + Nicotine	102.0 ± 8.2 (NS)	135.0 ± 5.9* (NS)	133.3 ± 5.2* (NS)	132.0 ± 4.3* (NS)
α7nAChR KO + VC	98.3 ± 7.4 (NS)	133.4 ± 8.1* (NS)	132.4 ± 5.7* (NS)	129.3 ± 5.4* (NS)
α7nAChR KO + Nicotine	110.5 ± 10.2 (NS)	139.5 ± 7.0* (NS)	138.2 ± 6.0* (NS)	134.9 ± 4.3* (NS)
$\alpha7^{f/f}$ + Nicotine	95.0 ± 10.7	129.0 ± 8.1*	131.0 ± 6.3*	125.7 ± 5.7*
$\alpha7^{f/f}$SM22Cre+ + Nicotine	99.8 ± 13.1 (NS)	129.4 ± 9.0* (NS)	129.4 ± 7.6* (NS)	127.6 ± 6.9* (NS)
$\alpha7^{f/f}$ + Nicotine	99.1 ± 9.4	127.4 ± 8.9*	129.2 ± 8.1*	129.2 ± 7.2*
$\alpha7^{f/f}$LysMCre+ + Nicotine	99.7 ± 10.1 (NS)	128.4 ± 10.6* (NS)	127.1 ± 9.0* (NS)	125 ± 6.5* (NS)
$\alpha7^{f/f}$ + Nicotine	99.7 ± 7.9	130.6 ± 8.2*	130.5 ± 10.0*	130.6 ± 11.0*
$\alpha7^{f/f}$Tie2Cre+ + Nicotine	98.6 ± 7.6 (NS)	132.2 ± 6.9* (NS)	132.0 ± 6.7* (NS)	132.2 ± 5.4* (NS)

*: $P < 0.05$ compared to the blood pressure at 1 week before aneurysm induction.
NS: no significant difference compared to the control group

Supplemental Table II. Aneurysm location and size

Location	VC	Nicotine	Nicotine + VC	Nicotine + MLA	VC	α7-agonist	Wild-type VC	Wild-type Nicotine	α7 KO VC	α7 KO Nicotine
Internal carotid artery	3	4	0	0	2	2	0.	2	3	2
Middle cerebral artery	3	3	5	5	4	5	4	1	2	3
Anterior cerebral artery	3	6	3	4	1	2	2	4	2	0
Posterior cerebral artery	4	3	3	2	2	3	3	3	1	3
Posterior circulation[#]	2	3	0	2	2	0	2	3	3	3
P-value (Chi-square test)	0.90		0.53		0.62		0.80			

Location	α7f/f + Nicotine	α7f/f SM22Cre + Nicotine	α7f/f + Nicotine	α7f/f LysMCre + Nicotine	α7f/f + Nicotine	α7f/f Tie2Cre + Nicotine
Internal carotid artery	3	2	0	3	4	3
Middle cerebral artery	3	1	2	4	2	2
Anterior cerebral artery	4	2	4	4	1	2
Posterior cerebral artery	2	3	5	2	3	1
Posterior circulation[1]	0	2	4	4	1	3
P-value (Chi-square test)	0.41		0.30		0.64	

Size of aneurysm (um)	VC	Nicotine	Nicotine + VC	Nicotine + MLA	VC	α7-agonist	Wild-type VC	Wild-type Nicotine	α7 KO VC	α7 KO Nicotine
< 200	3	2	2	3	3	5	1	1	1	1
200 - 300	3	7	1	2	3	3	4	6	3	2
300 - 400	3	6	3	1	2	3	4	4	2	1
400 - 500	3	3	3	3	1	1	2	2	2	0
500 - 600	2	0	0	2	1	0	3	0	1	4
600 - 700	0	1	1	1	0	1	0	0	2	2
700 - 800	1	0	1	0	0	0	1	0	0	0
≥ 800	0	0	0	1	0	0	0	0	0	1
Mean ± SD	343 ± 171	324 ± 132	383 ± 196	426 ± 287	282 ± 131	280 ± 167	388 ± 143	304 ± 94	349 ± 172	483 ± 191
P value (t-test or ANOVA)	0.95		0.92		0.77		0.01			

Size of aneurysm (um)	α7f/f + Nicotine	α7f/f SM22Cre + Nicotine	α7f/f + Nicotine	α7f/f LysMCre + Nicotine	α7f/f + Nicotine	α7f/f Tie2Cre + Nicotine
< 200	3	2	3	5	4	4
200 - 300	3	3	5	4	3	5
300 - 400	1	2	5	5	2	0
400 - 500	2	1	0	1	1	1
500 - 600	2	1	1	1	1	1
600 - 700	1	0	0	0	0	0
700 - 800	0	1	1	0	0	0
≥ 800	0	0	0	1	0	0
Mean ± SD	315 ± 149	334 ± 202	241 ± 108	312 ± 171	273 ± 189	303 ± 156
P value (t-test or ANOVA)	0.98		0.20		0.52	

#: Posterior circulation includes vertebral, basilar, superior cerebellar, and posterior communicating arteries.

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