Allergic lung inflammation aggravates angiotensin II-induced abdominal aortic aneurysms in mice

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MATERIALS AND METHODS

Mouse AAA and allergic lung inflammation production

Eight- to ten-week-old male *Apoe*$^{-/-}$ mice (C57BL/6) from The Jackson Laboratory (Bar Harbor, ME, USA) were used to produce AAA and ALI sequentially, or together. We infused 1000 ng kg^{-1} min^{-1} Ang-II (Sigma, St Louis, MO, USA) subcutaneously with an Alzet model 2004 osmotic minipump (DURECT Corp., Cupertino, CA, USA) to produce AAA according to established protocol.[1] Post-operative analgesia (buprenorphine, 0.05 mg/kg/12 h, intraperitoneal) was administered every 12 h for 48 h. Mice were weighed and systolic and diastolic blood pressures were measured using the CODA non-invasive blood pressure system (Kent Scientific Co., Torrington, CT) before and after AAA and/or airway allergic inflammation production. It took 28 days to produce AAA in these mice. On day 28 after a minipump implantation, mice were used for ALI production or sacrificed with carbon dioxide narcosis, followed by cardiac puncture blood collection. We did not perform baseline or repetitive measurement of mouse abdominal aortas for these mice. The maximal suprarenal aortic diameter of each aneurysm was measured from post-mortem mice after the peri-adventitial tissue was carefully removed from the aortic wall and when mice were remained under a physiological condition, followed by aortic tissue harvest. Aortic diameters of mice were measured using a surgical microscope (Zeiss Stemi SV11) equipped with a micrometer eyepiece (14 mm/0.1, SG02.T0218c, Motic Instruments, Inc, Vancouver, British Columbia, Canada), which allowed for the reading of aortic diameters at any time during the surgical procedure or during tissue harvesting. Development of AAA was defined by the expansion of the abdominal aorta by 150% of the aortic diameter from normal mice.[2] This definition determined the percentage of incidence per group. Aorta segments for immunohistochemistry were cut at the maximal suprarenal outer aortic diameter and embedded vertically with an optimal cutting temperature compound, and at least 10-15 serial frozen sections covering the maximal dilated aorta were prepared for immunohistochemical analysis. For those with similarly enlarged AAA diameter throughout the thoracic-abdominal aortas, we selected the segment at approximately the same distance from the renal artery as those of others with maximal AAA expansions. In cases with AAA lesions at multiple locations, we selected the largest lesion as close as possible to the same distance from the renal artery as those of others with maximal AAA expansions. Aortic cross-sections were used for AAA lesion area measurements and AAA lesion characterizations.

To produce ALI, we immunized intraperitoneally mice with 50 μg OVA in 1 mg $Al(OH)_3$ in 200 μl PBS on days 0, 7, and 14 in *Apoe*$^{-/-}$ mice (C57BL/6). On day 21, 22, and 23, mice were nebulized with 300 mg OVA in 5 mL saline.[3,4] Blood and bronchioalveolar lavage fluid (BALF) were collected on day 24, or mice were used subsequently to produce AAA. Mice in the control group received intraperitoneal mock sensitization with saline-$Al(OH)_3$ emulsion and were challenged with an aerosol of saline without OVA. Each experimental group contained 8 to 14 *Apoe*$^{-/-}$ mice at the same age. Mice were grouped randomly after purchase.

We also generated experimental AAA in 10-week-old C57BL/6 wild-type mice by peri-aortic application of 0.5 M $CaCl_2$, as described previously.[5,6] On the second day after $CaCl_2$ injury, mice (n=14) were immunized intraperitoneally with 50 μg OVA in 1mg $Al(OH)_3$ in 200 μl

PBS on days 2, 9, and 16 to induce ALI. On days 23, 24, and 25, mice were nebulized with 300 mg OVA in 5 mL saline. Mice in the control group (n=13) received intraperitoneal mock sensitization with saline-$Al(OH)_3$ emulsion and were challenged with an aerosol of saline without OVA. Mice were harvested 30 days after the surgery. We measured the blood pressure and aortic diameters before aneurysm induction and at sacrifice, 4 weeks at the harvest. All animal procedures conformed to the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Harvard Medical School Standing Committee on Animals (protocol # 03759).

This study included six experiments with different combinations of ALI and AAA models to test the interactions between ALI and AAAs in *Apoe*$^{-/-}$ mice: (1) To produce ALI and AAA at the same time; (2) mice with concurrent ALI and AAA received anti-IgE antibody treatment; and (3) to produce ALI first followed by producing AAA. These three protocols tested whether development of ALI affected AAA formation and whether ALI promotes AAA formation via increased IgE production. (4) To produce AAA first, and then induce ALI; and (5) to produce ALI only, as a control. These two protocols tested whether consequent ALI exacerbated pre-established AAA and whether pre-existing AAA affected airway allergic response development; and (6) finally to produce ALI in peri-aortic $CaCl_2$ injury-induced AAA.

Anti-IgE antibody treatment

Ten-week-old male *Apoe*$^{-/-}$ mice (C57BL/6) were used to produce AAA and ALI at the same time. Half of these mice received intraperitoneal injection of rat anti-mouse IgE monoclonal antibody (n=11) in a dose previously validated in mice (330 μg in 200 μl of saline per 25 g body weight, BD Pharmingen)[7-9] 1 day before the surgery. Vehicle (matched rat IgG1 isotype, n=10, BD Pharmingen) was injected into the other half of the mice as a negative control. Mice received a second dose of the same antibody or vehicle (IgG1 isotype) 14 days after surgery. Mice were harvested 28 days after initial Ang-II infusion.

Mouse aortic tissue immunohistochemical analysis and lung tissue histology analysis

Serial cryostat cross-sections (6 μm) were used for immunostaining for macrophages (Mac-2, BD Biosciences, San Jose, CA, USA, 1:900), T cells (CD4, 1:90; and CD8, 1:100, BD Biosciences), mast cells (CD117, eBioscience, San Diego, CA, USA, 1:30, or toluidine blue), major histocompatibility complex class II (MHC-II, BD Biosciences, 1:250), elastin (Modified Verhoeff Van Gieson Elastic Stain Kit, Sigma-Aldrich), collagen (0.1% Sirius Red; Polysciences Inc., Warrington, PA), SMC (α-actin, Sigma-Aldrich, 1:750), Ki67 (cell proliferation marker, Vector Laboratories, Inc., Burlingame, CA, 1:500), and CD31 (angiogenesis marker, BD Biosciences, 1:1500). Apoptotic cells in lesions were determined with the *in situ* apoptosis detection kit according to the manufacturer's instructions (Millipore, Billerica, MA). Collagen content, elastin fragmentation and media SMC accumulation were graded according to the grading keys.[5,10] CD31-positive microvessels, Ki67-positive cells, T cells, mast cells, and apoptotic cells were counted blindly and quantified as numbers per aortic section. The relative macrophage and MHC-II contents within the aortas were quantified by measuring the immunostaining signal-positive area using computer-assisted image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD, USA). Investigators were blinded to the sources of samples, the assay, and quantification. Mouse lungs were fixed in formalin overnight for paraffin section preparation. Lung tissue sections (5 μm) were prepared for hematoxylin and eosin (H&E) staining.

Plasma and bronchioalveolar lavage fluid (BALF) analysis

The plasma and BALF were collected from mice. BALF cellular typing and numbers were determined by cytospin preparation, followed by counting blindly macrophages, lymphocytes, eosinophils, and neutrophils. Plasma and BALF supernatants were stored at -

80°C until analysis was performed. Plasma and BALF IgE (BD Biosciences), MCP-1 (PeproTech, Rocky Hill, NJ, USA), eotaxin (PeproTech), IL4 (eBioscience), IL5 (eBioscience), IL13 (PeproTech), TGF-β (eBioscience), IFN-γ (eBioscience), and TNF-α (PeproTech) were determined using ELISA kits according to the manufacturers' instructions. Plasma total cholesterol, triglyceride, and high-density lipoprotein (HDL) levels were determined using reagents from Pointe Scientific (Canton, MI) and low-density lipoprotein (LDL) levels were calculated as follows: LDL = total cholesterol - HDL - (triglycerides/5). Investigators were blinded to the sources of samples during the assay.

Statistical analysis

This study used the non-parametric Mann–Whitney *U* test for unpaired data sets to examine the statistical significance for all data from experimental AAAs. Fisher's exact test compared the differences in AAA incidence and mortality rate. $P < 0.05$ was considered statistically significant.

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