5-Lipoxygenase Pathway In Experimental Abdominal Aortic Aneurysms

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METHODS

Elastase perfusion model

The elastase perfusion model of abdominal aortic aneurysm formation in mice was utilized as previously described [1-3]. Congenic age, weight and gender matched male C57BL/6J (Stock #000664, Jackson Laboratories, Bar Harbor, Maine) (WT) or 5-LO–/– mice (Stock #004155, Jackson Laboratories, Bar Harbor, Maine) were used for experiments (N=16/group). 5-LO–/– mice have no externally visible abnormal phenotype. Mice underwent 75/1mg/kg intraperitoneal ketamine/medetomidine anesthesia. Aorta were perfused with either saline or elastase (Lot #078K7018, Sigma Aldrich Inc., St. Louis, Missouri) for 5 and a-half minutes [4]. Immediate mechanically induced aortic wall dilatation of 15-35% after perfusion with saline or elastase (0.47U/mL porcine pancreatic elastase) is followed by either no aneurysmal (perfused with saline) or aneurysmal (perfused with elastase) dilatation over 14 days. Mice were housed individually throughout experiments, and maintained at 70°F, 50% humidity, in 12-hour light-dark cycles per institutional animal protocols. All mice were fed ad libitum water and placed on diet with no restrictions on movement. All mice had unlimited daily access to the chow (control or 5-LO admixed) ad libitum in their cage, and had no restrictions on movement. Video micrometry measurements of the maximum diameter of the aortic wall distal to the renal arteries were performed *in situ* before perfusion, following perfusion, and at the time of harvest using a Q-Color3 Optical Camera (Olympus Corp., Center Valley, Pennsylvania) attached to an operating microscope (Leica Microsystems, Bannockburn, Illinois) using QCapture Pro Software version 6.0 (QImaging Inc., Surrey, Canada). Infrarenal aortic diameter that exceeded the baseline Infrarenal aortic measurement by 50% were considered aneurysmal across all experiments. Change in aortic dilatation from baseline (%) was calculated by subtracting the infrarenal control diameter from the infrarenal maximal diameter divided by the infrarenal control diameter as determined by video micrometry. At the time of harvest following antegrade perfusion, the entire infrarenal aorta was explanted. The aortas (or aneurysms, when present) were either: 1) snap frozen in liquid nitrogen for analyses by polymerase chain reactions or protein extraction, or 2) processed for histology or immunohistochemistry.

Angiotensin II infusion model

Osmotic pumps (Alzet® 2004, Durect Corp., Cupertino, California) containing either Ang II (1000 ng/kg/min, Sigma Aldrich Inc., St. Louis, Missouri) or saline were introduced in 10-week-old LDLr–/– male mice (Stock #002207, Jackson Laboratories, Bar Harbor, Maine) as previously described [5, 6] (N=8/group). Mice were housed individually throughout experiments, and maintained at 70°F, 50% humidity, in 12-hour light-dark cycles per institutional animal protocols. All mice were fed ad libitum water and placed on high fat diet (TD 88137, Harlan Teklad Inc., Indianapolis, Indiana) with no restrictions on movement. All mice had unlimited daily access to the chow (control or 5-

LO admixed) ad libitum in their cage, and had no restrictions on movement. Aneurysmal segments of the aortas (proximal to the renal arteries) were harvested after 28 days just like in the elastase perfusion model and processed for histology. Cardiovascular profile (heart rate, mean arterial pressure, systolic and diastolic blood pressure) was determined over 28 days. Conscious mice underwent measurements using Coda 8 (Kent Scientific Corporation, Torrington, Connecticut) tail-cuff system as described previously [7]. Acclimatization cycles (N=10) were discarded, and average of the ensuing cycles (N=20) are reported. Measurements were obtained for 7 days prior to exposure to the 5-LO inhibitor, and then subsequently on day 0, 1, 3, 5, 7, 10, 14, 21 and 28 of Ang II infusion. At day 28, video micrometry measurements of the aortic wall diameter (proximal to the renal arteries) were performed *in situ* using a Q-Color3 Optical Camera (Olympus Corp., Center Valley, Pennsylvania) using QCapture Pro Software version 6.0 (QImaging Inc., Surrey, Canada). LDLr–/– mice (N=6) were harvested prior to experiments to establish baseline aortic diameters and based on these findings the 0.5mm baseline aortic diameter size was determined. Video micrometry measurements were compared to a baseline aortic diameter of 0.5mm, which was established as the arbitrary baseline aortic diameter from age, gender and weight matched LDLr–/– mice that were harvested separately.

Pre-injury Pharmacological studies

All mice were single housed throughout experiments and exposed to 5-LO inhibitor admixed into chow for 5-7 days prior to injury (i.e.: elastase perfusion or Ang II infusion). AZD4407 (AstraZeneca PLC, London, UK), a unique potent orally available non-redox inhibitor of 5-LO that has greater than 800-fold selectivity over other lipoxygenases and cyclooxygenases was utilized for experiments [8]. Control chow and 5-LO inhibitor admixture chow at 3 mg/kg, 10 mg/kg and 30 mg/kg for experiments were formulated either at AstraZeneca R&D (Mölndal, Sweden) or through Research Diets Inc. (New Brunswick, New Jersey) based on a baseline 25g mouse weight. All mice had unlimited daily access to the chow (control or 5-LO admixed) ad libitum in their cage, and had no restrictions on movement. Pre-exposure time points were selected based on *in vivo* 5-LO inhibitor PK levels, and LTB_4 production determined in preliminary experiments (data not shown). Mice were weighed routinely throughout elastase (Day -5, 0, 7, and 14) and Ang II experiments (Day -7, 0, 1, 3, 5, 7, 10, 14, 21 and 28).

Post-injury Pharmacological studies

All mice were single housed throughout experiments and exposed to 5-LO inhibitor admixed into chow for 10 days after injury (i.e.: elastase perfusion). All mice had unlimited daily access to the chow (control or 5-LO admixed) ad libitum in their cage, and had no restrictions on movement. The post-injury time point was selected based on prior unpublished observations from our lab, which confirmed the presence of a small aneurysm at day 7 following elastase perfusion. Based on the pre-exposure

studies, we anticipate *in vivo* drug steady state to be reached by day 7 in mice exposed to the inhibitor at day 3 after elastase perfusion. Control chow and 5-LO inhibitor admixture chow at 3 mg/kg, 10 mg/kg and 30 mg/kg for experiments were formulated either at AstraZeneca R&D (Mölndal, Sweden) or through Research Diets Inc. (New Brunswick, New Jersey) based on a baseline 25g mouse weight.

LTB_4 assay

Whole blood obtained by cardiac puncture from mice (500 µl) using heparinized syringes and collection tubes containing 1 mg EDTA diluted in RPMI was incubated at 37°C for 30 minutes. Samples were subsequently incubated at 37°C for 20 minutes with DMSO (control) or 25µM calcium ionophore (A23187, Sigma Aldrich Inc., St. Louis, Missouri). The reaction was stopped using 10 µM AZD4407 in PBS. Microtiter plates were centrifuged at 2000 rpm, 4°C for 10 minutes. Supernatants were stored at -80°C until further processing. LTB_4 enzyme immunoassay (EIA), plate reading by iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories Inc., Hercules, California), and calculations to quantify absorbance were completed per EIA manufacturer recommendations (Cayman Chemical Co., Ann Arbor, Michigan).

Plasma 5-LO levels

Whole blood obtained by cardiac puncture from mice using heparinized syringes and collection tubes containing 1 mg EDTA was centrifuged at 2500 rpm for 10 minutes, 4°C. Plasma samples were stored at -20°C for further processing, and when ready were transferred to a pre-labeled plate for analysis by liquid chromatography mass spectrometry/ mass spectrometry (LC-MS/ MS) according to manufacturer's protocol (AstraZeneca R&D, Mölndal, Sweden).

Plasma blood and urine metabolite profile

Whole blood obtained by cardiac puncture from mice using heparinized syringes and collection tubes containing 1 mg EDTA was either immediately assayed for blood chemistry profiles or centrifuged at 2500 rpm for 10 minutes, 4°C. Complete blood count with differential in whole blood, and plasma cholesterol, high-density lipoprotein, and triglyceride levels were determined though the University of Virginia medical laboratories. Urine samples collected at day 14 following elastase perfusion studies were examined for urine LTE_4 by EIA per manufacturer's protocol (Cayman Chemical Co., Ann Arbor, Michigan).

Adoptive transfer bone marrow transplantation

Adoptive transfer bone marrow transplantation experiments were performed as previously described [1, 2, 9]. Briefly 6-week-old recipient mice were lethally irradiated and rescued through retro-orbital injection of 6 million bone marrow cells, isolated from

femurs of 6-week-old donor mice [10]. Mice were placed on antibiotic prophylaxis for 3 weeks in their drinking water (2 weeks trimethoprim, 1 week flouroquinolone) following adoptive transfer, and then subsequently switched to regular drinking water. Non-rescued control mice died between days 7 and 10 following irradiation. Chimeric mice underwent blood DNA analysis, obtained from saphenous vein aspiration 4 weeks following adoptive transfer. LTB_4 production in blood samples was quantified at 10-weeks following adoptive transfer for additional verification of successful bone marrow transplantation. Chimeric mice then underwent elastase perfusion as described following a two-week recovery period (N=12/group).

Gelatin zymography

Snap-frozen murine AAA samples were analyzed by gelatin zymography. Protein was extracted after harvest using Lysing Matrix D (MP Biomedicals, Solon, Ohio) and 1M Tris (hydroxymethyl) aminomethane buffer at pH 7.5, 5M Sodium Chloride, 1M Calcium Chloride and Triton X-100 homogenized at 4°C, while concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, Illinois). Electrophoresis was completed using 0.1% gelatin in a 10% sodium dodecyl sulfate polyacrylamide gel using equivalent volume of each fraction. Enzymatic activity was visualized as negative staining with Coomassie Brilliant Blue R-250 (Thermo Scientific, Rockford, Illinois). Relative densitometry analysis of lytic bands, adjusted for background, indicative of MMP activity was performed using Gel Doc™ XR+ System and Image Lab™ software (Bio-Rad Laboratories Inc., Hercules, California).

Histology, immunohistochemistry and confocal microscopy

Murine aortas were harvested at sacrifice for histological analysis after undergoing left ventricular puncture and 4% paraformaldehyde (PFA) followed by phosphate buffered saline (PBS) antegrade perfusion at physiologic pressure. Further fixation was achieved by overnight incubation in 4% PFA at 4°C followed by paraffin embedding and sectioning at 5μm. Human and murine paraffin embedded samples underwent microwave antigen retrieval, antibodies were bound and detected using VectaStain Elite Kit (Vector Laboratories Inc., Burlingame, California). Murine samples were embedded in the same orientation based on the identifying ligature from the aortotomy sight. The specimens were sectioned at 5μm through the entire length of the aorta and mounted (approximately 120 slides per aorta harvested). Screening stains with Hematoxylin and Eosin (H&E) and Modified Russell-Movat Pentachrome (Movat) for elastin were completed. These slides were used to determined the area of interest for further staining. Then Hematoxylin and Eosin (H&E), along with Modified Russell-Movat Pentachrome (Movat) for elastin layers, luna and toluidine blue staining for mast cells, Verhoeff-Van Gieson staining for elastin layers, and immunohistochemical (IHC) staining was completed. Antibodies for IHC staining were anti-rat Mac2 for macrophages (Cedarlane Laboratories, Burlington, Canada), anti-mouse anti-Neutrophil (Ly 6B.2) for PMNLs (AbD Serotec, Oxford, United Kingdom), anti-mouse CD3ε (M-20)

for T-lymphocytes (Santa Cruz Biotechnology Inc., Santa Cruz, California), anti-mouse cleaved caspase-3 (Asp175) for apoptosis (Cell Signaling Technology, Inc., Danvers, Massachusetts), anti-mouse SMαA (14A) (Santa Cruz Biotechnology Inc., Santa Cruz, California), anti-rat 5-LOX (Cayman Chemical Co., Ann Arbor, Michigan), anti-human BLTr1 (Cayman Chemical Co., Ann Arbor, Michigan), and anti-human CysLT1 (Cayman Chemical Co., Ann Arbor, Michigan). Visualization color development was completed using diaminobenzidine (Dako Corporation, Carpinteria, California). Positive controls were included as per antibody manufacturer recommendations where paraffin embedded mouse organ sections specific to augment the antibody were stained (e.g. lymph node or spleen). A negative control included the same conditions, without the antibody being used. Conditions for both sets of controls (i.e. positive and negative) were first calibrated and then re-run with the slides of interest from our experiments. This process was repeated for each lot/batch # of antibody used per stain during every staining session. Images were acquired using AxioCam Software version 4.6 via 4X, 10X, and 40X objectives and an AxioCam MRc camera (Carl Zeiss Inc., Thornwood, New York).

Paraffin sections were examined on a Zeiss LSM 510 confocal microscope equipped with purple diode laser for 405 nm excitation (blue); an argon laser with 458, 477, 514, and 488 nm lines (blue-green); a 543 nm HeNe laser (red); and a 633 nm HeNe laser (far-red) (Carl Zeiss Inc., Thornwood, New York). IHC antibodies were optimized with appropriate fluorescently labeled detection antibodies (Alexa fluorophores, Life Technologies Inc., Grand Island, New York) and excited by lasers for acquisition through a 40X objective. ZEN lite 2011 (Carl Zeiss Inc., Thornwood, New York) software was used to analyze, merge images, and optically zoom to 300X.

Real-Time PCR

RNA from snap frozen mouse aortic samples was isolated with TRIzol® (Invitrogen Corp., Carlsbad, California) after homogenization according to the manufacturer's instructions. Total RNA quantification was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, Delaware). Subsequently 0.5µg complimentary deoxyribonucleic acid (cDNA) was synthesized from RNA using RNase H+ iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, California). Then cDNA was amplified by qPCR on a MyiQ™ Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California). The 5-LO mutant (S: 5'-ATC GCC TTC TTG ACG AGT TC-'3), common (S: 5'-GCA GGA AGT GGC TAC TGT GGA-'3) and wildtype (S: 5'-TGC AAC CCA GTA CTC ATC AAG-'3) sequences were obtained from Jackson Laboratories, Bar Harbor, Maine. 5-LO pathway Taqman® primer and probe sets (Applied Biosystems Inc., Foster City, California) were used to study amplicons in the aneurysm tissue samples, and compared to the amplification of standards. Relative quantification with normalization to 18S using iQ5 optical system software, version 2.1 (Bio-Rad Laboratories, Hercules, California) was completed. Amplification by CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad

Laboratories, Hercules, California) was completed. Polymerase chain reactions amplified DNA transcripts of 5-LO and WT sequences in whole blood isolated from chimeric mice per manufacturer's recommendations (Qiagen Inc., Valencia, California). Amplicons were resolved on a 1-2% agarose gel and quantified by Gel Doc™ XR+ System and Image Lab™ software (Bio-Rad Laboratories Inc., Hercules, California). We achieved near complete abrogation (99%) of the WT band in WT recipients of 5-LO–/– donor bone marrow cells. All fold changes were calculated by the method of ΔΔCt and are expressed as mean±sem compared with controls (as indicated).

Immunohistochemistry quantification

A circumferential area of interest (AOI) from IHC images was drawn to include the media and adventitial layers of the aorta. Threshold gated positive signal was detected within the AOI and quantified using Image-Pro Plus version 7.0 (Media Cybernetics Inc., Bethesda, Maryland).

Cytokine measurements

Plasma samples collected and stored at -80°C underwent quantification by mouse cytokine multiplex immunoassay (Bio-Rad Laboratories, Hercules, California) according to manufacturer's protocol.

Human Samples

Male patients between the age of 55 and 75 years who underwent open elective aneurysmectomy for infrarenal AAA were identified, and tissue was taken from the anterior or lateral wall (N=9/group). De-identified (per IRB protocol) control abdominal aorta samples were obtained from organ donors between the age of 15 and 35 without clinical or macroscopic signs of aortic atherosclerosis at The University of Virginia, Charlottesville, Virginia. There are inherent limitations to interpreting results from comparisons between these two groups.

Study approval

Animal care and use were in accordance with the *Guide for the Care and Use of Laboratory Mice* [11]. The animal protocol was approved by the University of Virginia Institutional Animal Care and Use Committee (#3634) in compliance with the Office of Laboratory Animal Welfare [12]. Approvals for human sample investigations, including patient consent waiver, was obtained by the Human Investigation Committee at The University of Virginia, Charlottesville, Virginia (HSR #13178).

Statistics

Statistical analysis was performed using GraphPad Prism 5.0f for Mac OSX software (GraphPad Software, La Jolla, CA). Aortic dilation between groups was compared by single factor analysis of variance. When multiple groups were compared post hoc Tukey corrections were made to reduce false discovery rate. Histology and serology groups were compared using nonparametric 2-tailed *Mann-Whitney U* tests or by χ^2 as appropriate (as no parametric distribution of the data was assumed). Survival was computed using Kaplan Meier plots, and compared by log rank tests. Comparisons were made against controls and considered statistically significant when *P* value was equal to or less than 0.05. Numbers of mice per group used in experiments are shown in each figure, or within the methods section. Data are presented as mean±sem.

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