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Development of a novel murine model of aortic aneurysms using peri-adventitial elastase

Castigliano M. Bhamidipati, DO, PhD, MSc^a, Gaurav S. Mehta, MBBS^a, Guanyi Lu, MD, PhD^b, Christopher W. Moehle, MS^a, Carlos Barbary, BS^a, Paul D. DiMusto, MD^c, Adriana Laser, MD^c, Irving L. Kron, MD^a, Gilbert R. Upchurch Jr, MD^b, and Gorav Ailawadi, MD^a

^aDivisions of Thoracic and Cardiovascular Surgery, and Vascular and Endovascular Surgery, University of Virginia School of Medicine, Charlottesville, VA

^bDepartment of Surgery, University of Virginia School of Medicine, Charlottesville, VA

^cDepartment of Surgery, University of Michigan School of Medicine, Ann Arbor, MI

Abstract

Background—Our aim was to establish a novel model of abdominal aortic aneurysms (AAA) in mice using application of peri-adventitial elastase.

Methods—C57BL/6J male mice underwent infrarenal peri-adventitial application of either (1) sodium chloride (control; $n = 7$), (2) porcine pancreatic elastase (PPE; $n = 14$), or (3) PPE and doxycycline (PPE + doxycycline 200 mg/kg; $n = 11$) for 14 days. Aortas were analyzed by video micrometry, immunohistochemistry, qualitative polymerase chain reaction, and zymography. Groups underwent Mann–Whitney U comparisons.

Results—At day 14 compared with baseline, control animals had minimal aortic dilation, whereas fusiform aneurysms were seen in PPE (control, $20 \pm 3\%$; PPE, $82 \pm 15\%$; $P = .003$). Doxycycline abrogated aneurysm formation (PPE, $82 \pm 15\%$; PPE + doxycycline, $37 \pm 10\%$; $P = .03$). Compared with control and PPE + doxycycline, immunohistochemistry demonstrated greater elastin fiber degradation, macrophage infiltration, and matrix metalloproteinase-9 expression in PPE. Ki-67 and cleaved caspase-3 were lower in control versus PPE. The loss of smooth muscle marker expression seen with PPE was preserved in PPE + doxycycline. Zymography confirmed that both MMP-2 and -9 were more active in PPE than PPE + doxycycline.

Conclusion—Peri-adventitial application of elastase is a simple, reproducible in vivo model of aneurysm formation leading to consistent infrarenal aortic aneurysm development by day 14, with inflammatory cell infiltration and MMP upregulation. Doxycycline inhibits AAA progression in this model via limiting matrix degradation and preserving differentiated smooth muscle cells.

Abdominal aortic aneurysms (AAA) contribute to >15,000 operations a year in the United States, and is the thirteenth leading cause of death among men.¹ The matrix of the abdominal aorta consists of collagen and elastin that degrades, and are thought to contribute to aneurysm formation via several mechanisms.^{2–4} The destruction of the constitutive layers of the aortic wall by inflammatory and mesenchymal cell infiltration, which leads to the loss of smooth muscle cell function, are characteristic hallmarks of aneurysm formation.

Several small animal models of aortic aneurysm exist to help investigators study these mechanisms of disease.⁵ The ideal model to study aneurysms should allow a thorough evaluation of medial layer degeneration, degradation by chronic inflammatory components, and mural thrombus contribution to aneurysm formation. This type of unique in vivo animal model that replicates human aneurysms has myriad redundant pathways, and makes focused study challenging. As such, investigators have utilized combinations of isolated surgical and/or toxic manipulation in vivo injury models and in vitro assays to understand the role of various inflammatory and smooth muscle cells in aneurysms. A landmark contribution by Anidjar et al⁶ first described the elastase perfusion model in rats in 1990 and documented similar chronic inflammatory infiltrate as seen in humans. This model requires in vivo isolation of the abdominal aorta, cannulation of the aorta to administer elastase, and careful repair of the aortotomy. Several modifications of the original rat model have allowed for adoption and use in transgenic and knockout mice.^{7,8}

The elastase perfusion model has allowed much of our understanding of the mechanisms leading to AAA. This perfusion model, however, has limitations in that it is technically demanding with a steep learning curve. Specifically, isolation of the mouse aorta can be difficult with the adjacent inferior vena cava and small lumbar branch vessels. Moreover, there can be variability in vessel injury owing to variations in pressure during perfusion with elastase. Additionally, careful aortotomy closure while avoiding stenosis to reestablish antegrade blood flow is crucial. These maneuvers can be difficult to teach and may have limited its use to select laboratories. Thus, a simpler model that facilitates aneurysm formation reproducibility that is less challenging technically would provide a unique opportunity to augment aneurysm research. Herein, we describe a novel, simple, and efficient peri-adventitial elastase application in vivo murine aortic aneurysm model, and evaluate the protective effect of doxycycline, a known potent matrix metalloproteinase (MMP) inhibitor.⁹

METHODS

Murine peri-adventitial elastase model

This model was initially conceived and validated by Dr. Guanyi Lu while at the University of Michigan. Briefly, 8- to 12-week-old male C57BL/6J mice (WT, Jackson Laboratories, Bar Harbor, ME) that weighed between 20 and 28 g were assigned randomly to groups. Singly housed animals were exposed to a 12-hour day–night cycle in 50% humidity and 70°F temperature controlled rooms, and fed standard chow ad libitum (Harlan Laboratories, Indianapolis, IN).

We developed the model of peri-adventitial elastase application for experimental AAA formation (Fig 1), where animals first underwent intra-peritoneal 75 mg/kg ketamine and 1 mg/kg medetomidine anesthesia. A laparotomy was performed, and the abdominal aorta from just below the left renal vein to the iliac bifurcation was identified. The abdominal aorta was isolated in situ after retroperitoneal reflection. Circumferential exposure of the infrarenal abdominal aorta was achieved after careful dissection, and de novo branches of the aorta were left in situ. After anatomic identification, the aorta was bathed in either 10 μ L of 0.9% NaCl (control) or 100% porcine pancreatic elastase (PPE; Sigma-Aldrich Co., St. Louis, MO) for 10 minutes. After elastase exposure, the abdominal contents were replaced, the fascial layers were closed with interrupted 6-0 coated undyed polyglactin 910 (Ethicon Inc., Somerville, NJ), and skin with interrupted 6-0 polypropylene suture (Ethicon Inc.), after which mice received 0.1 mg/kg subcutaneous buprenorphine, intraperitoneal 1 mg/kg atipamezole, and were recovered after 1 mL subcutaneous 0.9% NaCl administration.

Video micrometry measurements of the aortic wall diameter were performed in situ before application, after application, and at the time of harvest using a Q-Color3 Optical Camera (Olympus Corp., Center Valley, PA) attached to a Leica MZ12 stereomicroscope (Leica Microsystems, Bannockburn, IL) using QCapture 6.0 Pro Software (QImaging Inc., Surrey, Canada). The entire infrarenal aorta was removed at the time of harvest. Aortas were divided and analyzed by histology or immunohistochemistry, and gelatin zymography. Animal care and use were in accordance with the *Guide for the Care and Use of Laboratory Animals*.¹⁰ Animal protocols were approved by the University of Virginia Institutional Animal Care and Use Committee (#3634). Elastase–time associations were anticipated to be reproducible with targeted aortic dilation between 30% and 40% when 10 μ L of elastase was applied to the abdominal aorta for 10 minutes (Fig 1, B).

Doxycycline treatment

Animals treated by doxycycline (PPE + doxycycline) were given 200 mg/kg oral doxycycline (Sigma Aldrich, Inc.) dissolved in de-ionized water administered through photosensitive bottles, and changed every 48 hours as described.¹¹ Residual volumes were logged to record homogenous consumption across the group; no animals were excluded from analysis based on doxycycline intake.

Histology and immunohistochemistry

Murine aortas were harvested at killing for histologic analysis after left ventricular injection of 4% paraformaldehyde solution in phosphate-buffered saline. Further fixation was achieved by overnight immersion in 4% paraformaldehyde at 4°C, and after paraffin embedding, blocks were sectioned at 5 μ m. Every 10th section underwent screening hematoxylin and eosin staining to identify the region of interest (AAA) among individual animals. Once the aneurysm segment was identified, slide sections underwent serial immunohistochemistry staining.

After microwave antigen retrieval, antibodies were bound and detected using VectaStain Elite Kit (Vector Laboratories Inc., Burlingame, CA). Modified Russell-Movat Pentachrome (Movat) for elastin layers, and immunohistochemical staining were completed. Antibodies for immunohistochemical staining were anti-Mac2 for macrophages (Cedarlane Laboratories, Burlington, Canada), anti-MMP-9 for MMP-9 (R&D Systems, Minneapolis, MN), anti-SM α A (14A) for smooth muscle α -actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-MHC-SM1 for smooth muscle myosin heavy chain (Kamiya Biomedical Company, Seattle, WA), anti-Ki-67 (M-19) for cell proliferation (Santa Cruz Biotechnology Inc.), and anti-cleaved caspase-3 (Asp175) for apoptosis (Cell Signaling Technology, Danvers, MA). Enzymatic color development was completed using diaminobenzidine (Dako Corporation, Carpinteria, CA). Appropriate controls verified staining procedures, and images were acquired using AxioCam 4.6 Software with a 10 $\times$ objective on an AxioCam MRc (Carl Zeiss Inc., Thornwood, NY). Threshold color gated image quantification of the positive signal within an area of interest was calculated in immunostained sections using Image Pro Plus (Media Cybernetics Inc., Bethesda, MD).¹² Relative proportions of positive signals were compared between groups.

Gelatin zymography

Snap-frozen murine aortic aneurysm samples were analyzed by gelatin zymography. Protein was extracted after harvest using 1 mol/L tris (hydroxymethyl) aminomethane buffer pH 7.5, and incubated for 30 minutes at 37°C, and concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL). Electrophoresis was completed using 0.8% gelatin in a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) using equivalent volume of each fraction. Enzymatic activity was visualized as negative staining with

Coomassie Brilliant Blue R-250 (Thermo Scientific). Relative densitometry analysis adjusted for background, of lytic bands indicating MMP activity was performed using Gel DocXR+ System with a charge-coupled device camera and Image Lab software (BioRad Laboratories, Inc., Hercules, CA).

Statistical analysis

Pair-wise comparisons were examined after undergoing outlier evaluation as defined by Grubbs.¹³ Data underwent unpaired nonparametric analysis, with 2-tail probabilities at an alpha of 0.05 considered statistically significant. All calculations were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Fusiform aneurysmal formation with elastase application to adventitia of aorta

At the beginning of the operative procedure, the aorta was exposed after retroperitoneal separation. With peri-adventitial elastase application, there was a notable and visible change within the initial 3–5 minutes in the aortic wall with increasing exposure to elastase (Fig 2, *A*). During this period, the aorta was monitored for phenotypic changes, where adventitial digestion was noted. The vessel sheath that envelopes the inferior vena cava and the distal aorta separated after elastase exposure with minimal dissection. Additionally, the aorta dilated by nearly 30–40% after elastase application, which was then followed subsequently by aneurysmal dilation over 14 days. Specifically, at the end of 10 minutes (10.4 ± 0.5), once 10 μL ($9.4 \pm 0.6 \mu\text{L}$) of 100% elastase had bathed the abdominal aorta, early fusiform dilation ($33 \pm 2\%$ dilation over baseline) was evident (Fig 2, *B*). This degree of initial injury across the elastase groups ensured adequate exposure, and was comparable to the elastase perfusion model from our laboratory.⁴

Peri-adventitial elastase creates reproducible aneurysms

At day 14 compared with baseline, control animals exposed to saline had a $20 \pm 3\%$ increase in aortic diameter, whereas PPE animals exposed to elastase had an $82 \pm 15\%$ increase in aortic size with 60% incidence (Fig 2, *B*). Additionally, compared with PPE mice, animals exposed to PPE + doxycycline for 14 days developed less aortic dilation ($37 \pm 10\%$; $P = .03$).

Peri-adventitial elastase application has greater aortic wall degradation, which is limited by doxy-cycline treatment

Modified Russell-Movat Pentachrome stain in control animals showed preserved elastin fibers, which were degraded after PPE exposure (Fig 3, *A*). Importantly, degeneration of elastin fibers was prevented with doxycycline treatment. The anterior surface of the aorta seems to have greater elastic degradation than the posterior aspect of the aorta.

Immunostaining for activated macrophages with Mac2 and total MMP in control animals had lesser expression of inflammatory markers than PPE (Fig 3, *B* and *C*). Importantly, doxycycline treatment attenuated macrophage infiltration and total MMP expression (Fig 3, *D*). Similarly, staining for smooth muscle marker proteins SM-22 α , SM α A, and MHC-SM1 showed greater anterior smooth muscle loss with elastase application (Fig 4, *A–C*).

Ki-67 protein to assess the active phase(s) of the cell cycle were higher in the PPE group compared with PPE + doxycycline (Fig 4, *D*). Caspase-3 is an inactive zymogen that, after apoptotic signaling, is cleaved by an initiator caspase, and this cleaved caspase-3 expression that denotes apoptosis was increased from baseline only in PPE (Fig 4, *E*). Overall,

doxycycline treated mice had luminal smooth muscle cell preservation similar to control animals (Fig 4, *F*).

Gelatin zymography confirms higher active-MMP-2 and –MMP-9 activity with elastase application

SDS-PAGE gel electrophoresis showed little MMP activity in control animals, whereas more intense MMP activity in PPE exposed mice was noted, and was attenuated after doxycycline treatment (Fig 5, *A*). Relative densitometry of zymograms showed that compared with PPE, both pro-MMP-9 ($P = .049$) and active MMP-9 ($P = .04$) were decreased after doxycycline treatment (Fig 5, *B*). There were no differences in pro-MMP-2 expression among groups (Fig 5, *B*). Active-MMP-2 was greater after PPE application and decreased with doxycycline treatment (Fig 5, *B*).

DISCUSSION

This study describes a novel experimental model of aneurysm formation and evaluates the protective effect of doxycycline as seen in other *in vivo* models of AAAs.^{9,14–19} Our model achieved aneurysm formation by degradation of the elastic lamina, increased presence of activated macrophages, reduced smooth muscle protein expression, and increased active MMP activity as seen in other experimental models of aneurysm formation. Moreover, the marginally increased and expected apoptosis on the anterior surface of the aorta confirms that the current model does not overexpose the aorta to toxic levels of pancreatic elastase with destruction of the adventitial surface and mechanical aortic dilation. Additionally, cell proliferation was higher in the PPE-exposed, group as seen in small human aortic aneurysms, which supports the idea that inflammatory cells, fibroblasts, and smooth muscle cells are active around the adventitial wall and likely help to remodel the aorta. The fusiform aneurysms formed by this peri-adventitial application model are more consistent with those seen in human AAAs.

Matrix degradation enzymes are an important contributor to the study of aortic aneurysms, and in the current model, elastase exposure led to increased active MMP activity. Historical models of aneurysm research including the “Blotchy mouse model,” acetrizoate-induced destruction of the medial layer of the aorta, and disruption of smooth muscle cells during development by theophylline or β -aminopropionitrile have been supplanted with more sophisticated murine *in vivo* models. Moreover, *de novo* neutrophil elastase may be involved in the pathogenesis of aneurysms in humans, given the loss of elastic tissue and increased elastolytic activity in the media of human aneurysms. These changes suggest that the pathobiology of aortic aneurysms involve elastolysis of the aortic media and that elastase plays a major role in the destruction of elastin within the aortic wall. The pressure component of the perfusion model is important when pancreatic elastase is administered, because pressure perfusion with saline alone has been shown not to influence morphometry.^{2,4} Furthermore, the use of elastase experimentally, more so than papain, collagenase, trypsin, and chymotrypsin, is critical to macrophage activation and subsequent medial degeneration.^{6,20}

Technically, the challenges in the well-established elastase perfusion model of excluding every aortic branch in the infrarenal aorta by ligation, cannulating a 0.4-mm murine aorta, and subsequently closing the aortotomy without creating stenosis has limited the potential for widespread adoption to select institutions. Although we have extensive experience with the elastase perfusion model in our laboratory,^{2,4} we believe that there was a real need for a less complex and reproducible model of aortic aneurysm formation. For example, an important step in the elastase perfusion model involves separating the distal inferior vena cava from the aorta to pass a ligature around the aorta facilitating the stabilization of a

retrograde perfusion cannula. Our approach does not require individual vessel isolation or ligation, is easily teachable, and does not involve manipulation of the great vessels. Importantly, postoperative hind paw paralysis, which is lethal, is completely avoided.

Other murine models of experimental aortic aneurysms exist, including adventitial CaCl_2 and angiotensin II infusion. These models provide credence to the need for developing simpler experimental injury methods of aneurysm formation. These models have important limitations, specifically the peri-aortic application of CaCl_2 creates very modest aneurysms at 4 weeks with only 30–50% dilation without complete penetrance.⁵ A recent combination procedure combining intraluminal elastase and adventitial CaCl_2 has been touted to be a less difficult and reproducible rat model of aneurysms.²¹ However, adaptation to the mouse model is awaited. An alternate model where osmotic pump delivered angiotensin II infusion is performed in apolipoprotein E knockout mice, results in an aortic dissection with prominent thrombus that is distinct from the laminated version seen in mature human disease.²² Because this model is in an already genetically altered mouse, investigations that require an additional genetic deletion become very challenging to perform.

Physiologically, our novel model presented here is better suited to study the adventitial contribution to aneurysm disease because elastase-induced degradation of the abluminal surface induces inflammatory homing. The prevailing theory during aneurysm development is that aneurysms develop owing to an inflammatory infiltrate that initiates at the adventitia. In the current study, we show that adventitial inflammation is present similar to human disease. Both MMP-2 and -9 work in concert to produce aortic aneurysms.²³ MMP-2 is thought to contribute in large part in small aortic aneurysms and MMP-9 is believed to play a major role in larger aneurysms.²⁴ Preventing smaller aneurysms from disease progression is an important clinical event that shown some promise in human investigations.^{25,26} MMP-knockout mice have been resistant to aneurysm formation,²⁷ linking a central role of their contribution to disease progression.^{2,28,29} Doxycycline, a potent MMP inhibitor, has been shown in multiple studies to prevent murine aneurysm formation and progression.^{14,19} We utilized this known mechanism of aneurysm treatment to evaluate our model and found that orally administering doxycycline to animals, attenuated aneurysm development at 2 weeks.

This study has several limitations. First, the lack of atherosclerosis and intraluminal thrombus, which potentially contribute to human aneurysmal disease are unaccounted; however, these are not seen in the elastase perfusion model either. Second, the dosing and incubation strategies are not consistent compared with the other models of aneurysmal disease, albeit optimized by multiple dose response and time dependent preliminary work. As such, varying stock elastase concentrations can influence the degree of injury. Finally, there are no comparisons against the conventional elastase perfusion model presented, and so differences in species, gender, or mechanism are not fully explained, although they are under investigation by our laboratory.

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Fig 1.

(A) The peri-adventitial elastase application model. The infra-renal murine aorta is first circumferentially dissected, and 10 μ L of 100% PPE is topically applied to the adventitial aorta for 10 minutes. The aorta is monitored closely for phenotypic changes after which the animal is closed and recovered for 14 days. The AAA at day 14 is explanted for analysis. The in situ, naïve aorta at the beginning of the procedure has a fibrous matrix (*solid white arrows*). (B) Effects of elastase and time-dependent exposure on aortic dilation. Nonlinear regression curves for elastase volume and time of exposure where intersected with targeted aortic dilation between 30% and 40%. When considered with visual aortic changes during the operation, the most reproducible results were anticipated when 10 μ L of elastase was applied for 10 minutes.

Fig 2.

(A) Progression of aneurysm formation after peri-adventitial elastase application. The aorta at 5 minutes after PPE application (A) develops wall thinning and early qualitative changes. At 10 minutes after PPE application (B), the aorta develops an enriched red coloration, as the matrix seems to be visually degraded. The infrarenal murine abdominal aorta at 14 days after PPE application, has a sheen that is noted with fusiform changes (C). (B) Relative change in aortic diameter above baseline. The relative change in the aortic diameter over baseline is highest in the PPE group. An aneurysm was defined as an increase in the aortic diameter by 50% above baseline. * $P = .03$; ** $P = .003$.

Fig 3.

(A) Modified Russell-Movat Pentachrome (Movat). Movat staining (10×) revealed degraded elastin and smooth muscle layer(s) with PPE application (*solid white arrows* on anterior surface of aorta) that were preserved among doxy-cycline treated animals. (B) Activated macrophages (Mac2). Mac2 stain (10×) show increased presence with elastase administration (*solid white arrows*). Activated macrophages are reduced with doxycycline treatment. (C) Matrix metalloproteinase-9 (MMP-9). Total MMP-9 stain (10×) shows increased presence with elastase administration. MMP-9 expression is attenuated with doxycycline treatment at day 14. (D) Quantified immunostaining for activated macrophages (Mac2) and matrix metalloproteinase-9 (MMP-9). Threshold gated quantification of immunohistochemistry in aortic sections for Mac2 and MMP-9 show increased presence of macrophages, and MMP-9 at day 14 in the aortic wall after elastase administration. * $P < .05$; ** $P < .01$.

Fig 4.

(A) Smooth muscle 22- α (SM22 α). SM22 α immunostain (10 $\times$) shows that the smooth muscle layer are preserved among doxycycline-treated animals. The *solid white arrows* are on the anterior surface of the aorta. (B) Smooth muscle α -Actin (SM α A). SM α A immunostain (10 $\times$) shows increased smooth muscle degradation after topical elastase exposure (*solid white arrow*), which is decreased after treatment with doxycycline. (C) myosin heavy chain (MHC-SM1). MHC-SM1 immunostain (10 $\times$) shows increased smooth muscle degradation after topical elastase exposure, which is reduced after treatment with doxycycline. (D) Ki-67. Ki-67 immunostaining (10 $\times$) for cell proliferation, was greater in PPE group after peri-adventitial elastase application as shown by the *arrows* and decreased with doxycycline treatment. (E) Cleaved caspase-3 (Caspase). Caspase staining (10 $\times$) showed increased apoptosis from baseline after peri-adventitial elastase application as highlighted by the *solid black arrows*. (F) Quantified immunostaining for smooth muscle 22- α (SM22 α), Smooth muscle α -Actin (SM α A), myosin heavy chain (MHC-SM1), and antigen Ki-67. Threshold-gated quantification of immunohistochemistry in aortic sections for smooth muscle cell markers were reduced among elastase administered animals. Cellular proliferation with marked increase in Ki-67 protein was noted with peri-adventitial elastase application. ** $P < .01$; *** $P < .001$.

Fig 5.

(A) Gelatin zymography for MMP-2 and 9. Gelatin zymography was performed as described with Coomassie brilliant blue for visualization of lytic bands. Faint bands are seen in the control and doxycycline-treated animals, whereas PPE-treated animals had intense bands. Relative densitometry was completed on all groups. *B*, Relative densitometry of gelatin zymograms showed differences in protein expression between groups. Relative densitometry adjusting for background influence shows that PPE exposure increases pro-MMP9 expression, which is attenuated with doxycycline treatment (*A*). Similarly, cleaving of the MMP-9 protein to the active form was greater after PPE (*B*). Doxycycline treatment decreased active MMP-9 expression. (*C*, *D*) Although pro-MMP-2 expression is not different between groups, the active form of the protein is expressed in greater amounts after PPE, whereas doxycycline limited active MMP-2 expression. * $P < .05$; ** $P < .01$.