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A Novel Reproducible Model of Aortic Aneurysm Rupture

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Abstract

Introduction—Given the unknown biologic antecedents prior to aortic aneurysm (AA) rupture, the purpose of this study was to establish a reproducible model of AA rupture.

Methods—Seven-week-old apolipoprotein E deficient (ApoE^{−/−}) mice were fed a high-fat diet for 4 weeks and osmotic infusion pumps containing Angiotensin II (AngII) were implanted. AngII was delivered continuously for 4 weeks at either 1000ng/kg/min (n=25) or 2000ng/kg/min (n=29). A third group (n=14) were given AngII at 2000ng/kg/min and 0.2% β-aminopropionitrile (BAPN) dissolved in drinking water. Surviving mice were euthanized 28 days after pump placement, aortic diameters measured and molecular analyses performed.

Results—Survival at 28 days was significantly different among groups with 80% survival in the 1000ng/kg/min group, 52% in the 2000ng/kg/min group, and only 14% in the AngII/BAPN group (p=0.0001). Concordantly, rupture rates were statistically different among groups (8% vs 38% vs 79%, p<0.0001). Rates of abdominal AA were 48%, 55% and 93%, respectively, with statistically higher rates in the AngII/ BAPN group compared to both the 1000ng and 2000ng AngII groups (p=0.006 and p=0.0165, respectively). Rates of thoracic AA were 12%, 52% and 79% in the three groups with a statistically higher rate in the AngII/BAPN group compared to 1000ng group (p<0.0001).

Conclusions—A reproducible model of aortic aneurysm rupture was developed with a high incidence of abdominal and thoracic AA. This model should enable further studies investigating the pathogenesis of aortic rupture, as well as allow for targeted strategies to prevent human AA rupture.

INTRODUCTION

After atherosclerosis, aneurysm is the most common disease of the aorta with aneurysm prevalence increasing with age. The Centers for Disease Control list abdominal aortic

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aneurysm (AAA) as the 15th leading cause of death in adults between 60 and 64 years of age.¹ The most dreaded complication of AAA is aortic rupture which occurs in an estimated 12,000 to 15,000 patients per year in the United States and has an associated mortality rate of 50 to 80%.² These numbers may underestimate the true incidence, however, as patients with AAA are often asymptomatic, where rupture and death would occur before ever arriving at a hospital.

The study of aneurysm rupture in humans is limited in that human aortic tissue is usually only obtained for research during surgical repair. This does not allow great insight into the mechanisms leading to the initial development of an aneurysm, its gradual expansion or its eventual rupture. Researchers therefore rely on animal models of aneurysm in order to better investigate these events, though these models may not perfectly recapitulate the biology of human aneurysm formation. Aortic rupture is a devastating result of aortic aneurysmal disease, but there are no known biologic markers of impending rupture that allow physicians to predict which patients are at greatest risk of this catastrophic event. Additionally, there are few established, reproducible animal models of aortic rupture which would allow further investigation into the pathogenesis of rupture.

In contrast, there are many animal models used to study the pathogenesis of AAA that typically do not result in rupture. These models include the use of genetically engineered mice with defects in aortic wall structure or aberrant cholesterol homeostasis.³ Chemically-induced AAA models are also well-described in the literature and include utilization of periaortic or perfused elastase, periaortic calcium chloride or subcutaneous Angiotensin II (AngII) infusion.^{4, 5} Infusion of AngII at 1000ng/kg/min into ApoE ^{-/-} mice has previously been shown to cause suprarenal AAAs and ascending TAAs that are independent of the AngII-induced elevated blood pressure.⁶⁻⁹ High fat diets are used to further exaggerate the hyperlipidemic state in these knockout mice, resulting in greater atherosclerotic changes and aneurysm formation.³ AAAs in this model are believed to have accelerated inflammation with resultant macrophage infiltration and associated elastin degradation. In addition to significant atherosclerosis and thrombus formation, many mice also develop associated focal dissections.^{3, 8, 10}

Although much research has been done to investigate the pathogenesis and treatment of AAAs, little investigation has been done to elucidate the biologic antecedents prior to rupture. We therefore aimed to develop a novel, reproducible model of aortic aneurysm rupture to better understand the events causing aneurysms to progress to rupture.

MATERIALS AND METHODS

Angiotensin II Infusion

Seven-week-old ApoE ^{-/-} male mice (Jackson Laboratory, Bar Harbor, ME) were housed 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 a high-fat diet (TD 09682, Harlan Teklad Inc., Indianapolis, Indiana) with no restrictions on movement. Osmotic pumps (Alzet® 1004, Durect Corp., Cupertino, California) containing AngII (Sigma Aldrich Inc., St. Louis, Missouri) were then implanted into 3 groups of 11-week-old mice as

previously described.^{6, 11, 12} AngII was delivered at 1000 ng/kg/min (n=25) or 2000 ng/kg/min (n=29). A third group (n=14) was given 0.2% β -aminopropionitrile (BAPN) (Sigma Aldrich, St. Louis, MO) dissolved in drinking water 3 days prior to osmotic pump placement. These mice also received AngII at a rate of 2000 ng/kg/min and were continuously given BAPN until the end of the experiment. Any mouse that died prior to the study endpoint underwent autopsy to determine cause of death. After 28 days, surviving mice were euthanized under anesthesia by overdose and exsanguination. The aorta was dissected free from surrounding tissue and fully visualized (ascending to iliac bifurcation) for video micrometry measurements of the aortic diameter using NIS-Elements D.3.10 software attached to the microscope (Nikon SMX-800, Melville, NY). Suprarenal aortic samples were then collected and flash frozen and stored at -80°C or embedded in paraffin for histology.

The above experiment was repeated except with euthanasia 4 days after osmotic pump placement since a large proportion of mice in the AngII/BAPN group did not survive for the full 28-day time course. Day 4 was chosen as an end point as prior aneurysm experiments in our laboratory have shown peak infiltration of neutrophils and macrophages occurs between 3 and 7 days.⁴ These “day 4” mice (n=15/group) were cared for and harvested in the same manner as described in the previous paragraph.

Animal care and use were in accordance with the *Guide for the Care and Use of Laboratory Animals*. The animal protocol was approved by the University of Virginia Institutional Animal Care and Use Committee (#3848) in compliance with the Office of Laboratory Animal Welfare.

Histology

Murine aortas (n= 7–9/group) were harvested at sacrifice on day 4 for histology analysis after undergoing left ventricular puncture and 4% paraformaldehyde (PFA) 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 $5\mu\text{m}$. After microwave antigen retrieval, antibodies were bound and detected using VectaStain Elite Kit (Vector Laboratories Inc., Burlingame, California). Antibodies for IHC staining were anti-mouse anti-Neutrophil (Ly 6B.2) for neutrophils (1:10,000; AbD Serotec, Oxford, United Kingdom), anti-rat Mac2 for macrophages (1:10,000; Cedarlane Laboratories, Burlington, Canada), CD3 for T-cells (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, California), anti-mouse SM α A for smooth muscle α -actin (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, California), Verhoeff Van Gieson for elastin, as previously described.⁵ Visualization of color development was completed using diaminobenzidine (Dako Corporation, Carpinteria, California) for SM α A, Mac2, anti-neutrophil and CD3. Images were acquired using AxioCam Software version 4.6 via 10X, 40X, and 100X objectives and an AxioCam MRc camera (Carl Zeiss Inc., Thornwood, New York). Slides were then graded by 3 blinded reviewers using the following scales:

Elastin: (1) no degradation, (2) mild degradation, (3) moderate degradation, (4) severe degradation

SMCs: (1) no SMC loss, (2) mild SMC loss, (3) moderate SMC loss, (4) severe SMC loss

Macrophages/Neutrophils/Tcells: (1) none to few, (2) mild staining, (3) moderate staining, (4) severe staining

Protein Extraction

Protein was extracted from frozen aortic tissue samples with PBS and protease inhibitors (Roche Diagnostics, Indianapolis, IN) as previously described.¹¹

Cytokine Array

For the purpose of determining the effects of AngII and BAPN on pro-inflammatory cytokines in the aortic wall, mouse cytokine arrays (R&D Systems, Minneapolis, MN) were performed using isolated protein from mouse aortas at day 4 or 28 according to the manufacturer's instructions (n=3–5/group at 4 days and n=2/group at 28 days). Protein samples from each group were pooled for analysis, and all samples were run in duplicate.¹³ Given the high rate of rupture in the AngII/BAPN group at 28 days and the fact that aortic protein samples were pooled, these results were not statistically compared.

Zymography

Using protein isolated from the aortic samples, gelatin zymography was performed to determine matrix metalloproteinase (MMP) 2 and 9 activity. Individual mouse aortas (n=2/group at 28 days and n=3–5/group at 4 days) were used. Precast zymography gels (10%, Invitrogen, Carlsbad, CA) were loaded with 3µg of tissue protein from each aortic sample. Samples were diluted into 2x Tris-glycine SDS sample buffer and electrophoretically separated under nonreducing conditions. The gels were renatured for 30 minutes in renaturing buffer (Invitrogen) and incubated in developing buffer (Invitrogen, Carlsbad, CA) for 24 hours at 37 °C rocker. The gels were then stained in Simply Blue Safe Stain (Invitrogen, Carlsbad, CA). Pro- and active forms of MMP2 and 9 appeared as clear band against the blue background. Quantification was determined according the optical density using Bio-Rad Image Lab Software version 4.0 (Bio-Rad, Hercules, CA).

Statistical Methods

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Categorical data were assessed using a Chi-squared test. Kaplan-meier survival curves tracked the percentage survival of mice over the 28 day period and were assessed by log-rank (Mantel-Cox) test. Differences between groups for histologic grading for elastin degradation, SMC loss and macrophage, T-cell and neutrophil infiltration were tested with a 1- way ANOVA with Tukey's correction for multiple comparisons. Blinding to experimental groups was maintained for histologic grading.

RESULTS

AngII and BAPN Increase Aneurysm Formation

Exposure to continuous AngII with or without BAPN resulted in aneurysm formation in mice in all groups in both the suprarenal abdominal aorta and the ascending thoracic aorta. The incidence of AAA was higher in the AngII/BAPN group than in both the 1000ng (93% v 48%; $p=0.006$) and 2000ng (93% v 55%; $p=0.0165$) groups (Figure 1A). The rate of thoracic aortic aneurysm (TAA) was statistically higher in the AngII/BAPN group than the 1000ng group (79% v 12%; $p<0.0001$), although not statistically different from the 2000ng group (79% v 52%; $p=0.1107$) (Figure 1B). The incidence of TAA in the 2000ng group was significantly higher than the 1000ng group (52% v 12%; $p=0.0033$).

Survival Decreases with Increasing AngII and Addition of BAPN

The survival rate was significantly different among the 3 groups of mice with 80% survival in the 1000ng group, 52% in the 2000ng group and only 14% in the AngII/BAPN group ($p=0.0001$; Figure 2). Unadjusted comparisons between each group were also significantly different (all $p<0.05$). Although most early deaths were secondary to aortic rupture, there were mice (3 [12%] in 1000ng, 3 [10%] in 2000ng and 1 [7%] in AngII/BAPN groups) that died from unknown causes when examined during autopsy.

The rate of aortic rupture increased with higher AngII dosing and the addition of BAPN. The incidence of rupture in each group was 8%, 38% and 79% in 1000ng, 2000ng and AngII/BAPN groups respectively ($p<0.0001$) (Figure 3). Unadjusted comparisons between each group were significantly different (all $p<0.05$). The location of rupture was TAA in all of the 1000ng mice that died from rupture. In the 2000ng group, 55% had ruptured TAA, 27% had ruptured AAA, and 18% ruptured in both TAA and AAA locations. Finally, in the AngII/BAPN group, 27% had ruptured TAA, 45% had ruptured AAA, and 27% ruptured in both TAA and AAA locations.

Histologic Differences Are Not Appreciated Early in Rupture Model

Histologic analysis of aortas from mice harvested after 4 days showed no statistically significant differences (all $p>0.05$) in degree of elastin degradation, smooth muscle cell loss or neutrophil, macrophage or T-cell infiltration. However, there was a trend, toward more neutrophil (1.1 point increase) and macrophage (1.0 point increase on grading scale) infiltration in the aortas of mice in the AngII/BAPN group compared to those in the 2000ng group. Comparison suffered from not enough mice surviving to day 28 to allow histologic analysis.

Exposure to Increasing AngII and BAPN Upregulates Inflammatory Cytokines

In pooled aortic samples from mice that survived to day 28, most inflammatory cytokines were upregulated in the AngII/BAPN group when compared to both the 1000ng and 2000ng groups. Table 1 shows fold increases in cytokines in the AngII/BAPN group compared to the 2000ng group alone; similar changes were seen when compared to the 1000ng group. At day 4, however, only IL-1 β , IL-1RA, CXCL1, MCP-1, MIP-2, CCL17 and TREM-1 were more

elevated in the AngII/BAPN group (Figure 4A). These same cytokines are documented on day 28 in Figure 4B.

Increasing AngII and BAPN Exposure Upregulates Matrix Metalloproteinases

At 28 days, both pro- and active MMP9 and MMP2 were elevated in the AngII/BAPN group when compared to the other two groups (Figure 5). There was a statistically significant difference in active MMP9 and active MMP2 when compared to both the 1000ng and 2000ng groups (all $p < 0.01$). In day 4 mice, pro- and active MMP9 were again highest in the AngII/BAPN group ($p < 0.01$, data not shown in separate figure).

DISCUSSION

These experiments have demonstrated a new model of aortic aneurysm rupture using a combination of AngII and BAPN, with nearly 80% of mice succumbing to aneurysm rupture by day 28. The mice in this group developed high rates of both AAA and TAA, thus allowing further investigation into the differential pathogenesis of aortic aneurysms and their rupture in both the abdominal and thoracic aorta.

The AngII model is a well-established and accepted model of AAA, despite some of the typical limitations of animal models of human disease. It mimics human aneurysm development in that the model causes noted luminal dilation, degeneration of the media, thrombosis of the aorta.³ Additionally, the AngII mouse model is consistent with human disease where males have twice the rate of AAA as females.¹⁴ The experiments in this study were therefore performed only in male mice to maximize aneurysm formation and subsequent rupture. In the present experiment, the standard dose of AngII was doubled in two of the three test groups, in order to both increase the rate of AAA formation and to induce aneurysm ruptures. The addition of the irreversible lysyl oxidase inhibitor BAPN, which prevents elastin and collagen cross-linking in the elastic lamina, serves as another insult to aortic integrity in this rupture model.¹⁵ The combination of AngII delivered at 1000ng/kg/min and BAPN has been described previously as a means for inducing aortic dissection.¹⁶ In that study, 100% of mice developed an acute aortic dissection and 30% of mice died as a result of aortic rupture within 24 hours of AngII infusion. Although this rupture rate is far higher than in our 1000ng group, that study exposed mice to BAPN for 4 weeks prior to introduction of AngII, likely causing a greater extent of medial degeneration than in our model in which mice were started with BAPN only 3 days prior to AngII infusion. Other studies have used subcutaneous BAPN to induce aortic aneurysms and rupture in rats; however, these studies had high early rupture rates, likely secondary to acute inflammation.^{17, 18} Our lab has recently published a study utilizing lower dose BAPN dissolved in water (0.2%) to induce less acute aortic injury in order to develop a more chronic model of AAA.⁴ We elected to use this lower dose again in our rupture model with a goal of having increased survival to the experiment endpoint.

In this study, mice in the AngII/BAPN group developed more AAAs and TAAs than mice in either the 1000ng or 2000ng group. In initial studies using the AngII model, AAAs were noted in approximately 1/3 of mice at doses of 1000ng/kg/min.⁶ Our mice that were given the same dose of AngII developed AAAs at a rate of 48%. This difference may be

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attributable to the fact that they were also fed a high-fat, “western” diet which intensifies atherosclerotic changes in the aortas of these ApoE^{-/-} mice. A recent meta-analysis of all studies using the AngII model, however, showed that AngII dose and diet type had only moderate, non-significant effects on the reported incidence of AAA.¹⁹ The high incidence of ascending TAAs in our model will also allow further investigation into ascending aneurysms, particularly ruptured ascending aneurysms, in the future.

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Mice in the AngII/BAPN group experienced rupture more commonly with decreased overall survival compared to the other two experimental groups. Although some mice in each group died without an obvious aneurysm or aortic rupture at autopsy, the AngII model in ApoE^{-/-} mice is known to promote diffuse atherosclerosis, including coronary disease, and this serves as one possible explanation for cause of death in several of these animals.²⁰ BAPN has also been used in our laboratory in conjunction with periaortic elastase administration in order to establish a model of chronic, advanced-stage AAA.⁴ This 100-day model resulted in rupture in 46% of mice, with ruptures occurring either as early (~1 week after surgery) or late (60–80 days after surgery) events. Ruptures in our model using BAPN and AngII began as early as postoperative day 3 and continued until day 28. The higher rate of rupture throughout the entire experimental time course is likely related to the more inflammatory nature of the AngII model in addition to the aortic dissections seen in this model.

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In mice that survived to day 28, most inflammatory cytokines were upregulated in the AngII/BAPN group when compared to the 1000ng and 2000ng groups. These mice had the greatest insult to their aorta and therefore would be expected to have a more significant inflammatory response and greater influx of leukocytes. Because the aortas in each group were pooled in order to use the cytokine array kit, it was not possible to perform statistical analysis to test for significance. We did note, however, that 7 cytokines were markedly elevated in the aortas of mice in the AngII/BAPN group by day 4, suggesting that these cytokines may be most important in the onset of aneurysm formation and progression. Further focus on these specific cytokines will need to be performed in future studies to more clearly describe their role in AAA and rupture pathology.

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MMPs degrade elastin and collagen in the aortic media and several, including MMP2 and MMP9, have been implicated in the development of aortic aneurysms in both human and animal aneurysms.^{22–24} AngII infusion is thought to further augment MMP production in mouse models of atherosclerosis and AAA formation.⁷ In this study, pro- and active MMP2 and MMP9 were all elevated in the AngII/BAPN group when compared to the other two groups at 28 days. Likewise, on day 4, pro- and active MMP9 were again highest in the AngII/BAPN group. MMP2 and MMP9 have been demonstrated to peak at day 28 in prior AngII experiments in our lab.²⁵ Perhaps with the addition of BAPN, however, this rise in expression of MMP9 starts far earlier, potentially contributing to increased aneurysm and rupture rates in this group.

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There are limitations to the present study including the continued need to better define specific targets affecting the biology of aneurysm rupture. Additionally, although most mice in the AngII/BAPN group experienced the desired outcome of aortic rupture, the number of mice surviving to the study end was minimal, making intense investigation of the biologic

mechanisms limited. In the future, the protocol will need to be modified in order to increase survival and our ability to study these mechanisms using physiologic monitoring prior to aortic rupture. Another limitation is that mice in the AngII model do not form aneurysms or develop ruptures at a uniform time or anatomic location; these events can happen as early as day 3 and as late as day 28. The addition of blood pressure monitoring may assist in detection of rupture prior to the animals' deaths, again, perhaps, allowing the identification of important biologic precursors.

CONCLUSION

This model of 2000ng/kg/min AngII plus 0.2% BAPN created a model with 79% aneurysm rupture in the abdominal and/or thoracic aorta. The high penetrance of both abdominal and thoracic aneurysms leading to aneurysm rupture makes it an ideal model for studying this pathology. This novel, reproducible model will allow for investigation into the antecedent events leading to rupture in order to identify targets that allow prediction or even prevention of rupture in mice and, hopefully, eventually, in humans.

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A.

B.

Fig. 1. Increased aneurysm incidence in AngII/BAPN group

A. Abdominal aortic aneurysm (AAA) incidence. AAA was more common in the AngII/BAPN group than in both the 1000ng (93 v 48%, $p=0.006$) and 2000ng (93 v 55%, $p=0.0165$) groups. **B.** Thoracic aortic aneurysm (TAA) incidence. TAA incidence was higher in the AngII/BAPN group than the 1000ng (79 v 12%, $p<0.0001$) and the 2000ng groups (79 v 52%, $p>0.05$) groups. The 2000ng group also had more TAAs than the 1000ng group (52 v 12%, $p=0.0033$).

Survival in AngII Rupture Model

Fig. 2. Decreased survival in AngII/BAPN group

With increasing AngII dosing and the addition of BAPN, survival at 28 days decreased from 80% survival in the 1000ng group to 52% in the 2000ng group and 14% in the AngII/BAPN group ($p=0.0001$).

Fig. 3. Increased AAA and TAA rupture in AngII/BAPN group

With increasing AngII dosing and the addition of BAPN, rupture increased with 8% in the 1000ng group, 38% in the 2000ng group and 79% in the BAPN group ($p<0.0001$).

Fig. 4. Increased inflammatory cytokines in AngII/BAPN group

All tested inflammatory cytokines appeared to be upregulated in the pooled aortas of mice in the AngII/BAPN group when compared to the 1000ng and 2000ng groups at day 28. **A.** In day 4 aortas, only IL-1 β , IL-1RA, CXCL1, MCP-1, MIP-2, CCL17 and TREM-1 were more elevated in the AngII/BAPN group than the other two groups. **B.** In day 28 aortas, these 7 cytokines were also elevated in the AngII/BAPN group when compared to the 1000ng and 2000ng groups.

Fig. 5. Increased MMP activity in AngII/BAPN group

In day 28 aortic samples, pro- and active forms of MMP9 and 2 were increased in animals in the AngII/BAPN group compared to those in the 1000ng and 2000ng groups (seen as white bands on blue background). *p <0.01

Table I

Summary of cytokine activities at 28 days in AngII/BAPN group.

Amount greater than 2000ng group	Cytokines
> 1×	IL-3, IL-5, IL-10, IL-12 p70
> 2×	GM-CSF, CCL11, sICAM-1, IL-2, IL-6, IL-23, MIP-1 β /CCL4, CCL17
> 5×	CXCL13, G-CSF, IL-4, IL-7, IL-13, IL-17, IL-27, CXCL11, MIP-2, TIMP-1, TNF- α , TREM-1
> 10×	CCL1, IL-1 α , IL-1 β , CXCL10, CXCL1, M-CSF, CCL12, CXCL9, MIP-1 α /CCL3
> 20×	C5/C5a, IFN- γ , MCP-1, RANTES
> 50×	IL-1RA, IL-16, CXCL12

IL: Interleukin, GM-CSF: Granulocyte macrophage colony-stimulating factor, CCL: Chemokine (C-C motif) ligand, sICAM: Soluble intercellular adhesion molecule, MIP: Macrophage inflammatory protein, CXCL: Chemokine (C-X-C motif) ligand, G-CSF: Granulocyte colony-stimulating factor, TIMP: Tissue inhibitor of metalloproteinase, TNF: Tumor necrosis factor, TREM: Triggering receptor expressed on myeloid cells, M-CSF: Macrophage colony-stimulating factor, C5/C5a: Complement component 5/5a, IFN- γ : Interferon gamma, MCP: Monocyte chemoattractant protein, RANTES: Regulated on activation, normal T cell expressed and secreted, IL-1RA: Interleukin-1 receptor antagonist