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Differential Gender And Species Specific Formation Of Aneurysms Using A Novel Method Of Inducing Abdominal Aortic Aneurysms

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Abstract

Background—The objective of this study was to test a novel model of inducing AAA in different mouse strains and genders.

Materials and Methods—Male and female C57BL/6 and B6129 mice (N=5 per group) underwent peri-aortic dissection and porcine pancreatic elastase (30μl) or inactivated elastase application (5 min) to aorta. Aortic measurements were taken on day 0 and 14. Aortic samples were analyzed for histology, and zymography for MMP activity. Comparison statistics performed using unpaired t-test.

Results—AAA phenotype (50% aortic increase), occurred in external elastase treated males (100%) and females (90%). No control animals developed AAAs. The aortic diameter was larger in C57BL/6 and B6129 elastase treated versus control males (p=0.0028 and p<0.0001, respectively) and females (p<0.0001 and p=0.0458, respectively). Histology verified phenotype via disrupted internal elastic laminae. Macrophage counts in elastase treated animals were >6 fold higher than in controls (all groups significant). MMP9 activity was greater in elastase treated males and females in C57BL/6 (p=0.0031, p=0.0004) and B6129 (p=0.025, p=0.2) mice; MMP2 activity was greater in C57BL/6 vs. B6129 ME.

Conclusions—This rodent model produced AAAs in both genders and strains of mice. This model is simple, has little variability, and occurs in the infrarenal aorta, substantiating the external elastase model for future studies.

Introduction

Several experimental methods have been used to induce experimental abdominal aortic aneurysms (AAAs) in animal models. While there is no perfect model for murine development of AAAs that has universal applicability, we developed a model of topical

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elastase application to produce aneurysms in the infrarenal aorta which is reproducible and easy to perform. To date, the external elastase model has not been tested in mice.

Various experimental AAA models have had various incidences of AAA development amongst different murine strains. For example, Thompson et al. showed AAA resistance in wild types B6129/SvEv, SvJ, and CBA using the elastase perfusion model. Intermediate susceptibility to AAAs was found in Balb/c, among others, while C57BL/6 was highly susceptible to AAA formation¹. Also, as many knock out strains are on different genetic backgrounds, it must be known if the control animals of the same background develop AAAs for comparisons and conclusions to be valid. Therefore, models should be tested on more than one strain of mouse.

In addition, confirmation that the female resistance to AAA formation is maintained with external elastase application is necessary for utilization of this model in further studies of gender differences. There have been several studies from our lab utilizing the elastase perfusion model demonstrating that females are protected from AAA formation^{2,3,4,5}. The mechanisms underlying this gender difference are not completely clear. Some evidence exists that macrophage infiltration is inhibited, thus decreasing the inflammatory reaction in females as compared with males. MMP9, a crucial proteinase in AAA development released by macrophages, is decreased in females³. Cho et al. and Sinha et al. have demonstrated that neutrophils and certain proinflammatory mediators, such as chemokines and cytokines, are decreased in females compared with males during AAA formation^{4,5}. Thus, both direct and indirect mechanisms have been documented showing estradiol exerts protection from AAA formation for females.

The objective of this study was to test this novel model of AAA formation in different murine strains, in both male and female mice.

Methods

Animal Surgery

After induction of general anesthesia with isoflurane, male and female mice, both C57BL/6 and B6129 strains obtained from Jackson Laboratories (Bar Harbor, ME) (eight weeks, 20–30g) (N=5 for each of eight groups) had their abdominal aorta exposed via mid-line laparotomy. Aortic diameter was measured using a video-micrometer (Nikon Elements software, Nikon, Melville, NY) in three locations: just below the renal arteries, mid-abdominal aorta, and at the iliac bifurcation. The infrarenal aorta was treated with 30µl of highly concentrated type I porcine pancreatic elastase reconstituted with normal saline (5U/mg of protein) (LOT #098K7008; Sigma, St. Louis, MO) or 30µl of heat-inactivated elastase (the above elastase at 90°C for 30 minutes) as a control group. The topical application was accomplished by dropping the elastase on the anterior aorta from a 2cm height for 5 minutes. The elastase was not washed off prior to closing the abdomen. Aortic diameter was determined again on day 14 prior to aortic harvest. All experiments were approved by the University of Michigan Universal Committee on the Use and Care of Animals (UCUCA No.09679).

Histology and Immunohistochemistry

Harvested aortas were analyzed by immunohistochemistry for structural integrity, migratory macrophages, neutrophils, and CD3+ T cells. Aortic tissue was fixed in 10% buffered formaldehyde for 2 hours, transferred to 70% ethanol, and subsequently embedded in paraffin for sectioning. Sections were prepared with Hematoxylin and Eosin and Verhoeff-VanGieson (elastin) stains for elastic lamina evaluation. Further sections were stained using the following procedures^{6,7,8}.

The aortic sections were deparaffinized in xylene and rehydrated in graded alcohols. Heat-induced antigen retrieval using 10 mM sodium citrate, pH 6.0, was performed in a microwave. The sections were subsequently incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, followed by a blocking buffer to prevent nonspecific binding. Purified anti-Mouse Mac-2 Monoclonal Antibody (1:200, Cedarlane, Ontario, Canada) was used for staining, followed by an anti-Rat IgG biotinylated secondary antibody (1:500) and an avidin-biotin-HRP complex, available in the Rat IgG Elite Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Sections were then visualized using a DAB Peroxidase Substrate Kit (Vector Laboratories) and counterstained with Hematoxylin QS (Vector Laboratories).

For neutrophil staining, rat anti-mouse neutrophil, (1:1000 AbD Serotec, Raleigh, NC) and rat anti-mouse Mac-2 were used as primary antibodies. Alkaline phosphatase - conjugated anti-rat IgG (Sigma, St Louis MO) was employed as a secondary antibody. The signals were detected using Fast-Red (Sigma, St Louis MO). For negative controls, we used purified normal rat IgG (eBioscience Inc, San Diego, CA). For immunofluorescence staining, the cell nucleus was stained with 4, 6-Diamidine-2-phenylindole dihydrochloride (DAPI, Roche Diagnostics, Mannheim, Germany). After staining, the sections were dehydrated and pictures were taken with a Nikon microscope equipped with a CCD camera. The integrated optical density value of positive staining area of each section was randomly selected and measured. Twelve randomly selected 20x fields per aneurysm section were assessed for the density of staining. The positive signal in aneurysm for each group was semi quantified

Immunohistochemical staining of CD3+ T cells was performed according to previous publications^{7,8,9}. Briefly, the aorta sections (5µm) were dehydrated and incubated with 1% hydrogen peroxide followed by boiling in 1X unmasking solution (Vector Laboratories, Burlingame, CA) for 15 minutes and blocked with 10% serum. Immunostaining was performed with goat anti-mouse CD3e antibody (1:2000 Santa Cruz Biotechnology, Santa Cruz, CA) using Vectastain ABC kit (Vector). After incubation with an avidin-biotin complex, immunoreactivity was visualized by incubating the sections with 3,3'-diaminobenzidine tetrahydrochloride (DAKO Corp, Carpinteria, CA) to produce a brown precipitate. Sections were then counterstained with hematoxylin. The number of CD3+ T cells per high power field (100x) was assessed by immunohistochemical staining of at least five aneurysm sections.

The integrated density of the signal was acquired using the public domain software, Image J (U.S. National Institutes of Health, available at <http://rsb.info.nih.gov/niH-image>). All cell counts were done by a trained, blinded observer in HPFs of both the adventitia and media. A mean value was then calculated for positively stained cells per HPF in each animal to be used for statistical analysis.

Substrate Gelatin Zymography

Matrix metalloproteinase (MMP) 2 and 9 activity was determined by gelatin zymography of tissue homogenate. Gelatin substrate zymograms were run in pre-cast 10% SDS-PAGE gels containing 1 mg/ml of gelatin (Invitrogen, Carlsbad, CA). Equal amounts of protein were diluted into 2X tris-glycine SDS sample buffer and electrophoretically separated under non-reducing conditions. Proteins were renatured for 30 min in Renaturing Buffer (Invitrogen, Carlsbad, CA) and then the gels were incubated in the Developing Buffer (Invitrogen, Carlsbad, CA) for 30 min and again in the same buffer overnight at 37°C. The gel was stained in SimplyBlue SafeStain (Invitrogen, Carlsbad, CA) and the gelatinase activity was observed by clear bands against the blue background. Densitometry was performed using Image J software (National Institute of Health) program to quantify the levels of MMP activity.

Reverse Gelatin Zymography

Levels of TIMPs were detected by extracting total protein from aorta samples using proteinase extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 % (V/V) Triton X-100) using MP Fast-Prep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH). Protein concentrations were determined with BCA Protein Assay. Reverse Zymography was modified according to previous publications (3, 4). Briefly, equal amounts of proteins (30 µg/lane) were loaded onto SDS-polyacrylamide gels (12%), which co-polymerized with supernatant of smooth muscle cells and 1% gelatin. Mouse recombinant TIMP-1 was loaded as control. The washing, incubation, staining and destaining procedure are the same as gelatin zymography^{8,9}.

Statistical Analysis

Comparison statistics were determined using unpaired t-test with PRISM software (GraphPad Software Inc., La Jolla, CA). Mean values and standard error were graphed with significance values.

Results

AAA phenotype is confirmed in males and females in C57BL/6 and B6129 strains

AAA phenotype, as defined by a 50% increase in aortic diameter at harvest compared to baseline, occurred in 100% of males (5 of 5 for both strains) treated with external elastase (Fig 1). In addition, 90% of females (4 of 5 of the C57BL/6 and 5 of 5 of the B6129) also formed AAAs. Visually at harvest, treated animals had thin walled, fusiform AAAs, similar to those seen in humans. None of the control animals in any group (male and female, both strains) developed AAAs (0 of 20). The aortic diameter was significantly greater in male C57BL/6 and B6129 elastase treated (ME) versus control (MC) by 5 and 43 fold ($p=0.0028$ and $p<0.0001$, respectively). Of note, the B6129 ME had greater aortic diameters than C57BL/6 ME ($p=0.0392$); however, FEs were equivalent. Interestingly, female elastase treated C57BL/6 and B6129 strains (FE) also showed a significant increase in aortic diameter by 8 and 12.5 fold, compared with control animals (FC) ($p<0.0001$ and $p=0.0458$, respectively). Additionally, aortic diameters of ME were not significantly different from FE in either C57BL/6 ($p=0.135$) or B6129 ($p=0.1196$) strains.

Increased Macrophages and T-lymphocytes in Association with AAA Phenotype

Histology at 40x and 100x verified the phenotype and Van gieson stain confirmed a disrupted internal elastic lamina in animals which developed AAAs, whereas the internal elastic lamina was intact in control animals (Fig 2). The disrupted laminae was not completely confined to the outer layers of the wall, but was predominant in the portions of the wall corresponding to the anterior aorta, this amounted to approximately 180–270° of the circumference. Macrophage infiltration was measured by Mac-2 staining. Mac-2 stained cell counts in the elastase treated animals, regardless of strain or gender, were also higher than in controls (Fig 3). Unlike AAA phenotype, C57BL/6 ME Mac-2 cells were higher than B6129 ME ($p=0.0026$). In the C57BL/6 strain, ME and FE had 40 and 6 fold greater macrophage infiltration than MC and FC ($p=0.0031$ and $p=0.0004$, respectively). This strain maintained the traditional gender difference, with ME having more macrophages per HPF than FE ($p=0.0092$). However, the gender difference in the B6129 strain was consistent with the phenotypic findings, with no significant differences between ME and FE. B6129 ME were greater than MC ($p=0.025$) as with C57BL/6 mice, but FE and FC were equivalent ($p=0.236$).

T lymphocytes (CD3+) were found to be more prevalent in males than in females (Fig 4). In contrast, neutrophils were found to be equivalent in male and females. (Data not shown)

MMP9 not TIMP activity mirrors AAA phenotype changes

Zymography demonstrated increased active MMP9 activity in elastase treated animals over controls in both strains and genders of mice. This pattern, mirroring phenotypic data, was true in females (FE > FC, $p=0.0014$ and $p=0.041$ in C57BL/6 and B6129, respectively) and males (ME > MC, $p=0.0005$ and $p=0.0162$). Reflecting Mac-2 counts in ME, MMP9 activity in C57BL/6 elastase-treated mice was higher than B6129 elastase-treated mice in both males and females. Consistent with phenotype, MMP9 activity was equivalent in ME and FE in both C57BL/6 ($p=0.6638$) and B6129 ($p=0.3586$) strains (Fig 5). The only difference between groups that was significant in MMP2 activity was that C57BL/6 ME had greater activity than B6129 ME ($p=0.034$) (Fig 6).

Reverse zymography established no differences in TIMP1 or 2 activity levels in the aneurysm samples between male and females. (Data not shown)

Discussion

In the present study, we have documented that external elastase application to the infrarenal aortic results in the ability to generate AAAs in two strains of mice, and equally in male and female mice. As opposed to previous work, the ME B6129 mice formed larger aneurysms than their C57BL/6 counterparts. In this model the AAA phenotype (as determined by aortic diameter and disrupted elastic laminae by van gieson stain) occurs in association with an increase in Mac-2 stained cells in both the male and female C57BL/6 animals. However, the B6129 females did not have a significant increase in Mac-2 cells after elastase application, and B6129 ME had a muted increase as compared with C57BL/6 ME. CD+ lymphocytes were greater in C57 ME than FE, while PMNs were not found to be different. MMP9 activity was significantly higher in C57BL/6s than B6129s, but still significantly greater in treated vs. control groups in males and females and in both strains. However, unlike Mac-2 counts, MMP9 activity was equivalent between genders within the strains. MMP2 activity was less elucidating, with no clear significant pattern except C57BL/6 ME with greater activity than B6129 ME. The TIMPs also did not show any differences based on gender. These data suggest that this model therefore is good for inducing AAAs in a wider range of mouse strains, but may not be useable for helping us to understand gender differences. Further studies are needed to see if there are other methods of distinguishing the response of males versus females when the phenotype and proteinase activity are equivalent, but macrophages and lymphocytes show gender differences.

Presently, there are multiple methods for producing experimental *in vivo* AAAs, including topical calcium chloride, subcutaneous angiotensin II infusion, and elastase intraluminal perfusion. Each of these have advantages and disadvantages, as summarized in Table 1. However, none of these models completely recapitulate human disease. In addition, there have been knockout mice strains developed to produce AAAs, such as the *Lox* $-/-$, *MMP3* $-/-$, and *TIMP-1* $-/-$. These animals are either not hardy or have aortic wall destruction that is too generalized, and have other unknown phenotypic changes making translation to humans potentially problematic. An ideal model for experimental AAA development should replicate the shear stresses, hemodynamic forces, and perianeurysmal environment present in humans. While all of these animal models attempt to reproduce human disease with fidelity, it is difficult as information regarding early stage AAA development is lacking. External elastase application represents a novel, reliable and easily reproducible model for producing infrarenal AAAs. It produces the desired effect of destruction of elastic tissue within the adventitia and media, while eliminating the physical component of dilatation of the intra-luminal aorta that is needed in the standard elastase perfusion AAA model.

There are many different interrelated processes implicated in the development of AAAs, including chronic inflammation in the aortic adventitia, neovascularization, production and early recruitment of proinflammatory cytokines, excessive local production and dysregulation of MMPs leading to destruction of collagen, medial elastic lamellae, and other structural matrix proteins and depletion of medial SMCs. This leads to progressive weakening and dilatation of the aortic wall, partially due to an impaired capacity for connective tissue repair. Genetically altered mice have helped establish temporal expression of cytokines and hierarchy of inflammatory responses in AAA development. Thompson et al. reviewed the studies that were done in genetically altered mice in order to elucidate the roles of different molecular mediators, chemokines, proinflammatory cytokines, and cellular signaling molecules¹. With all of these processes involved, there are many possibilities for why different strains have different results in all models. Our data suggest the development of AAAs in B6129 mice may be through a different mechanism as compared with the C57BL/6 mice since the MEs have fewer Mac-2 positive cells and MMP9 activity, yet equal or greater AAA phenotype with C57BL/6 ME. Thompson summarized the fact that different wild type strains have different susceptibilities toward forming aneurysms as “consistent with an inherited, genetically determined susceptibility to the development of elastase-induced AAAs”¹. The differences documented in this study between C57BL/6 and B6129 could reflect that susceptibility is at least somewhat associated with genetically determined inheritable traits.

The gender disparities seen in AAA formation may also be due to genetic differences. Our lab has done many studies elucidating the mechanisms behind the gender differences in AAA formation. Female rats after elastase perfusion were found to exhibit an early five-fold decrease in proinflammatory TNF α superfamily ligands. Also, females had fourfold lower expression of TGF β and VEGF families, possible mediators of AAA development⁵. Interleukins, CC chemokine receptors, and CC ligand families were too low to be detected as compared with males⁵, again indicating possible mechanisms for less inflammation in female aortas. Another recent study from our lab correlatively documented decreased collagen types I and III along with increase in MMP-13 after elastase perfusion in male rats compared with females².

Many studies have looked at the estrogen receptor as having a role in protecting females from cardiovascular disease, including AAAs. It is now known that there are multiple estrogen receptors with possible overlapping and counter-acting functions. The effects are complex and involve eNOS, increased nitric oxide, G-protein pathways, and transcription regulated genomic changes. This complexity was witnessed when previously in our lab estrogen and testosterone were given to male and female rats before elastase perfusion. Males given estrogen experienced decreased aortic diameter, but oophorectomized females did not have increased aortic diameters⁴. The exact mechanisms for the elastase perfusion model maintaining a gender difference, but this topical elastase model forming AAA in both genders is not clear; the female protection may be largely overwhelmed by the topical method. An AAA phenotype may therefore be manifested in females, despite the expected decrease in Mac-2 cells, secondary to an altered inflammatory response.

Future studies include using the model to analyze IL-6, IL-1 β , TNF α , estrogen receptors, and EDPs (elastin-degradation peptides). These studies will hopefully serve to shed light on the pathogenesis behind the differences seen between strains and genders in AAA development.

Conclusions

In contrast to previous studies, which have documented vast variability in AAA formation between mouse strains and genders, the external elastase model of AAA formation produced significant aortic dilatation in males and females of two strains of mice despite differences in inflammatory cells. This study further serves to highlight the variability in rodent AAA models and substantiates the external elastase model for studying AAAs in future, as it can be easily duplicated in both genders and in multiple strains.

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Figure 1.

Percent increase in aortic diameter in elastase treated males (ME) and females (FE) compared with male (MC) and female (FC) controls. Aortic diameter was significantly greater in C57BL/6 ME>MC ($p=0.0028$) and B6129 ME>MC ($p<0.0001$). Aortic diameter was also significantly greater in C57BL/6 FE>FC ($p<0.0001$) and B6129 ($p=0.045$).

Figure 2.

Figure 2a. Representative C57BL/6 mouse aortas 14d after external elastase application. H&E staining shows intact aortic wall architecture in male controls (MC) and female control (FC) sections, while elastase treated males (ME) and females (FE) show inflammatory response and altered wall architecture. Van Gieson staining shows intact internal elastic lamina in MC and FC (long arrows) while elastic lamellae are clearly disrupted and fragmented in ME and FE (short arrows). Mac-2 staining highlights the increase in positively stained cells seen in the ME and FE aortas (arrows). Original magnification 40x, scale bars = 100 μ m.

Figure 2b. Representative B6129 mouse aortas 14d after external elastase application. H&E staining shows intact aortic wall architecture in male controls (MC) and female control (FC) sections, while elastase treated males (ME) and females (FE) show inflammatory response and altered wall architecture. Van Gieson staining shows intact internal elastic lamina in MC and FC (long arrows) while elastic lamellae are clearly disrupted and fragmented in ME and FE (short arrows). Mac-2 staining highlights the stark increase in positively stained cells seen in the ME and FE aortas (arrows). Original magnification 40x and 100x, scale bars = 100 μ m.

Figure 3.

Mac-2 stained cells per high power field (hpf). All elastase-treated groups had greater Mac-2 stained cells than respective control groups. C57BL/6 ME had significantly greater Mac-2 cells than all other elastase-treated groups.

Figure 4.

Figure 4a. Immunohistochemical staining of CD3+ T-cells in the AAA samples of C57BL/6 male and female mice. The slides were stained with anti- CD3 antibody. Cells stained brown or dark brown indicates CD3+ T-cell infiltration. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The magnifications (10X and 40X) were indicated in the representative pictures.

Figure 4b. The bar graph shows the analysis of positive immunostaining of CD3+ T-cell in the aneurysm on day 14. Data shown are the mean $\pm$ S.E. values for each group. HPF: High power field (40X).

Figure 5.

Matrix metalloproteinase MMP 9 activity by zymography densitometry analysis. All elastase groups had greater MMP9 activity than control groups. No gender differences within strains were seen, however C57BL/6 ME and FE had significantly higher activity as compared to B6129 ME and FE, respectively.

Figure 6. Matrix metalloproteinase MMP2 activity by zymography densitometry analysis. C57BL/6 ME had significantly higher activity than B6129 MEs. No other comparisons showed significance.

Table 1

Descriptive characteristics of different AAA animal models

	Topical CaCl₂ (gauze, direct)	Subcutaneous Angiotensin II Infusion	Intraluminal Elastase	Topical Elastase (direct)
Time course	Weeks	28d	14d	14d
Aneurysm location	Infrarenal, Thoracic	Suprarenal	Infrarenal	Infrarenal, could be Thoracic
Structural disruption	Media	Elastin degradation, thrombus	Medial elastic lamellae, adventitia	Elastin degradation adventitia, media
Presence of atherosclerosis/thrombus	No	No	Yes	No
Inflammatory response	Neutrophils, lymphocytes, Monocytes	Macrophages, then B&T lymphocytes then proteolytic cascade	Macrophages	Macrophages
Mechanical effects	None	None	Yes	None
Species used	Mouse, rabbit	Mouse, rat	Mouse, rat, rabbit	Mouse
Reported possible contamination	None	None	Yes	Yes
Ease of method	Very easy	Intermediate	Very hard	Very easy