Gender Differences in Experimental Aortic Aneurysm Formation


Objective—It is hypothesized that a male predominance, similar to that in humans, persists in a rodent model of experimental abdominal aortic aneurysm (AAA) via alterations in matrix metalloproteinases (MMPs).

Methods and Results—Group I experiments were as follows: elastase perfusion of the infrarenal aorta was performed in male (M) and female (F) rats. At 14 days, aortas were harvested for immunohistochemistry, real-time polymerase chain reaction (PCR), and zymography. Group II experiments were the following: abdominal aorta was transplanted from F or M donors into F or M recipients. At 14 days, rodents that had undergone transplantation underwent elastase perfusion. In group III, male rats were given estradiol or sham 5 days before elastase perfusion. In group I, M rats had larger AAAs with higher frequency than did F rats. M rat aortas had more significant macrophage infiltrates and increased matrix metalloproteinase (MMP)-9 production and activity. In group II, M-to-M aortic transplants uniformly developed aneurysms after elastase perfusion, whereas F-to-F aortic transplants remained resistant to aneurysm formation. F aortas transplanted into M recipients, however, lost aneurysm resistance. In group III, estradiol-treated rats demonstrated smaller aneurysms and less macrophage infiltrates and MMP-9 compared with M controls after elastase.

Conclusions—These data provide evidence of gender-related differences in AAA development, which may reflect an estrogen-mediated reduction in macrophage MMP-9 production. (Arterioscler Thromb Vasc Biol. 2004;24:2116-2122.)

Key Words: aorta ■ aneurysm ■ genetic ■ estrogen ■ metalloproteinase

Abdominal aortic aneurysms (AAAs) are potentially life-threatening, accounting for 150,000 hospital admissions yearly.1 Clear gender differences exist, with a prevalence in men 4-times that in women.2,3 The diminished risk of AAA development is lost in women after menopause, suggesting that reproductive events, including circulating estrogens, may play a protective role.4,5

Prominent local inflammatory cell infiltration, aortic wall cytokine production, medial wall destruction by proteases, and smooth muscle cell depletion characterize most AAAs. Destruction of elastin and collagen in the media by various matrix metalloproteinases (MMPs) is considered an essential element of AAA formation.6–8 MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, as well as tissue inhibitor of metalloproteinase-1, are all upregulated in the walls of human AAAs.9–14 Two of these, MMP-2 and MMP-9, have been extensively studied. MMP-9 has attracted particular attention in that it is highly expressed in human AAA wall and is present in serum from AAA patients.14,15 Mice with deletion of the gene responsible for the MMP-9 protein are resistant to the development of experimental AAAs.10 In addition, MMP-2, derived from aortic mesenchymal cells, appears necessary for experimental aneurysm formation.16

Many studies implicating MMPs in AAA evolution have used a rat or mouse model with porcine pancreatic elastase perfusion of the infrarenal aorta. This model causes an initial influx of macrophages and lymphocytes leading to destruction and remodeling of the aortic wall matrix, and subsequent aneurysm development.17 Atherosclerosis, only once considered an essential to aneurysm development, is not thought to be the mechanism responsible for AAA formation.18,19 Importantly, nearly all previous studies have been performed using male rodents. To date, the influence of gender on experimental AAA formation has received little attention. Furthermore, although estrogen is known to affect collagen and elastin matrix remodeling in rats,20 its role in AAA formation has not been studied. This investigation was designed to determine the relevance of male and female gender on experimental AAA formation and to define local and systemic events that might influence any anticipated differences related to gender.
Elastase Perfusion Aneurysm Model

Pancrotic porcine elastase perfusion of the rat aortas was performed as described previously. Male and female rats (n=15, each) were anesthetized with 2 to 2.5% isoflurane inhalation, and the infrarenal abdominal aorta was isolated under sterile conditions. Digital video micrometry was performed to directly measure outer aortic diameter. Specifically, images of the aorta were obtained using a Spot Insight Color Optical Camera (Diagnostic Instruments, Sterling Heights, Mich) attached to an operating microscope (Nikon, Melville, NY). Aortic diameters were then measured at the level of the left renal vein, the mid-infrarenal aorta, and the aortic bifurcation in triplicate using Image Pro Express software (Media Cybernetics Inc, Silver Spring, Md). Temporay proximal and distal aortic control was obtained using temporary 4-0 cotton suture loops, following which an aortotomy was made near the aortic bifurcation with a 30-gauge needle. The infrarenal aorta was cannulated with PE-10 tubing and perfused with 12 μL of porcine pancreatic elastase diluted to a total volume of 2 mL with sterile normal saline (Lot #032K7660 or Lot #102K685; Sigma, St. Louis, Mo) over 60 minutes. Subsequently, the tubing was removed and the aortotomy repaired with 10-0 monofilament suture. Patency was assured in all cases. Aortic diameter measurements were repeated immediately after perfusion. The intestines were replaced; the abdominal wall was closed; and the rats were recovered. At 7 or 14 days, aortas were re-exposed and aortic diameters were re-measured in vivo. Aneurysm formation was defined as a 100% increase in an individual animal’s pre-elastase perfusion aortic diameter. The infrarenal aorta was then removed and subjected to histological study, immunohistochemistry, and quantitative polymerase chain reaction (PCR).

Aortic Transplantation

In additional rats, transplantation of the infrarenal rat aorta was performed as previously described. Briefly, male and female donor rats were anesthetized and the abdominal aortas isolated. Donor rats were anticoagulated with 300 U of heparin and the abdominal aorta was rapidly removed and placed in cold 0.9% normal saline. The recipient rats’ infrarenal abdominal aortas were similarly isolated and proximal and distal aortic control was obtained with temporary 4-0 cotton suture loops. The recipient abdominal aorta was excised and donor abdominal aorta was transplanted into the infrarenal position of a size-matched recipient using a running 10-0 monofilament suture in an end-to-end fashion. After aortic patency was assured, the abdominal incision was closed and rats were recovered.

Female donor aortas were transplanted into male recipients (n=7) with controls including female aortas transplanted into female recipients (n=7) and male aortas transplanted into male recipients (n=9). Fourteen days after transplantation, the transplanted aortas were subjected to pancreatic porcine elastase perfusion and harvested after 14 days as previously noted.

Estrogen Pellet Implantation

In other experiments, male rats were randomized to implantation of an estrogen pellet or sham implantation (n=11005). In other experiments, male rats were randomized to implantation of an estrogen pellet or sham implantation (n=11005). In additional rats, transplantation of the infrarenal rat aorta was performed as previously described. Briefly, male and female donor rats were anesthetized and the abdominal aortas isolated. Donor rats were anticoagulated with 300 U of heparin and the abdominal aorta was rapidly removed and placed in cold 0.9% normal saline. The recipient rats’ infrarenal abdominal aortas were similarly isolated and proximal and distal aortic control was obtained with temporary 4-0 cotton suture loops. The recipient abdominal aorta was excised and donor abdominal aorta was transplanted into the infrarenal position of a size-matched recipient using a running 10-0 monofilament suture in an end-to-end fashion. After aortic patency was assured, the abdominal incision was closed and rats were recovered.

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Histological Analysis

All excised aortas were fixed in 10% formalin for 18 hours, followed by immersion in 70% ethanol for 24 hours. Aortas were then imbedded in paraffin and 4-μm sections were prepared with hematoxylin and eosin and Verhoff-Van Gieson stains.

Immunohistochemistry was undertaken after deparaffinization, rehydration, and unmasking using Trilogy (Cell Marque Corp, Hot Springs, Ariz) in a Princess model pressure cooker (Cell Marque). Endogenous peroxidase activity was then blocked using 3% hydrogen peroxide in methanol. To help ensure that rejection was not occurring in elastase-perfused or transplanted animals, anti-T lymphocyte immunohistochemistry was performed. Specifically, antirat CD3 monoclonal antibody (BD Pharmingen, San Diego, Calif) was used as the primary antibody and mouse IgG Vectastain (Vector Laboratories, Burlingame, Calif) as the secondary antibody. Rat spleen was used as the positive control for anti-CD3 staining. ED-1 macrophage staining was performed using mouse antirat ED-1 primary antibody (Serotec, Raleigh, NC) and mouse IgG Vectastain secondary antibody (Vector Laboratories). MMP-9 immunohistochemistry was performed using rabbit antirat MMP-9 polyclonal primary antibody (Chemicon International, Temecula, Calif) and rabbit IgG Vectastain secondary antibody (Vector Laboratories). Staining for all these antibodies was performed using Vector Red alkaline phosphate (Vector Laboratories) followed by hematoxylin Q5 counterstain (Vector Laboratories). Colocalization studies involved deparaffinization, rehydration, and unmasking as previously described. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol. Staining for ED-1 was performed using mouse antirat ED-1 primary antibody (Serotec), mouse IgG Vectastain secondary antibody (Vector Laboratories), and Vector Blue alkaline phosphate. Samples were stored in PBS at 4°C overnight. The next day, staining for MMP-9 was performed using rabbit antirat MMP-9 polyclonal primary antibody AB19016 (Chemicon International), rabbit IgG Vectastain secondary antibody (Vector Laboratories), and Vector Red alkaline phosphate stain.

Quantitative PCR

Expression of MMP-9 and β-actin mRNA was determined using quantitative PCR. Messenger RNA was isolated by exposure of aortas to TRIzol reagent and reverse-transcribed by incubating with oligo-dT primer (Life Technologies, Grand Island, NY) and M-MLV Reverse Transcriptase (Life Technologies, Grand Island, NY) at 94°C for 3 minutes, followed by 40°C for 70 minutes. The resultant cDNA was amplified by Taq Polymerase (Promega, Madison, Wis) in a SmartCycler quantitative PCR system (Cepheid, Sunnyvale, Calif). SYBR intercalating dye (Roche, Indianapolis, Ind) was used to monitor cDNA amplification for each gene. MMP-9 and β-actin primer sequences were derived using Primer Premieir software (PREMIER Biosoft International, Palo Alto, Calif) based on cDNA sequences from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). Primer sequences are as follows: MMP-9 forward primer, CCG CAA CTA TGA CCA GGA TA; MMP-9 reverse primer, CTT CAT GAG GTA GTC AGT CAG GTC. Results were stored in PBS at 4°C overnight. The next day, staining for MMP-9 was performed using rabbit antirat MMP-9 polyclonal primary antibody AB19016 (Chemicon International), rabbit IgG Vectastain secondary antibody (Vector Laboratories), and Vector Red alkaline phosphate stain.

Expression of the target gene in ratio to β-actin expression was calculated using the formula:

$$\Delta C_t = C_{\text{target gene}} - C_{\beta-actin}$$

Quantitative Gel Zymography

MMP-9 distribution after elastase perfusion was determined by zymography as previously described. Gelatinase activity was evident by clear bands against a dark blue background. The molecul-
ular weight of each band was determined by comparison of the bands against samples containing human recombinant MMP-9 (Oncogene, Boston, Mass). In previous studies, these bands were inhibited by EDTA and are thus metalloproteinases. Semiquantitative measurements were performed using densitometry as described and normalized to total protein.

**Densitometry**

Gels were imaged with a FOTO/Analyst charge-coupled device CAMERA (Fotodyne, Hartland, Wis). Band strengths were quantified using GEL-Pro Analyzer software version 3.1 (Media Cybernetics, Silver Springs, Md).

**Total Protein Assay**

Total cellular protein was determined by a bicinchoninic acid protein assay (Pierce, Rockford, Ill) in aortas on which MMP-9 activity assays were performed after they had been solubilized in 0.1% sodium dodecyl sulfate.

**Data Analysis**

Data are represented as mean±SE. Data were assessed by nonpaired t test or ANOVA with statistical significance assigned as P<0.05. When significance was reached, post hoc Tukey test was used to compare individual groups. Statistical analysis was performed using Prism software (GraphPad Software, San Diego, Calif).

**Results**

**Baseline Histology in Male Versus Female Rats**

Male and female rats’ aortas not subjected to any intervention were harvested and subjected to histological analysis. Male and female aortas were nearly identical in wall thickness and aortic lamellar structure (data not shown) by Verhoeff-Van Gieson stain. Aortas from both genders were indistinguishable by an experienced pathologist (E.P.K). CD3 immunohistochemistry demonstrated little to no lymphocytic infiltrate in the aortas of either males or females (data not shown).

**Experimental AAA Formation in Male Versus Female Rats**

Preperfusion baseline aortic diameters were not different (P=0.20) between male and female aortas (1.41±0.16 versus 1.32±0.07, respectively). The mean increase in aortic diameter 14 days after elastase perfusion in male aortas was 200±37.6%, whereas female aortas had a mean aortic diameter increase of 69.4±26.5% (P=0.0234; Figure 1a). The incidence of AAAs defined as an increase in aortic diameter at least 100% from preperfusion diameter was 82% in male rats compared with 29% in female rats (P=0.0229; Figure 1a).

Male aneurysmal aortas exhibited circumferential disruption of the elastic lamellae 14 days after elastase perfusion, whereas the aortas of female rats after elastase perfusion had largely intact elastin fibers (please see www.ahajournals.org). CD3 staining demonstrated minimal lymphocyte infiltration in either male or female aortas (data not shown). ED-1–positive cells were located primarily in the adventitia and media, consistent with previous reports. Male aortas exhibited increased MMP-9 expression by quantitative PCR compared with female aortas (males, 0.39±0.09 versus 0.003±0.001 MMP-9 mRNA copies; P=0.001; please see www.ahajournals.org). Total MMP-9 activity by zymography was 369% greater in male than female aortas (P=0.022; please see www.ahajournals.org).

**Aneurysm-Resistant Phenotype Is Lost After Transplantation of the Female Into the Male**

All male and female aortas, when transplanted into male recipients and subsequently subjected to elastase perfusion, evidenced by immunohistochemistry (Figure 1b and 1c). Colocalization of ED-1 and MMP-9 demonstrated increased costaining of macrophages and MMP-9 in male aortas versus female aortas (Figure 1d and 1e). Similarly, male aortas exhibited increased MMP-9 expression by quantitative PCR compared with female aortas (males, 0.39±0.09 versus 0.003±0.001 MMP-9 mRNA copies; P=0.001; please see www.ahajournals.org). Total MMP-9 activity by zymography was 369% greater in male than female aortas (P=0.022; please see www.ahajournals.org).
planted into male rats, the observed female resistance was lost. Male-to-male transplants revealed near-total destruction of the aortic medial elastic lamellar structure, whereas female-to-female transplanted aortas had more elastin preservation (data not shown). Female-to-male transplants followed a similar pattern as male-to-male transplants with near-total destruction of the elastic lamellar structure (please see www.ahajournals.org). Importantly, CD3 staining demonstrated minimal lymphocyte infiltration in any of the transplanted groups (data not shown). However, ED-1–positive macrophage staining was prominent in the media and adventitia of male-to-male and female-to-male transplanted aortas and less evident in female-to-female transplanted aortas (please see www.ahajournals.org). ED-1–positive macrophages, when quantified, were significantly higher in the male-to-male (68.5±7.4 cells/HPF) and female-to-male transplanted aortas (36.0±1.2 cells/HPF) when compared with female-to-female transplanted aortas (22.4±2.0 positive cells/HPF; P=0.0002). MMP-9 staining was more prominent in male-to-male and female-to-male transplanted aortas transplanted aortas than female-to-female transplanted aortas (Figure 2a to 2d). Co-localization of ED-1 and MMP-9 demonstrated more co-staining in male-to-male transplanted aortas and female-to-male transplanted aortas compared with female-to-female transplanted aortas (Figure 2e to 2g). MMP-9 mRNA, assessed by quantitative PCR, was also higher in the former aortas (male-to-male transplanted aortas, 0.050±0.002 mRNA copies; female-to-male transplanted aortas, 0.034±0.007mRNA copies) than in female-to-female transplanted aortas (0.005±0.002 mRNA copies, P=0.0175; please see www.ahajournals.org).

**Estradiol Effects on Aneurysmal Development**

Moderate aortic expansion at 7 days occurred in male rats receiving estradiol and sham control rats being 124%±19% versus 197%±39%, respectively (P=0.010). By 14 days, male rats receiving estradiol had significantly smaller aneurysms (241%±57) compared with sham rats (538%±105, P=0.0226; Figure 3a). Elastin fragmentation was less prominent in estradiol treated rats’ aortas (please see www.ahajournals.org). ED-1–positive cell counts were 1.8±0.3 cells/HPF in those receiving estradiol versus 5.2±0.5 cells/HPF in sham rats (P=0.0006). Aortic MMP-9 staining was also less evident in the estradiol treated rats compared with the sham rats (Figure 3b and 3c). Colocalization of aortic ED-1 and MMP-9 demonstrated less prominently stained cells in rats treated with estradiol compared with sham rats (Figure 3d and 3e). By 7 days after elastase perfusion, estradiol-treated rats exhibited less aortic MMP-9 mRNA expression (0.0017±0.004 mRNA copies) compared with sham rats (0.12±0.04 mRNA copies, P=0.11, please see www.ahajournals.org).

**Discussion**

This investigation documents that female rats are partially protected from experimental AAA formation, and male rats consistently form larger AAAs. Female rat aortas subjected to elastase perfusion exhibited less medial wall destruction, fewer infiltrating macrophages, and decreased MMP-9. Furthermore, MMP-9 expression was also decreased in the aortas of these female rats. The apparent protection that female aortas exhibited in situ was lost after their transplantation into the male rat, whereas the female aortas transplanted into other female rats main-
increased prostacyclin levels, resulting in improved vasore-

AAA formation receives indirect support from a number of

MMPs, it did suggest a gender-related effect of nitric oxide

Lee et al demonstrated no protection from experimental AAA

male and female animals in this experimental AAA model.

This suggests that estrogens may not act in aortic develop-

but rather to maintain structure and prevent aortic
dilatation. This is supported by the observation that women
appear to have a delay in their development of abdominal
aortic aneurysms until after menopause.

It is generally accepted that macrophages are the primary
source for MMP-9 in experimental and human AAAs. In the
current investigation, estradiol inhibited aortic macrophage
infiltration and MMP-9 production. Thus, estradiol may
effect AAA development by indirectly inhibiting the influx of
macrophages and directly by its inhibitory effect on macro-
phage and smooth muscle cell production of MMPs. Re-
cently, estrogen treatment of U937 cells have been shown to
decrease MMP-2 production. Estradiol may effect MMP-9
similarly.

The effect of estradiol on macrophages and the immune
system is not novel. For instance, it has been shown that
estrogen has a direct inhibitory effect on macrophage recruit-
ment, as well as on monocyte chemoattractant protein-1. In a
d mouse encephalitis model, estrogen inhibited monocyte
infiltration into the inflamed tissue. Furthermore, increased
estrogen levels in women, including those using estrogen
replacement therapy, correlated with reductions in circulating
monocyte chemoattractant protein-1 levels. These data sup-
port speculation that estrogens may inhibit macrophage infil-
tration, MMP-9 expression, and subsequent destruction of the
aortic wall.

Certain limitations exist in the current investigation. First,
elastase perfusion of the aorta has been argued to be an acute,
rapid model of AAA formation that may not mimic human
AAA development. Nonetheless, the primary cell involved in
the elastase model is the macrophage, which is the primary
source for MMP-9 in human AAAs. Other cells that may
be involved, such as smooth muscle cells, were consequently
not examined in this investigation. In addition, many other
proteases known to be upregulated in human AAAs and that
are consistently elevated in elastase-perfused experimental
AAAs were not examined in the present study. This does not
preclude a role for other cell types or other proteases in the
observed gender-related differences in experimental AAA
formation. Second, transplantation of the aorta, although
designed to alter the hormonal environment of the donor
aorta, may in and of itself result in a local inflammation in the
retroperitoneum. Previous work by Ailawadi et al suggests
that ED-1-positive cells are increased after transplantation
compared with native aortic explants. The lack of CD3-
positive lymphocytes and the few architectural differences

Figure 3. Estradiol-treated male aortas subjected to elastase
perfusion. a, Percent increase in aortic diameter in male sham
(MS) and male estradiol-treated (ME) rats before elastase perfu-
sion and at 7 and 14 weeks after elastase perfusion. By 14
days, ME had significantly smaller AAAs ($P < 0.0226$). Immuno-
histochemical staining for MMP-9 (red, arrow) was more exten-
sive in MS (b) compared with ME (c) (100×). Colocalization of
ED-1 (blue) and MMP-9 (red) demonstrates more prominent
staining in MS (d) than ME (e) (100×).
other than those described does suggest that rejection is not involved in this process after transplantation or elastase perfusion. Despite this lack of perceived differences, comparisons between transplanted elastase-perfused aortas and nontransplanted elastase-perfused aortas cannot be made. Third, 2 different lots of elastase were used in the present investigation and may have resulted in varied results. The first lot was used for elastase perfusion of aortas in the first and second group of experiments (intact and transplanted animals), whereas the latter lot was used for the third group of experiments (those treated with estradiol). These elastase lots were quite different, in as much as the nontransplanted and transplanted elastase-perfused male aortas increased their aortic diameter by \( \approx 200\% \), whereas elastase-perfused male aortas used in the estradiol-treatment experiments developed almost 500% increases in their aortic diameter using a different lot of elastase. Such variation has been reported with different lots of elastase despite uniform dose and activity.\(^4\) As a consequence, groups treated with different lots of elastase should not be compared, with comparisons limited only to animals treated with the same lot of elastase.

Despite these limitations, this investigation supports the theory that gender differences in experimental AAA formation exist that may be related to estrogenic effects on macrophages and MMPs. Gender differences in other cell lines and proteases, as well as cytokines, must be better-evaluated to further completely characterize the disparity between men and women with regard to AAA formation. The efficacy of estradiol will need to be better-delineated before the institution of this or related hormones as a potential therapeutic agent.

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References


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