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BASIC RESEARCH STUDIES

Pharmacologic blockade and genetic deletion of androgen receptor attenuates aortic aneurysm formation

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Background: **Testosterone is theorized to play a major role in the pathophysiology of abdominal aortic aneurysms (AAAs) because this disease occurs primarily in men. The role of the androgen receptor (AR) in the formation of AAAs has not been well elucidated, and therefore, it is hypothesized that androgen blockade will attenuate experimental aortic aneurysm formation.**

Methods: **Aortas of 8- to 12-week-old male C57Bl/6 wild-type (WT) mice or male AR knockout ($AR^{-/-}$) mice were perfused with purified porcine pancreatic elastase (0.35 U/mL) to induce AAA formation. Two groups of WT male mice were treated with the AR blockers flutamide (50 mg/kg) or ketoconazole (150 mg/kg) twice daily by intraperitoneal injection. Aortas were harvested on day 14 after video micrometry was used to measure AAA diameter. Cytokine arrays and histologic analysis were performed on aortic tissue. Groups were compared using an analysis of variance and a Tukey post hoc test.**

Results: **Flutamide and ketoconazole treatment (mean ± standard error of the mean) attenuated AAA formation in WT mice (84.2% ± 22.8% [$P = .009$] and 91.5% ± 18.2% [$P = .037$]) compared with WT elastase (121% ± 5.23%). In addition, $AR^{-/-}$ mice showed attenuation of AAA growth (64.4% ± 22.7%; $P < .0001$) compared with WT elastase. Cytokine arrays of aortic tissue revealed decreased levels of proinflammatory cytokines interleukin (IL)-α, IL-6, and IL-17 in flutamide-treated and $AR^{-/-}$ groups compared with controls.**

Conclusions: **Pharmacologic and genetic AR blockade cause attenuation of AAA formation. Therapies for AR blockade used in prostate cancer may provide medical treatment to halt progression of AAAs in humans. (J Vasc Surg 2016;63:1602-12.)**

Clinical Relevance: **Abdominal aortic aneurysms (AAAs) carry a significant mortality and morbidity. Currently, a medical cure is not available for AAAs. This work presents a potential target for medical treatment of AAAs: the androgen receptor (AR). Pharmacologic agents approved for prostate cancer reveal attenuation of experimental aneurysm formation and decreased proinflammatory cytokine profile in aortic tissues. Furthermore, genetic deletion of AR reveals similar results. Therefore, AR appears to play a critical role in aneurysm formation, and pharmacologic agents currently used for prostate cancer treatment may play a role in inhibition of aneurysm progression and prolong time to definitive repair.**

The incidence of abdominal aortic aneurysms (AAAs) is ~1.2% to 1.4% and is most prevalent among elderly male smokers.[1-4] Aortic aneurysms are a significant clinical problem because this disorder was primarily responsible for nearly 10,500 deaths in 2009.[5] The pathophysiology of aneurysm degeneration of the aorta involves increased elastin degradation, increased cytokine production, smooth muscle cell apoptosis, and dysregulation of matrix metalloproteinases, although the specific pathophysiology is unclear. Androgens, specifically testosterone, have been

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thought to play a role in aortic aneurysm formation[6,7]; however, the role of the androgen receptor (AR) in AAA development has not been well elucidated.

The androgen pathway has been a therapeutic target for male-specific diseases such as prostate cancer. Flutamide is an AR antagonist that historically has been shown to be effective at androgen depletion when combined with gonadotropin-releasing hormone agonists such as leuprolide.[8-10] Ketoconazole has also been shown to be an effective therapy for prostate cancer given its inhibition of the conversion of cholesterol to pregnenolone, which is a critical step during androgen synthesis.[11-13]

AR blockade has certainly proven beneficial in androgen-dependent disease states such as prostate cancer. Given the male preponderance for AAA, it is hypothesized that there is a fundamental difference in AR expression between male and female AAA tissues and that pharmacologic blockade and genetic deletion of the AR will influence the formation of experimental AAA in a murine model.

METHODS

The animal experimental protocols in this study were approved by the University of Virginia Institutional Animal Care and Use Committee (IACUC Protocol #3648 and #3634) in compliance with the Office of Laboratory Animal Welfare.

Mice. Wild-type (WT) C57Bl/6 mice and AR knockout mice ($AR^{-/-}$; strain B6.Cg-A^{w-J} $Eda^{Ta-6J+/+}$ $ArT^{fm/J}$, stock No. 001809) were ordered from Jackson Laboratories (Bar Harbor, Me) and maintained in-house. All mice were fed a minimal phytoestrogen diet (2016 Teklad Diet; Harlan Laboratories, Indianapolis, Ind) to decrease any anti-inflammatory effects of excess dietary estrogen.[14]

Aneurysm model. All mice underwent elastase perfusion, as previously described.[15-18] Male C57Bl/6 WT mice (8-12 weeks old) underwent induction of anesthesia with inhaled isoflurane, and anesthesia was maintained by inhaled isoflurane. The infrarenal aorta was isolated via midline laparotomy and perfused with 0.35 U/mL porcine pancreatic elastase (Sigma-Aldrich, St. Louis, Mo) for 5 minutes. Lower extremity perfusion was resumed after aortotomy repair and evacuation of residual elastase. Postoperative analgesia was provided with buprenorphine.

After 14 days, the mice underwent reoperation where video micrometry was used to estimate the size of maximal aortic dilation relative to an unperfused suprarenal aortic control segment. Blood was drawn from the inferior vena cava and left renal vein junction, and aortas were harvested from the level of the renal vein to the iliac bifurcation at euthanasia.

Negative controls were created by heating elastase at 99°C for 30 minutes, creating heat-inactivated elastase (HIE) and using it in place of active elastase or saline for perfusion. Elastase perfusion allows for chemical proteolysis of the aortic media, as well as mechanical disruption of the media, while perfusion with HIE or saline allows for mechanical dilation only.

All harvested aortas were (1) snap frozen in liquid nitrogen for real-time polymerase chain reaction analysis or protein extraction or (2) incubated overnight in 4% paraformaldehyde solution for immunohistochemistry or histology. Blood was centrifuged, and the serum was frozen at −80°C.

Human aortic tissues. AAA tissue was procured from patients undergoing elective open AAA repair at the time of surgery. Control abdominal aortic tissue was taken from transplant donors. Patients who donated aortic samples did not have a known collagen vascular disorder or dissection. Aortic samples were explanted and immediately flash frozen in liquid nitrogen. Patients gave written consent for collection of aortic aneurysm tissue in compliance with the University of Virginia Human Subjects Review Committee (Institutional Review Board #13178).

Quantification of ARs in aortic tissues. Normal human infrarenal abdominal aortic tissue and AAA tissue underwent analysis for messenger RNA (mRNA) expression of the AR (10 male and 10 female patients). C57Bl/6 WT mice (8-12 weeks old) were perfused with elastase or saline (n = 6 per group). Tissues were harvested on days 0, 3, 7, and 14 and underwent analysis for levels of mRNA specific for AR and for 18s.

Pharmacologic blockade of ARs. C57B/Bl6 mice (8-12 weeks old) underwent aortic perfusion with elastase (n = 7) or saline (n = 9). Two experimental treatment arms of perfused mice then underwent subcutaneous implantation with osmotic pumps (Alzet, Cupertino, Calif) containing flutamide (50 mg/kg) or ketoconazole tablets (150 mg/kg; Fig 1, *A*) at the time of the aortic elastase perfusion to give steady states of drug delivery. Additional mice were perfused with HIE for negative controls (n = 9). To examine an alternate route of drug delivery, two additional groups of mice were given 50 mg/kg of flutamide (n = 7) and 150 mg/kg ketoconazole (n = 8) by intraperitoneal injection twice daily for 14 days after elastase perfusion (Supplementary Fig 1, online only). It is important to note the variance of the mechanism of action of each pharmacologic agent: flutamide is an AR blocker, whereas ketoconazole inhibits the conversion of cholesterol to pregnenolone, an upstream step important in the formation of testosterone.

Genetic deletion of ARs. C57B/l6 mice (8-12 weeks old) underwent aortic perfusion with elastase (n = 7) or HIE (n = 9). An additional arm of 8- to 12-week-old $AR^{-/-}$ mice underwent aortic perfusion with elastase (n = 8; Fig 1, *B*).

Cytokine array. For the purpose of determining the effects of pharmacologic blockade and genetic deletion of ARs and to determine its effect on the proinflammatory cytokine milieu of the aortic wall, mouse cytokine arrays (R&D Systems, Minneapolis, Minn) were performed using isolated protein from mouse aortas harvested at day 14 after elastase perfusion. Protein samples from each group were pooled for analysis, and all samples were run in duplicate.[15-18]

Histology. Infrarenal aortas were harvested at euthanasia and irrigated with normal saline. Fixation was achieved with overnight incubation in 4% paraformaldehyde, followed by paraffin embedding and sectioning at 5 μm. Microwave antigen retrieval was performed, and antibodies were bound and detected using Vecta Stain Elite Kit (Vector Laboratories, Burlingame, Calif). Verhoeff-Van Gieson stain was used to evaluate aortic elastin content. Antibodies used for immunohistochemistry included (1) anti-rat Mac2 for macrophages

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