Cardiovascular Pathology 23 (2014) 152–159

Contents lists available at ScienceDirect

Cardiovascular Pathology

Original Article

Inadequate reinforcement of transmedial disruptions at branch points subtends aortic aneurysm formation in apolipoprotein-E-deficient mice

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form 26 December 2013
Accepted 30 December 2013

Keywords:
Aneurysm
Abdominal aortic aneurysm
Angiotensin-II
Apolipoprotein e-deficient mice

ABSTRACT

Introduction: Infusion of angiotensin-II (Ang-II) in apolipoprotein-E-deficient mice (Apo-$E^{-/-}$) results in suprarenal abdominal aortic aneurysm (AAA) in 30–85% of cases. This study identifies the apparent mechanism by which some animals do, but others do not, develop AAA in this model.
Methods: Male Apo-$E^{-/-}$ mice were infused with Ang-II (n=21) or saline (n=6) and sacrificed at 4 weeks. Aortas were excised, embedded in paraffin, sectioned (250 μm intervals), and stained. Sites of transmedial disruption (TMD) were identified and characterized, and their relationship to the 4 major aortic side branches (celiac, superior mesenteric, and renals) were determined.
Results: The frequency of TMDs in Ang-II-infused mice that formed AAA (n=9) was similar to those that did not (n=12) (AAA vs. no-AAA: 25 of 36[69%] vs. 28 of 48[58%] branches, P=.3 by chi-square). All TMDs were at branch points. However, in animals with AAA, the mean maximum length of the TMDs was significantly larger (1.94±1.6 vs. 0.65±0.5mm, P=.007 by Mann Whitney U test), the #mac-2^+ macrophages per 0.01mm^2 of defect area was greater (32±10 vs. 19±11, P<.02 by Kruskal–Wallis with Conover–Inman post hoc), the % area of attempted repair occupied by collagen was less (17±13% vs. 44±15%, P=.0009 by Mann Whitney U test), and the density of collagen per unit length of media missing was also markedly less (0.13±0.2 vs. 1.14±1.0, P=.0001 by Mann Whitney U test).
Conclusions: Reinforcement of transmedial defects at branch points by wall matrix is a key intrinsic player in limiting AAA formation in the Ang-II-infused, Apo $E^{-/-}$ mouse and a potentially important mechanism-based therapeutic target for management of small, slowly progressing aneurysms.

1. Introduction

Histopathologic studies of human abdominal aortic aneurysms (AAA) have been performed primarily on severely advanced aneurysms excised at autopsy or resected at surgery after exceeding 5–6 cm maximum anterior-posterior diameter. Such specimens, frequently complicated by mural thrombus, extensive calcific deposits, intramural hemorrhage, and necrotic pultaceous debris, provide very little information concerning the initial structural changes in the arterial wall at the beginning of aneurysm formation [1–8].

Findings from a variety of animal models of aneurysm formation suggest an inflammatory background of the initial events [9]. Infusion of angiotensin-II (Ang-II) in the apolipoprotein-E-deficient (Apo $E^{-/-}$) mouse results in suprarenal AAA in 30–85% of cases [10]. In this model, early medial disruption accompanied by inflammatory infiltrates was found to be the initial detectable event in aneurysm formation. This is followed by progressive lumen expansion, transmural disruption, media-adventitial dissection, fibromuscular hyperplasia, and intramural hemorrhage and thrombus formation [10–12].

In previous studies using high-frequency ultrasound with correlative histomorphometry, we showed that low-level laser (LLL) phototherapy, a non-thermal, near-infrared radiation, prevents AAA formation in the Ang-II-infused ApoE$^{-/-}$ model by enhancing matrix reinforcement and modification of the inflammatory response at sites of transmedial injury [13,14] (i.e., defects traversing all smooth muscle layers of the media and intervening elastica). However, in that study, only half of the angiotensin-infused control animals (without LLL) developed aneurysms. In the current study we sought to

Funding: This work was supported by The Israel Science Foundation [grant no. 1298/10]; The Rosetrees Trust Fund of the United Kingdom; and Professor Eliyahu Kelman of Jerusalem. SDG is the Brandman Foundation Professor of Cardiac and Pulmonary Diseases of The Hebrew University of Jerusalem.
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http://dx.doi.org/10.1016/j.carpath.2013.12.005

determine why some angiotensin-infused ApoE$^{-/-}$ mice do, and others do not, develop aortic aneurysms in this model (none of which received phototherapy) and to point out the relevance of these findings to the pathogenesis of AAA in man.

2. Methods

2.1. Study design

This study was performed on 27 C57/Black6, Apo E$^{-/-}$ mice age 12–13 weeks. Angiotensin-II (Calbiochem, La Jolla, CA, USA) was infused in 21 of the 27 mice via osmotic minipumps implanted subcutaneously on the right flank through an incision in the scapular region (Alzet, model 2004, Durect, Cupertino, CA, USA; infusion rate, 1000 ng/kg per minute). The abdominal aortas of all angiotensin-infused mice were exposed retroperitoneally through a left subcostal incision at the outset of the experiment since these mice served as the non-treated control group for the study of the effect of LLLI on aneurysm progression [13,14]. Six additional mice were infused with saline instead of angiotensin. All animals were sacrificed after 28 days. Angiotensin-infused animals that developed suprarenal AAAs (n=9) were compared with angiotensin-infused animals that did not develop aneurysms (n=12) and to animals infused with saline (n=6) (Table 1).

2.2. Mice

The mice were bred in-house from stock originating from Jackson Laboratories. The mice were housed in a specific pathogen-free environment. Water and food (normal diet) were available ad libitum. Prior to the procedure, mice were injected subcutaneously with ketamine (200 mg/kg) and xylazine (10 mg/kg) for anesthesia, tramadol (2 mg/kg) for analgesia, and cefamizine (30 mg/kg) for prophylaxis. Chloramphenicol ointment (5%) was applied to the eyes to prevent corneal damage and infection. This investigation conforms with the Guide for the Care and Use of Laboratory Animals (NIH Publication 85–23, revised 1996). Animal care and experimental procedures were approved by the Ethics Committee of the Faculty of Medicine of The Hebrew University, Jerusalem, Israel (MD-07-10349-3).

2.3. Sacrifice and macro-photography

At 28 days, the animals were sedated (sodium pentobarbital) and perfused with saline (0.9%, 100mmHg) followed by paraformaldehyde (4% in phosphate-buffered saline). The abdominal aorta was exposed through an anterior thoraco-abdominal incision and marked in situ with tissue dyes (Polysciences, Warrington, PA, USA) for identification of the level of the diaphragm, renal bifurcation, and infrarenal region. A 25G needle was placed next to the vessel for reference. The aorta and reference needle were photographed using a mounted Canon EOS DS126151 (Canon Japan) with a Canon EF-S 60mm, 1:2.8 USM macro-lens (Canon Taiwan) (Fig. 1).

Table 1
Experimental groups

Group	Infusion	Aneurysm	n
AngII-AAA	Angiotensin-II	+	9
AngII-no AAA	Angiotensin-II	-	12
Sal	Saline	-	6
Total			27

n=number of animals.

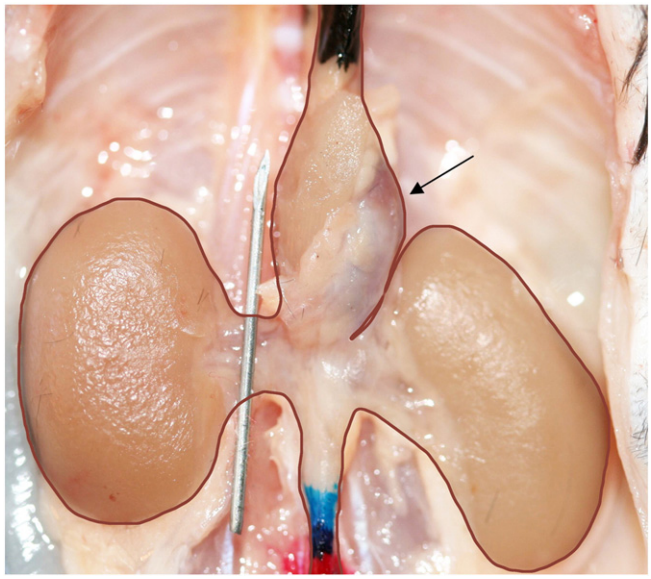

Fig. 1. Suprarenal AAA 4 weeks after angiotensin-II infusion in the Apo-E$^{-/-}$ mouse. Black tissue dye = level of diaphragm. Needle standard is 0.5 mm. diameter. Note hemorrhage into the wall of the aneurysm (arrow).

2.4. Tissue sampling and preparation for histology

The abdominal aorta, including the suprarenal aneurysm-prone (SR) and infrarenal (IR) segments of all animals were excised, postfixed, dehydrated, embedded in paraffin, and sectioned (5μm thick at 250-μm intervals). The mean number of aortic sections per mouse = 30±5. Sections were stained with H&E, Movat pentachrome, and picrosirius red (PSR) for collagen. PSR-stained sections were studied using bright field and polarized microscopy (BX51, Olympus Japan) with the polarizer mounted on a U-AN360 rotator disc for optical adjustment to the axis of polarization.

2.5. Immunofluorescence

Immunofluorescent staining was performed to identify macrophages and smooth muscle cells. Rat anti-mouse Mac2 (Galectin-3) (M3/38 hybridoma cell line, American Type Culture Collection, Manassas, VA, USA) was used as the primary antibody for detection of macrophages, and rabbit anti-α-smooth muscle cell actin (ab5694, Abcam, Cambridge, MA, USA) was used for detection of vascular smooth muscle cells (SMC). The matching secondary antibodies were Cy2-Goat anti-Rat IgG and Cy3-Goat anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Product#112-225-167 and 111-165-144 respectively, both 10 mg/ml). Hoechst solution-33258 was used for staining nuclei (#94403 Sigma-Aldrich, St. Louis, MO, USA).

Sections were deparaffinized and rehydrated. Antigen retrieval was performed in 10 mmol/l citrate buffer (pH 6.0, boiling, 15 min) After cooling, sections were washed and blocked for 2 h (room temperature) with 10% normal goat serum with 1% bovine serum albumin. Sections were double stained by incubation with Mac2 (dilution 1:10) and α-SMC-actin (1:300) overnight at 4°C. The sections were then incubated with the secondary antibodies for 1hr in the dark (room temperature) followed by staining with Hoechst. The slides were covered and studied by fluorescence microscopy (BX51, Olympus Japan).

2.6. Identification of aneurysm

Aneurysms were defined by reference to saline-infused animals: an animal was considered to have an aneurysm when the maximum suprarenal (SR) cross-sectional diameter (CSD) was at least 50% greater than the mean maximal SR-CSD of the saline infused group. The aortic