

Adrenomedullin Does Not Contribute toward the Development of Abdominal Aortic Aneurysm in Mice

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Abstract

Abdominal aortic aneurysm (AAA) develops based on advanced atherosclerosis; however, the underlying pathogenesis remains unknown. Adrenomedullin (AM) is widely expressed in various human and rodent tissues, and has been reported to protect blood vessels. We have previously reported that AM is produced in mast cells of human AAA and that AM exhibited the antifibrotic activity of mast cell-derived AM on fibroblasts. In the present study, we investigated the role of AM in the development of AAA in 12-week-old male apolipoprotein (apo)E^{-/-} mice bred with AM heterozygous, or its role when recombinant human (rh) AM was administered to the apoE^{-/-} male mice, which was infused with 1000 ng/kg/min of angiotensin II (Ang II) for 28 days. The incidence of the development of AAA in Ang II-infused apoE^{-/-} AM^{+/-} mice did not change compared with that in apoE^{-/-} mice (33.3% vs. 47.4%, $p = 0.2333$). In addition, rhAM administration had little effect on the incidence of the development of AAA formation (AM: 0 ng/kg/hr 47.4%; 300 ng/kg/hr 36.4%; 3000 ng/kg/hr 27.3%; $p = 0.2595$). In conclusion, this study suggests that AM does not contribute toward the development of AAA.

Keywords

Aneurysm, Angiotensin II, Aortic Rupture

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1. Introduction

Abdominal aortic aneurysm (AAA) is a relatively common disorder among individuals who are >60 years of age [1]. AAA develops based on advanced atherosclerosis [2], and it is pathologically characterized by an enlarged aortic lumen accompanied by a degenerated medial layer [3]. Chronic inflammation is predominantly observed in the outer-media and adventitia of the aneurysm wall [4] [5], and immune and inflammatory responses have been suggested to contribute toward pathogenesis of AAA [2] [6] [7]. However, the underlying mechanism of AAA progression remains unclear. Currently, medical therapy to attenuate or regress the development of AAA is unavailable.

Adrenomedullin (AM) was originally isolated from human pheochromocytoma [8]. It is a 52-amino acid peptide containing an intramolecular disulfide bond that forms a ring-like structure of six residues. AM is widely distributed among various tissues and organs, including the heart and aorta [9]-[11]. AM decreases the blood pressure and it inhibits cellular proliferation in the heart and blood vessels [8] [12] [13]. In addition, AM attenuates both the oxidative stress and inflammatory responses [14] [15]. We reported that AM was produced in mast cells present in the outer media and adventitia of AAA in humans, and discussed its possible involvement in the development of AAA by decreasing collagen synthesis *in vitro* [11].

Lipoprotein disorders are suggested to modify the atherogenesis and increase the cardiovascular risk [16]. Apolipoprotein E (apoE) can clear the cholesterol-enriched lipoprotein, and plays a protective role against atherosclerosis [17]. Mice inactivating the gene exhibit a marked increase in plasma level of total cholesterol, accompanied by progression of atheromatous plaque [18] [19]. Furthermore, administration of angiotensin II (Ang II) to the mice is susceptible to develop the AAA formation [20].

On the basis of previous studies, we hypothesized that AM might be involved in the development of AAA. In the present study, we conducted two experiments to clarify the role of AM in the development of AAA. In the first method, we administered subcutaneous Ang II to AM^{+/-} and apoE^{-/-} double knockout (apoE^{-/-} AM^{+/-}) mice. In the second, we administered recombinant human (rh)AM to Ang II-infused apoE^{-/-} mice.

2. Materials and Methods

2.1. License for Experiment

This study was conducted in accordance with the Animal Welfare Act and with the approval of both the University of Miyazaki Institutional Animal Care and Use Committee (#2007-541-5) and the University of Miyazaki Committee for Genetic Modification (#151-3 and #230). This investigation also conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Mice

Paired apoE^{-/-} mice (B6.129P2-Apoe^{tm1Unc}/J, background strain C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA), and were bred at our institution. AM^{+/-} mice were developed as previously reported [15]. ApoE^{-/-} AM^{+/-} mice were bred at our institution. Mouse genotyping was performed using genomic DNA extracted from tail biopsies. Specific genotyping for the apoE^{-/-} mice was performed by polymerase chain reaction (PCR: Gene Amp@ PCR System 2700, Life Technology Japan, Tokyo, Japan) with the following primers: oIMR0180 (5'-GCCTAGCCGAGGGAGAGCCG-3') as the wild-type sense primer, oIMR0181 (5'-TGTGACTTGGGAGCTCTGCAGC-3') as the antisense wild-type primer and oIMR0182 (5'-GCCGCCCGACTGCATCT-3') as the apoE mutant antisense primer (Jackson Laboratory). The PCR was performed in the following three phases: 94°C for 3 min; 35 cycle of 95°C for 20 s, 68°C for 40 s, and 72°C for 1 min; and 72°C for 7 min. A 245-base pair (bp) PCR fragment was generated from a mutated apoE locus, whereas a 155-bp PCR fragment was generated from the wild-type locus (Figure 1). Specific genotyping for the AM knockout mice was performed by PCR with the following primers: (5'-GGCTCCTTAAGTTGCGCA-3') as the sense primer and (5'-ACGTAGAAGAAGTATTAAACCGCA-3') as the antisense primer [15]. The PCR was performed in the following three phases: 94°C for 3 min; 35 cycles of 95°C for 30 s, 63°C for 1 min, and 72°C for 1 min; and 72°C for 7 min. A 380-bp PCR fragment was generated from a mutated AM locus, whereas a 300-bp PCR fragment was generated from the wild-type AM locus (Figure 1).

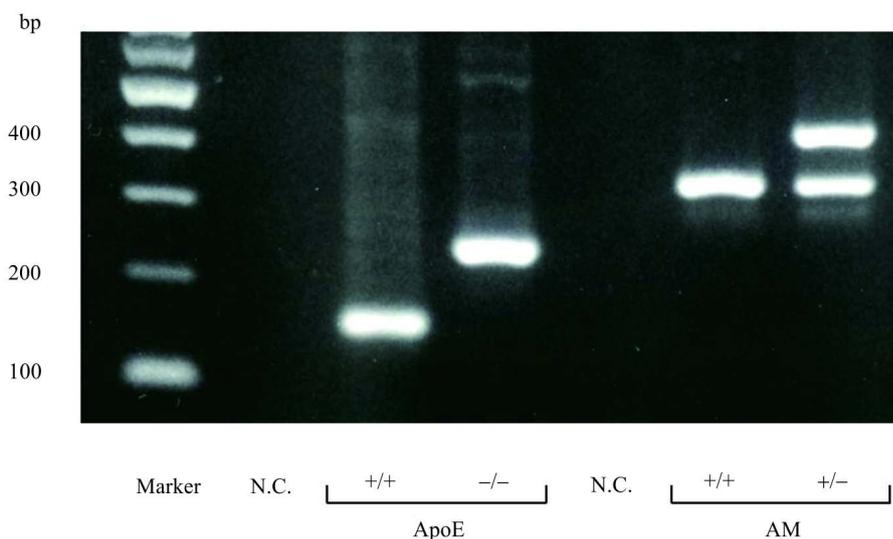


Figure 1. PCR amplification electrophoretogram of apoE^{+/+} (155-bp), apoE^{-/-} (245-bp), AM^{+/+} (300-bp) and AM^{+/-} (300- and 380-bp). bp, base pair. N.C. indicates negative control.

2.3. Induction of AAA

Mice were housed in a temperature- and light-controlled room (25°C ± 1°C; 12/12-hour light/dark cycle) with free access to normal chow and water until the end of the experiment. At 12 weeks of age, male mice were anesthetized by intraperitoneal administration of 2,2,2-tribromoethanol (Avertin) at 200 mg/kg, and osmotic mini-pumps (Alzet Model 1004; DURECT Co., Mountain View, CA, USA) were subcutaneously implanted.

Protocol 1: Osmotic mini-pump filled with either saline or Ang II (1000 ng/kg/min, Sigma Chemical Co., St. Louis, MO, USA) was implanted in apoE^{-/-} mice and apoE^{-/-} AM^{+/-} mice for 28 days.

Protocol 2: Osmotic mini-pumps filled with either saline or Ang II (1000 ng/kg/min) in the presence or absence of rhAM (300 or 3000 ng/kg/hr SHIONOGI & CO., LTD., Osaka, Japan) were subcutaneously implanted into the apoE^{-/-} mice for 28 days.

At the end of 28 days, the mice were anesthetized by intraperitoneal administration of 85 mg/kg pentobarbital sodium: their aortas were harvested and perfused with 4% paraformaldehyde.

2.4. Blood Pressure and Heart Rate

Tail-cuff blood pressure was recorded on day 28 using a noninvasive computerized tail-cuff system (BP98A Softron, Tokyo, Japan) while the animal was conscious. The mean values of blood pressure and heart rate were determined from at least three successful measurements after 2 days of training.

2.5. Analysis and Quantification of AAA

For quantifying the incidence of aneurysm, we defined an aneurysm as an increase in the external width of the suprarenal aorta by 50% or greater compared with aortas from saline-infused mice. The average diameter of the normal suprarenal aorta in control mice was 0.96 mm. Therefore, as evidence of the development of an aneurysm, we set a threshold of 1.44 mm. The diameter of the supra-renal aorta was measured by WinRoof software, version 5.6 (Mitani Co., Tokyo, Japan).

2.6. Statistical Analysis

All results are expressed as mean ± standard error of the mean (SEM). The Mann-Whitney test was used for comparing continuous variables. Fisher's exact test was used for comparing categorical variables. Survival curves were estimated using the Kaplan-Meier method, and data were compared by the log-rank test. Tests were conducted using PRISM 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Systolic Blood Pressure, Heart Rate and Body Weight

Table 1 shows that systolic blood pressure increased significantly ($p < 0.05$) in all mice infused with Ang II compared with those infused with saline. There was no significant difference in systolic blood pressure between apoE^{-/-} and apoE^{-/-} AM^{+/-} mice infused with Ang II. In addition, rhAM infusion (300 and 3000 ng/kg/hr) did not affect systolic blood pressure in Ang II-induced apoE^{-/-} mice. There was no significant difference in heart rate and body weight among five groups.

3.2. Mortality and Cause of Death

The 28-day survival rate did not differ significantly among the five groups (log-rank test, $p = 0.2735$) (**Figure 2(a)**), although Ang II-infused apoE^{-/-} mice treated with 3000 ng/kg/h of AM tended to have a high mortality rate and a high incidence of aortic rupture compared with the other groups (**Figure 2(b)**). The causes of death were as follows: pleural hemorrhage ($n = 1$) in apoE^{-/-} mice infused with Ang II; pleural hemorrhage ($n = 1$) and unknown cause ($n = 1$) in apoE^{-/-} AM^{+/-} mice infused with Ang II; pleural hemorrhage ($n = 2$) and intra-abdominal hemorrhage ($n = 2$) in Ang II-infused apoE^{-/-} mice treated with 3000 ng/kg/h of rhAM.

3.3. Incidence of AAA

Figure 2(c) shows the incidence of the development of AAA in mice that survived for 28 days. Ang II-infused apoE^{-/-} AM^{+/-} mice and Ang II-infused apoE^{-/-} mice exhibited insignificant incidence of the development of AAA ($p = 0.2333$). In addition, development of AAA tended to decrease with rhAM infusion at 300 and 3000 ng/kg/hr; however, the difference was not statistically significant ($p = 0.2595$).

3.4. Size of Supra-Renal Aorta

Figures 3(a)-(e) showed the representative pictures of suprarenal aorta in each group. As shown in **Figure 3(f)**, the maximal diameter of the suprarenal aorta increased significantly ($p = 0.0353$) in Ang II-infused apoE^{-/-} mice compared with those infused with saline. However, the suprarenal aortic size did not significantly change in either Ang II-infused apoE^{-/-} AM^{+/-} mice or upon administration of rhAM in Ang II-infused apoE^{-/-} mice.

4. Discussion

This study was designed to evaluate the effect of AM on the development of AAA in two ways—through the use of a genetically altered AM gene; and by exogenous administration of rhAM to Ang II-infused AAA model in mice. Based on the anti-inflammatory, -atherosclerotic and -oxidative activities of AM [14] [15] [21], we postulated that AM would protect against AAA development and rupture. However, in this study, we found that AM had little effect on these phenomena.

There are several possible explanations for the lack of beneficial effects of AM on aneurysm development. First, the incidence of Ang II-induced AAA development in apoE^{-/-} mice in this study was 47%, and was lower than the incidence observed in previous studies (75% to 100%) [22]-[24]. Moreover the maximal diameter of the

Table 1. Characteristics of apoE^{-/-} and apoE^{-/-} AM^{+/-} mice in this study.

	ApoE ^{-/-} AM ^{+/+}	ApoE ^{-/-} AM ^{+/+}	ApoE ^{-/-} AM ^{+/-}	ApoE ^{-/-} AM ^{+/+}	ApoE ^{-/-} AM ^{+/+}
Ang II 1000 ng/kg/min	(-)	(+)	(+)	(+)	(+)
rhAM ng/kg/hr	0	0	0	300	3000
BW g	28.4 ± 0.3 (4)	27.0 ± 0.5 (17)	27.3 ± 0.3 (27)	28.2 ± 0.4 (11)	27.5 ± 0.6 (16)
SBP mmHg	91 ± 1 (4)	123 ± 3* (17)	124 ± 3* (27)	126 ± 7* (11)	125 ± 6* (16)
HR /min	580 ± 6 (4)	621 ± 17 (17)	599 ± 13 (27)	610 ± 12 (11)	554 ± 16 (16)

Data are expressed as mean ± SEM. Parentheses indicate the number of mice examined. * $p < 0.05$ vs. apoE^{-/-} AM^{+/+} with saline. rhAM, recombinant human adrenomedullin; BW, body weight; SBP, systolic blood pressure; HR, heart rate; Ang II, angiotensin II.

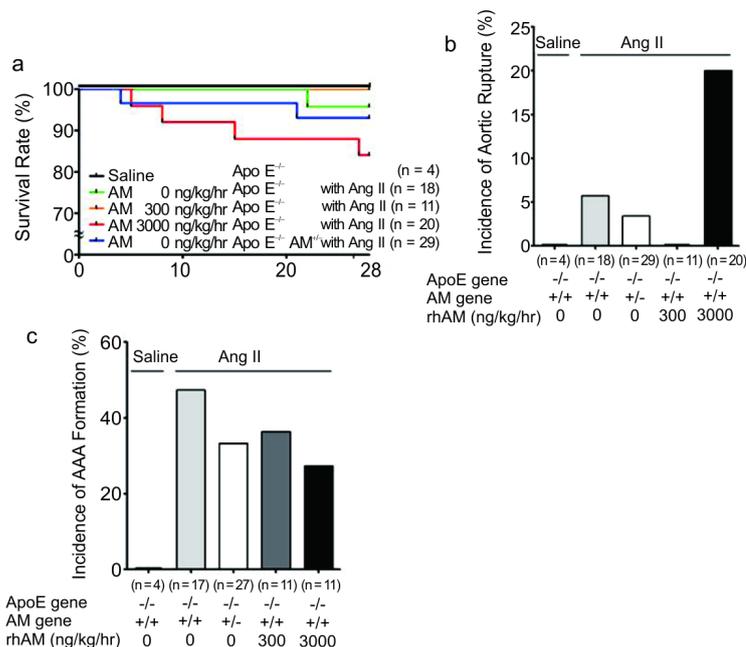


Figure 2. (a) Survival rates of: apoE^{-/-} mice infused with saline (n = 4, black) or 1000 ng/kg/min of Ang II (n = 18, green); apoE^{-/-} AM^{+/-} mice infused with 1000 ng/kg/min of Ang II (n = 29, blue); and apoE^{-/-} mice infused with 1000 ng/kg/min of Ang II and administered either 300 ng/kg/h rhAM (n = 11, orange) or 3000 ng/kg/h rhAM (n = 20, red). Incidence of aortic rupture (b) and development of AAA in mice that survived for 28 days (c) in apoE^{-/-} mice infused with either saline or 1000 ng/kg/min of Ang II; apoE^{-/-} AM^{+/-} mice infused with 1000 ng/kg/min of Ang II; and apoE^{-/-} mice infused with 1000 ng/kg/min of Ang II and administered either 300 or 3000 ng/kg/hr of rhAM. Parenthesis indicates the number of mice examined.

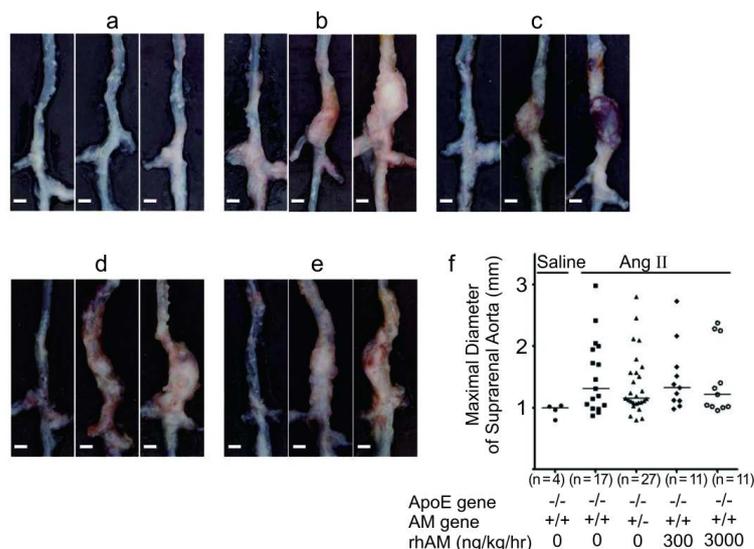


Figure 3. Representative pictures (a)-(e) and maximal diameter (f) of the suprarenal aorta in apoE^{-/-} mice infused with either saline (a) or 1000 ng/kg/min of Ang II (b); apoE^{-/-} AM^{+/-} mice infused with 1000 ng/kg/min of Ang II (c); and apoE^{-/-} mice infused with 1000 ng/kg/min of Ang II and administered either 300 (d) or 3000 ng/kg/hr (e) of rhAM. Parenthesis indicates the number of mice examined. Scale bar, 1 mm. Bars in **Figure 3(f)** indicate the median.

supra-renal aorta was widely distributed between 0.8 and 2.8 mm. These differences may have resulted from our use of younger mice that were fed normal chow. Secondly, we used heterozygous AM mice but not homozygous mice which are lethal due to the impairment of angiogenesis and lymph-angiogenesis during the embryonic development [25] [26]. Heterozygous AM mice exhibit AM levels in organs and plasma that are half the levels present in wild types [15]. Thus, we might have been unable to observe the full biological inactivity of AM in AM^{+/-} mice. We attempted to overcome this shortcoming by administering rhAM to Ang II-infused apoE^{-/-} mice. We chose to administer 300 ng/kg/hr of rhAM based on the report by Pan *et al.* [21], which showed that AM administration ameliorated vascular atherosclerotic lesions in rodents. We increased the dose up to 3000 ng/kg/hr and found that the dose rather increased the mortality. We speculate that the anti-fibrotic action of AM [11] [27] might have led to outcomes.

5. Conclusion

This study suggests that AM is unlikely to be involved in the pathogenesis of AAA development.

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Abbreviations

AAA: abdominal aortic aneurysm;
ApoE: apolipoprotein E;
AM: adrenomedullin;
rhAM: recombnant human adrenomedullin;
Ang II: angiotensin II;
BW: body weight;
SBP: systolic blood pressure;
HR: heart rate.