Pharmacologically Induced Thoracic and Abdominal Aortic Aneurysms in Mice

Yasuhiisa Kanematsu, Miyuki Kanematsu, Chie Kurihara, Tsung-Ling Tsou, Yoshitsugu Nuki, Elena I. Liang, Hiroshi Makino, Tomoki Hashimoto

Abstract—Aortic aneurysms are common among the elderly population. A large majority of aortic aneurysms are located at two distinct aneurysm-prone regions, the abdominal aorta and thoracic aorta involving the ascending aorta. In this study, we combined two factors that are associated with human aortic aneurysms, hypertension and degeneration of elastic lamina, to induce an aortic aneurysm in mice. Roles of hemodynamic conditions in the formation of aortic aneurysms were assessed using two different methods for inducing hypertension and antihypertensive agents. In 9-week-old C57BL/6J male mice, hypertension was induced by angiotensin II or deoxycorticosterone acetate-salt hypertension; degeneration of elastic lamina was induced by infusion of β-aminopropionitrile, a lysyl oxidase inhibitor. Irrespective of the methods for inducing hypertension, mice developed thoracic and abdominal aortic aneurysms (38% to 50% and 30 to 49%, respectively). Aneurysms were found at the two aneurysm-prone regions with site-specific morphological and histological characteristics. Treatment with an antihypertensive agent, amlodipine, normalized blood pressure and dramatically reduced aneurysm formation in the mice that received angiotensin II and β-aminopropionitrile. However, treatment with captopril, an angiotensin-converting enzyme inhibitor, did not affect blood pressure or the incidence of aortic aneurysms in the mice that received deoxycorticosterone acetate-salt and β-aminopropionitrile. In summary, we have shown that a combination of hypertension and pharmacologically induced degeneration of elastic laminas can induce both thoracic and abdominal aortic aneurysms with site-specific characteristics. The aneurysm formation in this model depended on hypertension but not on direct effects of angiotensin II to the vascular wall. (Hypertension. 2010;55:1267-1274.)

Key Words: aorta ▪ aneurysm ▪ hypertension ▪ angiotensin II ▪ lysyl oxidase ▪ hemodynamics ▪ remodeling

Aortic aneurysms are common among the elderly population, and their rupture results in severe mortality and morbidity. The primary purpose of surgical intervention for unruptured aortic aneurysms is to prevent future rupture. However, surgical intervention still carries significant risks of mortality and morbidity. Therefore, pharmacological stabilization of aneurysms that prevents growth and rupture of aortic aneurysms has been vigorously sought. To develop such a strategy, underlying mechanisms of aortic aneurysm formation and growth need to be elucidated in an animal model that recapitulates key features of human aortic aneurysms.

Clinically, systemic hypertension is closely associated with aortic aneurysm formations. However, a causal relationship between hypertension and aortic aneurysm has not been completely established. Degeneration and disorganization of elastic lamina are characteristic histological changes observed in both thoracic and abdominal aortic aneurysms in humans. Incidence of aortic aneurysms increases with age and aging-related degeneration of elastic lamina is often considered a precursory change that precedes aneurysm formation. Experimentally, degeneration of elastic lamina can be induced by administration of β-aminopropionitrile (BAPN), an inhibitor of lysyl oxidase. Lysyl oxidase cross-links elastin fibers and collagen fibers and plays a critical role in maintaining homeostasis of elastic lamina. With aging, lysyl oxidase activity decreases. BAPN is referred to as a lathyrorgen because its effects closely mimic human aging. Degeneration of elastic laminas has been observed in both lysyl oxidase knockout mice and Blotchy mice, which have decreased lysyl oxidase activity. Some of the mice show aneurysmal changes in large arteries. These findings suggest a possible mechanistic link between aneurysm formation and degeneration of elastic lamina caused by aging or reduction in lysyl oxidase activity.

In this study, we show that a combination of hypertension and degeneration of elastic lamina by lysyl oxidase inhibitor, BAPN, can cause both thoracic and abdominal aortic aneurysms in mice. We used two well-established methods of...
pharmacologically induced hypertension, angiotensin II–induced hypertension and deoxycorticosterone acetate (DOCA)-salt hypertension. Similar to human aortic aneurysms, aortic aneurysms in this model developed at the ascending thoracic aorta and abdominal aorta with site-specific morphological and histological characteristics. Furthermore, we assessed the roles of hypertension on aneurysm formation by using amloidipine, an antihypertensive agent. Potential contributions to aneurysm formation from angiotensin II locally produced in the vascular wall were assessed by using captopril (angiotensin-converting enzyme inhibitor) in the mice that received DOCA-salt treatment and BAPN.

Methods
Detailed methods are described in the online Data Supplement. Please see http://hyper.ahajournals.org.

Induction of Aortic Aneurysm by Angiotensin II and BAPN
In 9-week–old C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME), hypertension was induced by angiotensin II (1000 ng/kg per minute) or DOCA-salt treatment. BAPN (150 mg/kg per day), a lysyl oxidase inhibitor, was administered for the first 2 weeks through a subcutaneously implanted osmotic-pump (Alzet, Durect Corp) to induce degeneration of elastic laminas. Mice were euthanized 6 weeks after the surgery. Aneurysms were defined as a localized dilation of the aorta of >50% of its adjacent intact portion of aorta. One group of mice received an antihypertensive agent, amloidipine (5 mg/kg per day), in addition to angiotensin II and BAPN. Additional mice received captopril (angiotensin-converting enzyme inhibitor, 6 mg/kg per day) in addition to DOCA-salt treatment and BAPN.

Statistical Analysis
Data were presented as mean±SD. Differences between multiple groups were analyzed by 1-way ANOVA, followed by the Tukey-Kramer post hoc test. The χ² test was used to analyze categorical data. Statistical significance was taken at P<0.05.

Results
Combination of Angiotensin II–Induced Hypertension and Lysyl Oxidase Inhibition by BAPN Resulted in Aortic Aneurysm Formations
Forty-five mice received angiotensin II for 6 weeks and BAPN for 2 weeks. A total of 16 mice died before 6 weeks from ruptured aortic aneurysms (15 of 16) or dissecting aneurysm (1 of 16). A total of 64% of the mice (29 of 45) survived for 6 weeks. Including ruptured and unruptured aneurysms, 71% (32 of 45) of the mice developed aortic aneurysms during the 6-week period. Thirty-eight percent (17 of 45) of the mice developed thoracic aortic aneurysms (Figure 1A). Nine thoracic aortic aneurysms were found as ruptured aneurysms, indicating a 53% rupture rate (9 of 17). A total of 49% of the mice (22 of 45) developed abdominal aortic aneurysms (Figure 1A). Six abdominal aortic aneurysms were found as ruptured aneurysms, representing a 27% rupture rate (6 of 22). Seven of the 45 mice (16%) had both thoracic and abdominal aortic aneurysms.

One mouse had a dissecting aneurysm, which extended over the entire thoracic and abdominal aortas (Figure 1B1). Two animals developed small isolated aneurysms at the distal descending thoracic aorta. Except for these 3 aneurysms, all of the aneurysms were localized at the two distinct regions of the aorta that are known to be aneurysm-prone regions of the aorta in humans, the thoracic aorta involving the ascending aorta and the abdominal aorta. In the following sections, “thoracic aortic aneurysms” refer to aneurysms that involve the ascending aorta and arch. The 2 small aneurysms at the distal descending thoracic aorta are referred to as “descending thoracic aortic aneurysms.” Mice treated with angiotensin II (n=10), BAPN (n=10), or PBS (n=10) alone did not develop aneurysms.

Macroscopically, thoracic and abdominal aortic aneurysms in this model resembled human aortic aneurysms with their site-specific morphology (Figure 1B). Thoracic aortic aneurysms were saccular shaped with localized dilation at the great curvature (Figure 1Bb through 1Bd). In contrast, abdominal aortic aneurysms were fusiform-shaped aneurysms with a thick vascular wall and an intramural thrombus (Figure 1Bf through 1Bh). Figure 1Bi shows a dissecting aneurysm that extended over the ascending aorta and the abdominal aorta (1 of 45).

Systolic blood pressures of mice that received angiotensin II alone or a combination of angiotensin II and BAPN were significantly higher than those of the control group at 3 and 6 weeks (Figure 1C). The time course of aneurysm formation and growth in this model is presented in Figure S1.

Histological Characterization of Aortic Aneurysms
Control thoracic and abdominal aorta and representative thoracic and abdominal aneurysms are shown in Figure 2. Thoracic aortic aneurysms showed two distinct parts of the vascular wall: thin wall and thick wall parts (Figure 2B). The thin wall part of the aneurysm formed a saccular-shaped thoracic aortic aneurysm. The thin aneurysm wall had only 2 or 3 layers of elastic lamina (Figure 2B2 through 2B5). The thick wall part of the thoracic aortic aneurysm showed severe medial degeneration and adventitial thickening (Figure 2B9 through 2B12). Thickening and disorganization of the media were accompanied by fragmentation and disruption of elastic laminas with widening space between the elastic laminas (Figure 2B11). Thickened adventitia was collagen rich and contained large numbers of inflammatory-like cells (Figure 2B12). Neither atherosclerotic changes nor intramural thrombus was found. Smooth muscle cells were scarce in the thin wall (Figure 2B7), whereas smooth muscle cells were abundant in the thickened part of the media (Figure 2B14). Endothelial cell layer was intact in both thin and thick parts of thoracic aortic aneurysms (Figure 2B8 and B15). Fibroblasts were mainly present in the inner half of the thickened adventitia (Figure 2B13).

Abdominal aortic aneurysms showed thickening of the vascular wall throughout the entire circumference and presence of an intramural thrombus (Figure 2D), resembling human abdominal aortic aneurysms. Thinning of the vascular wall was not observed. Degeneration of elastic lamina in the abdominal aortic aneurysm was much less than that of the thoracic aneurysms (Figure 2D2 through 2D5 and 2D9 through 2D12). Inflammatory cells were observed in the adventitia, especially around the intramural thrombus. Oil red O staining showed the presence of lipids around the intramural thrombus, which is possibly an early sign of atherosclerosis (Figure S2). The majority...
of fibroblasts were present around the intramural thrombus, and some of them infiltrated into the media (Figure 2D13). Smooth muscle cell layers were mildly disorganized, losing tight alignment of the elastic lamina (Figure 2D7 and 2D14). Endothelial cell layer was generally intact (Figure 2D8 and 2D15). Similar to human aortic aneurysms, aortic aneurysms in this model showed inflammatory cell infiltration. At 1 week, numerous leukocytes were already detected in the adventitia, especially in the outer layer of the adventitia, in both the thoracic and abdominal aortas (Figure S3).

Differences in the distribution of inflammatory cells between thoracic and abdominal aortic aneurysms became apparent at 6 weeks (Figure 3). In thoracic aneurysms, numerous leukocytes were observed in the adventitia and media within both the thin and thick walls (Figure 3B2 and 3B6). In contrast, leukocytes were highly concentrated in the thick wall near the intramural thrombus in abdominal aortic aneurysms (Figure 3D6), and the wall without intramural thrombus contained only a small number of leukocytes (Figure 3D2). The majority of leukocytes (CD45+) appeared to be macrophages (CD68+; Figure 3B2 through 3B5, 3B6 through 3B9, 3D2 through 3D5, 3D6 through 3D9, and S5A). Helper T cells and B cells were present but scarce in both thoracic and abdominal aneurysms (Figure 3B4, 3B5, 3B8, 3B9, 3D4, 3D5, 3D8, and 3D9).

Thoracic and abdominal aortas with preaneurysmal changes (localized dilation of the aorta that did not reach the 50% cutoff) had similar structural and histological changes, including inflammatory cell infiltration, to those with mature aneurysms (Figure S3). Morphometric analysis, the grading of changes of elastic lamina, and semiquantification of leukocytes are shown in Figure S4 and S5B.

### Roles of Hypertension in Aneurysm Formation in This Model

Nonhemodynamic effects of angiotensin II could potentially have contributed to the formation of aneurysms independent

![Image](http://hyper.ahajournals.org/)

**Figure 1.** Combination of angiotensin II–induced hypertension and lysyl oxidase inhibition by BAPN resulted in aortic aneurysm formation in mice. A. Incidence of aortic aneurysms. A total of 38% and 49% of the mice that received angiotensin II and BAPN developed thoracic and abdominal aortic aneurysms, respectively. No aneurysm formation was found in the mice that received angiotensin II or BAPN alone. B. Representative aortic aneurysms. Macroscopically, thoracic and abdominal aortic aneurysms in this model resembled human aortic aneurysms with their site-specific morphology. Thoracic aortic aneurysms were saccular shaped with localized dilation at the great curvature, whereas abdominal aortic aneurysms were fusiform-shaped aneurysms with a thick vascular wall. a, normal thoracic aorta; b and c, unruptured thoracic aortic aneurysm; d, ruptured thoracic aortic aneurysm; e, normal abdominal aorta; f and g, unruptured abdominal aortic aneurysm; h, ruptured abdominal aortic aneurysm; i, dissecting aortic aneurysm. Scale bar: 1 mm. C, Systolic blood pressures of mice that received angiotensin II alone or a combination of angiotensin II and BAPN are significantly higher than systolic blood pressures of mice in the control group at 3 and 6 weeks. Mean ± SD. *P < 0.05 compared to control. #P < 0.05 compared to control at 6 weeks.
from its hypertensive effects. Therefore, to elucidate roles of hypertension and to assess potential contributions from nonhemodynamic effects of angiotensin II in aneurysm formation in this model, we performed two lines of experiments. First, we treated the mice that were receiving angiotensin II and BAPN with an antihypertensive agent, amlodipine (calcium channel blocker), to separate the hypertensive roles of hypertension and to assess potential contributions from nonhemodynamic effects of angiotensin II in aneurysm formation in mice resulted in the formation of aortic aneurysms. Histologically, thoracic and abdominal aortic aneurysms in the mice treated with DOCA-salt and BAPN (Figure 4C) were indistinguishable from the aortic aneurysms observed in mice that were treated with angiotensin II and BAPN. Because angiotensin II levels in the vessel wall can be elevated and potentially contribute to aneurysm formation in DOCA-salt–treated mice, we treated mice that were receiving DOCA-salt treatment and BAPN with captopril (angiotensin-converting enzyme inhibitor; n=13). Captopril did not cause a significant reduction of blood pressure compared with the group that only received DOCA-salt treatment with BAPN (143±12 versus 140±12 mm Hg). A total of 69% of the mice that received DOCA-salt, BAPN, and captopril (9 of 13) developed aortic aneurysms. Five mice had thoracic aneurysms, and 7 mice had abdominal aneurysms. Three mice had both thoracic and abdominal aneurysms. In total, 70% of the mice developed aortic aneurysms. Histologically, thoracic and abdominal aortic aneurysms in the mice treated with DOCA-salt and BAPN (Figure 4C) were indistinguishable from the aortic aneurysms observed in mice that were treated with angiotensin II and BAPN.

**Discussion**

In this study, we showed that the combination of hypertension and the degeneration of elastic lamina by lysyl oxidase inhibition in mice resulted in the formation of aortic aneu-
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rysms that recapitulate key features of human aortic aneurysms with site-specific phenotypes. Using this model, we showed critical roles of high blood pressure in the formation of aortic aneurysms, establishing a causal link between hemodynamic conditions and aortic aneurysm formation in animals.

Daugherty and colleagues\textsuperscript{23,24} pioneered an abdominal aortic aneurysm model in genetically atherosclerosis-prone mice by continuously infusing angiotensin II. They used apolipoprotein E–knockout mice and fat-fed, low-density lipoprotein–receptor knockout mice.\textsuperscript{23,24} Morphological and histological characteristics of angiotensin II–induced abdominal aortic aneurysms in these knockout mice were similar to the abdominal aortic aneurysms in our model, indicating that common molecular mechanisms potentially exist between these two models with respect to abdominal aortic aneurysms.

It should be noted that angiotensin II infusion in apolipoprotein E–knockout or low-density lipoprotein–receptor knockout mice did not cause thoracic aortic aneurysm.\textsuperscript{23,24} In contrast, aneurysm formation in our model occurred not only in abdominal aorta but also in the thoracic aorta involving the ascending aorta, the segment of the thoracic aorta in which most human thoracic aneurysms are located.

Previously, Ikonomidis et al\textsuperscript{25} showed that direct application of calcium chloride to the descending thoracic aorta through thoracotomy caused aneurysmal formation in the aortic segment that was exposed to calcium chloride. The advantage of their model is that aneurysmal dilatation occurred in almost all of the animals.\textsuperscript{25} However, the aneurysmal dilatation in their model was mild, that is, 25% dilatation compared with 50% in our model, and the location of aneurysms in thoracic aorta in their model differs from the common location of thoracic aneurysms in humans. More importantly, in our model, both abdominal and thoracic aneurysms were induced by the same pharmacological treatments. Our model may be more suitable for studying potential similarities and differences in the pathophysiology between thoracic and abdominal aortic aneurysms.

Although atherosclerosis is strongly linked to systemic hypertension, the majority of the patients with thoracic aortic aneurysms are often free from systemic or local atherosclerosis.\textsuperscript{19,26} Although many of the abdominal aortic aneurysms in our model showed signs of early atherosclerosis, such changes were absent in thoracic aortic aneurysms in this model. Interestingly, abdominal aortas from the earlier time point revealed preaneurysmal changes without any sign of atherosclerosis. Atherosclerosis observed in abdominal aortic aneurysms in this model may not be part of a causative factor but rather a secondary change that follows aneurysm, as suggested previously.\textsuperscript{27,28}

Figure 3. Inflammatory cells in thoracic and abdominal aortic aneurysms. Stainings for pan-leukocytes (CD45), macrophages (CD68), helper T lymphocytes (CD4), and B lymphocytes (CD19). In thoracic aneurysms, leukocytes were observed in the adventitia and media (B2 and B6). In contrast, leukocytes were highly concentrated in the thick wall near the intramural thrombus in abdominal aortic aneurysms (D6). In both thoracic and abdominal aneurysms, the majority of leukocytes appeared to be macrophages (B2 through B5, B6 through B9, D2 through D5, and D6 through D9). *Lumen. Scale bar: 0.1 mm. Arrows point to positive cells.
that differential responses to the combination of hypertensive and abdominal aortic aneurysms in this model may suggest morphological and histological differences observed between thoracic and abdominal aortic aneurysms in this model and in humans may be attributed to the differences in developmental origins of smooth muscle cells at these two segments of aorta.26,27

Another advantage of this new model is the use of wild-type mice, which makes it easier to examine roles of different signaling pathways compared with using knockout and transgenic mice. Although the successful induction of abdominal aortic aneurysms by angiotensin II in apolipoprotein E–knockout or low-density lipoprotein–receptor knockout mice has been validated by several groups, the incidence of abdominal aneurysms in apolipoprotein E–knockout or fat-fed low-density lipoprotein–receptor knockout mice in response to angiotensin II infusion,31 making it difficult to compare the differences between thoracic and abdominal aortic aneurysms at the respective locations. Morphological and histological differences between thoracic and abdominal aortic aneurysms in this model may suggest that differential responses to the combination of hypertension and lysyl oxidase inhibition at these two regions of the aorta lead to different phenotypes of aneurysms. Morphological and histological differences between thoracic and abdominal aortas in this model and in humans may be attributed to the differences in developmental origins of smooth muscle cells at these two segments of aorta.26,27

Embryologically programmed differences of vascular smooth muscle cells may determine different vascular responses to hemodynamic stimuli and degeneration of elastic lamina between thoracic and abdominal aortas,26,27,30 leading to site-specific phenotypes of aneurysms at the two regions. More importantly, phenotypic differences between thoracic and abdominal aortic aneurysms indicate that different pharmacological strategies may be needed to prevent growth and rupture of aneurysms at these two different locations.

Angiotensin II can exert various effects on the vasculature in addition to its hypertensive effect.20–22 For the formation of abdominal aneurysms in apolipoprotein E–knockout or fat-fed low-density lipoprotein–receptor knockout mice in response to angiotensin II infusion,31...
nonhemodynamic effects of angiotensin II, but not hypertensive effects, are required. In contrast, the aneurysm formation observed in our model depended on systemic hypertension. In our model, normalization of blood pressure by an antihypertensive agent dramatically reduced the incidence of aneurysms and almost completely abolished histological changes associated with angiotensin II and BAPN treatment. We were able to reproduce thoracic and abdominal aortic aneurysms when DOCA-salt hypertension was used instead of angiotensin II. However, in DOCA-salt–treated mice, endogenous angiotensin II that was produced in the vascular wall in response to systemic hypertension may have played a role in our model. Therefore, we treated the mice receiving DOCA-salt and BAPN with captopril, an angiotensin-converting enzyme inhibitor, to exclude potential confounding effects from the endogenous production of angiotensin II in DOCA-salt hypertensive mice. Captopril did not reduce the incidence of aortic aneurysm in DOCA-salt hypertensive mice, further suggesting critical roles for hypertension in this model.

Our data represent the first demonstration of the causal relationship between systemic hypertension and aortic aneurysm formation. One critical caveat to this study is that systolic blood pressure was measured under anesthesia, as previously described by others. Blood pressure measurement under anesthesia may underestimate the effects of hypertensive and antihypertensive agents.

It should be noted that, although our mouse model replicated key features of thoracic and abdominal aortic aneurysms in humans, aneurysms in this model did not form spontaneously but were induced by two pharmacological interventions, which potentially bypassed some of the early critical events that lead to aortic aneurysm in humans.

Perspectives

We showed that the combination of pharmacologically induced hypertension and degeneration of elastic lamina by lysyl oxidase inhibition caused aneurysm formation at two aneurysm-prone regions of aorta that are common locations of aortic aneurysms in humans. Using this model, we established critical roles of hypertension in the formation of aortic aneurysms. Phenotypic differences between thoracic and abdominal aortic aneurysms in this model and in humans may indicate that different pharmacological strategies may be needed to prevent growth and rupture of aneurysms at these two different locations. Our model may be suitable to study potential similarities and differences in the pathophysiology between thoracic and abdominal aortic aneurysms.

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Disclosures

None.

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Pharmacologically-induced thoracic and abdominal aortic aneurysms in mice

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Short Title
A new mouse model of aortic aneurysm

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Expanded Methods

All experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Measurement of blood pressure

Systolic blood pressure was measured at baseline, one, three, and six-week in animals that received phosphate buffered saline (PBS) (control) (n=10), beta-aminopropionitrile (BAPN) alone (n=10), angiotensin-II alone (n=10), or combination of angiotensin-II and BAPN (n=45). Systolic blood pressure was measured using a tail-cuff method (ADInstruments) as previously described.\(^1\)

In our preliminary experiments, we encountered that C57BL/6J mice have high locomotor activity and resistance to restrain even after training for blood pressure measurements, as previously described.\(^2\),\(^3\) Therefore, we anesthetized the mice with isoflurane, and systolic blood pressure was measured at the steady state (after 15 minutes of equilibration with the end-tidal isoflurane concentration at 1.5%) using a tail-cuff system (ADInstruments) to avoid confounding effects of locomotion and excitement on blood pressure measurement. A minimum of 10 measurements were obtained from each mouse.

Tissue harvesting

Mice were anesthetized with 5% isoflurane. Left cardiac ventricles were perfused with cold PBS (10 ml) under physiologic pressure with an exit through the severed right atrium. Hearts and aortas (portion between the heart and the bifurcation of iliac artery) were harvested. Harvested tissues were frozen in OCT compound immediately.

Definition of thoracic aortic aneurysm and abdominal aortic aneurysm

Thoracic and abdominal aortic aneurysms were defined as a localized dilation of the aortic wall with maximal outside diameter greater than 50% of its adjacent intact portion of aorta, which is the same set of criteria used for human aortic aneurysms.\(^4\),\(^5\) Image analyses were performed, using ImageJ software (National Institutes of Health) to measure the outer diameter of the aortic wall.

Histological and immunohistochemical examination

Serial cross-sections (10 µm thick) from both thoracic and abdominal aortas were mounted on microscope slides (Fisher Scientific Co.). In each group, 3 to 6 animals were examined for histology and immunohistochemistry. Sections were stained with hematoxylin and eosin (H&E), Elastica van Gieson (EVG), Gomori’s trichrome, and Oil red O for histochemical examination, elastin and collagen, smooth muscle fibers, and lipids, respectively. Other aortic cross-sections (10 µm thick) were prepared for immunohistochemical staining to identify pan-leukocytes, macrophages, helper T-lymphocytes, B-lymphocytes, endothelial cells, fibroblasts, smooth muscle cells, cell proliferation, and apoptosis.
The following reagents were used to detect specific cell types: rat monoclonal anti mouse CD45 (1:1000 dilution; 553076) for pan-leukocytes, rat monoclonal anti mouse CD68 (1:300; MCA1957; AbD Serotec) for macrophages, rat monoclonal anti mouse CD4 (1:250; 553043) for helper T-lymphocytes, rat monoclonal anti mouse CD19 (1:100; 553783) for B-lymphocytes, rat monoclonal anti mouse CD31 (1:250; 553370) for endothelial cells, rabbit polyclonal anti S100A4 Ab-8 (1:150; RB-1804-A1; Neomarkers/Lab Vision) for fibroblasts, rabbit polyclonal anti alpha smooth muscle actin (1:400; AB5694; Abcam) for smooth muscle cells, and rabbit polyclonal anti Ki67 (1:300, AB9260; Chemicon) for cell proliferation; these antibodies were purchased from BD Pharmingen, unless otherwise specified. The primary antibody was omitted for negative control.

Sections were fixed with cold acetone for 20 minutes, and endogenous peroxidases were quenched by incubating sections in 0.3% hydrogen peroxide in methanol. Sections were subsequently blocked with 10% normal serum from host species of the secondary antibody. Sections were incubated in primary antibody overnight at 4°C, followed by incubation with corresponding biotinylated secondary antibodies (Vector Laboratories) and with a complex of avidin-biotin-horseradish peroxidase (Vector Laboratories). Immunoreactivity was visualized by incubating the sections with 0.05% 3,3'-diaminobenzidine (DAB, Vector Laboratories). Nuclei were visualized by counterstaining with aqueous hematoxylin.

To identify the major type of inflammatory cells among pan-leukocytes positive cells, double immunofluorescence staining was also performed. The antibodies used were rabbit polyclonal anti CD45 (1:200; sc-25590; Santa Cruz Biotechnology, Inc.) for pan-leukocytes, and rat monoclonal anti mouse CD68 (1:300; MCA1957; AbD Serotec) for macrophages. Pan-leukocytes were observed with red fluorescent anti-rabbit IgG IgG (Alexa 594; A11012; Invitrogen), and macrophages were observed with fluorescein anti-rat IgG (Fluorescein; FI-4001; Vector laboratories). The images were visualized using fluorescence microscopy.

To assess apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using the ApopTag kit (S7101; ApopTag Peroxidase In Situ Oligo Ligation Apoptosis Detection Kit; Chemicon) according to the manufacturer’s instructions. To assess cell proliferation, we stained the tissues with anti-Ki67 antibody.

Quantification of inflammatory cells in aortic wall, thickness of vascular wall, and degree of degeneration of elastic lamina
Two blinded observers independently performed quantitative analysis. Quantification of inflammatory cells was performed as previously described. The thoracic and abdominal aorta or aortic aneurysm sections that were immunostained for CD45, CD68, CD4, and CD19, as mentioned above, were also used (n = 6 aortas). Positive-stained cells were counted at four different representative locations under high magnification (400x). The number of positive cells per area of 0.01 square millimeters was calculated using the following formula: number of positive cells per field / aortic area per field. The aortic area...
per field was measured by using ImageJ software (National Institutes of Health). Results from the two observers were averaged. Thickness of aortic wall and grading of the degree of elastic lamina degeneration were analyzed by using H&E stained cross-sections of the thoracic and abdominal aortas or aortic aneurysms. Thickness of total wall, media, and adventitia at four different areas (every 90 degrees) per cross section in each aorta were measured and averaged (n = 6 aortas). The same samples were graded to quantify the severity of changes in the elastic lamina using the method as previously described by others. The severity of changes in the elastic lamina was scored from 1 to 4; 1: completely intact elastic laminas, 2: mild degeneration (in less than 50% of area of the medial layers), 3: extensive degeneration or fragmentation (in more than 50% of area of the medial layers), and 4: complete disruption of the entire media as previously described. Four different areas per cross section in each aorta were evaluated and averaged (n= 6 aortas).

**Blood flow measurement**

Blood flow rates of the thoracic aortas, abdominal aortas, and common carotid arteries in non-transplanted mice, and grafted thoracic aortas and abdominal aortas in transplanted mice were measured using an ultrasound transit-time probe (Transonic Systems) as previously described. All measurements were performed six weeks after the initiation of treatment with or without angiotensin-II and BAPN (seven weeks after transplantation).

Mice were anesthetized with 1.5% isoflurane. Mice were intubated and ventilated using a small animal ventilator (Harvard Apparatus). Tidal volume of the pump was set at 0.4 ml, and the respiratory rate was set at 120 strokes per minute. A median sternotomy was performed, and a modified retractor was inserted to visualize the heart and the ascending aorta. Dissection along the ascending aorta was performed to free the vessel from the surrounding connective tissues. 1.5 PSL flow probe was placed under the aorta, and blood flow signal was recorded and analyzed with the PowerLab System using Chart 5 software (ADInstruments). For the measurement of abdominal aortic blood flow, a midline laparotomy was performed. Suprarenal abdominal aorta was exposed, and blood flow was measured.
Expanded Results

Time course of aneurysm formation and growth

To assess the time course of aneurysm formation and growth in this model, we prepared additional mice that were treated with angiotensin-II and BAPN and sacrificed them at two-weeks (n=10). As shown in Supplemental Figure S1A, incidence of thoracic aortic aneurysms was 30% and 38% at two and six-weeks, respectively. Supplemental Figure S1B shows an increase in the mean outer diameter of the thoracic aorta from control to two-weeks and two-weeks to six-weeks, suggesting continuous growth of thoracic aortic aneurysm over the six-week period (control vs. six-weeks: 0.92 ± 0.09 vs. 1.58 ± 0.54 mm, P<0.05; two-weeks vs. six-weeks: 1.23 ± 0.25 vs. 1.58 ± 0.54 mm, P <0.05).

In contrast, there was no difference in incidence of abdominal aortic aneurysms between two-weeks and six-weeks (50% vs. 49%) (Supplemental Figure S1A). However, similar to the thoracic aorta, the mean outer diameter of the abdominal aorta gradually increased from control to two-weeks, and two-weeks to six-weeks, suggesting continuous growth of aneurysm over the six-week period (control vs. six-weeks: 0.74 ± 0.04 vs. 1.77 ± 0.95 mm, P <0.05) (Supplemental Figure S1B). Possibly due to the faster rate of abdominal aortic aneurysm growth relative to that of thoracic aortic aneurysm, the incidence of abdominal aortic aneurysms reached plateau at two-weeks. There appeared to be discrepancies between the incidence of abdominal aortic aneurysms and the outer diameter. While there was continuous growth of the outer diameter between two and six-weeks, there was no difference in incidence of abdominal aortic aneurysms during that same period. This was thought to be due to the dichotomous nature of the diagnostic criteria that used an arbitrary cutoff at 50% increase of diameter to define as aneurysms. While both thoracic and abdominal aortic aneurysms appeared to continuously grow over the six-week period, more abdominal aortic aneurysms than thoracic aortic aneurysms reached the 50% increase cutoff within two-weeks (Supplemental Figure S1C). Localized dilation of the aorta that did not reach the 50% cutoff may be considered as pre-aneurysmal lesions. As described below, histological assessment of these pre-aneurysmal lesions showed similar histological changes to those of aneurysm tissues (Supplemental Figure S3).

Interestingly, none of the distal descending thoracic aortic samples, except for the two small isolated aneurysms that developed, showed significant increase in the outer diameter over the six-week period (Supplemental Figure S1B). There were no differences in the mean outer diameter of the distal descending thoracic aorta among the time points studied. These data may indicate that the distal descending thoracic aorta have different responses to the combination of angiotensin-II and BAPN compared to the thoracic and abdominal aortas, which were two regions of the aorta where the majority of aneurysms were found.

Oil red O staining to assess early atherosclerotic changes

Oil red O staining showed a presence of lipids around the intramural thrombus in abdominal aortic aneurysm (arrows indicate the positive staining of
lipids), possibly an early sign of atherosclerosis (Supplemental Figure S2B). However, atherosclerotic changes were not found in thoracic aortic aneurysm in our model (Supplemental Figure S2A).

**Aortic wall with pre-aneurysmal changes at one-week and six-weeks**

At one-week time point, histological changes started in both ascending and abdominal aorta, such as increase of fibroblasts, disorganization of medial layers, and accumulation of numerous inflammatory cells (Supplemental Figure S3). In both six-week aortas, these pre-aneurysmal changes developed more severely, in spite of lack of macroscopic aneurysm formation (Supplemental Figure S3). Unlike aneurysm samples, complete medial break or intramural thrombus was not found in aortas with pre-aneurysmal changes.

**Morphometric analysis of different stages of thoracic and abdominal aortic aneurysms**

Damages of the elastic lamina and accompanied changes in the media were more pronounced in the thoracic aortas than in the abdominal aortas. In addition, the thoracic and abdominal aortas revealed different time course of changes in wall thickness and in severity of damages of the elastic laminas.

In thoracic aortas, the total wall thickness gradually increased from control to one-week, to two-weeks, and to six-weeks (Supplemental Figure S4A, left). The gradual increase in wall thickness of the thoracic aorta was mainly due to the gradual thickening of the media, which was accompanied by the gradual increase in the severity of the damages in the elastic lamina (Supplemental Figure S4B, left). The time course of increase in the wall thickness and media in the thoracic aorta was similar to the time course of increase in the outer diameter (Supplemental Figure S1B), indicating that the gradual increase in outer diameter over the six-week period was mainly due to the increase in wall thickness.

In contrast, the increase in thickness of the vascular wall, media, and adventitia of the abdominal aorta reached a plateau at one-week, except for the region of the vascular wall with intramural thrombus (w IMT) (Supplemental Figure S4A right). Similarly, severity of damages in the elastic lamina reached plateau at one-week (Supplemental Figure S4B right). Faster rate of change in the wall thickness of the abdominal aortas coincided with faster rate of growth of the outer diameter presented in Supplemental Figure S1B.

**Inflammatory cell infiltration**

Double staining for CD45 (pan-leukocytes) and CD68 (macrophages) in aortic aneurysm tissues showed that the majority of pan-leukocytes in thoracic aortic aneurysms were macrophages (Supplemental Figure S5A).

Semi-quantification of leukocytes at different stages of aneurysm formation is presented in Supplemental Figure S5B.

**Cell proliferation and apoptosis**
Concurrent proliferation and apoptosis of vascular cells were observed in human aortic aneurysms.\textsuperscript{10, 11} Therefore, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and Ki67 staining were employed for assessment of apoptosis and proliferation, respectively (Supplemental Figure S6). At all time points we studied, abundant TUNEL positive cells were detected in both media and adventitia, regardless of whether aneurysms formed. However, there were far less Ki67 positive cells than TUNEL positive cells, indicating possible imbalance between the processes of cell proliferation and apoptosis.


Figure S1. Time course of aneurysm formation and growth.
A. Incidence of aortic aneurysms at two and six weeks. B. Outer diameter of the ascending, abdominal, and distal descending aortas at two and six-week. There was an increase in the mean outer diameter of the thoracic and abdominal aortas from control to two-week and two-week to six-week while the outer diameter of the descending thoracic aorta remained unchanged. C. Ratio of maximum outer diameter to normal outer diameter. All ascending, abdominal, and descending thoracic aorta of the mice that survived for two weeks and six weeks after the combination treatment of angiotensin-II and BAPN were examined for the ratio of maximum outer diameter to normal portion of aorta from the same mice using the following formula. Ratio: maximum outer diameter / ((outer diameter in normal portion of proximal aortic aneurysm + outer diameter in normal portion of distal aortic aneurysm)/2). In this study, thoracic and abdominal aortic aneurysms were defined as a localized dilation of the aortic wall with maximal outside diameter greater than 50% of its adjacent intact portion of aorta, which is the same criteria used for human aortic aneurysms. Mean ± SD. *: P < 0.05.
**Figure S2. Examination for lipids in aortic aneurysms.** To assess presence of atherosclerotic changes, oil red O staining was used to detect lipids. Oil red O staining showed a presence of lipids around the intramural thrombus in abdominal aortic aneurysm (arrows indicate the positive staining of lipids), possibly an early sign of atherosclerosis. But atherosclerotic changes were not found in thoracic aortic aneurysm in our model. Scale bars: 0.1mm. *: lumen; **: intramural thrombus.
Figure S3. Pre-aneurysmal changes in aortas without mature aneurysm formation at one-week and six-week. Representative stainings for H&E, fibroblasts (S100A4 Ab-8), smooth muscle cells (alpha smooth muscle actin), endothelial cells (CD31), pan-leukocytes (CD45), macrophages (CD68), Helper T-lymphocytes (CD4) and B-lymphocytes (CD19) in (A) thoracic aortas and (B) abdominal aortas without mature aneurysm formation (pre-aneurysmal lesion) at one and six-week time point. At one-week time point, histological changes started in both ascending and abdominal aorta, such as increase of fibroblasts, disorganization of medial layers, and accumulation of numerous inflammatory cells. In six-week aortas, these pre-aneurysmal changes developed severer in spite of lack of macroscopic aneurysm formation. Unlike aneurysm samples, complete medial break or intramural thrombus was not found in aortas with pre-aneurysmal changes. Scale bars: 0.1mm. *: lumen; arrows indicate positive cells.
Figure S4. Morphometric analysis of thoracic and abdominal aortic aneurysms. 
A. Quantification of thickness of the vascular wall, media, and adventitia at different stages of thoracic and abdominal aortic aneurysms. 
B. Grading of the degeneration of elastic lamina. TAA: thoracic aortic aneurysm; AAA: abdominal aortic aneurysm; w or w/o IMT: the aortic wall with or without
Figure S5. Inflammatory cell infiltration in aortic aneurysms.
A. Representative images of double immunofluorescent staining for macrophages (CD68 positive cells) and pan-leukocytes (CD45 positive cells) in aortic aneurysm at six-week time point are shown. The majority of pan-leukocytes in thoracic aortic aneurysms were macrophages. B. Quantification of inflammatory cells. TAA: thoracic aortic aneurysm; AAA: abdominal aortic aneurysm; w or w/o IMT: the aortic wall with or without intramural thrombus. Scale bars: 0.1mm. *:
Figure S6. Apoptosis and cell proliferation in aortic aneurysms. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and Ki67 staining were employed for assessment of apoptotic and proliferating cells, respectively. Both the thoracic and abdominal aortas with pre-aneurysmal changes showed abundant TUNEL positive cells in the adventitia and media as early as one-week time point. However, very few Ki67 positive cells were detected in both regions of aortas at one-week. At six-week time point after the treatment with angiotensin-II and BAPN, many Ki67 positive cells were detected in the smooth muscle cell layer of thoracic and abdominal aortas, regardless of whether aneurysm formed. TAA: thoracic aortic aneurysm; AAA: abdominal aortic aneurysm; w or w/o IMT: the aortic wall with or without intramural thrombus. Scale bars: 0.1mm. *: lumen; Arrows indicate positive cells.