

Aortic Dissection Precedes Formation of Aneurysms and Atherosclerosis in Angiotensin II–Infused, Apolipoprotein E–Deficient Mice

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Objective—We sought to define the temporal characteristics of angiotensin II (AngII)–induced abdominal aortic aneurysms (AAAs) and to provide mechanistic insight into the development of this vascular pathology in apolipoprotein E–deficient (apoE^{-/-}) mice.

Methods and Results—Male apoE^{-/-} mice were infused with AngII for 1 to 56 days. Suprarenal arteries were sequentially sectioned, and cellular features were defined by histologic and immunocytochemical techniques. The initial identified event was medial accumulation of macrophages in regions of elastin degradation. Subsequent medial dissection was associated with luminal dilation and thrombus formation. Thrombi were usually constrained by adventitial tissue, although ≈10% of mice died due to rupture. Thrombi led to profound inflammation that was characterized by infiltration of macrophages and T and B lymphocytes. Remodeling of the tissues was associated with regeneration of elastin fibers and reendothelialization of the dilated luminal surface. Aneurysmal tissue underwent profound neovascularization. Atherosclerotic lesions were only detected after development of the aneurysms.

Conclusions—The initial event in AngII–induced AAA is a focal dissection in the suprarenal region. The progression of AAA precedes the development of overt atherosclerotic lesions. (*Arterioscler Thromb Vasc Biol.* 2003;23:1621–1626.)

Key Words: angiotensin ■ aneurysms ■ atherosclerosis

Abdominal aortic aneurysms (AAAs) are permanent dilations of the artery that are normally defined as an increase of >50% of the normal diameter.¹ AAAs are a major cause of mortality in the elderly, with an anticipated increase in prevalence owing to the demographics of an increased proportion of aged individuals. Despite the prevalence of the disease, current therapy of AAA is restricted to surgical options, because there are no medicinal approaches with proven benefit.^{2–4}

The pathology of AAAs is largely defined from tissues acquired at the end stage of the disease. At this late stage of progression, the pathologic features of the tissue include degeneration of the medial elastic fibers, thinning of the media, adventitial hypertrophy with accumulation of macrophages and T and B lymphocytes, atherosclerosis, and thrombi.^{5–7} However, there is a paucity of data defining the sequential cellular events of human AAAs as they develop and progress. This lack of information hinders the ability to provide mechanistic insight into the initiating and propagating factors of the disease. The development of atherosclerotic lesions in the abdominal aorta has been proposed as an initiating factor for the formation of AAA.⁸ This is largely based on the presence of atherosclerotic lesions in aneurysmal tissue at the end stages of the disease.

However, although atherosclerotic lesions are frequently present at the site of AAA formation, they might not be a causal factor.

Animal models provide one mode to determine the sequential pathogenic factors critical to aneurysm development. The most commonly used mouse models of AAA are produced by calcium chloride,⁹ elastase,¹⁰ or angiotensin II (AngII).^{11–13} Previous studies in our laboratory have demonstrated that infusion of AngII into apolipoprotein E–deficient (apoE^{-/-}) or fat-fed, LDL receptor^{-/-} mice leads to reproducible formation of AAAs, particularly in male mice.^{11–13} In this study, the temporal sequence of events in AngII–induced AAAs was defined to provide mechanistic insight into AAA initiation and maturation. Results from these studies demonstrate that medial accumulation of macrophages and dissection are early events in AngII–induced AAA. Atherosclerotic lesions were only detected after the formation of AAAs, suggesting that atherosclerosis was not contributing to the development of AngII–induced AAA.

Methods

Mice

Male apoE^{-/-} mice (backcrossed 10 times onto a C57BL/6 background) were obtained from the Jackson Laboratory (Bar Harbor, Me) and housed in a specific, pathogen-free environment. Standard sterilized laboratory diet (Harlan Teklad catalog No. 2918) and water

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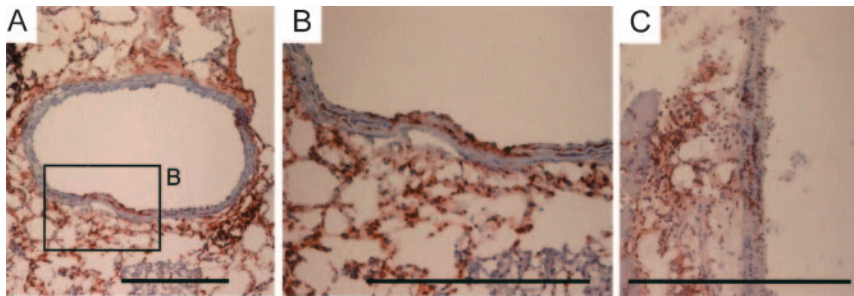


Figure 1. Macrophage migration into the aortic media was associated with elastin disintegration after 48 hours of Ang II infusion to apoE^{-/-} mice. Cross sections (A, B) and a longitudinal section (C) of the suprarenal aorta immunostained with rabbit antiserum against mouse macrophages illustrated the infiltration of discrete macrophages into the aortic media. Bars=400 μ m in this and all subsequent figures.

were available ad libitum. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

AngII Infusion

Mice (8 to 10 weeks old) were implanted with minipumps (Alzet model 2004, Durect Corp) that delivered AngII subcutaneously at a dose of 1000 ng \cdot kg⁻¹ \cdot min⁻¹, as described previously.^{11,12} Some mice were sequentially implanted with a second Alzet pump at day 28 to permit continuous delivery of AngII for 56 days. Mice (n=6) infused with saline were used as controls. Mice were killed on days 1 (n=8), 2 (n=14), 4 (n=4), 7 (n=13), 10 (n=6), 14 (n=7), 21 (n=3), 28 (n=5), and 56 (n=4) after AngII infusion.

Tissue Harvesting

Anesthetized mice were cut open ventrally. Left cardiac ventricles were perfused with phosphate-buffered saline (20 mL) under physiologic pressure with an exit through the severed right atria. Suprarenal regions of abdominal aorta were identified between the last pair of intercostal arteries and the right renal branch. The mesenteric and renal branches and the aorta distal to the right renal branch were ligated with silk sutures, and the suprarenal aorta was harvested. This portion of aorta, measuring \approx 5 mm in length, was infused with \approx 0.3 mL of OCT compound with a 21-gauge needle to attain full distension. Thoracic aortas between the left subclavian artery and the last pair of intercostal arteries were also harvested. The orientation of aortas was noted, and tissues were frozen immediately.

Pathology and Immunocytochemistry

Aortas were obtained at selected intervals after the initiation of AngII infusions and were sectioned longitudinally or by cross sections (10 μ m thick). For characterization of cross sections, aortic sections were collected serially from the proximal to the distal aorta. Histology was determined in sections that were taken at intervals of 200 μ m. For longitudinal examination of tissues, 10- μ m sections were also placed at 200- μ m intervals on slides. Standard histologic staining for neutral lipid, elastin, collagen, smooth muscle, erythrocytes, and nuclei were performed with oil red O, Verhoeff's, Gomori's trichrome, and Movat's pentachrome techniques, as described previously.^{14,15}

Immunocytochemical staining was performed to identify macrophages, T lymphocytes, B lymphocytes, endothelium, and neutrophils, as described previously.^{14,16} At least 2 slides, containing \approx 15 tissue sections, from each animal were examined for each cell type. The following reagents were used to detect specific cell types: rabbit antisera against mouse macrophages (1:3000 dilution; Accurate catalog No. AI-AD31240); monoclonal rat anti-mouse CD90.2 (1:100 dilution; Pharmingen catalog No. 553009) for T lymphocytes; monoclonal rat anti-mouse CD19 (1:100 dilution; Pharmingen catalog No. 553783) for B lymphocytes; biotinylated rat anti-mouse platelet endothelial cell adhesion molecule (PECAM)-1 (CD31, 1:1000 dilution; Pharmingen catalog No. 553371) for endothelium; and polyclonal goat anti-mouse neutrophil elastase (1:100 dilution; Santa Cruz catalog No. SC-9521) for neutrophils. Immunocytochemistry was performed with a commercially available system (Fisher Microprobe). Biotinylated secondary antibodies from the appropriate species were used (Vector Laboratories). A peroxidase-based ABC

system (Vector Laboratories) and the red chromogen AEC were used to detect the antigen-antibody reaction. Controls included isotype-matched antibodies and nonimmune sera.

Results

Implantation of osmotic minipumps containing saline into apoE^{-/-} mice did not produce overt changes in abdominal arterial tissue. In contrast, infusion of AngII (1000 ng \cdot kg⁻¹ \cdot min⁻¹) promoted rapid changes in the suprarenal region of the abdominal aorta, a region susceptible to the formation of AAA. The earliest changes noted were at 1 to 4 days of AngII infusion. During this interval, there was medial accumulation of macrophages that occupied discrete areas in the region that develops aneurysms, as demonstrated in both cross and longitudinal sections (Figure 1A–1C). At these sites of macrophage accumulation, we also frequently observed disruption of elastin fibers (online Figure I A and IB). Medial macrophage accumulation was not observed in the thoracic or sinus region of the aorta (data not shown and Daugherty et al¹²). However, AngII infusion led to an accumulation of macrophages in the adventitia of the suprarenal aorta (Figure 1), as well as other aortic regions (thoracic and sinus; data not shown and^{12,17}).

Between 4 and 10 days, a vascular hematoma was grossly observable in the majority of the mice (Figure 2A). Approximately 10% of the mice died during this interval of AngII infusion. On necropsy, these mice were found to have ruptured AAAs and were assumed to have died by exsanguination into the abdominal cavity (Figure 2B). The interruption of the media in the region of the thrombi was clearly evident on longitudinal and cross sections (Figure 2C–2D).

After development of the thrombi, there was subsequent development of an inflammatory reaction that prominently involved infiltrating macrophages. Macrophage accumulation was particularly evident at the edges of the thrombi, both in regions of disrupted and intact media (Figure 3A and 3B). In addition, at this interval of 4 to 10 days of AngII infusion, there were macrophages present within the thrombi (Figure 3C). No neutrophils were detected by immunocytochemistry (data not shown).

As aneurysmal tissue matured in the interval beyond 14 days of AngII infusion, there was increased deposition of extracellular matrix in regions previously occupied by thrombi (Figure 4A). Macrophages were also more ubiquitously present in this region (Figure 4C). The remodeled tissue also contained T (Figures 4B and online IIA) and B (Figures 4D and online IIB) lymphocytes. In some tissues,

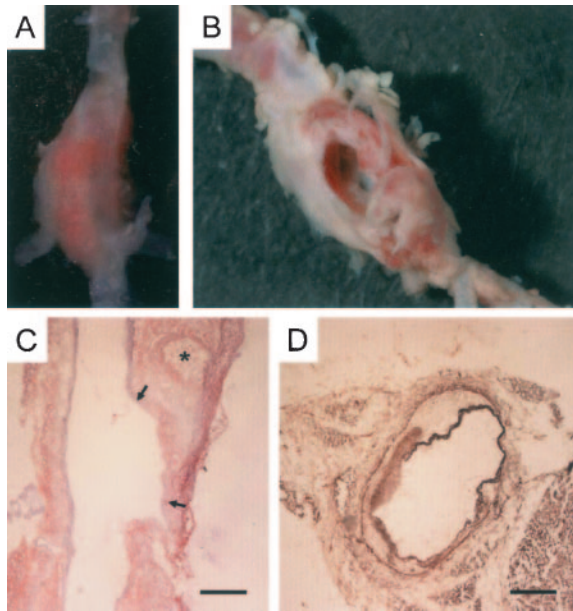


Figure 2. Medial destruction led to localized dissection and accumulation of intramural hematoma after 7 days of AngII infusion into apoE^{-/-} mice. A, Dissection resulted in a saccular accumulation of hematoma in the suprarenal aorta. B, In some mice, AngII infusion resulted in medial destruction that led to rupture of the aorta. C, Longitudinal section of a dissected aorta shows the localized break in the media (arrows) and the intramural hematoma (asterisk). D, Verhoeff's staining for elastin fibers of a cross section of aorta at the level of dissection shows the break in the media.

such as those illustrated in Figures 4 and online II, these 2 cells types were juxtapositioned.

Medial disruption, defined as breaks in elastin fibers, was still present beyond 14 days of AngII infusion (Figure 5A). However, staining with Movat's stain demonstrated the presence of disordered elastin fibers in the region between broken elastin fibers (Figure 5B). By ≈ 28 days of AngII infusion, there were marked changes in the distribution of endothelium, as defined by immunostaining for PECAM-1 (Figure 5C). The dilated lumen was completely reendothelialized over the region of medial disruption, and the endothelium was demonstrable adjacent to the original medial layer that was covered with remodeled tissue. There was also profound neovascularization throughout the aneurysmal tissue (Figure 5D).

Beyond 28 days of AngII infusion, atherosclerotic lesions were detected, as defined by the presence of neutral-lipid staining (Figure 6A) in a tissue region that also immuno-

stained for macrophages (Figure 6B). Atherosclerotic lesions were not detectable in this aneurysmal region at earlier intervals of AngII infusion.

Discussion

Contribution of Monocyte Infiltration to Elastin Destruction

The earliest event that we identified in the abdominal aorta after AngII infusion was the accumulation of macrophages in the media. This is contrary to early-stage accumulation of macrophages in atherosclerotic lesions that is restricted to the intima.¹⁴ Macrophage accumulation has also been demonstrated in the adventitia of all aortic regions examined (suprarenal, thoracic, and sinus) after AngII infusion.^{12,17} However, the suprarenal region is the only area of the aorta in which we have observed macrophage accumulation in the media in response to AngII administration.¹² Therefore, this regional accumulation is consistent with its being a direct determinant of disease evolution. This spatial specificity in response to AngII indicates a molecular mechanism within the suprarenal segment of the artery rather than in the infiltrating cells. There are well-characterized differences between the thoracic and abdominal aorta because of their differing origin, but heterogeneity within these regions has not been well defined.¹⁸ Future studies will be directed at determining molecular properties of the suprarenal aortic region that provides a rationale for the localization of AngII-induced macrophage accumulation.

AngII-induced medial accumulation of macrophages in the suprarenal region was associated with breaks in the elastin lamellae. Degradation of elastin would provide a chemoattractant gradient for recruitment of monocytes, which might be an initiating event.¹⁹ Conversely, monocyte recruitment might lead to elastin degradation. Degradation of elastin might be due to inappropriate activation of specific matrix metalloproteinases (MMPs) that have been implicated in aneurysm formation.^{10,20} Consistent with a role for MMPs, doxycycline, an inhibitor of broad specificity for MMPs,²¹ attenuates the incidence and severity of AngII-induced AAA formation.²²

Mechanism of Dissection

A dramatic manifestation of the early phase of AngII infusion was dissection, as evidenced by the presence of vascular hematomas. The thrombi were constrained by adventitial tissue in most mice, although in some, arterial rupture led to death through loss of blood into the abdominal cavity. We

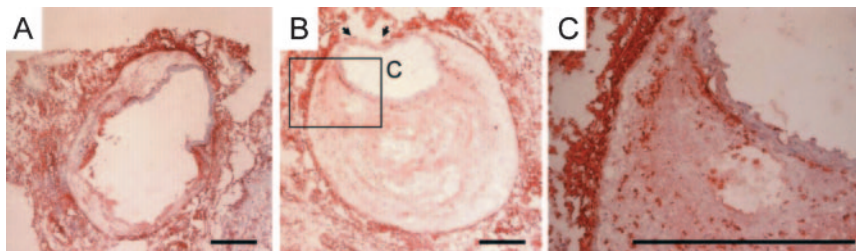


Figure 3. Intramural hematoma elicited a mononuclear inflammatory response in the periadventitial space. Immunostaining of cross sections of the suprarenal aorta with rabbit antiserum for mouse macrophages at the level of dissection (A) and below the level of dissection (B) demonstrates macrophage infiltration around the hematoma. Macrophages from the periadventitial space infiltrated the hematoma and the medial

elastic layers of the aorta (C). Macrophages did not infiltrate around the aortic wall that was not surrounded by the hematoma (arrowheads, B).

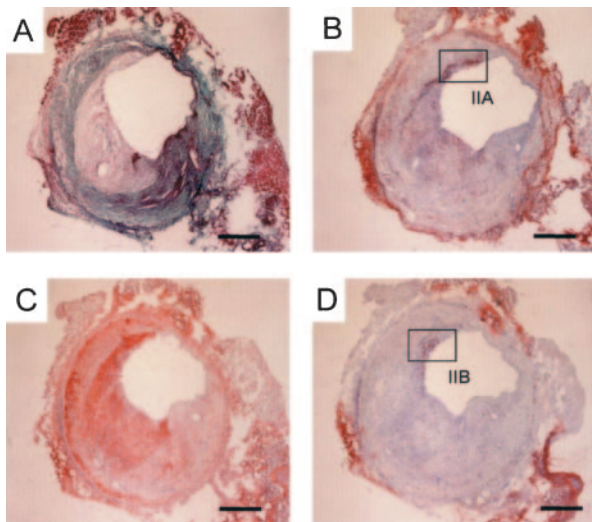


Figure 4. Adventitial remodeling occurred after dissection of the aortic wall, with accumulation of leukocytes and collagen at 28 days of AngII infusion to apoE^{-/-} mice. Sequential cross sections of suprarenal aortas were stained for collagen and leukocytes. A, Gomori's trichrome stain revealed circumferential accumulation of collagen in the adventitia. B, Immunostaining for T lymphocytes (CD90.2), (C) macrophages with rabbit anti-mouse serum for macrophages, and (D) B lymphocytes (CD19). Higher magnification can be viewed online (Figure IIA and IIB)

have previously observed that AngII infusion promotes a profound hypertrophy of the arterial adventitia at the sites of dissection.¹³ Therefore, the outcome of the dissection might be due to the balance of the effects of AngII on promoting the destruction of extracellular matrix versus its ability to promote fibrosis. MMPs have been commonly invoked as the primary mediators of medial destruction that leads to AAA formation.^{20,23} Although there is evidence that AngII promotes the synthesis of both MMP-2 and MMP-9,^{24–26} there are also contrary reports that AngII decreases MMP-2 synthesis.²⁷ Several pathways have been invoked for AngII-induced fibrotic responses, which include increases in synthesis due to effects on transforming growth factor- β and connective-tissue growth factor and decreases in degradation due to augmented plasminogen activator inhibitor-1.^{28–30} Therefore, the ultimate manifestation of AngII-induced dissection is caused by region-specific effects on the destruction and synthesis of extracellular matrix. We are currently using pharmacologic and genetic approaches to define the involvement of a specific MMP, or constellation of MMPs, in AngII-induced AAAs.

Contribution of Inflammation

A pronounced inflammatory response developed after the AngII-induced medial dissection and thrombus formation. AngII has many inflammatory properties, including stimulation of adhesion molecules, chemokines, and cytokines.³¹ However, it is likely that thrombi, rather than AngII, exerted the major influence on the inflammatory response.³² It will be of interest to determine whether coagulation inhibitors are able to antagonize the development of thrombi caused by AngII-induced medial dissection. Furthermore, it will be of

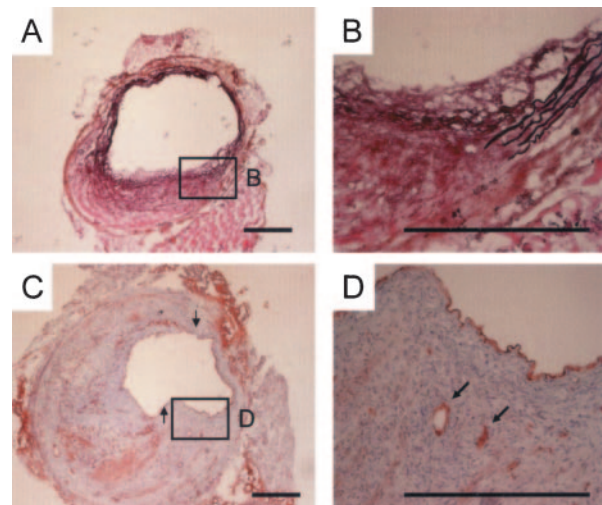


Figure 5. Medial remodeling and neovascularization of the adventitia occurred concomitant with adventitial remodeling at 28 days of AngII infusion to apoE^{-/-} mice. A and B, Movat's pentachrome staining of the suprarenal aorta shows medial elastin fibers in black. Although the normal elastin fibers in the media are arranged in a lamellar pattern, the newly laid fibers are not organized. The reparative process (B) in the media bridged the break in the original elastic lamellae. (C, D) Immunostaining for endothelium (CD31) demonstrated the reestablishment of aortic intima over the medial dissection. Arrows point to the break in the medial elastic layer. Media to the right of the arrows is intact, whereas that to the left is newly developed. The luminal surface was reendothelialized over the region of medial break (C), and pronounced angiogenesis occurred in the aneurysmal tissue (D).

interest to determine the effect of inhibiting thrombus formation on the evolution of aneurysmal disease.

This inflammatory response promotes the rapid infiltration of macrophages. At later stages, we were able to detect the presence of both T and B lymphocytes. The presence of T lymphocytes in AAAs is a feature in common with atherosclerotic lesions, in which they appear to exert a deleterious effect.^{33,34} In contrast, B lymphocytes have rarely been detected in atherosclerotic lesions but are common components of AAAs.^{35,36} The role of these cell types can be explored in mice that are deficient in mature lymphocytes, as described in atherosclerosis studies.^{16,33}

Maturation of Aneurysmal Tissue

After dilation of the arterial lumen, it became more difficult to distinguish the intimal, medial, and adventitial regions of the artery. However, remodeling was evident in aneurysmal tissue that corresponded to each region of the aorta. In the intima, a process of reendothelialization occurred, in which the entire dilated lumen became reseeded with PECAM-1-positive cells. In the media, there was evidence that poorly structured elastin fibers were re-forming in the regions between the dissection. Although this morphology would also be consistent with elastin degradation, our sequential pathology demonstrates that these structures were more likely to have originated from the synthesis, rather than degradation, of elastin. In the adventitia, there was a striking neovascular response at the later stages of disease evolution. The development of new blood vessels might be required for mainte-

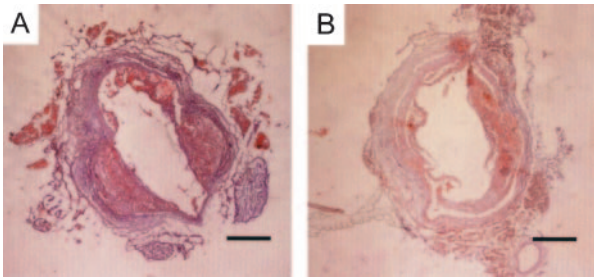


Figure 6. Atherosclerotic lesions developed subsequent to aneurysm formation at 56 days of AngII infusion to apoE^{-/-} mice. A, Oil red O staining illustrates the presence of neutral lipids in circumferential atherosclerotic lesions. B, Immunostaining for macrophages demonstrates the abundance of this cell type in these lesions.

nance of viable tissue in the markedly hypertrophied adventitia but might also assist in propagation of the disease. Therefore, there is a need to define the effects of inhibitors of angiogenesis on AAA development when the drugs are administered after initiation of the aneurysmal response.

Development of Atherosclerosis as a Consequence of AAA

We have previously reported that AngII infusion promotes development of AAAs in LDL receptor^{-/-} and apoE^{-/-} mice.^{11–13} The promotion of aneurysms in hyperlipidemic mice could lead to the assumption that the development of atherosclerosis is a precipitating factor in AAA development. However, although overt atherosclerotic lesions were present in mature aneurysmal tissue, several factors indicate that they developed independently, rather than as initiators of AAA. Although atherosclerotic lesions might occur in the abdominal aorta in mature animals,³⁷ we have not observed discernable atherosclerosis in the suprarenal region of young mice (2 months old at initiation of the protocol), as used in this study. Moreover, we did not observe lesions in the region of the AAA during the early stages of aneurysmal development. Also, it has recently been observed that AngII-induced AAAs can be generated in wild-type C57BL/6 mice, although the incidence was lower than in apoE^{-/-} mice.³⁸ Therefore, hyperlipidemia might augment AngII-induced AAA formation but not necessarily through promotion of atherosclerosis.

Relevance to AAA Formation in Humans

A salient difference between human AAAs and those formed in AngII-infused mice is their location in the infrarenal versus suprarenal region, respectively. The mechanism of location in human disease is unknown. One hypothesized explanation is hemodynamics caused by altered mechanical properties of the artery as a result of regional differences in the ratio of collagen to elastin.³⁹ Currently, there is no information on the relative composition of extracellular matrix throughout the mouse aorta. The suprarenal location that develops AngII-induced AAAs has been a consistent observation between laboratories.^{11,12,40,41} Interestingly, this is also the location for AAAs in both aged apoE^{-/-} mice fed a modified diet⁴² and mice with compound deficiencies of both apoE and endothelial nitric oxide synthase.⁴³ As noted earlier, the embryologic

lineage of vascular smooth muscle cells of the thoracic and abdominal aortas determines their response to various cytokines and matrix elements.^{18,44} This might be the reason for the site specificity of the aneurysmal response of the abdominal aorta to AngII.

Extrapolation of these temporal cellular events to defining the evolution of human disease is hindered by the limited data of sequential events in human AAAs. By analogy with atherosclerosis research, animal models provided substantial insight into the evolution of the human disease.^{45,46} These studies have been used in concert with human data from studies such as Pathobiological Determinants of Atherosclerosis in Youth to develop a sequence of cellular and biochemical change in the disease.⁴⁷ Therefore, further information is required on the early stages of human AAAs.

In summary, we have defined the temporal characteristics of the evolution of AngII-induced AAAs (online Figure III). The demonstration of medial dissection that precedes thrombus formation, inflammation, and atherosclerosis will have an impact in determining the molecular targets for prevention of the disease.

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