Genetically engineered resistance for MMP collagenases promotes abdominal aortic aneurysm formation in mice infused with angiotensin II

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Clinical evidence links increased aortic collagen content and stiffness to abdominal aortic aneurysm (AAA) formation. However, the possibility that excess collagen contributes to AAA formation remains untested. We investigated the hypothesis that augmented collagen promotes AAA formation, and employed apoE-null mice expressing collagenase-resistant mutant collagen (Col^{R/R}/apoE^{-/-}), heterozygote (Col^{R/+}/apoE^{-/-}), or wild-type collagen (Col^{+/+}/apoE^{-/-}) infused with angiotensin II to induce AAA. As expected, the aortas of Col^{R/R}/apoE^{-/-} mice contained more interstitial collagen than those from the other groups. Angiotensin II treatment elicited more AAA formation in Col^{R/R}/apoE^{-/-} mice than Col^{R/+}/apoE^{-/-} or Col^{+/+}/apoE^{-/-} mice. Aortic circumferences correlated positively with collagen content, determined by picrosirius red and Masson trichrome staining. Mechanical testing of aortas of Col^{R/R}/apoE^{-/-} mice showed increased stiffness and susceptibility to mechanical failure compared to those of Col^{+/+}/apoE^{-/-} mice. Optical analysis further indicated altered collagen fiber orientation in the adventitia of Col^{R/R}/apoE^{-/-} mice. These results demonstrate that collagen content regulates aortic biomechanical properties and influences AAA formation.

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The pathogenesis of abdominal aortic aneurysm (AAA) remains uncertain, and the lack of definitive insight suggests a complex, multifactorial process.^{1–4} AAA possesses major alterations in the content of extracellular matrix proteins: collagen and elastin.^{3,5–7} Interstitial collagen increases arterial stiffness, a property linked to AAA formation.^{8,9} Clinical evidence links increased arterial stiffness or collagen content to aortic aneurysms, whereas nonaneurysmal portions of aortas in AAA patients have showed increased collagen accumulation.^{10–20} However, whether collagen accumulation contributes causally to aneurysm formation or merely represents an epiphenomenon remains untested. To unravel the complex pathogenesis of AAA, we hypothesized that examination of both collagen content and its mechanical properties in mice with a genetic abnormality would provide novel insight.

To test directly *in vivo* whether aortic stiffness due to excess collagen accumulation promotes aneurysm formation, we used collagenase-resistant knock-in (Col^{R/R}) mice.^{21,22} This mouse strain bears a mutation in type I collagen, which comprises two-thirds of aortic collagen, at a cleavage site of the $\alpha 1$ (I) chain (Ile⁷⁷⁶ to Pro⁷⁷⁶) shared by collagenases of the matrix metalloproteinase (MMP) family (MMP-1/collagenase-1, MMP-8/collagenase-2 and MMP-13/collagenase-3). This mutation thus confers resistance to degradation, resulting in excess arterial collagen accumulation.²² Our study used angiotensin II (AngII) infusion to provoke AAA formation in apolipoprotein E-deficient (apoE^{-/-}) mice as established by Daugherty *et al.*²³ AngII also stimulates collagen synthesis *in vitro* and induces collagen turnover and remodeling in the aortic wall of apoE^{-/-} mice with features similar to human AAA.^{24–26}

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Our results revealed that augmented collagen content induced by collagenase resistance modified the mechanical properties of the abdominal aortas of apoE^{-/-} mice, and accelerated AAA formation. These data connect excess collagen accumulation and aortic stiffness to AAA formation, providing a novel potential mechanism for the pathogenesis of this complex disease process.

MATERIALS AND METHODS

Animal Experiments and Tissue Preparation

All mouse experiments conformed to protocols approved by the Standing Committee on Animals at Harvard Medical School. Col^{R/R} mice, backcrossed seven generations into C57BL/6, were crossed into apoE^{-/-} mice (C57BL/6), yielding littermates of Col^{R/R}/apoE^{-/-}, Col^{R/+}/apoE^{-/-}, and Col^{+/+}/apoE^{-/-} mice.²² Five-month-old male mice received AngII (1.44 mg/kg/day) infusions for 4 weeks with subcutaneously implanted osmotic mini pumps (Alzet). Mice were anesthetized and perfused with 4% paraformaldehyde at a constant pressure of 100 mm Hg through the heart. Aortas were dissected in the region of the superior mesenteric and right renal arteries and embedded with paraffin.

Histological Analyses

Sections (6-μm thick) were stained with Hematoxylin-Eosin, Masson trichrome, and picrosirius red. Aortic circumferences were measured on the histological sections by tracing the internal elastic lamina at the level of superior mesenteric artery in the aortas of AngII-infused Col^{+/+}/apoE^{-/-}, Col^{R/+}/apoE^{-/-}, and Col^{R/R}/apoE^{-/-} mice ($n=8$, $n=6$, and $n=8$, respectively), using the NIH Image software (Research Services Branch, National Institute of Mental Health). Aortic diameter of each mouse was then obtained based on the circumference, which determined the incidence of AAA (>1.5-fold greater than the aortas of mice untreated with AngII). Picrosirius red staining was viewed under polarized light to detect fibrillar collagen.^{22,27-29} Using picrosirius red- or Masson-stained sections, adventitial collagen was measured in the tissue within 0.1 mm of the adventitial-medial border. Immunohistochemistry employed mouse monoclonal antibodies against human α-smooth muscle actin (1A4; Dako) and human cathepsin S (Serotec) with a kit for immunohistochemistry using mouse primary antibodies on mouse tissues (Iso-IHC AEC kit; InnoGenex) and rat monoclonal antibody against mouse macrophages (Mac3; Pharmingen). Quantitative histological analysis on immunostaining and collagen staining used a digital imaging system (ImagePro Plus v5.1; Media Cybernetics).^{22,28}

RNA Extraction and Real-Time RT-PCR

Total RNA extraction employed RNA-Bee (Tel-Test) and RNeasy (Qiagen) followed by reverse transcription using SuperScript II reverse transcriptase (Invitrogen). Real-time RT-PCR employed PRISM7900 with SYBR green Master

Mix (Applied Biosystems). Primer sequences are available on request.

Mechanical Testing of Aortas

To examine the effects of collagenase resistance on the mechanical properties of the aortas, strain experiments employed freshly isolated, unfixed preaneurysmal abdominal aortas of Col^{+/+}/apoE^{-/-} ($n=3$) and Col^{R/R}/apoE^{-/-} ($n=4$) mice after AngII infusion (2 weeks). Parker MX80L electromechanical positioning system (Daedal Division) generated repetitive, controlled strains and ULC-1N load cell (Interface) recorded forces. Model SGA Strain Gauge Transducer Amplifier (Interface) passed through load cell signals. After determining a zero-point for each aorta, ie, when the aorta began to experience significant changes in restoring force, aorta length at zero-point was defined as the rest length. This measurement served to calculate subsequent strains for this aorta. The aorta was preconditioned with 300 cycles of 20% strain, then stressed for 10 cycles at 70% strain, both at 0.5 Hz frequency, and finally stretched to fracture (strain-to-break) by 5% strain increments up to 250% strain. The aorta's restoring forces were measured 20 times per cycle (10 times on extension and 10 times on relaxation), except for the strain-to-break experiment, which measured the forces at every 5% step. To determine the ultimate strength of the aortas, the strain-to-break experiment determined the stress and strain at the maximum force. The stresses, calculated by dividing the measured forces by the cross-sectional areas of the aorta, were reported as relative stresses to control stresses (which are normalized to 100%) for each experiment. Stiffness was approximated linearly using the stress at 70% strain, normalized to the control conditions.

Collagen Fiber Orientation

To probe further the mechanisms that link increased aortic adventitial collagen content to mechanical failure in collagenase-resistant mice, we performed additional qualitative analysis of collagen organization in the nonaneurysm portions (in Col^{+/+}/apoE^{-/-} and Col^{R/R}/apoE^{-/-} mice after 2 weeks of AngII treatment) and aneurysm portions (in Col^{R/R}/apoE^{-/-} mice after 4 weeks of AngII) of the aortic tunica adventitia in a subset of animals ($n=3$ for each group). Specifically, we examined and compared, using linearly polarized light and picrosirius red-stained sections, the collagen fiber extinction patterns seen in the three groups as the tissue sections were rotated on the microscope stage. When a fiber (or part of a fiber) is aligned parallel to the transmission axis of either of the microscope's two polarizing filters, it appears dark (said to be at extinction); at other orientations, the fiber appears bright.³⁰ Thus, the extinction pattern provides information on collagen fiber organization.

Statistics

Differences between two groups and among three groups were determined using the unpaired Student's *t*-test and one-

Table 1 Characteristics of mice

	<i>Col</i> ^{+/+} / <i>apoE</i> ^{-/-} (n = 8)	<i>Col</i> ^{R/+} / <i>apoE</i> ^{-/-} (n = 6)	<i>Col</i> ^{R/R} / <i>apoE</i> ^{-/-} (n = 8)	P
Body weight (g)	31.6 ± 0.6	32.6 ± 1.1	30.9 ± 0.9	NS
Cholesterol (mg/100 ml)	606.3 ± 27.5	635.3 ± 63.9	620.2 ± 28.5	NS
Triglycerides (mg/100 ml)	141.5 ± 10.9	118.0 ± 25.2	120.6 ± 14.5	NS
Systolic blood pressure ^a (mm Hg)	130.5 ± 17.0	134.6 ± 23.8	130.2 ± 9.5	NS

Values are mean ± s.e.m.

^aMeasured in four animals of *Col*^{+/+}/*apoE*^{-/-}, four animals of *Col*^{R/+}/*apoE*^{-/-}, and three animals of *Col*^{R/+}/*apoE*^{-/-}.

way ANOVA followed by Tukey's test, respectively. Pearson's test examined the correlation between collagen content and aortic size. Statistical testing for stretching experiments was determined using the unpaired Student's *t*-test. To compare relative changes, we used a single-group comparison *t*-test to test the hypothesis that the mean relative change differs from zero.

RESULTS

Collagenase Resistance Accelerated AAA Formation in AngII-Treated *apoE*^{-/-} Mice

Body weight, plasma total cholesterol, and triglyceride levels in all three mouse strains treated with AngII did not differ significantly (Table 1). Elevated blood pressure may promote AAA formation. Among *Col*^{+/+}/*apoE*^{-/-}, *Col*^{R/+}/*apoE*^{-/-}, and *Col*^{R/R}/*apoE*^{-/-} strains, conscious blood pressure did not differ (Table 1). AngII increased the diameter of abdominal aortas at the level of the superior mesenteric artery in *Col*^{+/+}/*apoE*^{-/-} mice (Figure 1a, left), as demonstrated by Daugherty *et al.*²³ However, *Col*^{R/+}/*apoE*^{-/-} mice exhibited greater aneurysm formation than did *Col*^{+/+}/*apoE*^{-/-} mice (Figure 1a, right). Histological sections showed that *Col*^{R/+}/*apoE*^{-/-} aorta dilated more than those of similarly treated *Col*^{+/+}/*apoE*^{-/-} mice (Figure 1b). Notably, the tunica intima rarely expanded in the abdominal aortas of these mice. We also observed no sign of rupture or thrombus formation in the aneurysmal aorta. Quantitative analysis demonstrated that the circumference of the aortas in *Col*^{R/+}/*apoE*^{-/-} mice exceeded that of *Col*^{R/+}/*apoE*^{-/-} or *Col*^{+/+}/*apoE*^{-/-} mice (Figure 1c). AAA (>1.5-fold increases in diameter) developed in 50% of AngII-infused *Col*^{+/+}/*apoE*^{-/-} mice (Figure 1d). The incidence was greater in *Col*^{R/+}/*apoE*^{-/-} (83%) and *Col*^{R/R}/*apoE*^{-/-} mice (100%). These results indicate that collagenase resistance accelerated AAA formation in a gene dosage-dependent manner.

Collagenase Resistance Induced Excess Collagen Content in The Aortic Adventitia

Defining whether collagenase resistance with AngII treatment promotes excess collagen accumulation entailed picosirius red staining for quantification of the collagen content in the aortas. Quantification of picosirius red staining viewed with polarization measures the content of fibrillar collagen,

which comprises more than 90% of aortic collagen.⁵ *Col*^{R/+}/*apoE*^{-/-} mice had more fibrillar collagen in aortas localized in the adventitia than *Col*^{+/+}/*apoE*^{-/-} mice (Figure 2a). Masson trichrome staining, another histological assay for collagen, also demonstrated more prominent collagen accumulation in the adventitia of *Col*^{R/+}/*apoE*^{-/-} mice (Figure 2b). Further, quantitative analysis of collagen content determined by picosirius red and Masson trichrome staining demonstrated more abundant adventitial collagen in the aortas of *Col*^{R/+}/*apoE*^{-/-} mice vs *Col*^{R/+}/*apoE*^{-/-} and *Col*^{+/+}/*apoE*^{-/-} mice (Figure 2c). Real-time RT-PCR showed slightly decreased levels of type I procollagen α 1 mRNA in the aortas of *Col*^{R/+}/*apoE*^{-/-} mice compared to those of *Col*^{+/+}/*apoE*^{-/-} mice (Figure 2d). These results suggest that impaired collagen degradation by collagenases of the MMP family, but not increased synthesis, accounts for the greater collagen accumulation in the aortic adventitia of *Col*^{R/+}/*apoE*^{-/-} mice.

Collagen Content in the Adventitia Correlated with Size of the Aorta

As *Col*^{R/+}/*apoE*^{-/-} mice had both increased collagen accumulation and accelerated formation of AAA, we tested the hypothesis that aortic collagen content correlates with abdominal aortic dimension. Regression analysis demonstrated that adventitial collagen content correlated positively with aorta circumferences in AngII-treated *apoE*^{-/-} mice, as determined by both picosirius red and Masson trichrome staining (Figure 3).

Collagenase Resistance Promoted Disruption of Medial Elastic Laminae, but did not Induce Expression of Elastases or Collagenases

Aneurysms characteristically have disrupted medial elastic laminae. In this study, the aortas of *Col*^{R/+}/*apoE*^{-/-} mice exhibited more prominent, but focal, medial disruption compared to the aortas of *Col*^{-/-}/*apoE*^{-/-} mice (Figure 4a). However, medial disruption of *Col*^{R/+}/*apoE*^{-/-} mice aortas did not colocalize with either macrophage accumulation, a major source of various elastolytic enzymes, or immunoreactive cathepsin S, a key elastase in vascular remodeling (Figure 4b).

Figure 1 Collagenase resistance accelerated AAA formation. **(a)** Representative gross appearance of abdominal aortas of AngII-treated $Col^{+/+}/apoE^{-/-}$ (left) or $Col^{R/R}/apoE^{-/-}$ (right) mice. **(b)** Representative Hematoxylin-Eosin staining of the abdominal aortas from $Col^{+/+}/apoE^{-/-}$ (top) and $Col^{R/R}/apoE^{-/-}$ (bottom) mice. The aortic wall protrudes and extends to the right in the $Col^{R/R}/apoE^{-/-}$ mouse. **(c)** Quantitative analysis of circumference of the internal lamina. $Col^{R/R}/apoE^{-/-}$ mice ($n = 8$) had greater abdominal aortas compared with $Col^{+/+}/apoE^{-/-}$ ($n = 6$) or $Col^{R/+}/apoE^{-/-}$ mice ($n = 8$). Bars and error bars represent mean and s.e.m, respectively. **(d)** Collagenase resistance also increased the incidence of AAA formation (> 1.5 -fold increase in diameter).

To address the potential role of the changes in smooth muscle cells, we performed immunohistochemistry for smooth muscle α -actin in the aortas of mice treated with AngII. In all nonaneurysmal regions, the majority of medial smooth muscle cells stained positively for α -actin in both groups (Figure 4c). In contrast, in aneurysmal segments of $Col^{+/+}$ and $Col^{R/R}$ mice, the disrupted medial smooth

muscle cell layer did not contain detectable levels of α -actin. These results were reproducible in all animals with no substantial differences between wild-type and collagenase-resistant mutant mice.

Germline manipulation of a gene in mice could cause 'compensatory' changes in other genes that share similar functions with the targeted gene. Such responses could

Figure 2 Collagen accumulates in the adventitia. **(a)** Representative cross sections stained with picrosirius red viewed under polarization. **(b)** Masson trichrome staining on cross sections adjacent to those shown in **(a)**. **(c)** Quantitative analysis of collagen content in the adventitia, determined by picrosirius red (left) and Masson trichrome (right) staining. Collagen increased in the adventitia of $Col^{R/R}/apoE^{-/-}$ ($n = 8$) compared to those of $Col^{+/+}/apoE^{-/-}$ mice ($n = 8$). **(d)** Real-time RT-PCR demonstrated that α -1 collagen expression did not increase in the aortas of $Col^{R/R}/apoE^{-/-}$ compared to those of $Col^{R/+}/apoE^{-/-}$ mice. The data were obtained from three independent pooled total RNA samples of the aorta ($n = 2$). Bars and error bars represent mean and s.e.m, respectively.

hinder data interpretation and require careful consideration. Indeed, our previous study of acute myocardial infarction in MMP-9-deficient mice yielded unexpected results likely due

to increases in other MMPs.³¹ Therefore, this study examined expression of several elastases including MMP-9, MMP-12 (MMP family elastases), and cathepsin S (a major non-MMP

elastase) to evaluate whether compensatory increases could contribute to elastolysis. Quantitative real-time RT-PCR demonstrated that GAPDH-adjusted levels of mRNAs encoding MMP-9, MMP-12, and cathepsin S in the AngII-treated aortas did not differ between Col^{R/R}/apoE^{-/-} and Col^{+/+}/apoE^{-/-} mice (Figure 5a). Additionally, we evaluated the expression of these elastases in macrophages, the major source of matrix-degrading proteinases in aortas. Collagenase resistance did not affect elastase gene expression in peritoneal macrophages from Col^{R/R}/apoE^{-/-} mice compared to those from Col^{+/+}/apoE^{-/-} mice (Figure 5b). We further evaluated expression of interstitial collagenases (MMP-8 and MMP-13). In particular, MMP-13, the major interstitial collagenase in mice (a species that lacks MMP-1), has elastase activity³² and converts pro-MMP-9 into its active form.³³ AngII-treated aortas and peritoneal macrophages of Col^{R/R}/apoE^{-/-} and Col^{+/+}/apoE^{-/-} mice expressed similar levels of MMP-8 and MMP-13 mRNAs (Figure 5a and b).

Collagenase Resistance Increased Aortic Stiffness and Promoted Susceptibility to Mechanical Failure

To test further the hypothesis that aortic stiffness due to excess collagen accumulation impairs resistance of the aorta to mechanical stresses, we examined the biomechanical properties of preaneurysmal aortas obtained from Col^{+/+}/

apoE^{-/-} and Col^{R/R}/apoE^{-/-} mice after 2 weeks of AngII infusion. The 70%-strain cycles assessed durability, whereas the strain-to-break experiments aimed to gauge the ultimate strength of the aortas (Figure 6a). As expected, the aortas of Col^{R/R}/apoE^{-/-} mice exhibited increased initial stiffness at the first 70% strain (after preconditioning) compared to those of Col^{+/+}/apoE^{-/-} mice (Figure 6b). However, stiffness decreased more rapidly in the aortas of Col^{R/R}/apoE^{-/-} mice after 10 cycles of the 70% strain than those of Col^{+/+}/apoE^{-/-} mice, suggesting higher susceptibility to fatigue-type failure in collagenase-resistant aortas (Figure 6c).

Examining whether the aortas of Col^{R/R}/apoE^{-/-} mice have impaired resistance to stretch required the strain-to-break test. Based on the stresses at failure, we found significantly lower ultimate stresses on the aortas of Col^{R/R}/apoE^{-/-} mice vs Col^{+/+}/apoE^{-/-} mice (Figure 6d). Thus, the collagen-enriched aortas of the collagenase-resistant animals are more fragile and more easily broken by deformation than wild-type aortas.

Collagenase Resistance Altered Collagen Orientation in the Aortic Adventitia

To address the potential mechanism for the paradoxical association of increased collagen accumulation in the aortas of Col^{R/R} mice and susceptibility to rapid fatigue and ultimate

Figure 3 Collagen content in the adventitia correlates with the size of aortas. Left, correlation between circumference of the internal lamina and percentage of picrosirius-red positive areas in the adventitia. Right, correlation between circumference of the internal lamina and the percentage of Masson trichrome-positive areas in the adventitia. The data were obtained from all apoE^{-/-} mice studied (n = 24).

Figure 4 Elastin disruption in aortas of Col^{R/R}/apoE^{-/-} mice following AngII infusion. (a) Elastica von Gieson staining demonstrates disrupted medial elastin in aortas of Col^{+/+}/apoE^{-/-} and Col^{R/R}/apoE^{-/-} mice. Note that Col^{R/R}/apoE^{-/-} showed marked loss of medial elastin (right side). (b) Immunohistochemistry for macrophages (Mac-3), smooth muscle cells (α -actin), and cathepsin S in aortic wall of Col^{R/R}/apoE^{-/-} mouse. The same section was stained with Elastica von Gieson. Disrupted medial elastin (arrow) did not colocalize with macrophages or immunoreactive cathepsin S. (c) In nonaneurysmal regions, the majority of medial smooth muscle cells stained positively for α -actin in both Col^{+/+} and Col^{R/R} mice. In contrast, in aneurysmal segments of both groups, the disrupted medial smooth muscle cell layer did not contain detectable levels of α -actin. These results were reproducible in all animals with no substantial differences between Col^{+/+} and Col^{R/R} mice (n = 8 each).

Figure 5 Collagenase resistance did not cause compensatory changes in elastolytic and collagenolytic enzymes. Real-time PCR demonstrated similar mRNA levels of MMP elastases (MMP-9 and MMP-12), MMP collagenase (MMP-8 and MMP-13), and cathepsin S (Cat-S) in aortas treated with AngII for 2 weeks from Col^{+/+}/ApoE^{-/-} or Col^{R/R}/apoE^{-/-} mice (**a**) and in thioglycollate-treated peritoneal macrophages (**b**) from Col^{+/+}/apoE^{-/-} or Col^{R/R}/apoE^{-/-} mice. The data were obtained from three independent pooled total RNA samples of the aorta ($n = 2$) or three different preparations of peritoneal macrophages. Bars and error bars represent mean cycle numbers (Δ CT: 40—threshold cycle numbers) and s.e.m.

disruption, we subsequently examined whether collagen orientation changed in collagenase-resistant mice. Figure 7 illustrates both schematic and actual representative extinction patterns seen with different collagen fiber architecture. The extinction pattern observed in the aneurysm wall corresponded to that expected for straight fibers (Figure 7a and b, top panels), whereas the banded extinction pattern frequently observed in the adventitia of Col^{R/R} aortas indicated two-dimensional fiber waviness (Figure 7a and b, middle panels). In contrast, the narrow band of extinction frequently observed to sweep across fibers in the adventitia of the wild-type samples echoed fibers whose waviness possesses a three-dimensional helical component (Figure 7a and b, bottom panels). These results reveal major differences in collagen fiber architecture in the adventitia. In contrast, we observed no structural changes or differences between wild-type and collagenase-resistant mutant mice in the medial collagen fibers of the nonaneurysmal segments of the aorta (data not shown).

DISCUSSION

Multiple biomechanical and biological factors, including smoking, hypertension, shear or circumferential stress, aging, and atherosclerosis, may contribute to the development of AAA.^{5,6,19} Such risk factors can influence the metabolism of the aortic extracellular matrix and promote ongoing structural matrix remodeling, a characteristic of AAA. Collagen and elastin comprise the two major extracellular matrix constituents of the aorta. Regulation of both elastin and collagen content in the abdominal aorta appears dynamic.^{5,6,19} Clinical evidence indicates an important role for aortic stiffness and/or excess collagen in aneurysm formation.^{10–16} Therefore, this study tested the hypothesis that excess collagen accumulation due to impaired collagen catabolism during aortic remodeling promotes AAA formation.

Daugherty *et al*²³ found that AngII treatment in apoE^{-/-} mice produces AAA. AngII also increases arterial collagen turnover: production and degradation.^{24–26} Our study used AngII-treated apoE^{-/-} mice bearing a mutation that renders collagen type I collagenase-resistant to induce greater aortic collagen accumulation than those expressing wild-type collagen and to test whether excess collagen accelerates AAA formation. Interstitial collagens, especially type I collagen, consist of a triple helix of polypeptide chains that resist degradation by most proteases except the interstitial collagenases.^{21,34} Unperturbed collagenase-resistant mice show no overt vascular phenotype.²¹ Indeed, without AngII treatment, Col^{R/R}/apoE^{-/-} mice do not develop aneurysm (data not shown). We found more prominent AAA formation and collagen accumulation in AngII-treated Col^{R/R}/apoE^{-/-} mice.

Collagen provides mechanical integrity to the arterial wall and regulates various biological functions of vascular cells.^{8,35} Adventitial fibrillar collagen generally confers tensile mechanical strength on the aorta.⁵ Collagen also contributes to aortic stiffness.⁸ Compared to the aortas of Col^{+/+}/apoE^{-/-} mice, increased collagen in the aortas of Col^{R/R}/apoE^{-/-} mice led to increased stiffness, as demonstrated by our mechanical testing (Figure 6b). However, this increased stiffness did not provide durability to the aorta (Figure 6c and d). Rather, compared to Col^{+/+}/apoE^{-/-} mice, the aortas of Col^{R/R}/apoE^{-/-} mice experienced more rapid loss of stiffness as well as mechanical failure at lower stresses, indicating that excess collagen accumulation promoted susceptibility to mechanical failure.

The apparent paradoxical association of increased aortic collagen accumulation in Col^{R/R} mice and susceptibility to rapid mechanical fatigue and eventual tissue disruption requires further consideration, even though our findings concur with clinical evidence linking aortic stiffness and increased collagen with aneurysm formation.^{10–15} Collagen's

Figure 6 Mechanical testing of the aorta. (a) The strain-device used to measure the mechanical response of aortas to cyclic strain and strain failure. The load cell is on the right and the electromechanical positioning system is on the left. The aorta is clamped in the center above the hole. (b) Aortic stiffness of Col^{R/R}/apoE^{-/-} mice is 65% higher, on average, compared to Col^{+/+}/apoE^{-/-} mice. (c) After 10 cycles of 70% strain, Col^{R/R}/apoE^{-/-} aortas showed a significant reduction in stiffness compared to Col^{+/+}/apoE^{-/-} aortas (Col^{R/R}/apoE^{-/-}: 32% decrease, Col^{+/+}/apoE^{-/-}: 8% decrease in stiffness before 70% strain test). (d) Aortas of Col^{R/R}/apoE^{-/-} mice failed under stresses at 48% of the failure, compared to Col^{+/+}/apoE^{-/-} aortas. These assays employed a subset of Col^{+/+}/apoE^{-/-} (n = 3) and Col^{R/R}/apoE^{-/-} mice (n = 4).

mechanical properties depend not only on its quantity and biochemical properties, but also on the fiber architecture. We indeed found substantial architectural differences between groups in the subset of mice studied in this regard. Normal arterial adventitial collagen organization displays a three-dimensional helical waviness,³⁶ the extinction patterns observed in our wild-type samples reflect such an organization (Figure 7). In our study, aortic adventitial collagen assumed a more two-dimensional, but still wavy, structure in Col^{R/R} mice (probably to accommodate the collagen increase). Additional remodeling resulted in fiber straight-

tening in the aneurysm wall. Assuming the absence of concurrent biochemical changes, such structural alterations would associate with increased tissue stiffness. In tissues subject to pulsatile or multiaxial deformation, the compliance provided by three-dimensional fiber waviness probably provides mechanical advantage. For example, skin possesses a three-dimensional structure and exhibits extinction patterns³⁷ similar to those of the Col^{+/+} adventitia. In contrast, collagen fibers in tendons, prone to rupture due to mechanical stresses, have a predominantly two-dimensional structure and extinction patterns³⁰ similar

to those seen in the adventitia and aneurysm of $Col^{R/R}$ tissue. We propose that these architectural changes in the adventitia agree with our mechanical data and explain why collagen accumulation induced by collagenase resistance paradoxically

increases susceptibility to mechanical failure. We observed no structural differences between groups in the collagen fibers or smooth muscle cells in the media of the nonaneurysmal aortic segments. Nonetheless, these observations were not

Figure 7 Collagen extinction patterns viewed with linearly polarized light provide an indication of fiber organization. (a) Schematic extinction patterns for three fiber configurations at three orientations (the cross indicates the alignment of the polarizers' transmission axes). When collagen is aligned parallel to either transmission axis, it appears dark. At all other orientations, it appears bright. The upper row of each schematic shows the extinction pattern that reflects fiber orientation illustrated in the lower row (dotted line connects fiber segments aligned parallel to a transmission axis to corresponding extinction pattern). Top: No portion of the straight fiber in the first column (0°) is parallel to a transmission axis and so appears bright. When rotated by 15° (second column), the entire fiber is parallel to the horizontal transmission axis and appears dark. When rotated an additional 15° (third column), the fiber again appears bright. Middle: Portions of the wavy fiber are parallel to a transmission axis, and hence a banded extinction pattern results. A 15° rotation brings different portions parallel to the transmission axes, producing a different banded pattern. Additional rotation changes the pattern again. Bottom: A fiber with three-dimensional waviness, ie, a helical structure (dashed line). In the first column, only a small portion of this fiber is aligned parallel to a transmission axis and a single narrow extinction band results; the rest of the fiber appears bright. Additional 15° rotations bring different regions to extinction—a narrow extinction band sweeps across the fiber. (b) Actual aortic tissue extinction patterns. Top: Aneurysm collagen from a collagenase-resistant mouse (4-week AngII). An arrow marks the fiber of interest and an arrowhead a fiber that appears bright in each panel. The entire marked fiber appears dark in the middle panel, but bright in the others—consistent with a straight fiber. Middle: Preaneurysmal Col^{R/R} tissue (2-week AngII). Different fiber portions appear dark at each orientation (white dots placed immediately below the dark area)—consistent with two-dimensional waviness. Bottom: Wild-type (2-week AngII). With rotation, a narrow extinction band sweeps across the marked fiber—consistent with three-dimensional waviness. The white dot lies approximately at the end of the band of extinction in each panel. These data represent three different mice per group that produced similar results.

made in regions directly involved in aneurysm formation and therefore whether medial changes occur during the initial development of the aneurysm remains to be determined.

Accumulating evidence suggests that MMPs participate in various aspects of cardiovascular remodeling including aneurysm formation.^{2,3,22,27–29,38–44} Previous studies including our own showed that MMP elastases and collagenases increase in the wall of human AAA.^{2,38–40} Degradation of the extracellular matrix in the aortic wall may cause aneurysmal changes, and animal studies suggest that MMPs participate in aneurysm formation.^{2,3,38–44} Understanding further mechanisms of aneurysm formation involving MMPs should provide important insights into therapeutic strategies for this disease. This study did not attempt to determine the role of MMPs in the pathogenesis of aneurysms, but tested the specific hypothesis that excess collagen accelerates AAA formation, using mice that resist cleavage by MMP family collagenases. We were also aware that if MMP collagenases play a role in AAA formation, introducing a loss-of-function phenotype due to collagenase resistance could decrease the incidence of AAA, contrary to our originally anticipated results. The data showed that collagenase resistance yielded foci of substantial aortic dilatation in AngII-treated apoE^{−/−} mice, supported further by mechanical testing data. In addition, collagenase resistance did not cause compensatory increases of mRNA that encode major elastases. Collectively, these results suggest that augmented formation of AAA in this particular study depends primarily on accumulation of uncleavable fibrillar collagen, but not directly on changes in matrix-degrading enzymes.

Our study demonstrates that collagenase resistance promotes excessive collagen accumulation in aortic adventitia and modifies aortic mechanical properties, in turn accelerating aneurysm formation. These results in genetically altered mice provide mechanistic insights into the pathogenesis of AAA formation, specifically linking collagen content and organization to the aorta's mechanical properties. Furthermore, these associations implied by our results combined with the novel finding of altered collagen architecture provide

a basis for future investigation of the structural and mechanical aspects of AAA formation.

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