Novel Arterial Pathology in Mice and Humans Hemizygous for Elastin

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Abstract

Obstructive vascular disease is an important health problem in the industrialized world. Through a series of molecular genetic studies, we demonstrated that loss-of-function mutations in one elastin allele cause an inherited obstructive arterial disease, supravalvular aortic stenosis (SVAS). To define the mechanism of elastin's effect, we generated mice hemizygous for the elastin gene (*ELN* +/−). Although *ELN* mRNA and protein were reduced by 50% in *ELN* +/− mice, arterial compliance at physiologic pressures was nearly normal. This discrepancy was explained by a paradoxical increase of 35% in the number of elastic lamellae and smooth muscle in *ELN* +/− arteries. Examination of humans with *ELN* hemizygosity revealed a 2.5-fold increase in elastic lamellae and smooth muscle. Thus, *ELN* hemizygosity in mice and humans induces a compensatory increase in the number of rings of elastic lamellae and smooth muscle during arterial development. Humans are exquisitely sensitive to reduced *ELN* expression, developing profound arterial thickening and markedly increased risk of obstructive vascular disease. (*J. Clin. Invest.* 1998. 102:1783–1787.) Key words: elastin • cardiovascular models • mice, transgenic • vascular disease • compliance

Introduction

Obstructive vascular disease is the predominant cause of mortality and morbidity in developed nations (1). Extensive research has implicated key pathogenic roles for lipids and growth factors, but it is likely that other factors participate in the pathogenesis of these disorders. In a series of molecular genetic experiments, we demonstrated that a human obstructive vascular disorder, supravalvular aortic stenosis (SVAS),[1] is associated with hemizygosity of the elastin gene (*ELN*) (2–6). Mutations in genes encoding other structural proteins, including collagen and fibrillin, have also been associated with vascular disease, but these genetic abnormalities cause dissection and degeneration, not obstruction (7, 8). The pathogenic mechanisms underlying *ELN* mutations are not understood.

Elastin is the dominant arterial extracellular matrix protein, comprising 50% of the dry weight of the aorta (9). Encoded by a single gene on human chromosome 7q11.23, elastin expression is largely confined to the third trimester of fetal development and early postnatal years. Elastin is synthesized by smooth muscle, secreted as a soluble monomer, tropoelastin, and organized into insoluble polymers that form concentric rings of elastic lamellae around the arterial lumen. Each elastic lamella alternates with a ring of smooth muscle, forming a lamellar unit. Elastic lamella provides the resilience that arteries need to absorb hemodynamic stress of cardiac systole and to release this energy in the form of sustained blood pressure during diastole. The number of lamellar units is thought to be species specific, fixed, and genetically predetermined (10, 11).

Previously, human pathologic studies of SVAS described medial necrosis, fibrosis, and disorganization, a common end-stage pathology of many vascular diseases (12, 13). Identifying the mechanism of disease through these studies was hampered by the inability to separate cause and effect. To define the pathogenic mechanism underlying SVAS, we characterized mice hemizygous for *ELN*. This work demonstrates that the number of lamellar units is neither fixed nor species specific, but is modulated by the level of *ELN* expression during development. We show that humans are extremely sensitive to reduced *ELN* expression, developing profound thickening of the arterial wall.

Methods

Northern analysis. Poly(A)$^+$ RNA was extracted from the visceral organs of the thorax from mice at birth using a Micro-Fast Tract Kit (Invitrogen, Carlsbad, CA). RNA was electrophoresed on a 1.0% denaturing agarose gel, transferred to Hybond filter (Amersham, Arlington Heights, IL), and hybridized with a ^{32}P-labeled 0.85-kb fragment of mouse *ELN* cDNA. Filters were rehybridized with a 1.5-kb fragment of human cardiac actin cDNA. Intensity of bands was measured by PhosphorImager analysis. *ELN* expression in *ELN* +/+ and *ELN* +/− mice was standardized to cardiac actin expression and compared.

Electron microscopy. Ascending thoracic aortae were dissected from mouse pups at birth after cardiac perfusion with 3% glutaraldehyde. Aortic segments were sequentially stained with osmium tetroxide, tannic acid, and uranyl acetate, then dehydrated and embedded in Epon. Thin sections (60-nm) were counterstained with uranyl acetate and lead citrate and examined on a Jeol 1200 electron microscope (14).

Histological examination. Mice were fixed overnight in either 4% paraformaldehyde or methyl Carnoys at 4°C and embedded in paraffin. Sections were stained with hematoxylin and eosin and Hart stain for elastin. Individuals blinded to the genotype counted lamellar units

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1. *Abbreviations used in this paper: ELN*, elastin gene; SVAS, supravalvular aortic stenosis.

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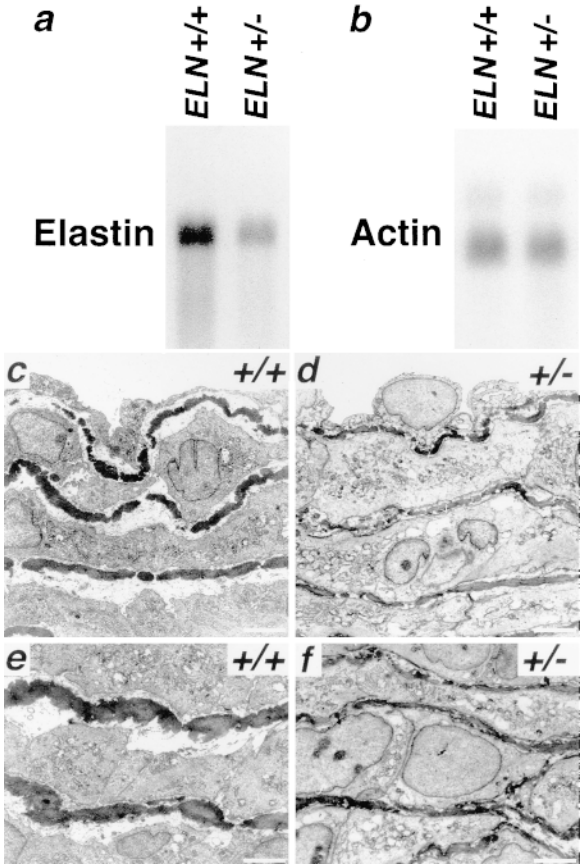

Figure 1. (*a* and *b*) Northern analysis of mRNA from *ELN* +/+ and *ELN* +/− mice at birth for *ELN* (*a*) and cardiac actin (*b*) expression. There is a 47% decrease in *ELN* mRNA in *ELN* +/− mice by PhosphorImager analysis. (*c–f*) Electron micrographs of ascending aortic cross-sections from *ELN* +/+ (*c* and *e*) and *ELN* +/− (*d* and *f*) mice at birth. Subendothelial (*c* and *d*) and medial sections (*e* and *f*) for both genotypes are compared. In *ELN* +/+ aortae, circumferentially oriented smooth muscle cells are interposed between well developed elastic lamellae. Note that elastic lamellae are abnormal and ~ 50% thinner in *ELN* +/− aortae. Bar, 3.0 μm.

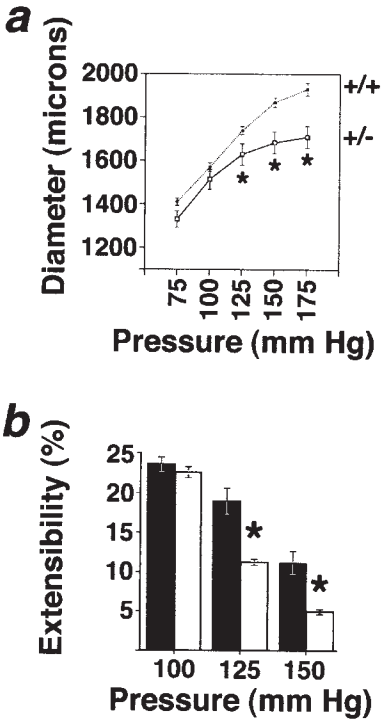

Figure 2. Extensibility of *ELN* +/+ and *ELN* +/− aortae. (*a*) Pressure–diameter curve. At pressures 100 mmHg and below, *ELN* +/+ aortae (*filled circles*) have diameters similar to *ELN* +/− aortae (*open squares*). At pressures above 100 mmHg, the diameters of *ELN* +/− aortae are significantly less than *ELN* +/+ aortae. (*b*) Extensibility at varying pressures. The extensibilities of *ELN* +/+ (*black bars*) and *ELN* +/− aortae (*white bars*) are similar at physiologic pressure (100 mmHg). However, at elevated pressures (125 and 150 mmHg) extensibility of *ELN* +/− aortae is significantly decreased compared with controls. *Statistically significant differences, $P < 0.05$.

on two separate occasions. The statistical significance was calculated by comparison of the means using *t* test analysis.

Vascular extensibility. Arteries studied were cannulated and mounted on the pressure myograph (15). The vessel was transilluminated under an inverted microscope connected to a CCD camera, allowing the continuous recording of the outer diameter of the vessel. In the ascending aorta, intravascular pressure was increased from 75 to 175 mmHg by steps of 25 mmHg (0–50 mmHg by steps of 10 mmHg for the left pulmonary artery), and the arterial diameter was recorded. Extensibility was calculated using the following formula with 125 mmHg as an example: *extensibility* = [(*diameter at 150 mmHg* − *diameter at 100 mmHg*)/(*diameter at 100 mmHg*)] × 100 (16). Diameter statistical analysis was assessed by a four-way ANOVA followed by least significance difference test for post-ANOVA paired comparisons. Extensibility statistical analysis was assessed by the nonparametric Mann-Whitney U test. *P* values below 0.05 were considered statistically significant.

Results

To define the role of *ELN* hemizygosity in arterial disease, we generated mice hemizygous (*ELN* +/−) for an *ELN* null mutation using homologous recombination in embryonic stem cells (17). Three independent recombinant cell lines containing a 4.0-kb deletion of the promoter and exon 1 of *ELN* were used to generate chimeras. Chimeras were mated to generate *ELN* +/− mice. *ELN* +/− mice were identical to *ELN* +/+ mice in gross appearance, behavior, and life expectancy. Northern analysis of *ELN* +/− mice revealed a 47% decrease in *ELN* mRNA when compared with *ELN* +/+ mice at birth (Fig. 1, *a* and *b*). To determine if the structure of elastic lamellae in *ELN* +/− mice was affected by reduced *ELN* expression, we examined aortic cross-sections by electron microscopy. Elastic lamellae in *ELN* +/− aortae (Fig. 1, *d* and *f*) were ~ 50% thinner than those in *ELN* +/+ aortae (Fig. 1, *c* and *e*). These data indicate that elastic lamellae in *ELN* +/− mice are structurally abnormal.

To determine the physiologic consequences of structural changes observed in *ELN* +/− mice, we measured aortic diameter and extensibility at varying intraluminal pressures. Despite differences in *ELN* mRNA expression and protein deposition, at a physiologic pressure of 100 mmHg *ELN* +/+ and *ELN* +/− aortae had similar extensibilities (23.6±0.9 vs. 22.5±0.7%) (Fig. 2) (18). At 125 mmHg and above, however, the pressure–diameter curves diverged with a marked reduction in extensibility of *ELN* +/− aortae (at 125 mmHg, 18.8±1.5% for *ELN* +/+ aortae vs. 11.2±4% for *ELN* +/− aortae, $P < 0.05$). The extensibility of *ELN* +/− pulmonary arteries was similar to controls within a broad pressure range (0–50 mmHg) surrounding normal physiologic pressure (mean pressure = 10 mmHg). These data indicate that *ELN* +/− mice maintain extensibility at physiologic pressures.

To understand the structural determinants enabling *ELN* +/− aortae to maintain normal extensibility at physiologic

Table I. Number of Lamellar Units in ELN +/+ and ELN +/− Aortae

Mouse ID	Genotype	Age	Ascending aorta	Descending aorta
BL6	*ELN* +/+	5 mo	9	6
390-3	*ELN* +/+	12 mo	8	5
390-10	*ELN* +/+	14 mo	8	6
390-11	*ELN* +/+	14 mo	8	5
1002-7	*ELN* +/+	4 mo	9	6
1029-8	*ELN* +/+	5 mo	8	5
1045-3	*ELN* +/+	5 mo	9	5
		Average	8.4±0.5	5.4±0.5
390	*ELN* +/−	12 mo	11	7
390-8	*ELN* +/−	14 mo	11	7
390-9	*ELN* +/−	14 mo	11	7
705-7	*ELN* +/−	7 mo	11	8
720-2	*ELN* +/−	7 mo	10	8
720-6	*ELN* +/−	7 mo	10	6
1029-4	*ELN* +/−	6 mo	11	8
1029-5	*ELN* +/−	6 mo	10	7
1029-6	*ELN* +/−	5 mo	10	7
1029-7	*ELN* +/−	5 mo	10	7
1045-1	*ELN* +/−	6 mo	11	7
1045-4	*ELN* +/−	6 mo	10	8
		Average	10.5±0.5	7.3±0.6

Differences in number of lamellar units between *ELN* +/− mice and *ELN* +/+ mice were statistically significant, $P < 0.005$.

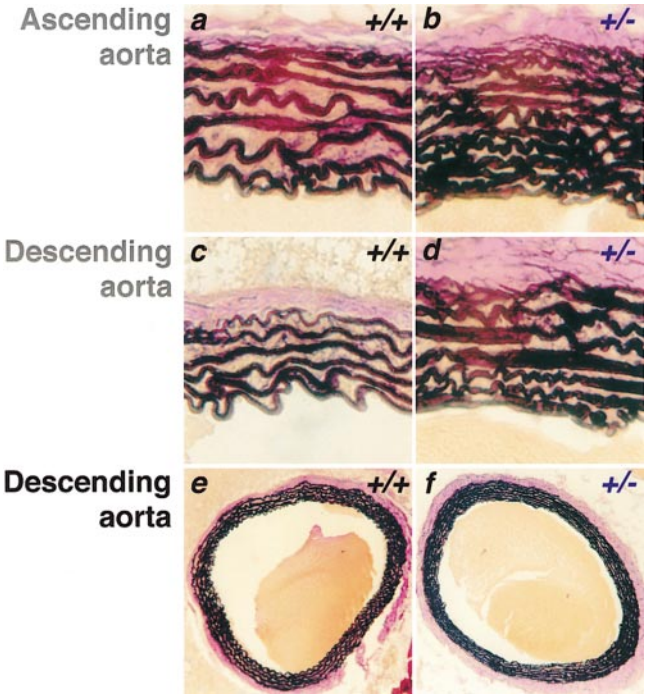

Figure 3. Hart stains of the ascending (*a* and *b*) and descending (*c–f*) aortae in *ELN* +/+ (*a*, *c*, and *e*) and *ELN* +/− (*b*, *d*, and *f*) mice. Cross-sections are at the level where the pulmonary artery courses behind the ascending aorta. In this example there are 8 and 6 elastic lamellae in the ascending and descending aortae of the *ELN* +/+ mouse, respectively, compared with 11 and 8 in the *ELN* +/− mouse, respectively, indicating an inverse relationship between *ELN* expression and the number of elastic lamellae. Lower magnification of the descending aortae (*e* and *f*) demonstrates that the inner and outer diameters of *ELN* +/+ and *ELN* +/− aortae are similar.

pressure despite a 50% decrease in elastin, we examined the arterial structure. We discovered that aortae dissected from *ELN* +/− mice (5–14 mo) had additional lamellar units. Consistent with previous work, we found that *ELN* +/+ aortae had 5.4±0.5 (descending) and 8.4±0.5 (ascending) layers of elastic lamellae (Table I and Fig. 3) (10, 19). By contrast, cross-sections of *ELN* +/− mice revealed an increase in the number of elastic lamellae to 7.3±0.6 (descending) and 10.5±0.5 (ascending) layers, respectively ($P < 0.005$). This represented an increase of 35% and 25% for the descending and ascending aortae of *ELN* +/− mice, respectively. Similar changes were observed in the pulmonary artery with an average of 7.5±0.7 lamellar units in *ELN* +/− mice vs. 6.1±0.2 in the *ELN* +/+ mice ($P < 0.005$). These changes were also apparent at birth. Our data demonstrate that *ELN* +/− mice develop additional rings of elastic lamellae and smooth muscle. Thus, the number of lamellar units in an arterial wall is not fixed or species specific.

To determine if developmental changes observed in the aortae of *ELN* +/− mice were also present in humans, we examined aortic segments from individuals with SVAS ($n = 2$ for affected, $n = 3$ for controls; Fig. 4 and data not shown). Previous studies focused only on arterial sections affected by discrete stenosis and showed subendothelial accumulation of cells, hypertrophy of smooth muscle, disruption of elastic fibers, and fibrosis (12, 13). We studied regions of the aorta that were free of discrete stenosis. The number of lamellar units in controls was consistent with previous reports (10, 20). By contrast, the aortic wall of individuals with SVAS was thicker and contained 2.5-fold more lamellar units (152±27.6 vs. 62±8.7; $P < 0.025$). These data indicate that humans, like mice, respond to reduced elastin content during development by increasing the number of lamellar units.

Discussion

It was thought previously that the number of lamellar units in a developing arterial wall is fixed and species specific (9, 10). Our work demonstrates that this concept is incorrect. We found that *ELN* +/− mice develop arteries with a 25–35% increase in the number of lamellar units. Examination of arterial specimens obtained from individuals with SVAS, a human disorder caused by *ELN* hemizygosity, revealed a 2.5-fold increase in the number of elastic lamellae. Thus, the number of lamellar units in an arterial wall is not fixed or species specific and is modulated by *ELN* expression.

Elastin's effect on arterial lamellar development likely involves smooth muscle cells sensing increased wall stress. The reduced *ELN* mRNA and thinning of each elastic lamella observed in *ELN* +/− mice would cause reduced extensibility in each lamella. These changes would lead to increased arterial wall stress, which is determined by arterial pressure and diameter and inversely proportional to the number of lamellar units and the tensile strength of each unit (21). However, the extensibility and diameter of *ELN* +/− arteries were normal at physiologic pressures. Thus, mice with abnormal elastic fibers maintain arterial extensibility by increasing the number of

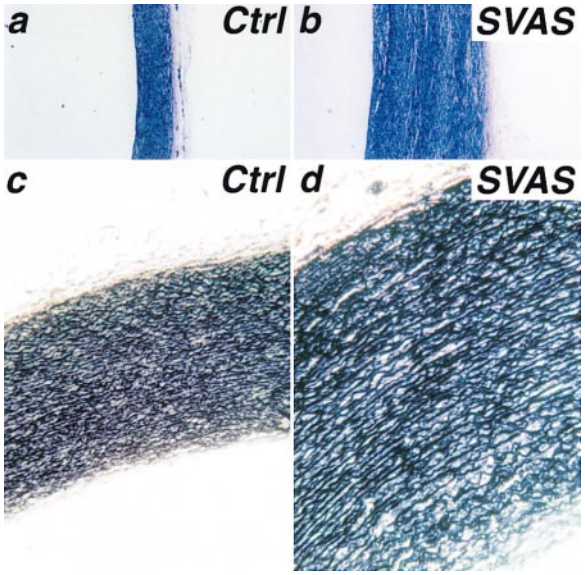

Figure 4. Elastin Van Gieson's stain of descending aortae cross-sections from a control (*a* nd *c*) and a human with SVAS (*b* and *d*). Descending aortae were examined 1.0 cm distal to the left subclavian artery and were free of discrete stenosis. Low magnification of aortic sections (*a* and *b*) demonstrates the marked increase in thickness of SVAS samples. Higher magnification (*c* and *d*) shows 2.5-fold more elastic lamellae in the aortae of an individual with SVAS, a disorder caused by *ELN* hemizygosity, indicating the sensitivity of humans to reduced developmental elastin.

lamellar units during development. Our model is further supported by anatomic and physiologic studies showing that the relationship between wall stress and the number of lamellar units in an artery is remarkably constant across species despite enormous variation in arterial diameter and stress (10). In addition, we observed no increase in the number of lamellar units when *ELN* +/− aortae were cultured in the absence of hemodynamic stress (17 and data not shown). Thus, the physiologic force of wall stress is a key determinant of arterial development.

Our work defines a novel pathology in a human obstructive arterial disease, SVAS. Because of arterial pathology in *ELN* +/− mice, we examined aortic sections from individuals with SVAS and found a compensatory increase in arterial lamellar units. As human arteries are larger and wall stress is greater (110,000 dynes/cm in humans vs. 7,800 dynes/cm in mice) the increase in humans is profound (2.5-fold increase in humans). The increase in the smooth muscle cells needed to form additional layers likely outstrips blood supply, leading to medial necrosis and fibrosis. These changes in the medial wall would stimulate recurrent injury and repair resulting in the focal stenosis observed in SVAS (12, 13).

Our data indicate that the number of lamellar units can only be modulated by wall stress early in development. During gestation when pulmonary vascular pressures are similar to systemic pressures, pulmonary arteries respond to *ELN* hemizygosity by increasing the number of lamellar units. Despite a sharp decrease in hemodynamic stress with onset of respiration at birth, the number of lamellar units in the pulmonary artery does not change. Later in life, lamellar structure is fixed and medial hypertrophy is the primary arterial response to increased hemodynamic stress (22). This concept is further supported by the failure to observe increased lamellar units in other disorders associated with increased arterial wall stress or decreased elasticity like hypertension, aortic aneurysms, or Marfan's syndrome.

Other factors may also contribute to the risk of obstructive arterial disease by reducing *ELN* expression during development. Hypervitaminosis D, for example, reduces *ELN* expression in in vitro and in vivo systems (23, 24). Animal models exposed to hypervitaminosis D gave birth to offspring that developed SVAS (25, 26). These data support our model of reduced gestational *ELN* expression resulting in abnormal vascular development and obstructive vascular disease. Future studies need to define the impact of genetic and environmental modulators of *ELN* expression on the risk of obstructive vascular disease.

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