Spatial Distribution and Mechanical Function of Elastin in Resistance Arteries
A Role in Bearing Longitudinal Stress

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Objective—Despite the role that extracellular matrix (ECM) plays in vascular signaling, little is known of the complex structural arrangement between specific ECM proteins and vascular smooth muscle cells. Our objective was to examine the hypothesis that adventitial elastin fibers are dominant in vessels subject to longitudinal stretch.

Methods and Results—Cremaster muscle arterioles were isolated, allowed to develop spontaneous tone, and compared with small cerebral arteries. 3D confocal microscopy was used to visualize ECM within the vessel wall. Pressurized arterioles were fixed and stained with Alexa 633 hydrazide (as a nonselective ECM marker), anti-elastin, or anti-type 1 collagen antibody and a fluorescent nuclear stain. Exposure of cremaster muscle arterioles to elastase for 5 minutes caused an irreversible lengthening of the vessel segment that was not observed in cerebral arteries. Longitudinal elastin fibers were demonstrated on cremaster muscle arterioles using 3D imaging but were confirmed to be absent in cerebral vessels. The fibers were also distinct from type I collagen fibers and were degraded by elastase treatment.

Conclusion—These results indicate the importance of elastin in bearing longitudinal stress in the arteriolar wall and that these fibers constrain vascular smooth muscle cells. Differences between skeletal muscle and cerebral small arteries may reflect differences in the local mechanical environment, such as exposure to longitudinal stretch. (Arterioscler Thromb Vasc Biol. 2011;31:2889-2896.)

Key Words: cell physiology ■ extracellular matrix ■ microcirculation ■ vascular biology ■ elastin

The extracellular matrix (ECM) contains a number of proteins, including collagen, elastin, laminin, fibronectin, vitronectin, glycoproteins, and proteoglycans. In addition to providing a mechanically dynamic structural scaffold, the ECM is involved in physiological processes such as cell growth, differentiation, and migration. With respect to the vasculature, recent studies have demonstrated a number of these ECM proteins to actively signal through outside-in means into both vascular smooth muscle and endothelial cells, particularly conveying mechanical signals. For example, fibronectin binding through cell surface integrins modulates the activity of smooth muscle cell (SMC) ion channels (voltage-gated Ca\(^{2+}\) channels and large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels) and affects local cellular contractions. Similarly, ECM protein-integrin activation of various intracellular signaling mechanisms underlies endothelial cell mechanotransduction to stimuli such as shear stress. Despite these demonstrated roles of the ECM in vascular cell signaling, relatively little is known of the complexities of the in situ arrangement between specific ECM proteins and arteriolar SMCs. Given the above examples, it is likely that the structural arrangement of the vessel wall ECM proteins, particularly at the microvascular level, has an impact on how local mechanical forces are transmitted, sensed, and responded to and, ultimately, how effectively a vessel is able to alter its diameter.

Adding to difficulties in understanding the complexity of the extracellular components of the vessel wall is an apparent regional heterogeneity. Importantly, regional differences involve both variation in matrix composition and structural arrangement. Thus, in contrast to skeletal muscle arterioles, small cerebral arteries lack an external elastic lamina that is present in many peripheral vessels. Both vessel types exhibit an internal elastic lamina (IEL), although recent studies have suggested that het-
In regard to specific ECM proteins, Baumbach and colleagues have provided information on the composition of the arteriolar wall, particularly as it relates to the cerebral circulation under physiological conditions and in experimental models of hypertension.\textsuperscript{10–12} Those studies have emphasized the significant contribution that elastin makes to the overall composition of the vessel wall. From a functional perspective, elastin is an important determinant of arterial distensibility, and hence stiffness, in conduit vessels. Elastin’s contribution is particularly relevant at low intraluminal pressures as compared with collagen, which exerts its effects with axial forces.

Elastin-rich fibers exist in the vessel wall that are predominantly arranged in a longitudinal fashion and thereby bear and exert longitudinal mechanical stress in the arteriolar wall. To examine this hypothesis, we predicted that abluminal exposure of isolated arterioles to elastase would cause vessel lengthening. Furthermore, we predicted that this response to elastase would be absent in vessels lacking an overt external elastic lamina (such as small cerebral arteries).

Materials and Methods

All studies were performed on arterial blood vessels obtained from male Sprague-Dawley rats (175–250 g, 6–9 weeks of age). Surgical and animal handling procedures were approved by the Animal Care and Use Committee of the University of Missouri.

Vessel Isolation, Cannulation, and Study

Cremaster first-order (1A) arterioles, small cerebral arteries, and mesenteric arteries were isolated from rats as previously described and cannulated for isobaric protocols\textsuperscript{15,16} (see supplemental material, available online at http://atvb.ahajournals.org). Arterioles were exposed for 5 minutes to porcine pancreatic elastase (0.05 U/mL) and then washed for 20 minutes. Vasomotor responses were then reassessed. In some experiments, vessels were superfused with a 0 mmol/L Ca\textsuperscript{2+} physiological salt solution containing 2 mmol/L EGTA to allow passive distensibility (pressure-diameter) relationships to be determined before and after elastase treatment. All elastase digestions were performed in the presence of a Ca\textsuperscript{2+}-containing physiological salt solution.

Imaging and 3D Reconstruction/Data Analysis

Pressurized arterioles were fixed (2% paraformaldehyde) and stained with Alexa 633 hydrazide (0.2 μmol/L, excitation 633/emission 700 nm; nonselective matrix staining dye), anti-elastin antibody (primary antibody dilution 1:100; secondary antibody, 491/515 nm) and a nuclear stain Yo-Pro-1 iodide (491/515 nm) or 4’,6-diamidino-2-phenylindole (500 ng/mL, excitation: 355, emission: 400–450 nm).\textsuperscript{17} A subset of vessels were stained for type I collagen using an anti-rat polyclonal antibody (Millipore; 1:1000, 1 μg/mL) and a goat anti-rabbit Alexa Fluor 488 secondary antibody (20 μg/mL).

3D confocal microscopy was used to visualize ECM within the arteriolar wall. Imaging was performed using a Leica TCS-SP5 microscope in conjunction with a Leica ×63 water immersion objective lens (numerical aperture 1.2) and Leica LAS AF image acquisition software. Images (512×512 pixels) were acquired at a resolution of 0.48 μm/pixel at 400 Hz. Line averaging (3 scans) was performed to reduce the low frequency noise. z-Dimension step size was 0.05 to 0.3 μm for all z-stacks. All acquisitions represent 8-bit TIFF gray scale images. Post-image-acquisition analyses were performed using ImageJ (National Institutes of Health, Bethesda, MD), Imaris (Bitplane Scientific Software, South Windsor, CT), and Image Pro (Media Cybernetics, Bethesda, MD).

See supplemental material for detailed Materials and Methods, including additional information relating to image acquisition and processing.

Chemicals and Reagents

Porcine elastase was obtained from Calbiochem (48 U/g, catalog #324682, lot #D00012477, EMD Chemicals, San Diego, CA) or from Sigma-Aldrich (5.9 U/mg, catalog #E1250, lot #041M7018V, St Louis, MO). Type II collagenase (553 U/mg) was purchased from Sigma-Aldrich. Anti-elastin antibody was obtained from Chemicon International (Millipore, Billerica, MA) and anti-collagen antibody directly from Millipore. Species appropriate secondary antibodies were obtained from Molecular Probes (Invitrogen, Carlsbad, CA). Alexa hydrazide, Yo-Pro-1 iodide, and 4’,6-diamidino-2-phenylindole were similarly obtained from Molecular Probes.

Figure 1. Effect of elastase treatment on the length of cannulated cremaster and cerebral small arteries. A, Example images of a cremaster and cerebral vessel. The numbers 1 to 5 refer to specific time points shown in B. Note that the cremaster vessel shows considerable lengthening, whereas the cerebral vessel is relatively unaffected. Note also that in image 5 for the cremaster vessel, the pipettes have been repositioned in the x-dimension to straighten the vessel. Images were collected in real-time using a stereomicroscope coupled to a charge-coupled device camera and digital video recorder. Video files (AVI format) were subsequently imported into Image Pro software for continuous measurement of vessel length. B, The length of the cremaster vessel is shown on the left y-axis, and the cerebral artery is shown on the right y-axis. C, Group data showing that abluminal application of elastase (0.05 U/mL, 5 minutes) led to a significant (P<0.05) increase in cremaster vessel segment length (n=10).
Results are presented as mean ± SEM. Simple comparisons between 2 means were performed using a 2-tailed Student t test, and multiple group comparisons were performed using analysis of variance (ANOVA) with the Tukey post hoc test. Statistical significance was assumed at the P < 0.05 level.

Results

Treatment of Cremaster Arterioles With Elastase Causes Irreversible Lengthening

After developing myogenic tone, cannulated and pressurized (70 mm Hg) cremaster 1As were subjected to a 5-minute exposure to elastase (0.05 U/mL) delivered to the adventitial surface. Length of vessels was assessed before enzyme exposure and again following elastase treatment (and 20 minutes of washout) by adjusting the calibrated x translational direction of the micromanipulator holding the cannulation pipette. Lumenal diameter was monitored continually during elastase treatment and subsequent washout. The arterioles responded to the elastase by significantly lengthening by 35.6 ± 2.3% (n = 10; P < 0.05) (Figure 1), whereas there was little apparent change in diameter (−5.2 ± 0.8%). The lengthening, evident as a lateral bowing of the vessel between the cannulation pipettes, was not reversed by washout of the elastase, consistent with a direct effect on the vessel structure.

Statistical Methods

Studies of reactivity were performed to demonstrate that the vessels retained viability and that cellular contractile function was intact. Vessels held pressure and did not display leaks. Furthermore, vessels retained steady-state myogenic tone, although acute myogenic reactivity was depressed (Figure 2A). No difference in ability to dilate to acetylcholine (10⁻⁶ mol/L) or contract to phenylephrine (10⁻⁶ mol/L) was observed (Figure 2B).

To assess the effects of elastase treatment on the passive properties of the cannulated cremaster arterioles, pressure-diameter relationships were measured after superfusion with physiological salt solution lacking Ca²⁺ and containing 2 mmol/L EGTA. Elastase caused a significant leftward shift in the pressure-diameter relationship particularly at low pressures (Figure 2C) consistent with a major effect on the elastin components of the vessel wall as opposed to the collagen fibers.13,14
To quantify the degree of cremaster arteriole lengthening and to determine its effects on elements of the vessel wall, cell width and number of cells per unit length were determined pre- and postelastase treatment. To facilitate these measurements, a dye exclusion technique was used as previously described. Cell-impermeable carboxyfluorescein was placed in the vessel bath from which it subsequently diffused into the spaces between vascular smooth muscle cells (VSMCs). Acquired images are then digitally inverted to reveal the SMCs, which allowed cell shape to be readily discerned (Supplemental Figure I). Cell width increased significantly (P<0.05) following elastase treatment, whereas the number of cells per unit length decreased significantly (P<0.05) (Table). Lengthening was also associated with a measurable change in VSMC width or the number of cells per unit length (Table). Furthermore, cerebral VSMC width and numbers of cells per unit length were not significantly different from those of cremaster VSMCs under control conditions (Table). The IEL hole long:short axis ratio in cerebral arteries was unaffected by the elastase treatment (Table).

### Cremaster Arterioles Show Longitudinal Adventitial Fibers That Contain Elastin and Are Degraded by Elastase

Cremaster 1A arterioles, stained with Alexa 633 hydrazide and counterstained for nuclei, showed longitudinally arranged adventitial (superficial) fibers that were branched in a dense and complex fashion forming a network traversing the circumference of the vessel (Figure 4A; Supplemental Movie Files I and II; Supplemental Figure IVa and IVb). From the reconstructed cross-sectional view, it is clear that the cremaster 1A has both an internal and external ECM layer (Figure 4A). The internal layer resembles previous descriptions of the IEL and exhibited holes that have previously been described to be sites where myoendothelial cell projections can be found (see also Supplemental Figure IV). The Alexa 633 hydrazide-stained adventitial ECM structure was absent in small cerebral arteries, consistent with the previous observation that these vessels lack a defined external elastic lamina (Figure 4B and Supplemental Movie Files III and IV).

Under the imaging conditions used, autofluorescence of ECM proteins did not contribute greatly to the images of

### In Contrast, Small Cerebral Arteries Do Not Lengthen In Response to Elastase

In additional studies, the effects of elastase treatment were compared between cerebral arteries (n=8) and cremaster 1As (n=8). Vessels (taken from the same animals) were cannulated and treated in a similar fashion, and all developed spontaneous myogenic tone. Although cremaster vessels again showed a lengthening response, the cerebral vessels were largely unaffected by elastase treatment (Figure 3). Example images and the time course of elastin exposure are shown in Figure 1.

Despite the absence of lengthening of cerebral vessels following elastase treatment, their passive pressure-diameter relationships showed a leftward and upward shift (Figure 3), consistent with the enzyme treatment exerting distinct effects on the 2 vessel types.

### Table. Quantitative Analyses of the Effects of Elastin Treatment on Vascular SMCs and IEL

<table>
<thead>
<tr>
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<th>Cremaster Arterioles</th>
<th>Small Cerebral Arteries</th>
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<tbody>
<tr>
<td></td>
<td>Control (n=4)</td>
<td>+Elastase (n=4)</td>
</tr>
<tr>
<td>SMC width</td>
<td>5.64±0.06</td>
<td>6.98±0.10*</td>
</tr>
<tr>
<td>SMC No./unit length†</td>
<td>34.0±1.6</td>
<td>30.3±1.2*</td>
</tr>
<tr>
<td>IEL holes (long:short axis)‡</td>
<td>2.56±0.52</td>
<td>3.63±0.39*</td>
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n indicates No. of vessels. For each vessel, measurements are based on approximately 30 SMCs, as noted in the Table. Because of differences in the distribution of IEL hole numbers between vessel types, measurements of long:short axis ratios are based on an average of 24 holes/cremaster vessel and 13 holes/cerebral artery. IEL indicates internal elastic lamina; SMC, smooth muscle cell.

*P<0.05 compared to the respective control value.
†Unit length=134 μm.
‡Long axis:short axis ratio as a measurement of IEL hole shape.
vessels stained with Alexa 633 hydrazide. Details of control experiments are shown in the supplemental material.

Staining with a specific elastin antibody showed overlap between its pattern and the structures stained by the Alexa hydrazide dye, suggesting that the adventitial fibers observed in cremaster vessels indeed contain elastin (Figure 5A–5C). Under the imaging conditions used, no fluorescence staining was detected when the elastin (primary) antibody was omitted (data not shown; also see supplemental material). Vessels treated with elastase showed only remnants of these fibers and areas of indentation where the fibers had likely been positioned before enzyme treatment (Figure 5D). It should be noted that, consistent with the observations of Briones et al., the conditions of the elastase treatment (addition to the adventitial surface, time of exposure, and concentration/activity of enzyme) were such that whereas the adventitial fibers were disrupted, the IEL remained largely intact.

Staining with a specific antibody for a Type 1 collagen showed an entirely different staining pattern from that of either Alexa 633 hydrazide or the elastin antibody (see Supplemental Figure V). In contrast to the distinct adventitial fibers shown by Alexa 633/elastin antibody staining, Type 1 collagen stained in a wavy, belt, or strap-like manner. A similar distribution of collagen was confirmed from autofluorescence images, as used by previous investigators (data not shown).

Small Mesenteric Arteries Contain Adventitial Fibers Resembling Those of Cremaster Arterioles

On the basis of the above, we hypothesized that elastin fibers support vessels prone to longitudinal stretch and as such predicted that small mesenteric arteries should contain an elastin fiber network more closely related to cremaster vessels than cerebral arteries. Similar to the cremaster vessels, small mesenteric arteries stained with Alexa 633 hydrazide and counterstained for nuclei showed longitudinally arranged adventitial (superficial) fibers that branched into a very dense and complex network traversing the adventitia (Figure 6 and Supplemental Movie Files V and VI).

Discussion

Despite consisting of only a few cell layers in thickness, the walls of small arteries and arterioles present a complex

Figure 4. Example pseudocolored images of a cannulated and pressurized cremaster arteriole (A) and a similarly prepared small cerebral artery (B). The images represent adventitial (Aa and Ba), midwall (Ab and Bb), intimal (Ac and Bc), and end view (Ad and Bd) sections. Vessel segments were fixed while pressurized (see text for details) and stained with Alexa 633 (for extracellular matrix [ECM] structures) and Yo-Pro-1 iodide (nuclei). Images are representative of n=11 (cremaster) and n=6 (cerebral). Movie files for the complete image stacks and 3D rotating representations are shown in the supplemental material.

Figure 5. A, Alexa 633 hydrazide staining of adventitial fibers in a cannulated cremaster 1A segment. B, The same vessel segment stained with a specific elastin antibody and a secondary antibody conjugated with Alexa Fluor 488. Inset, Secondary antibody-only control (additional details of controls are provided in the supplemental material). C, Overlay image for A and B. Results are typical of n=4. Yellow color indicates apparent colocalization, suggesting that the Alexa 633 hydrazide–stained fibers contained elastin. D, Elastase (0.05 U/mL, 5 minutes) cut the longitudinally arranged, elastin-containing fibers (typical of n=5).
biomechanical structure. Furthermore, as a contractile tissue, there is considerable interdependence of structure and cell function. Using confocal microscopy and 3D imaging approaches to reconstruct through-focus structural features of the vascular wall, the present study illustrates the complexity of the ECM while also demonstrating that particular matrix proteins may constrain or shield the environment within which SMCs reside and exert their contractile activity. In this regard, the data support a significant role for elastin-containing fibers in the mechanical properties of true resistance vessels and suggest that this property is not limited to larger conduit vessels. These conclusions are highlighted by brief elastase treatment causing degradation of adventitial matrix fibers and significant lengthening of cremaster muscle arterioles. Heterogeneity between vascular beds was, however, also observed. In particular, small cerebral arteries that are known to lack an external elastic lamina did not show lengthening to a similar elastase exposure.

In regard to the physiological significance of the apparent difference between adventitial structure of cremaster muscle and mesenteric arterioles versus small cerebral arteries, we speculate that this structure confers the ability to withstand longitudinal stretch. Thus, retraction and relaxation of the cremaster muscle would result in the centrally located arteriole being subjected to longitudinally directed strain, whereas cerebral vessels, located within a rigid skull, are largely protected from such elongation. To support this hypothesis, mesenteric arteries were examined as another vascular bed where vessels are commonly subjected to longitudinal changes in length. Similar to the cremaster 1A, small mesenteric arteries also showed a complex network of adventitial matrix protein fibers. Supporting structural heterogeneity within the vessel wall (in particular as it relates to the ECM) between vascular beds, elastin mRNA expression was significantly greater in cremaster and mesenteric vessels compared with small cerebral arteries (see Supplemental Figure VI). Further supporting heterogeneity, the relative amount of muscle cell protein (measured as α actin content) was greater in cerebral arteries as compared with both cremaster and mesenteric vessels (see Supplemental Figure VI). Conceivably, morphological differences could also exist at the level of the VSMCs; however, under control conditions, calculations of VSMC width and number of cells per unit length showed no differences between cremaster and cerebral small arteries.

Interestingly, although elastase did not cause lengthening of the small cerebral arteries both cremaster and cerebral vessels showed shifts in their passive pressure-diameter relationships on enzyme treatment. We speculate that this may be explained by differences in the longitudinal and circumferential properties of the vessel wall. Thus, the length changes are a function of the longitudinally arranged adventitial elastin fibers, whereas the shifts in the pressure-diameter relationships may relate to effects of elastase on other elements, such as the IEL.

IEL holes have recently received considerable attention as sites of endothelial-SMC connectivity via myoendothelial junctions. Additional analysis was undertaken to determine whether the elastase treatment affected the shape of IEL holes. To quantify changes, the long axis:short axis ratios of the holes were determined. For measurement of the IEL hole size, the vessel image stacks were processed to extract the image data associated with IEL layer alone. Elastase treatment of cremaster vessels led to a significant (P<0.05) increase in the long:short axis ratio of the IEL holes as the vessel lengthened. This could reflect either stretch of the IEL or a direct effect of the elastase on the IEL. In contrast to the cremaster arterioles, cerebral vessels neither lengthened nor changed the measured characteristics of the IEL holes. Indirectly, this supports the idea that cremaster arterioles are normally constrained in the axial direction by adventitially located elastin fibers. Cleavage of these fibers leads an overt increase in vessel length, an increase in cell width, and a stretching of wall structures such as the IEL.

The present studies did not consider any structural role played by other elements that contribute to the adventitia. Images stained with either 4′,6-diamidino-2-phenylindole or propidium iodide showed nuclei in the region of the adventitia. In addition, the orientation of these cells indicated that they were unlikely to be either smooth muscle or endothelial cells. As these cells were apparent in cremaster vessels before and after elastase treatment (data not shown) it is unlikely that these elements contributed to the lengthening process. Nev-
ertheless, future studies should be aimed at identifying the nature of such cells.

Consistent with elastase causing lengthening of the cremaster arteriole, arterial tortuosity has been reported in studies administering elastase in vivo to induce aneurysm formation\(^1\) and in transgenic animal models where elastin levels are genetically manipulated.\(^2\) Such studies have largely focused on conduit vessels because of the role of elastin in elastic recoil of arteries and the clinical significance of large vessel aneurysms. In addition to common findings with respect to vessel length, disruption of elastin appears to decrease the distensibility of both large arteries and, at least, first-order arterioles from skeletal muscle. Also consistent with a role for elastin in regulating small vessel dimensions, Briones et al\(^1\) showed elastase treatment to decrease the ex vivo distensibility of small mesenteric arteries. Interestingly, in the present imaging studies, mesenteric vessels exhibited a complex adventitial fibrillar network similar to that observed in the walls of the cremaster muscle arterioles.

Additional studies will be required to delineate the exact involvement of specific matrix proteins. We initially used Alexa 633 hydrazide staining as a nonselective marker for ECM proteins. However, it is evident from the specific elastin staining that this dye preferentially binds elastin-containing structures. Further supporting its restricted staining was the observation that a different staining pattern is observed with an antibody known to be specific for Type 1 collagen. Similarly, cleavage of the fibers with elastase led to a leftward shift in the passive pressure-diameter relationship (for cremaster arterioles) at low pressures, consistent with the contribution of elastin to vascular mechanics as compared with that of collagen.\(^3\) This, however, does not indicate that the stained adventitial fibers are composed of elastin alone and they presumably contain other proteins, such as fibrillin.\(^4\)–\(^6\)

A question that arose from the present studies is whether or not the staining pattern of elastin-containing fibers found in the cremaster arterioles is representative of small arteries and arterioles in other skeletal muscles. Relevant to this, we have observed a complex fiber network in arterioles isolated from hamster gracilis muscle, suggesting that a similar structure exists in differing skeletal muscles and across species (data not shown).

Important questions arising from our studies include how VSMCs interact with the matrix scaffold and whether the differences in regional matrix structure imply differences in how local mechanical forces are transmitted, sensed, and responded to. Clearly, the lengthening of cremaster muscle 1As and increasing SMC width following elastase exposure suggest that under resting conditions, SMCs are constrained along the long axis of the vessel or across the short axis of individual SMCs. Importantly, the enzyme treatment did not markedly alter vascular reactivity to vasoactive agents (acetylcholine and phenylephrine), and the vessels remained free of leaks to the intraluminal pressure. Furthermore, although the lengthening response was seen with elastase treatment, this was not seen following collagenase exposure. As such, we do not believe that our observations can be simply explained by nonselective destruction of vessel integrity per se.

In an earlier study, Spofford and Chilian\(^2\) reported differing responses of cerebral and mesenteric small arteries with respect to pressure-induced changes in c-fos expression. Increasing intraluminal pressure led to a decrease in cerebral vessel c-fos expression while causing an increase in mesenteric vessels. Interestingly the pressure-induced effect on c-fos expression was mediated through the elastin-laminin receptor and could be blocked by decoy peptides that are presumed to inhibit normal matrix protein binding to elastin. These authors further found differences in the matrix protein composition between the cerebral and mesenteric vessels suggesting that the differences in pressure responsiveness may reflect regional variation in the matrix environment. Specifically, consistent with the present study, these authors reported an absence of elastin in the adventitia of the cerebral vessels and a presence of elastin in the adventitia of mesenteric vessels. Cerebral vessels, however, were reported to show comparatively greater levels of elastin within the medial layer.

In addition to understanding the physiological structure of the vessel wall, the relevance to pathophysiology should also be noted. Although currently largely limited to studies of conduit vessels and subcutaneous resistance arteries, age-related changes in the ratio of elastin to collagen affect vascular stiffness.\(^2\)–\(^5\) Furthermore, in aging and metabolic disturbances (for example, in diabetes mellitus), long-lived proteins such as elastin and collagen are subject to modification by glycation and oxidation.\(^1\)–\(^3\) Presumably, such changes may alter not only the mechanical properties of the ECM proteins but also the relationship between the ECM and cellular elements of the vessel wall, thus potentially affecting matrix protein-mediated cellular signaling.

Collectively, the data support our hypothesis that elastin is an important ECM protein for bearing longitudinal stress in the arteriolar wall. Furthermore, when present, these fibers act to constrain or restrict the longitudinal dimensions of the vessel. Differences between arterioles from skeletal muscle and mesentery versus small arteries from the cerebral circulation may be related to differences in the local mechanical environments in which these vessels reside. Skeletal muscle and mesenteric tissues are continuously exposed to longitudinally oriented stretching forces that are transferred to the vasculature. By comparison, cerebral tissue is encapsulated and the brain vasculature is not normally exposed to these types of stretching forces. Knowledge of the 3D architecture of the ECM components of the vessel wall is also of importance to our understanding of how these elements are affected in aging and disease states, particularly where matrix proteins are damaged, degraded,\(^4\) or posttranslationally modified.\(^1\)–\(^3\)

Sources of Funding

This study was supported by National Institutes of Health Grants HL92241-02 (to M.A.H.), P01-HL-095486 (to G.A.M. and M.J.D.), and HL088105-02 (to L.M.L.). Dr Clifford was supported by a Research Career Enhancement Award from the American Physiological Society.
Disclosures

None.

References

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Arterioscler Thromb Vasc Biol. 2011;31:2889-2896; originally published online October 6, 2011;
doi: 10.1161/ATVBAHA.111.236570
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENT MATERIAL

1. Material and Methods

Vessel Isolation, Cannulation and Study

Excised cremaster muscles were placed in a cooled (-2° C) dissection chamber containing physiological saline solution (PSS) containing (in mmol/l) 145 NaCl, 4.7 KCl, 2 CaCl₂, 1 MgSO₄, 1.2 NaH₂PO₄, 4 glucose, 2 pyruvate. With the aid of a stereomicroscope (Olympus SZX12, Melville, NY), segments of 1A arterioles were microdissected. The vessel segment was transferred to a clear Lucite chamber filled with PSS, then each end of the vessel segment was cannulated with a glass micropipette (tip diameter approximately 80 µm) and secured with nylon mono-filament suture (11-0, Alcon, Ft. Worth, TX). The vessel and tubing were filled with PSS containing 1% albumin. The chamber was then transferred to the stage of an inverted microscope (model IX71, Olympus) coupled to a video camera (model TMC-7DSP, Pulnix, Glostrup, Denmark), electronic video caliper (Texas A&M, College Station, TX), and a data acquisition system (Powerlab, AD Instruments, Colorado Springs, CO) that allowed continuous measurement of internal diameter. The micropipettes were connected to independent reservoirs for control of luminal pressure by adjustment of reservoir height. Temperature of the bath was continuously monitored and maintained at 34° C by constant flow through the water jacket surrounding the vessel bath. A peristaltic pump (model Sci-Q 400, Watson Marlow, Wilmington, MA) was used for superfusion of the vessel with PSS at a rate of 3-5 ml/min. Arterioles were initially held at a pressure of 40 mmHg, then the pressure raised to 70 mmHg to induce spontaneous myogenic tone. To be considered viable, arterioles had to be free of pressure leaks, exhibit spontaneous tone, and reactive to agonists. Functionality of the vessels was
tested by myogenic responsiveness and dilation to the endothelium-dependent vasodilator, acetylcholine (1 μM).

Cerebral and mesenteric vessels were prepared in a similar fashion with the exception that superfusion buffers were maintained at 37°C.

**Experimental Protocols:**

**Sample Preparation:**

Vessels were isolated, mounted on glass cannula and checked for leaks as above. Vessels were then pressurized to 70 mmHg and lengthened to remove lateral bowing. For fixation 2% paraformaldehyde was added to the bath (20 minutes at room temperature). Vessels were subsequently permeabilized with 0.1% Tween 20 for 1 hour and then washed with a physiological salt solution.

**Antibody labeling:**

_Elastin:_ Primary antibody used was rabbit anti-rat elastin polyclonal antibody (Chemicon International) supplied as a stock concentration of 1 mg/ml. For immunostaining the antibody was used at a final concentration of 10 μg/ml and applied abluminally for approximately 12 hr, at 4°C. Goat anti rabbit IgG, Alexa fluor 488 – conjugated antibody (10 μg/ml) was subsequently used to visualize primary antibody binding. The secondary antibody was applied abluminally for 1 hr at room temperature. Vessels were maintained at an intraluminal pressure of 70 mmHg during the labeling procedure.

_Collagen:_ Primary antibody used was a rabbit anti-Rat Collagen Type 1 polyclonal antibody (Millipore) supplied as a stock concentration of 1 mg/ml. For immunostaining the antibody
was used at a final concentration of 1 \( \mu \text{g/ml} \) and applied abluminally for approximately 12 hr, at 4°C. Secondary antibody, goat anti-rabbit IgG, Alexa fluor 488 (final concentration 20 \( \mu \text{g/ml} \)) was applied abluminally for 1 hr at room temperature. As above, vessels were maintained at an intraluminal pressure of 70 mmHg.

**Indicator labeling:**

*Alexa 633 Hydrazide:* Vessels were incubated at room temperature in Alexa 633 hydrazide (0.2 \( \mu \text{M} \); Molecular Probes) for 20 minutes. The hydrazide was prepared in physiological salt solution. After washing, staining was visualized by excitation at 633 nm (HeNe laser) at emission wavelengths of 650-750 nm.

*4’6-diamino-2-Phenyindol (DAPI):* DAPI (500 ng/ml) was applied abluminally for 20 mins at room temperature. DAPI was prepared in physiological salt solution. After washing, staining was visualized by excitation at 350 nm (using 2 Photon laser at 700 nm) at emission wavelengths of 400-450 nm. DAPI nuclear stain was used only when tri-labeling (Alexa 633 hydrazide, specific elastin antibody; and nuclear localization) was performed to avoid spectral overlap with the secondary antibody. In other situations nuclei were labeled with Yo-Pro-1 iodide.

*Yo-Pro-1 Iodide:* Yo-Pro-1 Iodide (1 \( \mu \text{L/ml} \); Molecular Probes) was applied abluminally for 20 minutes at room temperature. The indicator was prepared as a stock (1 mM) in physiological salt solution). After washing, staining was visualized by excitation at 488 nm (Argon laser) at emission wavelengths of 500 – 550 nm.
Dicarboxyfluorescein: DCF was prepared in Krebs buffer at a final concentration of 100 μM. DCF. Prior to vessel cannulation, one pipette tip was filled with DCF. For imaging, the DCF was advanced into the arterial lumen while additional DCF solution was added to the vessel superfusate. Image acquisition was then performed with the dye remaining in the lumen and the bath. Excitation was at 488 nm and emission was set from 500 - 600 nm. As DCF diffused into gaps between the cells, digital image inversion using Imaris software resulted in a shadow image of the VSMC which was then pseudo-colorized (Martinez-Lemus et al., FASEB J., 2004; Supplementary Information Figure I).

Supplementary Figure I: Left hand image shows a cannulated cremaster arteriole exposed to cell-impermeable DCF. The middle image shows the same image digitally inverted to provide the ‘shadow image’ of the VSMCs that has been pseudo-colored. The right hand image shows an enlarged area of vessel image as would be used for measurements of cell width and number of cells per unit length.
Imaging Parameters:

Spatial: For routine studies, image resolution was set at 512 x 512 pixels with image dimensions of 246 x 246 µm. Line scans were averaged over 3 lines with no additional requirement for frame averaging. Additional higher resolution images were collected at 1024 x 1024 pixels.

Temporal: Laser illumination was scanned at 400 Hz

Z-stack: Stack depths were typically taken over 30-40 µm with step sizes of 0.05 - 0.3 µm.

Laser Power: Typical laser power levels employed were HeNe, 10%; Argon, 10-20%; and multiphoton, 50%

Objective : 60x water immersion lens, NA: 1.2

Microscope: Leica TCS-SP5

Image Processing

Image analysis was performed using Image J. Image stacks were independently saved according to the wavelength at which they were acquired. Depending on dual- or tri-labeling, individual stacks were merged on an image and pixel basis to obtain a composite stack. The composite was reduced to 256 colors and saved as an 8-bit avi file. The 3D viewer macro was used to visualize the composite stack both as a 3D rotating image and specific image planes. The orientation and axis of the blood vessel was adjusted to view the different layers of arterioles. In order to selectively visualize the EEL or IEL, images corresponding to those sections of the stack were summed on a pixel-to-pixel basis to obtain enhanced fluorescence signals and contrast of the structures.
**Antibody Specificity and Autofluorescence**

To demonstrate specificity of the staining procedure controls were performed where fixation, permeabilization and exposure to secondary antibody were performed in the absence of primary antibody. No staining resembling elastin fibers or collagen was evident in the absence of the primary antibody (Supplementary Figure II). Non-specific staining appeared limited to the outer vessel layer presumably reflecting binding to tissue damaged during vessel dissection (Supplementary Figure II).

**Supplementary Figure II:** Demonstration of elastin antibody specificity. Panel a. secondary antibody (goat anti-rabbit IgG, Alexa fluor 488) alone; Panel b. corresponding Alexa 633 hydrazide staining; Panel c. staining with primary elastin antibody; and Panel d. corresponding Alexa 633 hydrazide staining.
As autofluorescence approaches can be used for the imaging of elastin and collagen control studies were undertaken to determine any contribution of autofluorescence to our imaging data. To perform these studies permeabilized and fixed vessels were imaged in the absence of staining procedures. Laser power was initially set at levels used for the specific staining protocols (488 nm, 60 - 80 µW; 633 nm, 13-20 µW) after which illumination was increased to high levels (488 nm, 530-560 µW; 633 nm, 520-560 µW). At the laser powers routinely used in our studies no evidence of an autofluorescence component to images was detected (Supplementary Table I). Even at high laser powers only low-level autofluorescence signals were observed under the imaging conditions used (Supplementary Table I). In addition no bleed-through autofluorescence was detected between channels.

**Supplementary Table I**

<table>
<thead>
<tr>
<th>Excitation Wavelength</th>
<th>Low Laser Intensity</th>
<th>High Laser Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>488</td>
<td>No signal</td>
<td>+</td>
</tr>
<tr>
<td>633</td>
<td>No signal</td>
<td>No signal</td>
</tr>
<tr>
<td>488 and 633</td>
<td>No signal</td>
<td>+</td>
</tr>
</tbody>
</table>

2. Results

*Acute Effects of Collagenase on Cannulated Arterioles*

Exposure of cannulated arterioles to type II collagenase (30 units/ml) for 5 min resulted in a 34.1 ± 4.0% increase in diameter which was reversed after washing (Supplementary Figure IIIa). In contrast, a similar treatment with elastase (0.05 U/ml; 5 mins) did not cause vasodilation
Results are expressed as mean ± SEM of absolute vessel diameter (µm) (n = 13 for collagenase and 16 for elastase). Collagenase treatment caused an upward shift in the passive pressure-diameter relationship (Supplementary Figure IIIb) differing from the leftward shift caused by elastase (see Figure 2d main manuscript).

Supplementary Figure III: Panel a. - collagenase treatment caused dilation of myogenically active cremaster arterioles (n = 6). This effect was reversed by washing and is assumed to reflect the exposure of matricryptic sites or the generation of soluble vasoactive factors (see Davis G.E. et al., Am. J. Pathol. 156:1489-98, 2000). The effect of collagenase was in contrast to the effect of elastase which caused irreversible lengthening of the cannulated vessel segments. Panel b. – collagenase causes an upward shift in the passive pressure – diameter curve for cremaster arterioles (n = 6). This again contrasts with the actions of elastase that caused a leftward shift in the relationship, particularly evident at low pressures.
Supplementary Figure IV: Images of arterial wall illustrating Alexa 633 hydrazide staining of specific structural elements. Panel A shows the fibers (elastin) as viewed from the adventitial surface and B from the intimal view. Both images are from the same cremaster muscle arteriole. Panel C shows the IEL of a small cerebral artery. For each vessel, Z-dimension image slices were collected at a step thickness of 0.05 um.
**Distribution of Type 1 Collagen in the Arteriolar Wall**

Cremaster 1As were cannulated and pressurized as in the earlier studies. Vessels were fixed, permeabilized and incubated with an anti-type 1 collagen antibody followed by a FITC-labeled anti-rabbit IgG secondary antibody. Nuclei were stained with DAPI and advential/elastic fibers with Alexa-633. Type 1 collagen staining appeared more diffuse and restricted to the outer surfaces of the vessel. Importantly, the pattern of staining was distinct to the discrete fibers stained by either the Alexa-633 dye (*Supplementary Figure V*) or the specific elastin antibody (Figure 4, main manuscript).

![Images showing distribution of Type 1 collagen](image)

**Supplementary Figure V:** Staining of Type 1 collagen (green) in a cremaster arteriole shows a pattern distinct from the Alexa 633 hydrazide staining elastin-containing fibers. Panels a, b and c show differing optical planes through the vessel wall while Panel D shows an end-on view (see main manuscript for section details).
Biochemical Indices of Vessel Wall Heterogeneity Between Cremaster and Cerebral Vasculatures.

Given the apparent differences in the structure of the vessel wall between the cremasteric and mesenteric arteries versus those from the cerebral circulation it was expected that the latter vessels may show a higher ratio of muscle (as reflected by α actin content) to total protein. α actin content was determined using Western blotting and densitometry and expressed relative to total protein content. Total protein was measured using the BCA assay (Pierce, Thermoscientific Fisher, Rockford, IL). The amount of muscle protein in cremaster 1A and small cerebral and mesenteric arteries was expressed as arbitrary actin densitometry units per µg protein. Data were then expressed relative to the results obtained for cremaster 1A samples on a given Western blot. A significantly (p < 0.05) higher ratio was observed in small cerebral arteries (2.43 ± 0.52; n = 5) compared to either cremaster (1.0 ± 0.19; n = 5) or mesenteric (1.23 ± 0.11; n = 5) vessels (Supplementary Figure VIa). These data further support heterogeneity of the vascular wall between vascular beds but do not give direct insight into specific proteins.

Although a major component of elastin expression has been shown to occur during development (Mecham 2008) real time PCR was used to examine differences in the levels of mRNA for elastin in cremaster, mesenteric and cerebral vessels (as used in the imaging studies). PCR was performed using an Eppendorf Realplex cycler and SYBR green interchelation. Equal amounts of total vessel RNA extract (Melt Total Nucleic Acid isolation system, Ambion) were reverse-transcribed into cDNA using a Superscript III First-Strand synthesis system, (Invitrogen) according to the manufacturer's instructions. Sequence
specific primers for rat elastin (accession no. NM_012722) and rat β-actin (accession no. NM_031144) as a house keeping gene were designed as follows:

Elastin (forward): 5'-TTCTCCTATCTACCAGGTGG-3'; and
(reverse): 5'-AAGATCACTTTTCTTCCGG-3',

β-actin (forward): 5'-CCTCTATGCAACACAGTGTGCTGTCT-3'; and
(reverse): 5'-GCTCAGGAGGAGCAATGATCTTGA-3'.

Samples (n=4) were performed in triplicate and each run included a no template and no enzyme control to test for contamination of assay reagents. To verify that only specific product was amplified, a melting point analysis was performed after the final cycle. Data were collected using Realplex software (Eppendorf) and relative quantification was performed using the comparative threshold (Ct) method after determining the Ct values for reference (β-actin) and the target gene, elastin, in each sample sets according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

As shown in **Supplementary Figure VIb** mRNA levels for elastin were significantly (p < 0.05) lower in cerebral arteries compared to either cremaster or mesenteric vessels.
Supplementary Figure VIa: $\alpha$-actin:total protein ratios for cerebral arteries, cremaster 1A and small mesenteric arteries. Results are expressed as arbitrary actin densitometry units per $\mu$g protein and are shown as mean ± SEM, n = 5.

Supplementary Figure VIb: Elastin mRNA as measured by quantitative PCR. Results are expressed relative to $\beta$-actin as a housekeeping gene and are shown as mean ± SEM, n = 4. Similar results were obtained using an alternate housekeeping gene (GAPDH; data not shown).

Supplementary Movie Files

1. Rotating volume rendered 3D projection of a cannulated cremaster muscle 1A. The image has been reconstructed from a 3D image series with z sections taken at an interval of 0.3 $\mu$m. The playback speed of the movie is adjusted to 10 frames/sec

2. Movie file showing the individual images of the cremaster 1A used to produce the 3D image in Supplementary Movie File 1. The image stack begins at the adventitial surface
and progresses through to the intima. Alexa 633 hydrazide was used to stain the adventitial matrix fibers (elastin-containing; red) while the nuclei (green) were stained with Yo-Pro-1.

3. Rotating 3D image of a cannulated cerebral small artery. The image has been reconstructed from a 3D image series with z sections taken at an interval of 0.3 µm.

4. Movie file showing the individual images of the cerebral artery used to produce the 3D image in Supplementary Movie File 3. The image stack begins at the adventitial surface and progresses through to the intima. Alexa 633 hydrazide was used to stain the adventitial matrix fibers (elastin-containing; red) while the nuclei (green) were stained with Yo-Pro-1.

5. Rotating 3D image of a third order mesenteric artery. The image has been reconstructed from a 3D image series with z sections taken at an interval of 0.3 µm.

6. Movie file showing the individual images of the third order mesenteric artery used to produce the 3D image in Supplementary Movie File 1. The image stack begins at the adventitial surface and progresses through to the intima. Alexa 633 hydrazide was used to stain the adventitial matrix fibers (elastin-containing; red) while the nuclei (green) were stained with Yo-Pro-1.