Partial Off-Loading of Longitudinal Tension Induces Arterial Tortuosity

Zane S. Jackson, Dorota Dajnowiec, Avrum I. Gotlieb, B. Lowell Langille

Objectives—Arterial tortuosity is a frequent manifestation of vascular disease and collateral vessel growth, but its causes are poorly understood. This study was designed to assess the relationship between the development of tortuosity and the mechanical forces that are imposed on arterial tissue.

Methods and Results—Axial strain in rabbit carotid arteries was reduced from 62±2% to 33±2% by implanting an interposition graft, prepared from the contralateral carotid, at the downstream end of the artery. Axial strain remained unchanged for 12 weeks; however, all vessels became tortuous because of tissue growth and remodeling. After 7 days, there was a marked elevation in proliferation rates of endothelial and smooth muscle cells; however, increased apoptosis was also detected, and no net accumulation of DNA was observed. Significant accumulations of elastin (24%) and total collagen (26%) occurred by 5 weeks. Gelatin zymography detected upregulation and activation of matrix metalloproteinase-2 (MMP-2), and confocal microscopy revealed enlargement of fenestrae in the internal elastic lamina. MMP inhibition by treatment with doxycycline prevented enlargement of fenestrae and development of tortuosity, and it enabled normalization of axial strain by 5 weeks.

Conclusions—These findings indicate that substantial axial strain is necessary to sustain the morphological stability of arteries, and that a reduction in strain results in arterial tortuosity attributable to aberrant MMP activity. (Arterioscler Thromb Vasc Biol. 2005;25:957-962.)

Key Words: peripheral vasculature ■ vascular biology ■ tortuosity ■ remodeling ■ metalloproteinase

Arteries often become tortuous in response to potetial and persistent growth stimuli. For example, collateral vessels that expand after coronary or peripheral arterial occlusion often assume a meandering path; indeed, the presence of tortuosity is frequently used to identify these arteries. In addition, vessel wall hypertrophy associated with persistent hypertension frequently induces extreme tortuosity of central arteries, including the descending aorta, in the aged. Development of tortuosity is important because it represents a futile component of arterial growth that elevates flow resistance, it compromises the capacity of arteries to remodel appropriately in response to additional growth stimuli, and the flow disturbances it produces may predispose the vessel to atherogenesis.

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The causes of vessel tortuosity are unclear. The simplest explanation is that arterial growth inevitably involves some longitudinal elaboration of new tissues. Because the origins of most arteries at parent vessels and their terminations at daughter branches are anatomically fixed, longitudinal growth may ultimately force vessels to become tortuous. Tortuosity is generally seen only with very potent growth responses, but this may be because most arteries exhibit substantial in situ axial stretch of 40% to 60%. Consequently, it is possible that longitudinal tissue elaboration must first off-load this axial strain before tortuosity is manifest.

This paradigm presumes that axial loads on arterial tissue are unregulated; however, we recently found that large experimental increases in axial tension were completely corrected by remodeling within days. If off-loaded axial strain also elicited tight regulation through tissue remodeling, then growth responses would be completed without the development of tortuosity. In the current study, we assessed the effect of reducing axial stretch in rabbit carotid arteries from ≈60% to 30% using interposition grafts. No normalization of axial strain occurred within 12 weeks. Surprisingly, all of these arteries displayed tissue growth and remodeling that ultimately gave rise to marked vessel tortuosity, despite persistent axial stretch. When parallel experiments were performed in weanling rabbits, similar tortuosity ensued. Tortuosity apparently resulted from aberrant metalloproteinase activity because strain was normalized, and tortuosity did not occur in animals that were treated with the matrix metalloproteinase (MMP) inhibitor doxycycline. These findings indicate that neither remodeling of mature arteries nor...
growth modulation of developing vessels normalized reductions in axial strain. Therefore, substantial axial strain is required for normal arterial development and to maintain the anatomic stability of mature vessels.

**Methods**

Experiments were approved by the animal care committee of the University Health Network and were conducted in accord with the standards of the Canadian Council on Animal Care.

**Surgical Off-Loading of Axial Strain**

Adult (body weight 3.1 ± 0.1 kg), and 6-week-old (1.7 ± 0.1 kg) rabbits were anesthetized by intramuscular xylazine (0.8 mL/kg, 2 mg/mL) and ketamine (90 mg/mL) and then intravenous infusion of the anesthetic (0.02 to 0.03 mL/min). Both carotid arteries were exposed by a midline cervical incision, and 2 marker sutures (8-0 polypropylene) were sewn into the adventitia of the left carotid arteries and used to measure changes in axial strain (percent strain = $100 \times$ (in situ length – unstressed vessel length)/unstressed vessel length) by measuring percent retraction of the tissue between the markers on vessel excision. After intravenous infusion of heparin (800 U), the right carotid artery was ligated as far upstream and downstream as possible and excised. The left carotid artery was then exposed, and marker sutures were sewn into the adventitia of the vessel. Clamps were placed on the left carotid artery at its upstream and downstream ends, the vessel was transected downstream from the marker sutures, and 2 end-to-end anastomoses were performed to insert a segment of the right carotid as an interposition graft. To generate a recoverable model, we estimated the length of graft that would be needed to reduce axial strain from measured values in control rabbits (62 ± 2% strain adults and 52 ± 3% in weanlings) to 30%. Final strain was then measured according to the equation in the preceding paragraph using the separation of the marker sutures before and after transecting the artery and after interposing the graft. Longisil (150 000 IU/mL benzathine penicillin G, 150 000 IU/mL procaine penicillin G, 0.9 mg methyparaben, and 0.1 mL propyl paraben) was injected intramuscularly before surgery, and 0.1 mL of the analgesic Buprenex (0.3 mg/mL buprenorphine HCl) was injected subcutaneously 1 hour after surgery. Five adult rabbits underwent sham surgeries to control for surgical manipulations, scarring, and adhesions that might affect subsequent changes in axial strain. Grafts were transplanted as described above, except that a segment of the host carotid matching the length of the graft was excised. Possible changes in axial strain because of these procedures were assessed at 1 week after surgery. As an alternate sham procedure for later time points, the right carotid artery was ligated as far upstream and downstream as possible. Marker sutures were placed on the left carotid artery, and then the left carotid artery was clamped at its upstream and downstream ends for the same time that arteries were occluded in experimental animals (1 hour). Data for this latter sham procedure were reported in a previous publication and indicated that elastin, collagen, and DNA contents were unaffected.

**Determinations of Blood Flow Rate**

Blood flow rates were measured in anesthetized rabbits at 1 day, 5 weeks, and 12 weeks after surgery, and in rabbits 5 weeks after sham surgeries using an ultrasonic transit time flowmeter (Transonics model T206).

**Axial Strain and Arterial Tissue Constituents**

Rabbits were killed by intravenous infusions of T-61 (0.1 mL Hoechst) immediately or 5 or 12 weeks after surgery, and final axial strains of carotid arteries were measured as described above. Carotid tissues were stripped of adventitia and DNA, and elastin and collagen contents were determined. DNA contents were determined using a fluorometric assay that exploits the enhancement by DNA of the fluorescence of bisbenzimidazole (Hoechst 33258; Sigma). Total collagen and insoluble elastin contents were determined after treatment with cyanogen bromide (CNBr), which cleaves proteins at methionine residues. Because elastin contains no methionine residues, it is resistant to CNBr digestion, whereas collagen and proteins are solubilized. Insoluble elastin in the residue was determined using a ninhydrin assay. Collagen content was determined by measuring 4-hydroxyproline content in the CNBr extract, assuming that collagen contains 12.77% 4-hydroxyproline.

DNA, elastin, and collagen contents were expressed as micrograms per centimeter of in situ vessel length. Experimental data were compared with control data that had been corrected for the concentration of tissue that occurs when axial strain was surgically off-loaded (see dashed bars in Figure 6). This was done to ensure that statistically significant differences truly reflected net tissue accumulation. Tissue contents for experimental arteries were corrected for fractional growth of the distance between suture markers that had occurred since surgery as the vessels became longer and tortuous, so tissue accumulation would not be masked by the diluting effects of axial growth. Remodeling was inferred from changes in in vitro vessel length from changes in amounts of DNA, elastin, or collagen and from development of vessel tortuosity.

**Vascular Casting**

In separate experiments, rabbits were killed 5 weeks after experimental or sham procedures. The aorta was cannulated retrogradely, and Batson’s No. 17 methylmethacrylate casting compound (Polysciences) was infused at a pressure of 100 mm Hg. After the casting material had set, the carotid casts were removed and the length of left carotid was measured from its origin to the carotid bifurcation. A tortuosity index (TI) was calculated as the ratio of the path length defined by the vessel (Lp) minus the straight-line distance between its 2 ends (Lo) divided by Lp: $[TI=(L_p - L_o)/L_p]$, as described previously.

**Tissue Fixation, Confocal Microscopy, and Vessel Morphometry**

Carotid arteries of rabbits killed at 3, 7, or 35 days after surgery were fixed by perfusion, and samples were harvested for histology and for en face confocal microscopy. Medial thickness (h) at 4 sites and internal circumference (C) were measured from 3 nonserial sections of each artery (n=8) using commercial software (C-Imaging; Compix). Medial cross-sectional areas (h×C) and internal diameters (C/π) were then calculated.

Samples were prepared for en face confocal microscopy as follows. Vessel segments were opened longitudinally, mounted lumen side up on slides under glass cover slips, and examined with a Bio-Rad confocal microscope (MRC-1024). Autofluorescence of elastin allowed visualization of the internal elastic laminae (IEL) and the fenestrae that perforate them. Image analysis software (C+ Imaging; model 640; Compix) was used to determine the mean areas and densities (fenestrae/mm²) of the fenestrae. For this analysis, fenestrae <3 μm were rejected because they contained too few pixels (<20) for accurate area measurement and because they were frequently difficult to discriminate from noise in the image signal. No other selection criteria were applied. Morphometry of the IEL was performed on arteries of control rabbits (n=5) and on arteries from rabbits at 3 (n=5) and 7 (n=5) days, and 5 weeks (n=5) after off-loading of axial strain.

**Doxycycline Treatment**

Additional animals were given 80 mg/kg per day of the MMP inhibitor doxycycline in drinking water from the day of surgery until euthanasia at 5 weeks after surgery. Axial strain, vessel diameter, and tortuosity were assessed in 5 of these rabbits, and morphometry of the IEL (previous section) was performed on an additional 6 animals.

**Cell Proliferation**

To assess daily cell replication rates, 5-bromodeoxyuridine (BrdU; Sigma) was injected intramuscularly at 17, 9, and 1 hour before
BrdUrd was detected by immunostaining of histological sections (Oncogene; BrdUrd staining kit; HCS24). Slides were counterstained with hematoxylin. Percent cells replicating per day were determined for endothelium and medial smooth muscle cells. Counts were made in segments of the artery that were well proximal to the upstream graft anastomosis site to avoid artifacts associated with handling of the graft or with suturing the anastomosis site.

**Gelatin Zymography and Immunoblotting**

Arteries were harvested at 1, 3, and 7 days after surgery, frozen in liquid nitrogen, and ground to a fine powder. Protein was extracted in lysis buffer and then equal amounts of protein from each extract were electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.1% type I gelatin (Sigma). Gels were washed with 2.5% Triton X-100, incubated overnight in 0.05 mol/L Tris with 2.5 mmol/L CaCl₂ and 0.02% NaN₃, and then stained with Coomassie blue. Gelatin degradation was observed as white lytic bands.

Proteins extracted as described above were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were probed with monoclonal anti-human MMP-2–purified IgG antibody (ICN) and appropriate secondary antibody (Amersham Life Science), then treated with enhanced chemiluminescence detection reagent (Amerham). Bands were visualized by exposing the membranes to Kodak X-OMAR film.

**Detection of Apoptotic Degradation of DNA Into Oligonucleosomes**

Arteries were harvested at 1, 3, and 7 days after surgery, and DNA was extracted by overnight incubation in DNA lysis buffer followed by a phenol/chloroform extraction. Arterial DNA (5 μg) plus 30-bp oligonucleotide (to control for variability in loading of wells) were incubated with [³²P] dCTP and 10 U of Klenow polymerase (Phar-macia Biotech). DNA was electrophoresed on a 1.8% agarose gel and blotted onto Hybond nylon membrane (ICN). End-labeled DNA fragments were visualized by exposing the membranes to Kodak X-OMAR film.

**Statistical Analysis**

Significant differences were determined by ANOVA followed by Dunnett’s tests. A value of P<0.05 was considered significant (n=5 to 7 rabbits for all groups). Data are presented as mean±SEM. Use of animals for various aspects of this study is summarized in Table I (available at http://atvb.ahajournals.org).

**Results**

**Partial Off-Loading of Axial Strain Induces Arterial Tortuosity**

Axial strains of adult rabbit carotid arteries were off-loaded from an initial mean value of 62±2% to 33±2%. Axial strain was not subsequently normalized (ie, strains measured at 5 weeks [26±3%] and 12 weeks [31±4%] were not significantly different from values measured immediately after surgery [Figure 1]). The same outcome was observed in immature rabbits in which axial strains were off-loaded from 52±3% to 30±3%. No subsequent changes in strains after 5 weeks (32±3%) or 12 weeks (30±3%) were statistically significant.

Surprisingly, all arteries with reduced strain were highly tortuous at 5 weeks even though ~50% axial stretch persisted (Figure 2). A modest TI of ~3% was observed for control arteries because the proximal artery bends at ~2 cm from its origin. TI then increased by ~5-fold at 5 weeks postoperatively. TI was unaltered for sham-operated animals. Measurements of strain using multiple marker sutures in the adventitia of some arteries confirmed that even highly tortuous segments of the vessel remained under 30% strain.

The development of tortuosity was accompanied by an increase in arterial diameter of 7% at 7 days (1.42±0.03 mm versus 1.52±0.02 mm; P<0.05; n=5 per group) and a further increase to 30% at 5 weeks (1.85±0.04 mm; P<0.05; n=5 per group). Medial cross-sectional area was increased by 14% over this time (0.36±0.02 mm² versus 0.41±0.02 mm²; P<0.05; n=5 per group). This tissue growth was not attributable to changes in carotid blood flow rate. Volume flow rate of blood was unaffected by the surgeries (Figure I, available online at http://atvb.ahajournals.org), and consequently, there was a decrease in shear stress in these vessels that was...
Net accumulation of elastin (24%) and collagen (26%) was detected at 5 weeks postoperatively (Figure 1), even though no recovery of strain occurred in unmanipulated carotid arteries (C; n=5) and in arteries 3 days (n=5) and 7 days (n=5) after surgery. Asterisk indicates significant difference from control (P<0.05). B, Artery 7 days after axial strain was off-loaded. C, Percentage of endothelial (black bars) and smooth muscle (white bars) cells replicating per day in unmanipulated carotid arteries (C; n=5) and in arteries 3 days (n=5) and 7 days (n=5) after surgery. Asterisk indicates significant difference from control (P<0.05). D, Autoradiogram of DNA gel illustrating degradation of arterial DNA into oligonucleosomes, a hallmark of apoptosis, in control arteries and at 1, 3, and 7 days after axial strain was off-loaded (n=3 for each group). The bottom band (arrow) in each lane represents a 30-bp oligonucleotide that was added to samples to control for variability in loading of the gels. Lane S contains DNA size markers at multiples of 100 bp.

Vascular Tissue Growth After Reduction of Axial Strain
Endothelial cell replication rate increased by >20-fold, and medial smooth muscle cell replication rate increased by 6-fold at 3 days after surgery (Figure 3A through 3C). Replication rates were further increased by 7 days to 37-fold for endothelial cells and 10-fold for smooth muscle cells. Smooth muscle cell replication was also randomly distributed over the intimal surface. Despite this cell replication, vessel wall cell number per mm² at 5 weeks (2340±440; n=5) were not different from control (3000±1004; n=5 per group; P>0.05). Densities of fenestrae after off-loading axial strain for 3 days (3070±857; n=5) and 7 days (3230±530; n=5) also were not significantly different from that observed in control vessels (P>0.05).

Gelatin zymography of extracts from control arteries revealed a strong lytic band with a molecular weight of 70 kDa and a faint band at 62 kDa (Figure 6A) that were identified in immunoblots as the latent and active forms of MMP-2 (Figure 6B). A slight elevation in activated MMP-2, and an additional lytic band at 88 kDa was observed in tissue extracted from arteries harvested at 1 day when compared with control (Figure 6A). The 88-kDa band was not detected at 3 and 7 days postoperatively, but higher levels of active MMP-2 were observed.

Aberrant MMP Activity Prevents Normalization of Axial Strain and Induces Vessel Tortuosity
When rabbits were treated for 5 weeks after off-loading axial strain with the MMP inhibitor doxycycline, tortuosity was almost totally prevented (Figure 2). Remarkably, we also found that axial strain was fully normalized after 5 weeks (Figure 1), even though no recovery of strain occurred in unmanipulated carotid artery (A) and artery 5 weeks (wk) after axial strain was off-loaded (B). Marker=50 μm. C, Graph of mean area of fenestrae in unmanipulated carotid arteries (control; n=5) or arteries 3 days (3d), 7 days, or 5 weeks after axial strain reduction (n=5 per group). Also shown are data for rabbits treated with doxycycline (Dox) for the 5 weeks after axial strain reduction (open bar; n=6). Asterisk indicates significant difference from control (P<0.05).
Mechanically induced arterial remodeling is often more pronounced in the developing than in the mature vasculature, and in vascular diseases. The capacity of changes in blood pressure to induce remodeling of arterial wall thickness and blood flow rate to influence vessel diameter are well established and have a proven influence on the pathogenesis and progression of hypertension and atherosclerosis. Arterial growth is also stimulated by increases in axial strain, was associated with tissue growth and remodeling. Cell turnover (proliferation and apoptosis) was much accelerated, and there was net accumulation of elastin and collagen despite marked upregulation MMP-2 activity. Matrix remodeling was manifest as marked enlargement of fenestrae that pass through the IEL after off-loading of axial strain, even though passive recoil during off-loading should have caused their shrinkage. This enlargement of fenestrae occurred while substantial amounts of new elastin accumulated in the vessel wall, a finding that is especially noteworthy given that newly synthesized elastin is preferentially deposited at fenestrae. Prevention of enlargement of fenestrae by treatment with the doxycycline is consistent with participation of this matrix reorganization in development of tortuosity.

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however, this is unlikely. Flow disturbances propagate downstream, but tissue growth responses and arterial tortuosity were observed well upstream of the anastomosis site; furthermore, sham procedures involving implantation of grafts that replaced equal lengths of host vessel did not cause tortuosity.

In summary, we have found that partial off-loading of axial arterial strain induces arterial tortuosity, without further alteration in axial strain, that involves a tissue growth response and that depends on production of MMP activity. Accordingly, axial strain appears to be normalized only when this mechanical load is increased, a finding that has important implications for remodeling of arteries that invest tissues that undergo involution or atrophy.

Acknowledgments
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References
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Figure I

Blood Flow (ml/min/kg)

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</tr>
<tr>
<td>18</td>
<td>5 WEEK DOXYCYCLINE</td>
<td>11 adult</td>
<td>Measurements of diameter, medial area and morphometry of IEL</td>
<td>1,2,5</td>
</tr>
<tr>
<td>19</td>
<td>12 WEEK</td>
<td>5 adult</td>
<td>axial strain, blood flow measurement</td>
<td>1</td>
</tr>
</tbody>
</table>

Table indicating use of animals and the incorporation of data derived from them into journal figures.