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Short Communication

Eliminating Arterial Pulsatile Strain by External Banding Induces Medial but Not Neointimal Atrophy and Apoptosis in the Rabbit

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We have examined the role of vessel pulsation and wall tension on remodeling and intimal proliferation in the rabbit infrarenal abdominal aorta. A rigid perivascular polyethylene cuff was used to reduce vessel systolic diameter by 25%, producing a region of reduced circumferential strain. At 6 weeks postoperatively, reduced circumferential strain caused medial atrophy, with 45% reduction of medial area and 30% loss of medial smooth muscle cells. Apoptotic cell death was indicated by DNA fragmentation, propidium iodide staining, and cell morphology. Cuffing the aorta after balloon denudation produced medial atrophy but did not inhibit neointimal growth. At 1 week postoperatively, intimal thickness was slightly decreased in regions with reduced strain; however, intimal thickening in regions of reduced strain was not different from control segments at 3 weeks postoperatively (intimal area was 0.37 ± 0.05 mm^2 with reduced strain and 0.50 ± 0.08 for controls, mean ± SEM). We conclude that circumferential strain is a major factor controlling medial structure and cell number, whereas growth of the neointima after injury is not significantly affected by either reduced strain or extensive medial cell death. Vessel cuffing represents a new model of blood vessel remodeling *in vivo* that involves extensive smooth muscle cell apoptosis. *(Am J Pathol 1998, 153:1723–1729)*

Both pathological processes and surgical interventions can severely alter the normal mechanical behavior of blood vessels. For example, vascular grafts, stents, angioplasty, and atherosclerotic plaque all change the normal pulsatile behavior of arteries.[1–3] These changes in wall mechanics can affect the progression of vascular disorders because altered mechanical tension induces marked structural changes in the artery wall. Thus, in animal models of hypertension, hypertrophy of smooth muscle leads to increased wall mass of peripheral vessels and is associated with an increased passive arterial wall stiffness in elastic arteries.[4,5] Veins implanted in the arterial circulation exhibit intimal hyperplasia and medial thickening, and this arterialization is thought to be a response to increased wall tension.[6] Also, reducing circumferential strain by perivascular cuffing of vein grafts markedly reduces the medial thickening and structural remodeling of veins implanted in the arterial circulation.[7–9]

In vitro studies have demonstrated that smooth muscle cells in culture respond to cyclic changes in strain of the substrate on which they are grown. Pulsatile strain upregulates mitogenesis and release of fibroblast growth factor (FGF)-2, and it alters actin, myosin, and h-caldesmon expression[10–16]; however, it has been impossible to reproduce in cell culture the three-dimensional strain and complex matrix interactions that smooth muscle cells experience *in vivo*. Thus, the role that cyclic mechanical strain plays in arterial remodeling in physiological and pathological conditions has not been fully elucidated.

To study the role of wall tension and strain on arterial remodeling, we have developed a model in which an external cuff was used to reduce vessel diameter and eliminate cyclic strain produced by pulsatile pressure in

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the rabbit abdominal aorta. A marked medial atrophy, smooth muscle cell loss, and DNA fragmentation was observed under the cuff, implying that a reduction of physiological strain levels can be transduced into signals that induce apoptotic cell death. Thus, mechanical signals play an important role in determining cell number and structural remodeling.

Surprisingly, reducing circumferential strain after balloon denudation did not effect the development of a neointima even in the presence of severe medial atrophy. Thus, intimal proliferative responses appear to be independent of tensile loads and independent of extensive medial remodeling and atrophy.

Materials and Methods

A total of 56 New Zealand White rabbits were premedicated with intramuscular injections of Ketamine (100 mg) and Xylazine (16 mg). Anesthesia was then maintained with intravenous injections of Ketamine and Xylazine as required. A laparotomy was performed, and a portion of the abdominal aorta immediately proximal to the caudal mesenteric artery was isolated. Cuffs manufactured from sterile, medical-grade polyethylene tubing (3.9-mm inner diameter) were passed around the aorta and tied closed with 3–0 silk. Cuffs produced an approximately 25% reduction in arterial diameter at systolic pressure, as measured in four rabbits in which the arterial system was cast by infusion of Batson's casting compound at systolic pressure from 1 to 6 weeks postoperatively. Cuffed aortic segments were compared with segments of aorta immediately upstream from the cuff.

We performed a separate series of experiments to control for tissue reactions to the foreign material comprising the cuff. For these experiments, 30% of the circumference was removed from the upstream half of the cuff (region A in Figure 1). Aorta in this region of the cuff was free to distend under pressure but was in contact with polyethylene for most of its circumference. The downstream half of the cuff was identical to the standard cuffs described above.

After closure of the incisions, all rabbits were given the analgesic buprenorphine (0.03 mg).

For histology, 11 standard cuffs and 6 control cuffs were implanted in 17 rabbits that were sacrificed by anesthetic overdose at 6 weeks postoperatively. The aorta was perfusion fixed at 100 mm Hg with phosphate-buffered 3% paraformaldehyde, and transverse aortic sections from the downstream limit of the cuff to 1 cm upstream from the cuff were prepared.

Role of Cell Death in Remodeling

To examine the role of cell death in remodeling responses to cuffing, standard cuffs were implanted in 12 additional rabbits. Three animals were sacrificed at each of 1, 2, 3, and 6 weeks postoperatively. Cuffed and upstream segments of the abdominal aorta were excised for DNA extraction and electrophoresis. A small cross-sectional segment was also taken from each specimen for transmission electron microscopy.

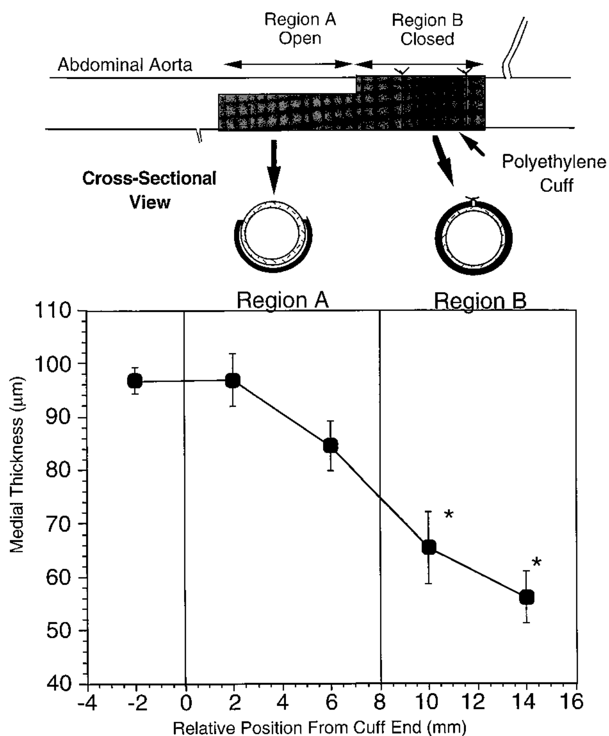

Figure 1. An open-ended control cuff was implanted around the abdominal aorta of rabbits as depicted in the line drawing. In region A, the cuff material was in close contact with the adventitia; in this region, the circumferential strain in the vessel was not reduced. In region B, the closed portion of the cuff produced a reduction in circumferential strain. Morphometry of vessel cross sections revealed a marked thinning of the media in region B only. Results are mean ± SE (ANOVA, Fisher PLSD at $P < 0.01$ for both sections in region B compared with all others).

DNA was extracted from arterial tissues and purified using a standard extraction method. The proximal and cuff portions of abdominal aorta were minced and incubated overnight in DNA lysis buffer (20 mmol/L Tris/HCl, pH 7.4, 1% SDS, 5 mmol/L EDTA, 100 μg/ml proteinase K) at 50°C. DNA was extracted with salt-saturated phenol and chloroform and precipitated with 100% ethanol. Optical measurements were used to estimate the purity, and total DNA was extracted.

Fragmentation of DNA was measured with a modified end-labeling procedure.[17] Five micrograms of arterial tissue DNA was incubated with [^{32}P]dCTP (10 μCi; ICN Biomedical Canada, St. Laurent, Quebec, Canada) and Klenow polymerase (10 U; Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) for 15 minutes at 30°C. The end-labeling reaction was terminated by addition of 10 mmol/L EDTA. Unincorporated nucleotides were removed using a Magic DNA Clean-up system (Promega Corp., Madison, WI). Radiolabeled DNA was electrophoresed on a 1.8% agarose gel and blotted onto Hybond nylon membrane, and the membrane was exposed to Kodak X-Omat x-ray film. In a separate lane, we loaded 1 μg of DNA size markers consisting of multiples of 100-bp fragments (Gibco BRL Life Technologies, Gaithersburg, MD) that was similarly end-labeled with [^{32}P]dCTP. The appearance of bands of labeled DNA from arterial tissue

Table 1. Vessel Morphometry 6 Weeks after Implantation of a Perivascular Cuff

Group	*n*	Medial Area (mm^2)	Cells/section	Cells/mm^2
Under cuff	11	0.50 ± 0.04	2170 ± 130	4400 ± 230
Outside cuff	11	0.89 ± 0.05	3170 ± 190	3600 ± 170
Paired *t*		$P < 0.0001$	$P = 0.0004$	$P = 0.007$

at multiples of approximately 200 bp indicated apoptotic fragmentation DNA into oligonucleosomes.

In a separate group of four rabbits, the aorta was cuffed for 1 or 2 weeks at which time propidium iodide (5 μmol/L/kg) was injected intravenously 15 minutes before sacrifice and perfusion fixation. An *en face* preparation of the abdominal aorta was examined from each rabbit with confocal microscopy to detect nonviable cells, as propidium iodide is excluded from viable cells.[18]

Circumferential Strain and Remodeling after Balloon Denudation

To investigate the role of reduced circumferential strain in arterial response to injury, the rabbit infrarenal abdominal aorta was denuded of endothelium using a balloon catheter before cuff placement. A 4 F Fogarty balloon catheter was introduced through the femoral artery and advanced to the renal bifurcation, and the balloon was inflated and pulled back to the iliac bifurcation. This procedure was performed two more times before removing the catheter and ligating the femoral artery. To examine the early response to injury, four rabbits were balloon denuded and cuffed. A control group ($n = 4$) was balloon denuded, and a sham laparotomy, but no cuff placement, was performed. These control aortas were compared with cuffed vessels and with segments of aorta upstream from cuffs to test whether cuff placement affects upstream neointimal formation. The rabbits were sacrificed 1 week postoperatively. Proliferating cells were labeled over the entire implantation period by intraperitoneal implantation of an osmotic pump (ALZET, 2ML1) loaded with 5′-bromodeoxyuridine (BrdU), which delivered 1.6 mg of BrdU/hour. The healing response at 3 weeks postoperatively was examined in 11 rabbits in which control cuffs were implanted. In this group, cell proliferation was labeled over the last 24 hours before sacrifice by administration of an intravenous loading dose of BrdU (28 mg) and subcutaneous implantation of a 24-hour osmotic pump (ALZET, 2001D) loaded with BrdU. The bolus injection was used to counteract variable pump start-up times, which were deemed inconsequential for the 1-week infusions.

All rabbits were killed by anesthetic overdose and perfusion fixed at 100 mm Hg with phosphate-buffered 3% paraformaldehyde. Consecutive sections of abdominal aorta were taken from the area underneath the cuff and extending to 1 cm proximal of the cuff. For animals with no cuff, three sections were taken immediately proximal to the caudal mesenteric artery. BrdU immunohistochemistry and quantitative morphometry were performed to calculate cell numbers, dividing cells and medial and intimal areas.

Results

Aortic sections from under standard cuffs were compared with regions of aorta proximal to the cuff. At 6 weeks postoperatively, all regions under the cuff displayed decreased medial area, decreased medial cell number, and increased cell density (Table 1). No intimal thickening was observed at any location when the cuff was placed on uninjured aorta. Histological sections revealed numerous patent small distributing vessels between the cuff and the adventitia of the aorta, indicating that the cuffs did not block blood flow to the adventitia.

DNA extracted from regions of vessel under the cuff displayed a distinctive laddering pattern at 1, 2, 3, and 6 weeks postoperatively (Figure 2). Extensive DNA fragmentation was not observed in DNA extracted from regions of the aorta proximal to the cuff. Transmission electron microscopy revealed irreversibly damaged cells

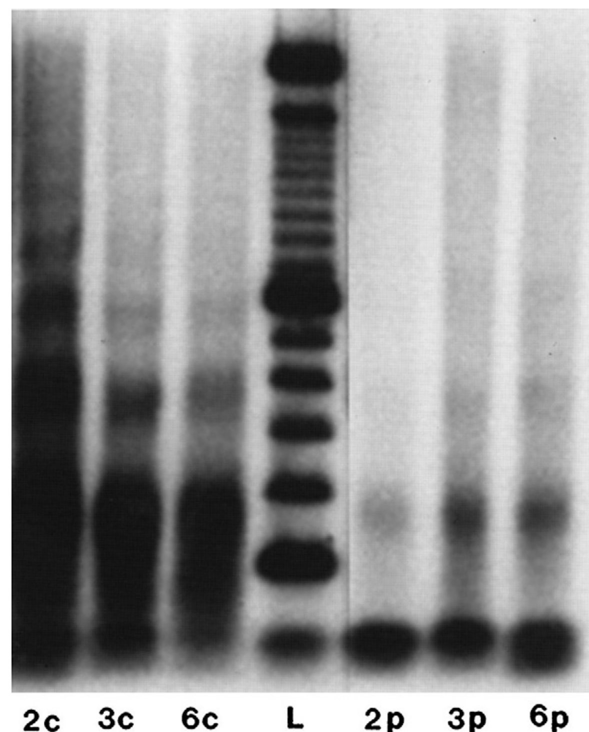

Figure 2. Gel electrophoresis of end-labeled DNA extracted from rabbit abdominal aortas. Regions under the perivascular cuff at 2 weeks (2c), 3 weeks (3c), and 6 weeks (6c.) after implantation display a distinctive laddering pattern indicative of internucleosomal DNA fragmentation (L represents a standard 100-bp DNA ladder). In paired sections of proximal aorta (2p, 3p, and 6p) minimal DNA fragmentation is observed. The amount of low molecular weight DNA from the proximal artery was always much less than that observed from vessels under the cuff. Under the cuff, DNA fragmentation appeared to be maximal at the earlier time points and slightly diminished by 6 weeks after cuff implantation.

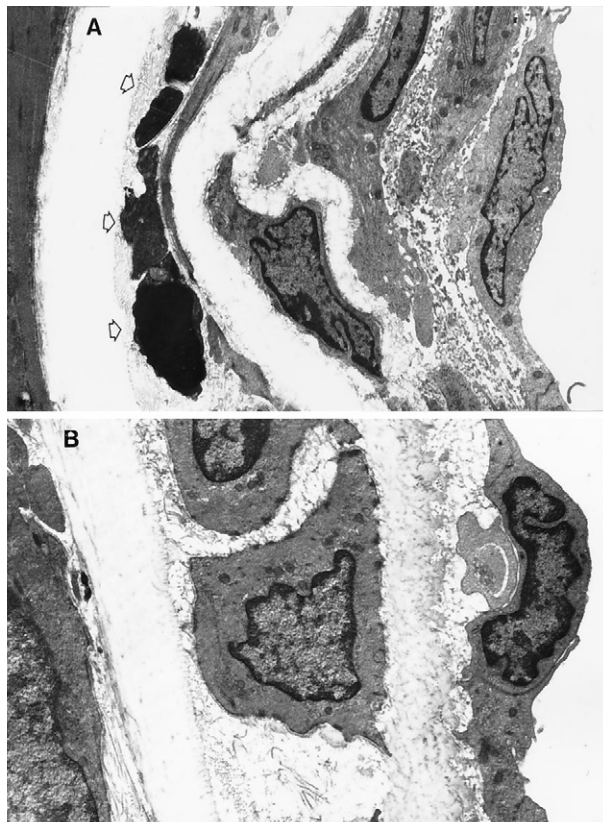

Figure 3. Transmission electron micrographs (magnification, ×10,000) of rabbit abdominal aorta 3 weeks after implantation of a perivascular cuff. In regions under the cuff (A), the elastic lamellar units appeared to be collapsed with smooth muscle cells displaying abnormal morphology, including cell condensation and fragmentation (arrows). Regions proximal to the cuff displayed normal vessel morphology (B).

in the portion of the vessel beneath the cuff, nuclear and cytoplasmic condensation, swelling of intracellular organelles, large cytoplasmic inclusions and membrane blebbing in scattered cells throughout the media (Figure 3).

Propidium iodide is excluded from viable cells and when injected intravenously it labels nuclei in cells that are not viable. In rabbits treated with propidium iodide, *en face* preparations of the aorta from cuffed regions displayed scattered cells in which the dye had been internalized (Figure 4). These cells also displayed a distinct nuclear fragmentation typical of apoptotic cell death.

In control cuffs (30% of circumference removed from upstream half) all regions under the closed portion of the cuff produced a marked thinning of the media when compared with regions in the open cuff or proximal vessel (Figure 1). No decrease in medial thickness was observed under the open cuff. There was no significant effect of the open region on medial area or total cells per cross section; medial cells per section were 3230 ± 280 in the open region *versus* 3260 ± 200 in the region of the vessel outside the cuff. Although the open and closed cuffs produced some perivascular fibrosis, this did not extend into the vessel wall. Inflammatory infiltrates were not observed in the adventitia under the cuff.

Balloon Denudation

At 1 week after balloon denudation and cuff implantation, a significantly smaller intima was produced under the cuffed aorta when compared with animals with no cuff (Table 2). The amount of neointima in uncuffed arteries was not significantly different from that seen upstream from cuffs (data not shown). The percentage of proliferating (BrdU-positive) cells was elevated over uninjured control vessels in both the media and intima. No apoptotic cells were detected by propidium iodide staining in arteries subjected to ballooning but not cuffing, whereas 0.16 ± 0.02% of medial cells displayed propidium iodide uptake with apoptotic nuclear morphology at 1 week after surgery. No apoptotic intimal cells were detected.

At 3 weeks postoperatively, the cuffs produced a marked, statistically significant reduction in medial cross-sectional area compared with regions upstream from the cuff in the same animals (Table 3); however, the cuff produced no statistically significant effect on the size of the intima at 3 weeks. (A power analysis of the data indicated that the experimental design was robust enough to determine a 20% or greater difference in intimal area.) Also, no statistically significant difference was found between cuffed and upstream vessels for the total intimal cells per section, or medial and intimal cell density.

There was no statistically significant difference, between cuffed and upstream segments, in the percentage of intimal or medial cells proliferating over the 24 hours before sacrifice at 3 weeks. There was, however, considerable variability in the labeling indices.

Discussion

A perivascular cuff placed around the rabbit abdominal aorta to reduce circumferential strain caused a marked thinning and loss of cells in the media over 6 weeks. The smooth muscle cell loss showed classical hallmarks of apoptotic cell death, including DNA fragmentation into multiples of 200 bp, incorporation of propidium iodide *in vivo*, and a distinctive cellular morphology that included condensation and fragmentation into apoptotic bodies.[19–22] Taken together, these results indicate that a perivascular cuff induces extensive smooth muscle cell apoptosis. This response was not seen under the open regions of control cuffs, which maintained close perivascular contact yet did not reduce strain, indicating that remodeling in the media was not a response to a foreign body or to tissue irritation due to the cuff. Extensive medial cell loss under a cuff has not been reported previously. The most likely explanation for this observation is that smooth muscle cells of the media are responding to changes in tensile load. By cuffing the vessel, the average strain in the wall is reduced and the pulsatile strain eliminated. It was not possible to distinguish the independent roles of reduced mean strain level *versus* the loss of pulsatile strain in arterial remodeling. As mentioned earlier, *in vitro* models suggest that vascular smooth muscle cells display a wide range of responses

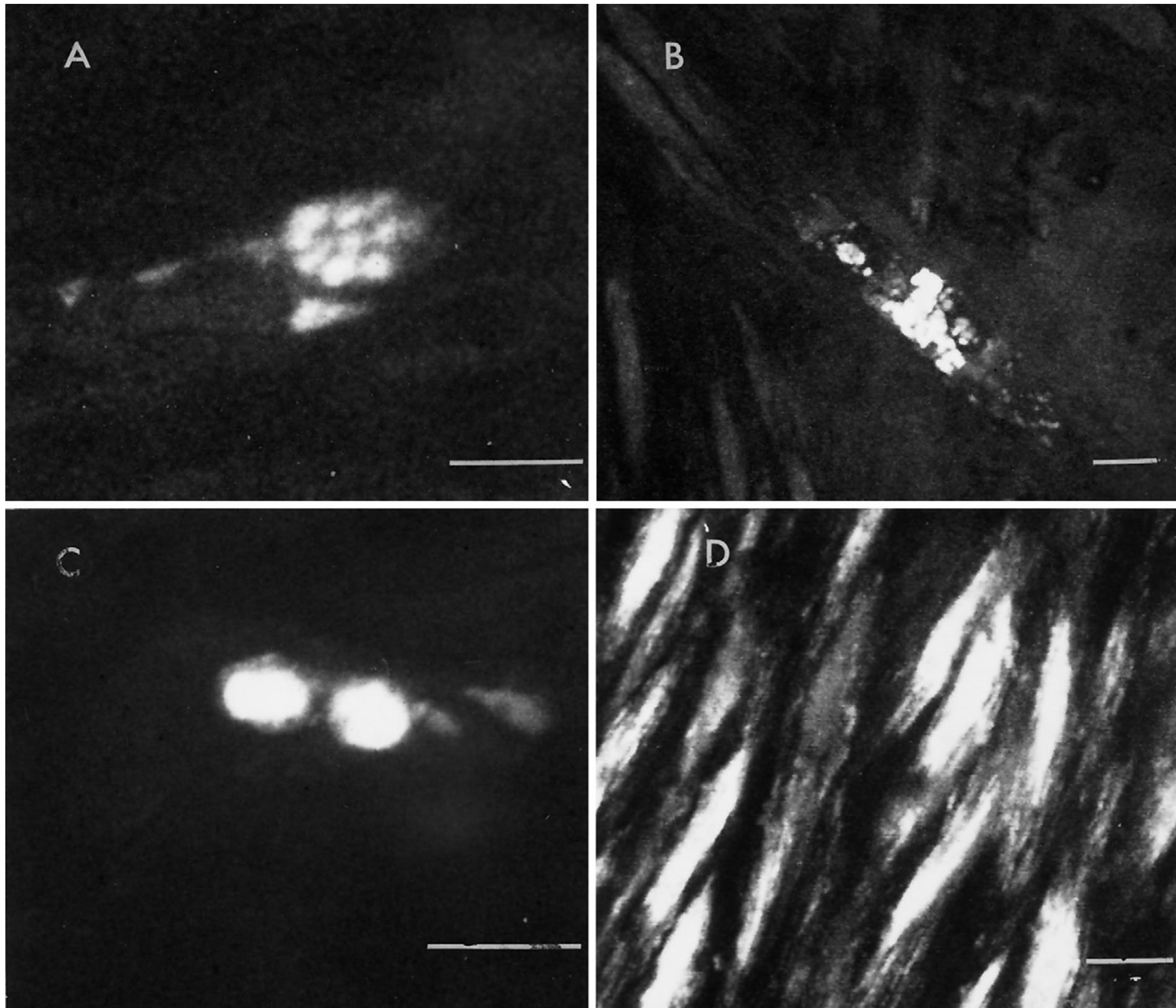

Figure 4. Before sacrifice and 1 week after the aorta was cuffed, the fluorescent dye propidium iodide was injected intravenously. *En face* preparations of perfusion-fixed aortas were viewed with confocal microscopy. Propidium iodide uptake was observed in scattered cells throughout the media only in regions under the cuff (A–C), indicating that these cells were not viable. The cells displayed shrunken and fragmented nuclei when compared with a control segment of vessel permeabilized with detergent before dye application (D).

to cyclic strain; however, it is also possible that a step reduction in the mean strain level will produce profound cellular responses and vessel remodeling.

In the media of uncuffed arteries, hydrostatic pressure decreases from arterial blood pressure at the intimal side of the vessel wall to near atmospheric pressure at the adventitial side of the media. Our standard cuffs will cause hydrostatic pressures throughout the media to approach arterial pressure, as the constricting cuff will bear most of the wall tension, but tissue pressure cannot exceed arterial blood pressure. As innermost smooth muscle cells in unmanipulated arteries are untraumatized by this level of pressure, it is unlikely that any injury associated with hydrostatic forces can account for medial atrophy in our model.

Cuffs may induce other perturbations to the vessel wall, including possible disruption of adventitial innervation, vascularization, and transport of fluid across the media. However, such perturbations occur after application of loose-fitting perivascular cuffs with sealed ends. The latter cuffs cause intimal proliferation without substantial changes, eg, atrophy, to the media.[23,24] Consequently, this previous work argues against a role for such perturbations in the atrophy seen in the current work. A

Table 2. Vessel Morphometry 1 Week after Balloon Denudation and Perivascular Cuff Implantation

Group	Area (mm^2)		Cells/section		Proliferation %/week	
	Media	Intima	Media	Intima	Media	Intima
Cuff (n = 4)	0.49 ± 0.08	*0.02 ± 0.01	2818 ± 280	*200 ± 130	62 ± 22	78 ± 5.3
Control (n = 4)	0.67 ± 0.05	0.16 ± 0.05	2837 ± 200	975 ± 240	31 ± 4	49 ± 7.4

*$P < 0.05$, t-test.

Table 3. Vessel Morphometry 3 Weeks after Balloon Denudation and Perivascular Cuff Implantation

Group	n	Area (mm^2)		Cells/section		Proliferation %/day	
		Media	Intima	Media	Intima	Media	Intima
Under cuff	11	0.56 ± 0.05	0.37 ± 0.05	1010 ± 120	1180 ± 100	4.2 ± 1.3	10.0 ± 3.0
Outside cuff	11	0.86 ± 0.05	0.50 ± 0.08	1530 ± 90	1010 ± 120	2.6 ± 1	5.8 ± 1.1
Paired t		$P = 0.008$	$P = 0.2$	$P = 0.009$	$P = 0.29$	$P = 0.5$	$P = 0.17$

decrease in nutrient delivery produced by the cuff would be most pronounced on the adventitial side of the media, and smooth muscle cells immediately adjacent to the lumen would receive adequate nutrient supply. In our experiments the morphology of cell loss and the propidium staining in cuffed vessels clearly showed that smooth muscle cell apoptosis was equally distributed throughout the vessel media. These findings argue against a primary role for reduced nutrient delivery as the primary cause for the apoptotic cell loss. We have not, however, proven conclusively that reduced nutrient delivery may be involved in the remodeling process in cuffed vessels.

DNA fragmentation data indicated that cell death through apoptosis in cuffed regions occurs over an extensive postoperative period, with evidence of elevated cell death present even at 6 weeks postoperatively. This implies a chronic turnover of the cell population in the media. The cuffed regions of vessel also displayed marked remodeling of the extracellular matrix, evidenced histologically by a compacting of the elastic lamellar units and an extensive loss of non-cell-associated area. Loss of attachment to the extracellular matrix has been associated with apoptosis,[25,26] and matrix-integrin interactions have been implicated in mediating cell survival.[27] It is possible that loss or restructuring of extracellular matrix in response to reduced wall tension is a stimulus for apoptosis in our model.

Although changes in tensile strain are the most likely stimulus for remodeling under the cuff, the reduction in diameter produced by the cuff would also elevate the luminal shear stress under the cuffed portion of the vessel. Increased shear stress can induce release of endothelial-derived relaxing factor (nitric oxide). Cuffing may have led to a frustrated vasodilatory response in which nitric oxide was chronically released. Nitric oxide is an important mediator of cell death in a number of cell culture systems, including vascular smooth muscle.[28–31] Consequently, it is possible that chronically elevated levels of nitric oxide released from the endothelium may lead to medial smooth muscle cell loss; however, our balloon denudation experiments in the rabbit do not support this hypothesis. Loss of medial smooth muscle cells was evident even after extensive balloon denudation in which the endothelium was stripped from the vessel. At 3 weeks postoperatively, medial cell loss was evident despite the absence of endothelium. Also, an intact endothelial cell monolayer is a primary determinant of fluid flux through the wall,[32] and therefore, disruption of the endothelium is likely to have a marked impact on fluid flux. As similar medial cell loss was observed with and without balloon denudation, it is unlikely that changes in fluid flux are a primary stimulus.

The application of a perivascular cuff delayed the onset of intimal thickening after balloon denudation; however, the intimal area under the cuffed vessel was similar to the unconfined regions by 3 weeks postoperatively. Delayed onset of intimal thickening under the cuff may have been the result of increased shear stress on the luminal surface. A 20% to 25% decrease in diameter in the cuffed region would have doubled the peak luminal shear stress (assuming Poiseuille flow, for which shear stress varies as the inverse cube of vessel diameter), and elevated shear stress slows neointimal thickening. Intimal proliferation induced by balloon injury in rat carotid arteries was inhibited by shear stress transiently (2 to 4 weeks) but not at steady state 8 weeks after injury.[33] Thus, elevated shear stress may have contributed to the delayed smooth muscle cell migration into the intima of the cuffed vessels.

Medial trauma after balloon injury has been linked to neointimal formation; however, our data clearly indicate that chronic medial cell apoptosis in response to vascular cuffing does not influence intimal thickening after balloon denudation. Unlike the necrotic cell death produced by ballooning, apoptosis is much less likely to produce an inflammatory reaction even if present in large numbers,[21,22] and consequently the release of cytokines that could contribute to intimal proliferation is probably modest. However, recent evidence has suggested that apoptotic cell death may not be completely benign. In cell co-cultures of endothelium with fibroblasts, induction of apoptosis enhanced tissue factor procoagulant activity, possibly due to increased exposure of phosphatidylserine.[34] In our model, extensive medial cell loss under the cuff did not promote any observable thrombosis, inflammation, or increased intimal growth. These data show that extensive vascular smooth muscle cell death can take place within the vessel wall without promoting or initiating these pathological processes.

When the cuff was applied after balloon denudation, it promoted medial atrophy yet did not affect long-term neointimal formation. Possibly, the very organized structure of the extracellular matrix in the media is important in the transduction of mechanical stimuli. Alternatively, it may be that phenotypic differences between the smooth muscle cell populations in media *versus* within the neointima[35,36] account for differences in responses to cuffing. Finally, it should be noted that inferences concerning circumferential tensions imposed on neointima should be made with caution, regardless of whether a cuff is present or not. Neointima in uncuffed vessels forms on a

prestressed substrate (the media), but it is not clear that intimal tissues acquire wall tension (become stretched) as they are elaborated. Indeed, if medial stretch and structure are unchanged during neointimal formation, then medial tension may remain in equilibrium with intraluminal pressure.

Finally, our findings may be relevant to remodeling of atherosclerotic vessels in humans, in which lesion tissues may reduce tensile loads on adjacent media. They may also be relevant to many surgical interventions that alter blood vessel mechanics and promote intimal hyperplasia. The cuff procedure we present here represents a novel model in which to study, *in vivo*, the role of medial smooth muscle cell apoptosis in blood vessel response to injury.

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