Smooth muscle phenotypic modulation is an early event in aortic aneurysms

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Objectives: Vascular smooth muscle cells can undergo profound changes in phenotype, defined by coordinated repression of smooth muscle cell marker genes and production of matrix metalloproteinases in response to injury. However, little is known of the role of smooth muscle cells in aortic aneurysms. We hypothesized that smooth muscle cells undergo phenotypic modulation early in the development of aortic aneurysms.

Methods: Abdominal aortas from C57B6 mice (n = 79) were perfused with elastase or saline (control) and harvested at 1, 3, 7, or 14 days. Aortas were analyzed by means of quantitative polymerase chain reaction and immunohistochemistry for smooth muscle cell marker genes, including SM22A, smooth muscle α-actin, and matrix metalloproteinases 2 and 9. In complimentary experiments human aneurysms (n = 10) and control aorta (n = 10) were harvested at the time of surgical intervention and analyzed.

Results: By 14 days, aortic diameter was larger after elastase perfusion compared with control diameter (100% $\pm$ 9.6% vs 59.5% $\pm$ 18.9%, $P = .0002$). At 7 days, elastase-perfused mice had a 78% and 85% reduction in SM22α and smooth muscle α-actin expression, respectively, compared with that seen in control animals well before aneurysms were present, and these values remained repressed at 14 days. Immunohistochemistry confirmed less SM22α and smooth muscle α-actin in experimental aneurysms at 14 days in concert with increased matrix metalloproteinase 2 and 9 expression at 7 and 14 days. Similarly, human aneurysms had less SM22α and smooth muscle α-actin and increased matrix metalloproteinase 2 and 9 staining, compared with control values, as determined by means of quantitative polymerase chain reaction.

Conclusions: Aneurysms demonstrate smooth muscle cell phenotypic modulation characterized by downregulation of smooth muscle cell marker genes and upregulation of matrix metalloproteinases. These events in experimental models occur before aneurysm formation. Targeting smooth muscle cells to a reparative phenotype might provide a novel therapy in the treatment of aortic aneurysms. (J Thorac Cardiovasc Surg 2009;138:1392-9)

The mechanisms involved in aortic aneurysm formation are not well understood. An unknown inciting event results in aortic wall injury, whereby leukocytes are recruited into the aortic wall.[1] In response to local inflammatory cytokine production, macrophages and smooth muscle cells (SMCs) release proteases, including matrix metalloproteinases (MMPs), leading to destruction of the extracellular matrix proteins collagen and elastin.[1] Late in the course of aneurysms, SMCs undergo apoptosis, thereby losing the cells primarily responsible for the synthesis of extracellular matrix proteins. The ongoing shift toward degradation of collagen and elastin is thought to be an important concept in the progression of aneurysms.[1]

It is well established that vascular SMCs have the ability to undergo profound changes in phenotype in response to changes in their extracellular environment, as occurs after vascular injury and in atherosclerosis in both preclinical animal models and human subjects.[2] These changes often include enhanced proliferation and migration, as well as marked changes in gene expression patterns, including coordinated repression of SMC marker genes and induction of MMPs in response to inflammatory mediators.[2,3] Specifically, repression of SM22α, smooth muscle (SM) α-actin, and smooth muscle myosin heavy chain in concert with increased MMP-2, MMP-3, and MMP-9 expression is seen in patients with atherosclerosis.[2] These and other alterations in SMC phenotype are thought to play a key role in the pathophysiology of multiple disorders, including atherosclerosis, restenosis, tumor metastasis, and hypertension.[2,3] The focus of much of the work related to the development of aortic aneurysms has been on the role of macrophages and leukocytes, whereas little is known of the role of SMCs.

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Abbreviations and Acronyms

CABG = coronary artery bypass grafting
KLF = Krüppel-like factor
MMP = matrix metalloproteinase
qPCR = quantitative polymerase chain reaction
SM = smooth muscle
SMC = smooth muscle cell
TGF = transforming growth factor

We hypothesized that SMCs undergo phenotypic switching early during aneurysm formation and contribute to the destruction of aortic wall matrix in response to local inflammatory mediators. Our overall hypothesis is that SMCs, by altering the phenotype to a differentiated or reparative state, might be a target to treat aortic aneurysms.

MATERIALS AND METHODS

Murine Aneurysm Model

The elastase perfusion model to create abdominal aortic aneurysms in mice has been well described.[4-6] In this well-accepted model aortas dilate by 30% to 50% initially after perfusion with elastase or saline. Elastase perfusion results in progressive aortic dilation such that an aneurysm of 100% over baseline develops by 14 days (Figure 1, *B*).[4-6] In contrast, saline-perfused aortas do not dilate following perfusion. Briefly, the infrarenal abdominal aorta is isolated in situ. Branches are ligated with 7–0 monofilament sutures. After occluding the aorta proximally and distally, an arteriotomy is made at the aortic bifurcation with a 31-gauge needle. The aorta is cannulated and perfused with porcine pancreatic elastase (0.47 U/mL) for 5 minutes at a pressure of 100 mm Hg (Figure 1, *A*). Control animals are perfused with normal saline (0.9%) for 5 minutes. The arteriotomy is repaired with interrupted 10–0 monofilament sutures, and flow is reestablished in the aorta. The abdominal contents are replaced, the abdominal wall is closed, and the mice are recovered. Video micrometric measurements of the aortic wall diameter are made in situ before perfusion, after perfusion, and at harvest. All animal protocols described were approved by the University of Virginia Animal Care and Use Committee (protocol no. 3634).

The aortas from wild-type C57B6 mice ($n = 79$) were perfused with elastase or saline (control). Saline-treated aortas were harvested at 1 ($n = 9$), 3 ($n = 9$), 7 ($n = 8$), or 14 ($n=10$) days. Similarly, elastase-treated aortas were harvested at 1 ($n = 9$), 3 ($n = 10$), 7 ($n = 12$), or 14 ($n = 12$) days. Aortas were analyzed by means of real-time quantitative polymerase chain reaction (qPCR) or immunohistochemistry.

qPCR

Aortas were harvested, flash-frozen in liquid nitrogen, and held at –80°C until extraction. RNA was extracted with Trizol (Invitrogen, Carlsbad, Calif). Briefly, for mice, RNA was extracted from frozen tissue by pulverizing in Trizol with the Pellet Pestle handheld homogenizer (Thermo Fisher Scientific, Waltham, Mass). Human tissue was crushed under liquid nitrogen with a mortar and pestle and further homogenized in Trizol by using a bead mill (Fast Prep 24; MP Biomedicals, Solon, Ohio). cDNA was synthesized from extracted RNA with the QuantiTect Reverse Transcription Kit (Qiagen, Inc, Valencia, Calif). qPCR was performed with TaqMan Gene Expression probe sets in conjunction with TaqMan Gene Expression Master Mix (Applied Biosystems, Inc, Foster City, Calif). Primers and probes used were as follows: 18s (both mouse and human) forward, CGGCTACCACATCCAAGGAA; 18s reverse, AG CTGGAATTACCGCGGC; FAM-labeled probe, TGCTGGCACCA GACTTGCCCTC. Probe sets were for murine SM22A (reference no. Mm00441660-m1), murine SM α-actin (reference no. Mm01546133-m1), and murine MMP-2 (reference no. Mm00439506-m1). Human SM α-actin (reference no. Hs00909449_m1) and human MMP-2 (reference no. Hs00234422_m1) were purchased from Applied Biosystems. Gene expression was calculated by using the relative quantification method according to the following equation: $2^{(-\Delta CT)}$, where ΔCT = (Average gene of interest) – (Average reference gene), where ribosomal 18s was used as the reference gene.

Immunohistochemistry

For immunohistochemistry, murine and human aortas were cut into 5μm paraffin sections. Antibodies for immunohistochemistry were as follows: MMP-2 (R&D Systems, Inc, Minneapolis, Minn), MMP-9 (R&D Systems), SM22α (ABCAM, Inc, Cambridge Mass), and SM α-actin (Sigma Cy3-labeled clone 1A4, Product number C6198). To assess for SMC apoptosis and proliferation, caspase-3 (Cell Signaling, Danvers, Mass) and Ki-67 (Santa Cruz, Santa Cruz, Calif) staining was performed. In brief, after antigen retrieval (Vector Laboratories, Burlingame, Calif) with heat-induced epitope retrieval, the primary antibodies were detected with the Vectastain Elite Kit (Vector Laboratories). Visualization was done with DAB (DAKO North America, Inc, Carpinteria, Calif). For murine SM α-actin, DAPI (Vectashield HardSet Mountin Medium with DAPI, catalog no. H-1500) counterstaining was used (Vector Laboratories). For human SM α-actin, Permanent Red Tables and Substrate Buffer (DAKO) were used. Counterstaining was done with Harris Hematoxylin 1 (Richard-Allen Scientific, Kalamazoo, Mich). For caspase-3, Methyl Green (DAKO) counterstaining was used. Negative controls were run with the omission of the primary antibody. Images were acquired by using the 4×, 10×, and 25× objectives on a Zeiss microscope (Zeiss, Peabody, Mass) equipped with an AxioCam digital camera (Zeiss) with the AxioCam version 4.6 software program in the University of Virginia Cardiovascular Research Center.

For histochemistry, 5μm paraffin sections were assessed for histopathology by using a modified Russell-Movat pentachrome method and hematoxylin and eosin (Laboratory Methods in Histotechnology, Armed Forces Institute of Pathology). Movat- and hematoxylin and eosin–stained slides were imaged as above at the Academic Computing Health Sciences Center at the University of Virginia.

Human Tissue Harvest

The collection of human aortic aneurysm tissue was approved by the University of Virginia institutional review board (no. 13178). Patients undergoing open surgical operations for ascending or abdominal aortic aneurysm repair or coronary artery bypass grafting (CABG) were consented. The ascending or abdominal aortic aneurysmal wall ($n = 10$ each) was resected at the time of the operation and immediately snap-frozen and later analyzed by means of qPCR and immunohistochemistry. Control tissue was only obtained from the ascending aorta during CABG procedures ($n = 10$) from the proximal aortotomy. Patients who had evidence of atherosclerotic ascending aorta, ascending aortic aneurysm, or any known history of collagen vascular disease or aneurysm were excluded from collection as control subjects.

Statistical Analysis

Comparisons were made between elastase- and saline (control)–perfused animals. Similarly, human aneurysms and control aortas were compared. Statistical analysis was performed with an unpaired, 2-tailed, Mann–Whitney test by using GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif). Data are represented as the mean (± standard error of the mean) of the subject group versus the mean (± standard error of the mean) of the control group. Data were subjected to the Grubb's test to detect any outliers (GraphPad Software).

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