Mechanical Strain Induces Specific Changes in the Synthesis and Organization of Proteoglycans by Vascular Smooth Muscle Cells*

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The vascular smooth muscle cell plays a prominent role in development and maintenance of arterial structure. Vascular smooth muscle cells are the primary source of arterial extracellular matrix (ECM), including collagens, elastic fibers, and several proteoglycans (1). Proteoglycans serve several functions in the artery wall, including regulation of cell adhesion, migration, and proliferation (2–4). The major proteoglycan in the arterial ECM synthesized by arterial smooth muscle cells (ASMC) is the large chondroitin sulfate proteoglycan versican, also known as PG-M (5–9). Versican is a member of a family of proteoglycans, including brevican, neurocan, and aggrecan that can bind hyaluronan and form large aggregates (10, 11). These large aggregates contribute to tissue mechanical properties, providing a hydrated sponge-like matrix that resists or cushions against deformation (12). Arteries also contain smaller proteoglycans that contain dermatan sulfate glycosaminoglycans such as decorin and biglycan, which interact with other ECM proteins and with macromolecules that enter the vascular wall such as low density lipoproteins (13). In addition, blood vessels contain perlecan, which is a heparan sulfate proteoglycan associated with basal lamina surrounding ASMC (2).

Vascular smooth muscle cells are under dynamic mechanical stresses from arterial pressure, and their responses to mechanical stimuli have therefore been of long-standing interest. In the past decade, improvements in bioengineering have provided much more precise and uniform methods of cell deformation (14). Using DNA microarrays and a device that provides a precise and uniform biaxial strain profile, we have shown that small mechanical deformations, well below the amplitudes that cause cell injury, induce highly specific molecular events in ASMC (15). These events include induction of several genes that may affect arterial extracellular matrix, including tenascin-C and plaminogen activator inhibitor-1. In addition, small deformations specifically suppress matrix metalloproteinase-1, an enzyme that can initiate degradation of fibrillar collagen (16).

These studies indicate that deformation regulates smooth muscle cell ECM metabolism and suggest that ASMCs may modify their biomechanical environment in a manner that limits potential biomechanical injury. Because proteoglycans are ECM molecules that can play a prominent role in tissue mechanics, this study was designed to determine whether mechanical strain induced specific changes in the synthesis of proteoglycans and altered their ability to interact with other ECM molecules.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—ASMC were prepared from explants of aorta or cardiac tissue from the donor at the time of organ harvest for orthotopic cardiac transplantation at Brigham and Women's Hospital. ASMC were maintained in Dulbecco's modified essential medium, 10% fetal calf serum and 1% penicillin/streptomycin (16) at 37 °C, 5% CO₂ up to passage 6–7 for experiments. ASMC were obtained from the Brigham and Women’s Hospital Committee for Human Research approved the protocol.

Mechanical Strain—Mechanical deformation was applied to a thin and transparent membrane on which cells were cultured, an approach that provides a nearly homogeneous biaxial strain profile. Each culture dish consists of a plastic cylinder and a circular silicone elastomeric membrane, which is the culture surface. The membrane undergoes cyclic tensile deformation as the platen assembly moves sinusoidally. We have previously measured membrane strains with a high-resolution video device (17); for this study, all experiments were performed with

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§ The abbreviations used are: ECM, extracellular matrix; ASMC, arterial smooth muscle cells; PAGE, polyacrylamide gel electrophoresis; LDL, low density lipoprotein.
4% cyclic strain, a magnitude of strain that does not lead to cell injury but reproducibly induces a restricted set of genes (15). Culture membranes were precoated with 2 μg/ml serum fibronectin in 13 ml of Hank’s solution for 24 h at 4 °C and then washed twice with 10 ml of phosphate-buffered saline. ASMC were plated on the coated membrane at a density of 6 × 10⁴ cells/dish in 13 ml of Dulbecco’s modified essential medium containing 10% fetal bovine serum and incubated for 24 h. Before mechanical strain was applied, 10 ml of fresh medium was exchanged. To eliminate the variable of time-dependent changes because of cell age or effects of adhesion to fibronectin or the membrane in each experiment, all cells were cultured on the membrane for an identical period of time, and cells and media from all samples were harvested at the same time. For example, in a time course experiment with strain, the time point represents the time prior to strain that was initiated, such that the strain sample and control sample were harvested at the same time.

**Metabolic Labeling and Proteoglycan Isolation.—**To assess proteoglycan synthesis, cells were labeled with 100 μCi per ml Na₂³⁵SO₄ for various times. The medium was combined with a 0.1 volume of 10× protease inhibitors dissolved in 8 ml urea buffer (8 mM urea, 2 mM EDTA, 0.25 mM NaCl, 50 mM Tris-HCl, and 2% Triton X-100 detergent, pH 7.4, Ref. 8). The cell layer was washed with phosphate-buffered saline and scraped into 8 ml urea buffer with 1× protease inhibitors (5 mM benzamidine, 10 mM 6-aminohexanoic acid, and 1 mM phenylmethylsulfonyl fluoride Ref. 18). Total [³⁵S]sulfate incorporation into proteoglycans was determined by CPC precipitation (19). Medium and cell layer extracts were purified and concentrated by ion exchange chromatography on DEAE-Sepharose in 8 mM urea buffer and eluted with 8 mM urea buffer containing 3 mM NaCl (20).

**Enzymatic Digestion and Electrophoresis.—**Aliquots of DEAE-purified material containing 30,000 dpm were precipitated in 80% ethanol and 1.3% potassium acetate. The resulting pellet was then applied directly to SDS-PAGE or digested by incubation prior to chromatography with 2.3 units per ml chondroitin ABC lyase (Sigma) in Tris-buffered solution (45 mM Tris, 0.09 mg/ml bovine serum albumin, 2.7 mM sodium acetate, pH 8.0, Ref. 21), or in heparitinase I and II (Sigma) in Tris-buffered solution (45 mM Tris, 0.09 mg/ml bovine serum albumin, 10 mM calcium acetate, pH 7, Ref. 22).

**Column Chromatography.—**To determine the size classes of [³⁵S]sulfate-labeled proteoglycans synthesized and secreted by the cells, medium and cell layer extracts, purified and concentrated over DEAE-Sepharose, were applied to an 8 mm × 113 cm-Sepharose CL-2B molecular sieve column in 4 mM guanidine buffer (4 mM guanidine, 0.1 mM EDTA, 0.5 M NaCl, 0.5 M sodium acetate, pH 7.4) and collected in 0.5-ml fractions (23).

**Northern Analysis.—**Total RNA was isolated using guanidine isothiocyanate solubilization followed by phenol extraction at pH 4 and subsequent precipitation in isopropyl alcohol (24). Purified samples were fractionated on 1.0% formaldehyde agarose gels, alkali denatured in 50 mM NaOH, 10 mM NaCl, and transferred to nylon blotting membranes (Zeta Probe; Bio-Rad Laboratories, Richmond, CA). Blots were hybridized with cDNA probes to the following matrix molecules: human versican, clone 7 (25); human biglycan (26), clone p16; human collagen type I, clone HF677 (27); human perlecan, clone HS-1 (28), and bovine decorin (29). These were generous gifts from Erkki Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA; Larry Fisher, National Institute of Dental Research, Bethesda, MD; Francisco Ramirez, Mt. Sinai School of Medicine, New York, NY; Renato Iozzo, Thomas Jefferson University, Philadelphia, PA, and Marian Young, National Institute of Dental Research, respectively. For quantitation of mRNA levels autoradiograms were scanned, analyzed using NIH Image software, and normalized to the amount of 28 S RNA as revealed by ethidium bromide staining.

**Western Analysis.—**Cell layers were harvested as for proteoglycan analysis into 8 ml urea buffer with protease inhibitors, concentrated over DEAE-Sepharose, and precipitated with ethanol. They were then digested with chondroitin ABC lyase and heparitinase I and II, and applied to SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA) for detection of biglycan and decorin. Versican and perlecan-containing gels were transferred using a Bio-Rad Trans-Blot Semi-Dry Transfer Cell. The transferred proteins were then detected with a series of primary antibodies and enhanced chemiluminescence (Western-Light Chemiluminescent Detection System with CSPD substrate; Tropix, Bedford, MA). Antibodies specific for biglycan (LF-51) and decorin (LF-136) (30) were a gift from Larry Fisher, Bone Research Branch, NIDR, National Institutes of Health, Bethesda, MD. Antibodies to versican (31) and perlecan (R14, Ref. 32) were from, respectively, Richard LeBaron, The University of Texas at San Antonio; and Gerardo Castillo, University of Washington, Seattle, WA. Antibodies to TSG6 were the generous gift of H-G Wiesniewski of New York University (33).

**Proteoglycan Aggregation.—**Medium containing [³⁵S]sulfate-labeled proteoglycans from cells exposed to 4% strain for 48 h, or controls, was changed into an associative buffer (500 mM sodium acetate, 0.25% CHAPS detergent, pH 5.6) by passage through a 0.5 × 12 cm size exclusion column of Sephacry G50 (Amersham Pharmacia Biotech). The void peak was taken to eliminate free [³⁵S]sulfate, split into two aliquots and applied to a 0.6 × 50 cm Sepharose CL-2B (Amersham Pharmacia Biotech) molecular sieve column in the same associative buffer. Prior to chromatography, one of the two aliquots was digested with 2 units of Streptomyces hyaluronidase (Sigma) at 37 °C for 24 h. Under associative conditions, proteoglycans containing aggregates will elute in the void volume. Digestion of components of the aggregate, for example by hyaluronidase, will shift the elution position of the proteoglycans to a later fraction (34, 35).

**RESULTS**

**Mechanical Strain Selectively Increases Proteoglycan mRNA Expression.—**Human ASMC were exposed to 4% strain for 0, 12, 24, 36, or 48 h prior to harvest of all samples at the same time, with no media changes in the 48-h period. Northern analysis revealed a time-dependent increase in mRNA levels for versican, biglycan, perlecan, and type I collagen (Fig. 1). The greatest increase occurred in versican mRNA levels, reaching 3.2-fold at 48 h. Levels of versican and biglycan mRNA were greatest at 48 h whereas those of perlecan were greatest at 24 h, indicating that the synthesis of different proteoglycans may be differentially regulated by strain. In contrast, decorin mRNA dropped rapidly by 12 h after the application of strain and remained low throughout the experiment. In additional zero strain controls, we observed no significant changes in gene expression during the 48 h of culture.

**Strain Increases the Accumulation of [³⁵S]Sulfate-labeled Proteoglycans.—**To investigate the effect of strain on proteoglycan synthesis, ASMC were metabolically labeled with [³⁵S]sulfate for the last 12 h of 12, 24, 36, or 48 h of strain. A modest increase in total incorporation of [³⁵S]sulfate into proteoglycans, as determined by CPC precipitation, peaked at 24–36 h (47% increase, p < 0.005; Fig. 2A). The increase in proteoglycans was apparent in both the cell layer and the medium.
increased the accumulation of total [35S]sulfate-labeled proteoglycans, fate into proteoglycans. Equal aliquots were assayed per dish. Strain harvested for determination of total incorporation of radiolabeled sul-
were treated identically except for the absence of strain. They were then
 elongation of GAG chains as has been observed for the synthe-
crease in radiolabeled chondroitin/dermatan sulfate synthesis followed by CPC precipitation (Fig. 2
droitin ABC lyase digestion of the cell and medium fractions,
A, ASMC. The chondroitin/dermatan, and heparan sulfate portions of total radiolabeled proteoglycans were determined by chon-
droitin ABC lyase digestion of the cell and medium fractions, followed by CPC precipitation (Fig. 2B). The maximum in-
crease in radiolabeled chondroitin/dermatan sulfate synthesis was in the medium at 36–48 h (62% increase, p < 0.05), whereas that of the heparan sulfate proteoglycans in the me-
dium was at 12–24 h (87% increase, p < 0.005), consistent with the early increase in perlecan mRNA levels (Fig. 1). In the cell
layer, heparan sulfate incorporation reached a maximum at 36–48 h (58% increase, p < 0.05). Because the heparan sulfate
component of the medium was approximately twice that of the cell layer, the total of cell and medium heparan sulfate peaked at 12–24 h.

Hydrodynamic Size of Proteoglycans—To determine whether strain altered the relative quantities or average size of the different classes of proteoglycan synthesized by ASMC, radiolabeled proteoglycans collected after 24–36 h were isolated by ion affinity exchange and subjected to molecular sieve chromatography on Sepharose CL-2B. Two major peaks eluted at \( K_v \approx -0.28 \) and \(-0.67 \) in the medium and 0.42 and 0.78 in the cell layer (Fig. 3). The major component of the large peak is versican and the smaller peak is a mixture of biglycan and decorin (8). No major differences were seen in the elution positions of these radiolabeled peaks between control or strain cultures. The relative amount of radiolabeled material secreted into the medium was \(-80\% \) greater in the larger, versican-containing peak, indicating increased synthesis in response to strain. This is consistent with increased levels of versican mRNA (Fig. 1).

The absence of a change in hydrodynamic size in the versican peak suggests that the increased accumulation of [35S]sulfate-labeled material is a result of increased synthesis of the proteoglycan core proteins and their GAG chains rather than elongation of GAG chains as has been observed for the synthe-

siss of these proteoglycans by ASMC when stimulated by cyto-
kines (8). A small shift in the \( K_v \) of the second peak in the medium to \(-0.63 \), indicating a greater hydrodynamic size, was probably because of an increase in the ratio of biglycan to decorin in that peak, because biglycan is a larger macromolecule (8). This is supported by the increase in mRNA and protein expression of biglycan by strain (Figs. 1 and 4) and the decrease in mRNA and protein for decorin (Figs. 1 and 4).

Strain Reduces the Accumulation of [35S]Sulfate-labeled Decorin—Proteoglycans from cells exposed to strain for 24–36 h were applied to 4–12% gradient SDS-PAGE. Decorin and biglycan run as separate bands in the resolving gel whereas versican remains in the stacking gel (8). No change in the relative amount of biglycan could be detected by this method. On the other hand, the relative intensity of the decorin band in the medium was greatly decreased, to \(-30\% \) of the control level (Fig. 4A). This is consistent with reduced production of decorin mRNA by cells exposed to strain (see Fig. 1).

Synthesis of Proteoglycan Core Proteins by ASMC Is Altered by Strain—Western analysis was performed on medium and cell layer extracts from ASMC exposed to strain for 24–36 h (Fig. 4B). Increases were observed in the levels of versican, biglycan, and perlecan. Versican core protein was increased to 126% of control levels in both medium and cell layer. Perlecan was increased to 144 and 413% of control levels in the medium and cell layers, respectively. The percent increase in biglycan levels could not be determined by scanning, because detectable levels were only found in medium after application of strain. In contrast, the level of decorin core protein was reduced to 13 and 89% of control levels in medium and cell layer, respectively.

Strain Increases Expression of TSG6—In DNA microarray experiments with the Affymetrix HU6800 microarray that were ongoing at the same time as these experiments, we found that the gene for the hyaluronan-binding protein TSG6 was among a small set of genes increased by 4% strain in ASMC. Northern analysis confirmed that mRNA for TSG6 was reproducibly increased by strain (Fig. 5). Both TNF-\( \alpha \) (10 ng/ml) and strain led to modest increases in a single band (\(-90 \) kDa) recognized by anti-TSG6 antibody in a Western analysis under reducing conditions (data not shown). This corresponds to a size that is larger than the deduced molecular mass of TSG6.
These findings demonstrate that mechanical strain induces a 1.0-fold increase in TSG6 mRNA (24 h).

**Fig. 5.** TSG6 is increased by strain in ASMC. A, SDS-PAGE reveals reduced accumulation of the proteoglycan core proteins versican, biglycan, decorin, and perlecan. B, confluent cells were subjected to strain for 36 h or kept as controls in identical conditions lacking strain. Medium and cell layer were then harvested and subjected to Western analysis with antibodies against versican, biglycan, decorin, and perlecan. Equal fractions of the total proteoglycans per dish were loaded for comparison between strain and control treatments. Different quantities were used for cell layer and medium. Accumulation of the core proteins of these proteoglycans was increased in response to strain in all cases except decorin, which declined.

**Fig. 6.** Strain increases the aggregation of proteoglycans to hyaluronan. Medium containing [35S]sulfate-labeled proteoglycans from cells exposed to strain for 48 h, or controls, was subjected to chromatography with Sephadex G50. Prior to chromatography, one of two aliquots was digested with 2 units of Streptomyces hyaluronidase (Sigma) at 37 °C for 24 h. Left panel, disintegrations per min per fraction (DPM) in medium from control cells without hyaluronidase (C) and with hyaluronidase (S). The presence of a void volume peak in the strain cells that is eliminated by hyaluronidase indicates that strain causes aggregation with hyaluronan.

**FIG. 5.** Accumulation of proteoglycan core proteins is altered by strain in ASMC. A, SDS-PAGE reveals reduced accumulation of the proteoglycan core proteins versican, biglycan, decorin, and perlecan. [35S]sulfate-labeled proteoglycans, prepared as for Fig. 3 from control and strain-treated ASMC, were applied to 4–12% gradient PAGE with a 3.5% stacking gel. B, confluent cells were subjected to strain for 36 h or kept as controls in identical conditions lacking strain. Medium and cell layer were then harvested and subjected to Western analysis with antibodies against versican, biglycan, decorin, and perlecan. Equal fractions of the total proteoglycans per dish were loaded for comparison between strain and control treatments. Different quantities were used for cell layer and medium. Accumulation of the core proteins of these proteoglycans was increased in response to strain in all cases except decorin, which declined.

**DISCUSSION**

In this study, we found that small, highly controlled biomechanical deformation induces the synthesis of specific vascular proteoglycans including versican, biglycan, and perlecan, whereas decorin expression was suppressed. We also found increased expression of the hyaluronan-binding protein TSG6. Furthermore, mechanical strain increased proteoglycan aggregation, suggesting that the cellular response to deformation is highly coordinated. Because DNA microarray experiments demonstrate that relatively few genes are altered by mechanical strain in these cells (15), these experiments highlight the restricted and specific molecular regulation of ASMC by biomechanical stimuli.

Vascular smooth muscle cells perform a sentinel role in arterial mechanics. Under disturbed conditions such as hypertension, the vascular smooth muscle cell must sense the changes and respond appropriately. These responses include changes in the extracellular matrix, and prior studies have demonstrated that mechanical strain increases collagen and proteoglycan synthesis by these cells (36–38). However, the molecular specificity of the proteoglycan increase has not been characterized previously. Because different proteoglycans can have markedly varied biological roles, we anticipated that not all vascular proteoglycans would be regulated similarly. These experiments demonstrate that decorin is specifically decreased by strain. Because decorin binds to collagen (39) and regulates collagen cross-linking (40), the decrease in decorin could promote disorganization of collagen and a loosening of ECM.

One of the key potential defenses of cells against excess deformation is secretion of proteoglycans. Proteoglycans, through negatively charged glycosaminoglycan chains, can serve as molecular sponges that cushion against mechanical forces. The increase of versican-hyaluronan complexes shown in this study may provide a matrix environment that thickens intima and protects the cells against mechanical stimuli. This consistent with the response of chondrocytes to deformation; chondrocytes increase aggregation of the large proteoglycan aggrecan to hyaluronan following deformation, which provides a highly hydrated mechanical environment around the cell (41).

The mechanisms by which strain differentially regulates vascular proteoglycan synthesis are unclear. Our studies indicate that although remarkably few genes are induced by biomechanical stimulation of vascular smooth muscle cells, these responses are reproducible in different cell sources (15). Mechanical signals activate different signal transduction pathways, but currently no single transcriptional regulatory pathway.
element or combination of elements can explain cell-specific mechanical responses.

Among the relatively restricted subset of genes induced by the small biomechanical stimulus of 4% biaxial strain is TSG6. This hyaluronan-linking protein is primarily known as a pro-inflammatory response gene that is induced by cytokines, but it is intensely overexpressed in mechanically injured arteries (42). The impact of mechanical strain on hyaluronan synthesis will need to be explored to address the hypothesis that increased aggregation may be because of increased hyaluronan synthesis.

In some circumstances, excess accumulation of vascular proteoglycans may be detrimental. Williams and Tabas proposed that retention of atherogenic lipoproteins in the artery is a central process in atherogenesis, a hypothesis known as the Response-to-Retention hypothesis (43). Proteoglycans may serve as the reservoir for arterial lipoproteins through a specific association with the apo-B100 protein; a single point mutation in the apo-B100 protein inhibits binding to proteoglycans without affecting low density lipoprotein (LDL) receptor binding (44). Immunohistochemical studies have shown that biglycan colocalizes with apo-B in the atherosclerotic intima, and both versican and biglycan can bind LDL (45). Thus, whereas accumulation of proteoglycans may contribute to mechanical properties of the artery, it may also provide a subendothelial retention reservoir for lipoproteins. In this study, we demonstrate that biomechanical strain selectively up-regulates expression of biglycan and versican, two vascular proteoglycans that can serve as LDL binding reservoirs. Thus, enhanced proteoglycan synthesis may serves as an initial defense against mechanical stress that can nonetheless prove deleterious as LDL infiltrates the vessel wall.

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