Elevated cyclic stretch alters matrix remodeling in aortic valve cusps: implications for degenerative aortic valve disease

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Elevated cyclic stretch alters matrix remodeling in aortic valve cusps: implications for degenerative aortic valve disease. Am J Physiol Heart Circ Physiol 296: H756–H764, 2009. First published January 16, 2009; doi:10.1152/ajpheart.00900.2008.—Matrix metalloproteinases (MMPs) and cathepsins are proteolytic enzymes that are upregulated in diseased aortic valve cusps. The objective of this study was to investigate whether elevated cyclic stretch causes an increased expression and activity of these proteolytic enzymes in the valve cusp. Circumferentially oriented fresh porcine aortic valve cusp sections were stretched to 10% (physiological), 15% (pathological), and 20% (hyperpathological) in a tensile stretch bioreactor for 24 and 48 h. The expression and activity of MMP-1, MMP-2, MMP-9, tissue inhibitor of MMP-1, and cathepsin L, S, and K were quantified and compared with fresh controls. Cell proliferation and apoptosis were also analyzed. As a result, at 10% physiological stretch, the expression and activity of remodeling enzymes were comparable with fresh controls. At 15% stretch, the expression of MMP-1, -2, -9 and cathepsin S and K were upregulated, whereas the expression of cathepsin L was downregulated compared with controls. A similar trend was observed at 20% stretch, but the magnitudes of upregulation and downregulation of the expression were less than those observed at 15%. In addition, there were significantly higher cell proliferation and apoptosis at 20% stretch compared with those of other treatment groups. In conclusion, elevated mechanical stretch on aortic valve cusps may detrimentally alter the proteolytic enzyme expression and activity in valve cells. This may trigger a cascade of events leading to an accelerated valve degeneration and disease progression.

cathepsin; matrix metalloproteinase

THE NATIVE AORTIC VALVE functions in an environment where it is exposed to a myriad of hemodynamic forces such as pulsatile pressure, steady and oscillatory shear stresses, as well as cyclic stretch (29). Altered or nonphysiological hemodynamics are often implicated in the initiation and progression of aortic valve disease (22), leading to a compromised valve structure and function (16). Several studies have demonstrated the effects of pressure and shear stresses on the biosynthetic activity and disease progression of aortic valve cusps and cells (6, 16). However, the response of aortic valve tissue or cells to cyclic stretch has not been very extensively studied. Cyclic stretch is one of the forces experienced by the aortic valve during the cardiac cycle that allows the valve cusps to extend and form a coaptive seal with each other during diastole (33, 36, 37). The valve, under normal physiological conditions, experiences ~10% stretch during diastole (17, 32). Preliminary studies in our laboratory (unpublished), using an in vitro flow loop and a native porcine aortic valve, have also shown that for every 40 mmHg increase in pressure, there is a 5% increase in cyclic stretch.

Cyclic stretch has also been shown to modulate valve biosynthetic activity. Ku et al. (14) reported that cyclic stretch upregulated collagen synthesis in valve interstitial cells as well mesenchymal stem cells, which was also recently demonstrated by us on whole valve cusps (2). Merryman et al. (19) reported that valve interstitial cells respond to local tissue stresses by altering cellular stiffness via collagen biosynthesis. They also reported that the presence of cytokines, such as transforming growth factor-β (TGF-β), in a cyclic stretch environment could potentially result in an altered matrix architecture and compromised valve function, underlining the importance of cyclic stretch in regulating valve structure and function (18).

Aortic stenosis and regurgitation, which are clinical manifestations of aortic valve disease, have been correlated in several patients with an overexpression of proteolytic enzymes such as matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs), and cathepsins (9). During normal homeostasis in the aortic valve, there is a balance between extracellular matrix biosynthesis and degradation maintained by these enzymes (5). A perturbation of this delicate equilibrium can lead to a pathological remodeling of the tissue matrix and a compromised valve function (9). A number of these cathepsins, MMPs and TIMPs, are also involved in key cellular processes such as apoptosis, proliferation, and cell differentiation and have demonstrated roles in valve disease pathways (7). Cathepsins K, L, and S, which are potent elastolytic proteases, have been associated with atherosclerotic plaque progression (31) and myxomatous heart valves (26).

While the link between an altered expression of extracellular matrix remodeling enzymes and valve disease progression has been well established, there is no study linking an altered cyclic stretch to an altered expression of MMPs and cathepsins. Elucidating this would strengthen the link between an altered cyclic stretch and degenerative valve disease. The studies alluded to earlier, therefore, led us to hypothesize that cyclic stretch is a key regulator of aortic valve remodeling and that an elevated cyclic stretch results in an increased expression of MMPs and cathepsins while decreasing TIMPs. The approach of this study was to investigate the acute effects of cyclic stretch in regulating MMP and cathepsin expression, MMP activity, and TIMP inhibitory activity in the aortic valve. Our...
results show that cyclic stretch is a regulator of key matrix remodeling enzymes.

MATERIALS AND METHODS

Porcine aortic valve culture under cyclic stretch. Fresh porcine aortic valves were obtained from a local abattoir (Holifield Farms, Covington, GA) following an on-site dissection of the hearts within 10 min of slaughter. The pigs were between the ages of 12–24 mo. Dulbecco’s phosphate-buffered saline (PBS; Sigma, St. Louis, MO). Upon arrival to the laboratory, a rectangular section of tissue with an aspect ratio of 15 × 10 mm was isolated from the central region of each valve cusp, proximal to the line of coaptation. Circumferentially oriented tissue sections were extracted to be studied (Fig. 1A). These samples were then randomized and assigned to one of five treatment groups. The sections were either allocated as fresh or static controls or cyclically stretched in a tensile stretch bioreactor to three levels of strain magnitudes: 10% (physiological, see Ref. 34), 15% (pathological/hypertensive), and 20% (hyperpathological/severely hypertensive) at 1.167 Hz, which corresponds to 70 beats/min (2). Fresh cusp sections were extracted from the same location in separate aortic valves. Statically incubated cusp sections were prepared as stretched cusps but were not stretched for the experimental duration. We ensured that each cusp sample originated from a different animal and that there was a random mix of left, right, and noncoronary cusps for each treatment group. The waveforms imposed on the cusp are depicted in Fig. 1B. Each experiment was run for either 24 or 48 h, with 12–15 cusp sections for each experimental run. These time points were chosen based on previous studies as necessary and sufficient to observe acute responses in valve cusps (2, 13, 16, 20, 38). All samples were stretched in normal Dulbecco’s modified Eagle’s medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (Fisher-Scientific), 50 mg/l ascorbic acid, 3.7 g/l sodium bicarbonate, 1% (vol/vol) nonessential amino acid solution, and 1% (vol/vol) antimycotic-antibiotic solution (all Sigma). Upon completion of the stretch analysis, experiments were run with 5 mg/l bromodeoxyuridine in ice-cold sterile PBS and either frozen in optimum cutting temperature medium (Bio-Scientific) or flash frozen in liquid nitrogen, or dried in a vacuum desiccator in preparation for analyses.

Cell proliferation and apoptosis. Anti-bromodeoxyuridine (Sigma) immunohistochemistry (38) was used to visualize proliferating cells, whereas a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling fluorescence kit (Roche Applied Science, Indianapolis, IN) was used to identify apoptotic cells. For the cell proliferation analysis, experiments were run with 5 mg/l bromodeoxyuridine included in the DMEM for the last 24 h of the experiment. Immunopositive cells were manually counted for each valve sample (over 3 image fields and separately by 2 users) and normalized by unit surface area of the tissue in that image field. A distinction was not made between endothelial and interstitial cells during the cell counting.

Valvar collagen content. A colorimetric collagen assay (Bio-color) was used (2) to quantify the total enzyme soluble collagen content in the cusp samples under all the conditions. This assay takes into account both the synthesis of new collagen as well as any degradation of mature collagen fibers. Briefly, the cusp sections were dried for 24 h in a vacuum desicator, weighed, and digested over 24 h at room temperature in pepsin (Sigma) with frequent agitation. After centrifugation at 12,000 rpm, the supernatant was then used for the collagen assay. The collagen amount in the assay sample was then normalized by the dry weight of the tissue and reported in this study.

Immunohistochemistry analysis. After appropriate treatment, the cusps were flash frozen in optimum cutting temperature media and cut into 5-μm sections. Fluorescence immunohistochemistry was used as before (2) to identify cells positive for cathepsin L (1:50), S (1:50), and K (1:100; all Santa Cruz Biotechnology, Santa Cruz, CA). Semi-quantitative assessments of cathepsin expression were performed using the ImageJ program (National Institutes of Health, Bethesda, MD) over three image fields per sample and separately by two users. The area coverage of cathepsin immunopositive expression was measured and normalized by the total area coverage of tissue in that image field (2).

Immunoblotting analysis. Immunoblotting was used to analyze the expression of MMP-1 (1:100; Santa Cruz), MMP-2 (1:200; Calbiochem, San Diego, CA), MMP-9 (1:100; Calbiochem), TIMP-1 (1:100; Calbiochem), and β-actin (1:1,000; Sigma). Briefly, after the appropriate culture duration, the aortic valve cusp tissues were pulverized using a mortar and pestle in liquid nitrogen and homogenized in ice-cold radioimmunoprecipitation assay buffer (Santa-Cruz) and centrifuged at 15,000 rpm to pellet extracellular matrix debris. The
supernatant was assayed for protein concentration using the BCA Protein Assay (Pierce, Rockford, IL). Equal amounts (30 μg) of tissue lysates were resolved by reducing SDS-PAGE. After the transfer to a polyvinylidene difluoride membrane (Millipore), the blots were blocked with 3% BSA, probed with primary antibody, and with an appropriate biotinylated secondary antibody. The membranes were finally incubated in horseradish peroxidase-conjugated streptavidin. The immunopositive bands were then detected using a luminol-based chemiluminescence reagent (Pierce) and analyzed by densitometry using the ImageJ program.

Collagenase activity assay. A commercially available collagenase assay (Millipore, Billerica, MA) was used to quantify the activity of collagenases within the valve samples. Briefly, equal amounts of cell lysates (10 μg) and MMP-1 standards were first activated using 4-aminophenylmercuric acetate at 35°C for 60 min. All noncollagenolytic activity is inhibited. 200 μl of 1 × FITC-collagen is added to each sample, and the samples and standards are incubated at 35°C for 60 min. After the enhancement and extraction of the collagenase degradation products using reagents supplied in the kit, the fluorescence intensity was determined at fluorescence emission (λex ) of 520 nm and fluorescence excitation (λem ) of 490 nm. The data were normalized by individual protein concentrations. Each sample was analyzed in triplicate.

Gelatin zymography analysis. Gelatin zymography was used to quantify the activities of gelatinases within the valve samples. Equal amounts (5 μg) of tissue lysate were resolved at 4°C by 10% nonreducing SDS-PAGE containing 1 mg/ml gelatin. After electrophoresis, the gels were cleared of SDS by incubating for 30 min with two changes of 2.5% (vol/vol) Triton X-100 (Sigma). Gels were then incubated in substrate buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 10 mM CaCl2, and 0.05% Brij 35 at 37°C for 8–10 h. The gels were then rinsed with deionized water and stained with 0.5% Coomassie brilliant blue and destained. Proteolytic activity was observed as clear bands against a blue background and were analyzed using densitometry. Each zymography experiment was performed in duplicate.

Reverse zymography analysis. Gelatin reverse zymography was used to analyze the activity of gelatinase inhibitors. Equal amounts (50 μg) of tissue lysate were resolved at 4°C by 12.5% nonreducing SDS-polyacrylamide gels containing 1 mg/ml gelatin and 150 ng/ml MMP-2/MMP-9 (Millipore). After electrophoresis, the gels were cleared of SDS by incubating for 30 min with two changes of 2.5% (vol/vol) Triton X-100 (Sigma). The gels were then incubated in substrate buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 10 mM CaCl2, and 0.05% Brij 35 at 37°C for 24 h. The gels were then rinsed with deionized water and stained with 0.5% Coomassie brilliant blue and destained. Inhibitory activity was observed as dark blue bands against a lighter blue background, and these bands were then analyzed using densitometry. Each reverse zymography experiment was performed in duplicate.

Statistical analysis. All quantitative data were expressed as means ± SE. The sample size was at least n = 8 (from different animals) for all analyses. The data were first analyzed using ANOVA to see whether there was a significant contribution by stretch level and culture duration on the data measured. This was followed by post hoc pairwise comparisons with the Tukey test. A P value of <0.05 was used as a measure of statistical significance. All statistical analyses were performed using Minitab (Minitab R14, Minitab State College).

RESULTS

Cell proliferation and apoptosis increased in a cyclic-stretch magnitude-dependent manner. We first determined whether cyclic stretching regulated cell proliferation and apoptosis of aortic valve cusps. The number of proliferating cells in the valve samples was dependent on the magnitude and duration of applied stretch (Fig. 2A). At 10% stretch, there were low numbers of proliferating cells even after 48 h of culture. At 15%, proliferating cells were numerous and significantly (P < 0.05) higher in number than 10% after 48 h of culture but not after 24 h. In contrast, significantly (P < 0.05) large numbers of proliferating cells were observed at 20% after just 24 h, compared with 10% stretch for the same time point. Statically incubated samples did not have a significant difference in number of proliferating cells compared with 10% 24 h stretch. Cell proliferation, where observed, was evenly distributed throughout the tissue and not localized to any surface.

Cell apoptosis (Fig. 2B) was increased by cyclic stretch in a magnitude-dependent manner. The observed response was strongly exponential (R2 = 0.997, P < 0.05) with initially no significant difference (P > 0.05) in immunopositive apoptotic cell number from fresh to 10% stretch and rapidly increasing in number beyond 15%. The number of apoptotic cells was significant (P < 0.05) at both 15% and 20% levels of stretch. Additionally, the number of apoptotic cells in the static controls was not significant compared with 20% stretch samples and significant (P < 0.05) compared with fresh controls, 10% stretch, and 15% stretch.

Cyclic stretch increased collagen content in a magnitude-dependent manner. We next studied the effect of cyclic stretch on the collagen content of aortic valve cusps. Collagen content (Fig. 3) was significantly increased at 15% (29.2% increase; P < 0.05) and 20% (23.8% increase; P < 0.05) cyclic stretch compared with that of fresh control. At 10% stretch, collagen content was significantly decreased compared with that of fresh controls (P < 0.05). There was no significant difference (P > 0.05) in collagen content between 15% and 20% stretch.

Cathepsin S and K expressions were upregulated, whereas cathepsin L was downregulated by 15% cyclic stretch. To determine the mechanisms by which cyclic stretch regulates collagen content of the cusps, cathepsin L, S, and K expressions were studied. Immunohistochemical staining revealed that cathepsin S and K expressions were upregulated by 15% cyclic stretch, whereas cathepsin L expression was downregulated when compared with fresh controls (Fig. 4A). The expression of cathepsin S was mainly localized to the fibrosa of the valve cusp, whereas cathepsin K expression was broadly distributed throughout the entire cusp. Cathepsin L distribution in the fresh valve cusp depicted a broad distribution throughout the cusp. Up until the 24-h time point, there was no difference in cathepsin L expression compared with fresh control, but at 48 h, no cathepsin L expression was observed.

The semiquantification of the 48-h samples (Fig. 4B) revealed a significant decrease (P < 0.05) of all cathepsins at 20% stretch, compared with 15%. Cathepsin L and K expressions were comparable between fresh controls and 10% stretch, whereas cathepsin S expression was significantly reduced (P < 0.05) at 10% stretch compared with fresh controls.

MMP-1, -2, and -9 expression was increased by stretch, whereas TIMP-1 expression was reduced. Western blot analyses were carried out to investigate whether the changes in MMP and TIMP activities induced by cyclic stretching was due to alterations in their expression levels. The blots (Fig. 5A) clearly demonstrated that MMP expression was increased by cyclic stretch compared with those of controls. The greatest expression of MMP-1, -2, and -9 was observed at 15% stretch, compared with that of fresh controls. Conversely, for TIMP-1...
expression, cyclic stretch resulted in a reduced expression compared with that of fresh controls.

TIMP-1 expression was strongly, negatively correlated with MMP-1 expression (Fig. 5B; \( R^2 = 0.755, P < 0.05 \)). MMP-1 expression was normalized by the corresponding TIMP-1 expression for each sample to obtain a dimensionless parameter representing the overall matrix remodeling potential of the valve cusp in response to stretch. Plotting this MMP-1-to-TIMP-1 ratio against stretch level (Fig. 5C) revealed that the remodeling potential peaked at 15% stretch (at all time points) and was significantly different \((P < 0.05)\) compared with 10% and 20% stretch and compared with unstretched valves. **MMP activity was increased and TIMP activity reduced by 15% and 20% cyclic stretch.** We next studied whether cyclic stretch regulated MMP and TIMP activity. Gelatin zymography (Fig. 6A) and reverse zymography (Fig. 6B) revealed the modulation of MMP and TIMP activity in a time- and magnitude-dependent manner. After 24 h, there was no significant change \((P < 0.05)\) in MMP activities for the 15% and 20% stretch groups compared with fresh controls. After 48 h, MMP activity was increased significantly \((P < 0.05)\) at 15% (62% increase) and 20% cyclic stretch (62% increase) compared with that of fresh controls. The active form of MMP-2 was observed only in the static 15% and 20% stretch groups; MMP-9 activity was observed in the 15% stretch group only. TIMP activity was reduced significantly \((P < 0.05)\) by all levels of cyclic stretch when compared with that of fresh controls. When comparing within-stretched valve cusps, a 10% stretch resulted in the lowest levels of TIMP inhibitory activity. Inhibitory activity was significantly higher at 15% and 20% stretch \((P < 0.05)\) compared with 10% stretch. Collagenase activity (Fig. 6C) was increased significantly \((P < 0.05)\) compared with that of fresh controls after 15% (4.21-fold increase) and 20% (4.02-fold increase) stretch. There was no statistical difference between fresh controls, static controls, and samples stretched to 10% or between 15% and 20% stretch. It was also observed in this study that enzyme activity correlated \((R^2 = 0.597, P < 0.05)\) with enzyme expression (Fig. 6D).

**DISCUSSION**

The objectives of this study were to understand the acute effects of different levels of cyclic stretch on the activity and expression of matrix remodeling enzymes in porcine aortic valve cusps. The key findings of the study were as follows: 1) normal cyclic stretch (10%) maintained the native levels of matrix remodeling activity; 2) cellular proliferation and apoptosis increased as cyclic stretch was increased from normal to

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**Fig. 2.** A: cell proliferation was increased by cyclic stretch in a magnitude-dependent manner. Proliferation as measured by bromodeoxyuridine (BrdU) staining (arrows) is highest at 20% stretch after 48 h, showing numerous proliferating cells across the thickness of the cusp. V, ventricularis; n, number of valve cusps. B: cell apoptosis was increased by cyclic stretch in a magnitude-dependent manner. Apoptotic cell number as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (arrows) was significantly higher at 20% (70 immunopositive cells/unit area of tissue) compared with 10% and 15% stretch (10 and 27 immunopositive cells/unit area of tissue respectively) after 48 h. These immunopositive cells at 20% were distributed throughout the thickness of the tissue.
pathological levels; 2) cathepsin L expression was reduced by elevated cyclic stretch, whereas cathepsin S and K expression was increased; and 4) elevated cyclic stretch (15% and 20%) results in acutely increased collagenase and gelatinase activity compared with controls. Our results therefore demonstrate the importance of cyclic stretch in regulating extracellular matrix remodeling. These findings suggest an important role for elevated cyclic stretch in the progression of degenerative aortic valve disease.

Understanding the mechanobiology of proteolytic enzymes such as MMPs and cathepsins is clinically important. Matrix proteolytic enzymes are expressed both early in valve disease progression and are also highly overexpressed in explanted diseased valves (12). Degenerative aortic valve disease involves a progressive degradation of valve mechanical properties and also changes in the hemodynamic environment in the valve (9, 21). Progressive changes in stiffness of the valve can result in changes in the strain environment that might result in a feedback loop of accelerated valve degeneration. It is also acknowledged that hypertension, a risk factor for valve disease (27), can increase the strain environment on the valve cusps (28). The effect of elevated levels of cyclic stretch observed in this study can therefore have important implications for hypertension-related valve degeneration.

Normal 10% stretch was able to maintain the expression and activity of the remodeling enzymes to native (fresh) levels, whereas elevated levels of cyclic stretch resulted in elevated levels of expression of matrix remodeling enzymes. The altered levels of some of these enzymatic proteins suggest an acute disruption in the overall equilibrium between matrix synthesis and degradation under elevated levels of cyclic stretch. Interestingly, there was also a significant upregulation of collagen content in cusps cultured to 15% and 20% stretch, suggesting that there was a net synthesis of collagen under these experimental conditions. These findings underline the ability of the aortic valve cusp to continuously adapt and remodel its load-bearing components to function adequately in a changed mechanical environment. This observation is in agreement with the stress overload theory proposed by Robicsek and Thubrikar (28). Additional studies are required to identify which specific collagen isoforms were synthesized in response to cyclic stretch.

Our study demonstrated increases in both cell proliferation and apoptosis at 15% and 20% stretch, suggesting a disruption in normal homeostasis of the valve as the stretch on the valve increases. Physiologically, the valve experiences 10–12% stretch in the circumferential direction (33). Beyond 15–17%, the collagen fibers are fully stretched and “locked up” (17, 37). Therefore, at 20%, there is a possibility of damage to collagen fibers and the attached interstitial cells (3, 4). We speculate that

Fig. 3. Cyclic stretch increased collagen in aortic valve cusps. Collagen content was significantly reduced \( (p < 0.05) \) at 10% stretch but significantly increased \( (p < 0.05) \) at 15% and 20% stretch after 48 h compared with fresh controls \( (n = \text{number of valve cusps}) \). amt, Amount.

Fig. 4. A: effect of cyclic stretch on cathepsins L, S and K. There was a clear stretch magnitude dependence in the regulation of cathepsins. Among the three cathepsins analyzed, cathepsin L was the dominant cathepsin in the fresh valve, whereas 15% stretch significantly increased expression of cathepsins S and K. F, fibrosa; \( n \), number of valve cusps. B: effect of stretch magnitude on cathepsin expression.
the high levels of proliferation and apoptosis at 20% is an injury-response mechanism to counter the higher mechanical strains imposed on the valve, leading to increased cell apoptosis and compensatory proliferation to replace damaged cells. Additionally, cellular apoptosis is also known to be a hallmark of degenerative aortic valve disease (11). The above speculation is also consistent with the generally reduced level of remodeling observed at 20%, as the priority of the cell is thought to be proliferation. In vivo, the pressure load (and therefore the stretch) on the aortic valve cusp is not likely to increase in such an acute manner, and we can expect a constant adaptive remodeling of the valve to remain functional. However, hypertensive emergencies as a result of the failure of normal autoregulation and an abrupt rise in systemic vascular resistance can occur (1), which may result in a situation of an acute increase in stretch on the valve.

We showed by immunohistochemistry that cathepsin S and K expression was upregulated by high levels of stretch. High levels of cathepsin L expression, which was downregulated by stretch, were observed in fresh valve cusps. Cathepsin L may potentially be a key regulator of normal matrix turnover in the native valve, and if nonphysiological (15% and 20% stretch) stimuli are applied, a disease-remodeling pathway may be activated, resulting in other cathepsins (S and K observed in this study) to be preferentially expressed. Indeed, in a recent study by Helske et al. (10), it was revealed that cathepsin S, K, and V expression and activity were the cathepsin subtypes that were upregulated in stenotic aortic valves. The associated
...increase in collagen content at 15% and 20% stretch led us to further speculate that cathepsin L is a key enzyme involved in normal collagen turnover in valve cusps, wherein its down-regulation under 15% and 20% stretch resulted in increased collagen content. Previous studies in our laboratory showed that cathepsin L was downregulated by altered levels of cyclic pressure (25) and laminar shear stress (23, 24), further lending strength to this speculation.

Our study also showed that high stretch resulted in the increased expression of MMP and the reduced expression of the TIMPs. It is known that cytokines such as tumor necrosis factor-α can contribute to the increased expression of MMPs and cathepsins in diseased aortic valves (12). Although we did not specifically study inflammatory cytokines in this study, the overexpression of MMPs that we observed in response to 15% and 20% stretch could be speculated to be concurrent with cytokine expression. This hypothesis is further strengthened by the increased proliferation and apoptosis that we observed at these levels of stretch. In addition, we presented a parameter that can be used to represent this altered remodeling by normalizing proteolytic and inhibitory expressions (MMP-to-TIMP ratio). We acknowledge that this is an idealized metric to assess the tissue remodeling potential of the aortic valve under cyclic stretch, as there are numerous other MMPs and cathepsins involved in its remodeling in vivo. In addition, proteolytic activity, which is a subset of total proteolytic expression, is likely to be more relevant for the actual degradation of the valve, though we did observe a good correlation between enzyme expression and activity ($R^2 = 0.597$, $P < 0.05$). The value of stretch (15%) where the highest remodeling was observed is also in good agreement with a similar study on valve interstitial cells reported by Ku et al. (14).

The stretch bioreactor used in this study has been designed and validated in previous work (2, 8). It can be used to provide
a uniform stretch environment to the valve cusps. Stainless steel springs were used in this study to couple the aortic valve samples to the stationary and moving posts of the tensile stretch bioreactor. This method has several advantages over other clamping methods. First, the springs were threaded through the tissue at least five to six times, and this allows for a more uniform force distribution across the width of the tissue. There was no incidence of the tissue being ripped through the springs due to the cyclic motion of the linear actuator. Second, due to the stiffness of the springs, the off-axis deformation (radial direction in this case) can be assumed to be negligible. Indeed, such a strip biaxial (30) deformation is more advantageous than a uniaxial deformation for mechanobiological studies where any observed biological responses may be confounded due to the influence from the off-axis component.

Finally, while our study is limited to 48 h, our results support the claim that under nonphysiological loading, the valve interstitial cell transforms from a quiescent phenotype to an activated one. If other exogenous factors favorable to valve disease progression were present, accelerated degeneration and even progression were likely. Merryman et al. (18) demonstrated that the interstitial cells in aortic valve cusps stretched to 15% in the presence of the cytokine TGF-β1 transform to an activated phenotype (18). Liu and Gotlieb (15) demonstrated that TGF-β signaling regulates response to injury of wounded valve cells.

In conclusion, we have shown that elevated levels of cyclic stretch result in a highly activated cell phenotype resulting in an altered cell turnover and a MMP and cathepsin expression setting the stage for valve degeneration. However, the downstream signaling pathways require further elucidation.

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