

Differential Immediate-Early Gene Responses to Elevated Pressure in Porcine Aortic Valve Interstitial Cells

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Background and aim of the study: Cardiovascular risk factors are believed to play a role in the pathogenesis of aortic valve disease. In the present study the hypothesis was proposed that elevated pressure would cause a change in the expression of prototypical pro-inflammatory genes. Hence, the expression of MCP-1, osteopontin (OPN), VCAM-1, GM-CSF and PAI-1 was examined using semi-quantitative real-time RT-PCR.

Methods: Porcine aortic valve interstitial cells at passage 1 were exposed to constant pressures of 100, 140, or 170 mmHg or cyclic pressures of 80-120, 120-160, or 150-190 mmHg for 2 h. Static cultures at atmospheric pressure served as controls. Total RNA from pooled experiments was isolated for analysis of gene expression. Single tube primer-mediated RT-PCR was performed directly on the RNA.

Results: Cells responded differently to constant and cyclic pressure. The most notable response was the expression of OPN, which was significantly up-regu-

lated under steady conditions but down-regulated under cyclic conditions. The opposite was true in VCAM-1 expression, which was significantly down-regulated at 170 mmHg static pressure, but up-regulated at 140 and 170 mmHg mean cyclic pressure. There was no clear proportional correlation between pressure magnitude and expression of MCP-1, GM-CSF, or PAI-1. However, elevated cyclic pressure caused a proportional increase in VCAM-1 expression and a proportional decrease in OPN expression. **Conclusion:** Elevated cyclic pressure is a potent stimulus for the up-regulation of VCAM-1 expression and the down-regulation of OPN expression. This demonstrates an association between hypertension and aortic valve stenosis and calcification. The regulation of the chemotactic genes MCP-1 and GM-CSF is not correlated to a change in compressive forces.

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The initiation and progression of aortic valve stenosis has a strong correlation with inflammatory processes (1-3). This is true of rheumatic, congenitally bicuspid and previously healthy valves (4,5). The inflammatory reaction is most often associated with a response to injury, where the tissue has been damaged and then incites a repair process (6). This process is instigated by the presence of specific molecules that are responsible for the adhesion, migration and accumulation of monocytes and T cells. These include intracellular adhesion molecules (ICAM) and vascular

cell adhesion molecules (VCAM). These act in conjunction with chemotactic molecules, such as monocyte chemoattractant protein 1 (MCP-1) and osteopontin (OPN), that are expressed by the endothelium and smooth muscle cells to attract monocytes and T cells into the tissue. Monocytes migrate into the subendothelial space and differentiate into macrophages (7). There are few, if any, neutrophils present in the aortic valve lesion, as is the case with atherosclerosis (8). Monocyte-derived macrophages are scavenging antigen-presenting cells, which secrete cytokines, chemokines, growth-regulating factors and metalloproteinases. The continual entry and survival of monocytes depends in part, on the expression of macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (9). Exposure to these molecules permits macrophages to survive in vitro and possibly to multiply within lesions. If this chronic inflammatory response is unabated and excessive, it can result in an

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aortic valve lesion, similar to an atherosclerotic plaque, and ectopic calcification. There are several proposed theories for the cellular commencement of valvular pathogenesis: (i) mechanical shear stress leading to calcific injury as in bicuspid aortic valves; (ii) autoimmune phenomena causing degeneration; and (iii) cardiovascular risk factors initiating an injury response similar to those seen in atherosclerosis (10). Population-based studies show a strong correlation between aortic stenosis/calcification and hypertension (5,11-14).

The pressure difference between the systemic circulation and the left ventricle produces a compressive force to be exerted on the leaflets. Under normal physiological conditions, the diastolic and systolic blood pressures will be less than 80 and 120 mmHg, respectively. The valve has developed to withstand this environment through the continuous renewal of extracellular matrix proteins and glycosaminoglycans (GAG), especially in areas of high stress (15). Under stage 1 and stage 2 hypertension, the systolic blood pressure increases to 140-159 and ≥ 160 mmHg, respectively, leading to an increase in the compressive forces that are exerted on the leaflet. Valve leaflets have been shown to respond to elevated pressure by increasing collagen and GAG synthesis (16,17). The heart valve can be considered to be incompressible (18), and the onset of hypertension may cause mechanical deformation of the leaflet tissue and could be an important proximal signal for the development of aortic stenosis.

The aim of the present study was to determine if elevated pressure would induce an up-regulation in pro-inflammatory gene expression in aortic valve interstitial cells (VICs) *in vitro*. Cells were exposed to static and cyclic pressures within normal and hyper-

tensive ranges for 2 h. The relative expression of MCP-1, OPN, VCAM-1, GM-CSF and Plasminogen Activator Inhibitor 1 (PAI-1) was determined using real-time reverse transcriptase (RT) PCR.

Materials and methods

Cell isolation and culture

Aortic valves were obtained from adult pigs within 30 min of slaughter at a local abattoir (Holifield's Farm, Covington, GA, USA) and transported to the laboratory in ice-cold Dulbecco's phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA). Cells were isolated as previously described (19). In brief, after removal of the endothelium, the tissue was digested overnight with collagenase (~300 U/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C and 5% CO₂ with gentle agitation.

Porcine aortic VICs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Mediatech Inc., Herndon, VA, USA) and 1% Anti-biotic/Anti-mycotic solution (Sigma), using standard tissue culture methods. Cells used in all experiments were at passage 1.

Pressure studies

Cells were seeded into six-well plates and grown to confluence. The medium was completely changed and the plates were then placed into a pressure chamber, as described previously (16,17). A water tray was also placed in the chamber to maintain humidity. The chamber was sealed, placed into a 37°C incubator and pressurized using air plus 5% CO₂. The cells were exposed to static pressures of 100, 140 and 170 mmHg

Table I: Primers used for RT-PCR.

Gene	Primer sequence	Product length (bp)	Annealing temperature (°C)
MCP-1	5'-GTCCTTGCCCAGCCAGATG-3' (F) 5'-CGATGGTCTTGAAGATCACTGCT-3' (R)	78	60
OPN	5'-GGTCTATGGACTGAGGTCAAATCTA-3' (F) 5'-TCCGAGGAAATAGTATTCTGTGGC-3' (R)	140	60
VCAM-1	5'-GAGATATGGTGACTGGGGTGGTG-3' (F) 5'-GAACAAACAACATCATAAAAA-3' (R)	462	55
GM-CSF	5'-GCTGTGATGAATGAAACCGTAGAC-3' (F) 5'-TGGGTTTCACAGGAAGTTTCCTC-3' (R)	117	60
PAI-1	5'-CACTCACCACCGCCTCTCCACAA-3' (F) 5'-TGCCGCTCTCATTACCTC-3' (R)	449	55

or cyclic pressures of 80-120, 120-160 and 150-190 mmHg with a frequency of 1.167 Hz (equivalent to 70 beats per minute) for a duration of 2 h. Control cultures were incubated under atmospheric pressure. These pressure conditions were chosen to represent normal, stage 1 and stage 2 hypertension, respectively. Upon completion of the experiment, the plates were removed from the chamber; the cells were then trypsinized (0.25% trypsin; Sigma), centrifuged (8 min; 1500 rpm), and the pellet washed twice with sterile PBS to remove all residual media. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and stored at -20°C prior to gene expression analysis.

Real-time PCR

Semi-quantitative RT-PCR was performed to measure the relative change in mRNA expression of five candidate genes: MCP-1, OPN, VCAM-1, GM-CSF and PAI-1. These genes were chosen as they represent several of the critical phases in inflammation and are known to be responsive to mechanical forces (20-25). RT-PCR was carried out using the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). A master mix was created using 0.25 µl SYBR Green One-Step Enzyme Mix, 6.25 µl 2X SYBR Green Reaction Mix, 0.25 µl of forward and reverse primers (see Table I) and RNase/DNase-free distilled water to give a final volume of 11.5 µl per sample. The master mix was pipetted into a 96-well PCR plate, and 1 µl of total RNA for each sample (between 250 and 750 ng) was added, giving a final reaction volume of 12.5 µl. cDNA synthesis and PCR amplification was performed using the following steps: 50°C for 30 min; the reaction mixture was then heated to 95°C for 5 min; a 45 cycle two-step PCR was then performed consisting of 95°C for 15 s followed by 1 min at the annealing temperature (see Table I). Reactions were carried out using an i-Cycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA).

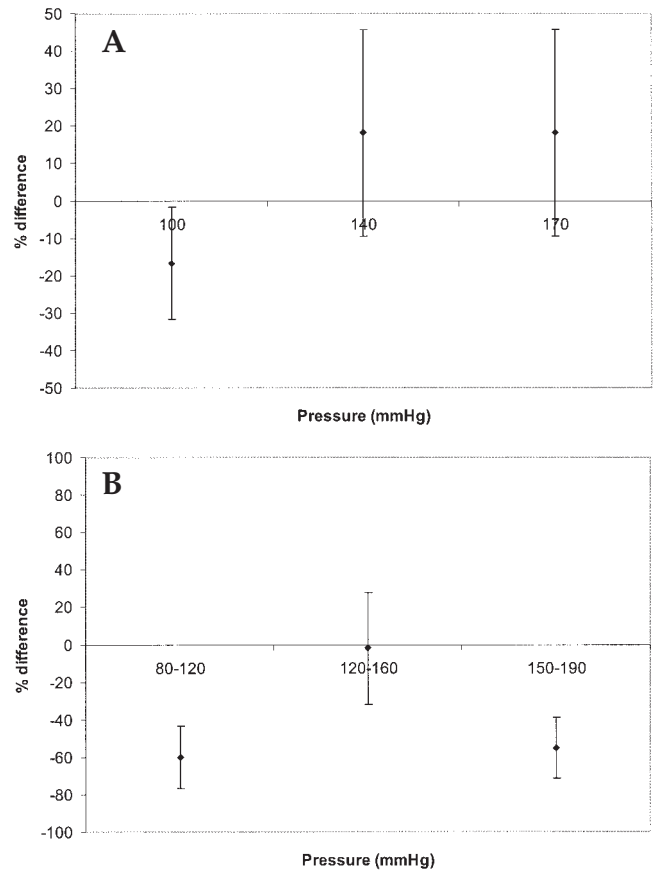


Figure 1: Expression of MCP-1 in porcine aortic valve interstitial cells exposed to static (A) and cyclic (B) pressure, relative to static controls. Data represent mean values ± 95% CI. Each experiment contained three pooled samples; four experiments were performed at each condition.

Data analysis

Four experiments were run for each condition, and each experiment contained RNA pooled from three samples. For ease of interpretation, all data were represented as 45-cycle threshold (C_T) - that is, the total number of PCR cycles - for each PCR. Relative expression was then calculated assuming a 100% PCR effi-

Table II: Summary of statistically significant changes in gene expression relative to static control; ↑, up-regulated; ↓, down-regulated; -, unchanged.

Gene	Pressure (mmHg)					
	100	140	170	80-120	120-160	150-190
MCP-1	↓	-	-	↓	-	↓
OPN	↑	↑	↑	-	↓	↓
VCAM-1	-	-	↓	-	↑	↑
GM-CSF	↓	↓	↓	↓	↓	
PAI-I	-	↑	↑	↑	-	-

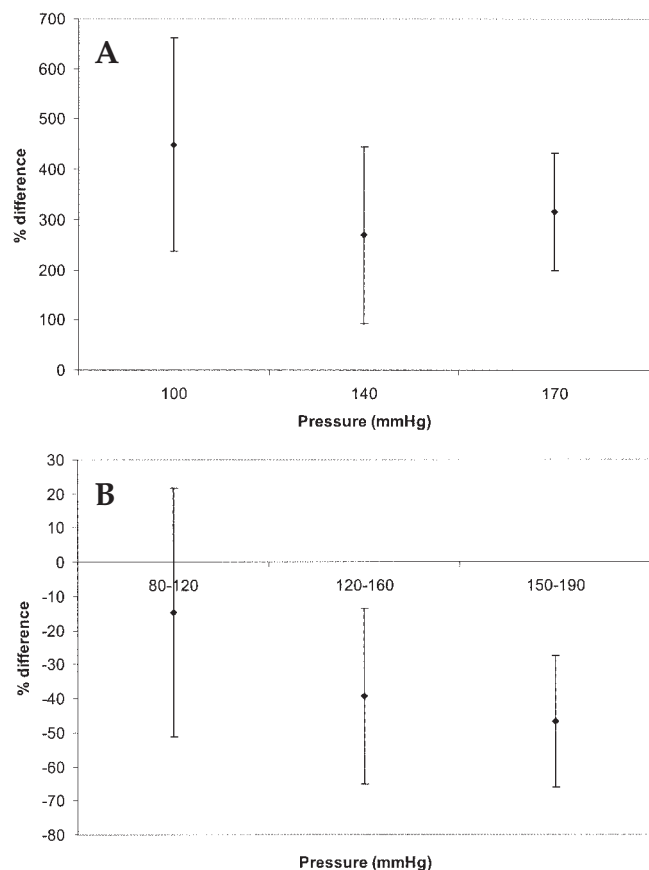


Figure 2: Expression of osteopontin in porcine aortic valve interstitial cells exposed to static (A) and cyclic (B) pressure, relative to static controls. Conditions as Figure 1.

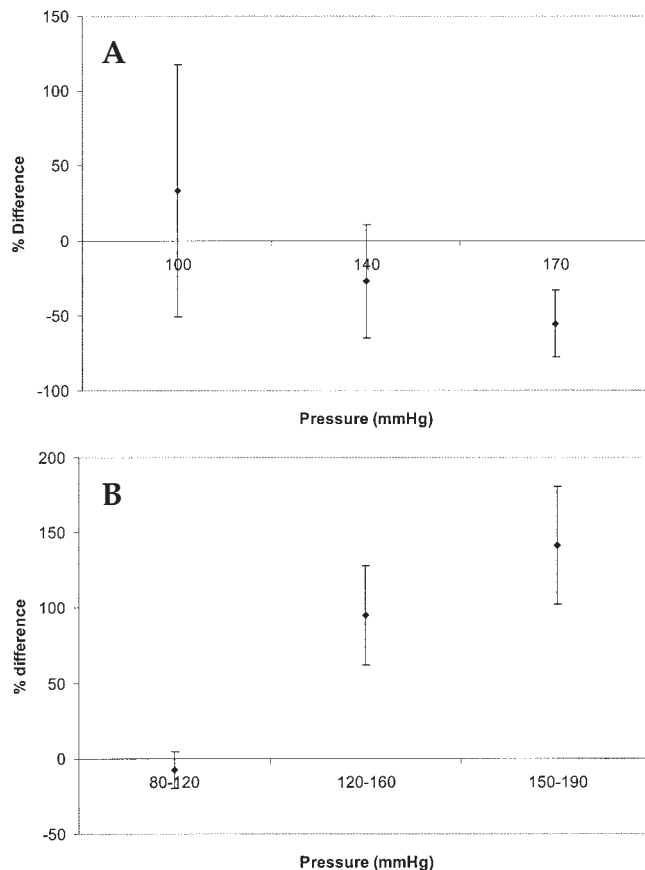


Figure 3: Expression of VCAM-1 in porcine aortic valve interstitial cells exposed to static (A) and cyclic (B) pressure, relative to static controls. Conditions as Figure 1.

ciency using $2^{(\Delta C_T)}$ where ΔC_T is the difference between the control and the experimental groups. This was then converted to a percentage difference between the experimental samples and controls. Means and 95% confidence intervals (CI) were then determined.

Results

The statistically significant changes in expression of all genes tested are summarized in Table II. At 100 mmHg static pressure there was no significant change in VCAM-1 or PAI-1 expression; however, OPN was significantly up-regulated ($450 \pm 259\%$), while MCP-1 and GM-CSF were down-regulated ($16.8 \pm 14.9\%$ and 39.6 ± 12.7 , respectively). As shown in Figure 2A, OPN was significantly increased at 140 and 170 mmHg static pressure ($269 \pm 176\%$ and $316 \pm 117\%$, respectively), as was PAI-1 ($8296 \pm 7419\%$ and $2656 \pm 2393\%$, respectively; see Fig. 5A). The data presented in Figure 1A show that MCP-1 was unchanged at 140 and 170 mmHg, whereas VCAM-1 was significantly down-regulated at 170 mmHg ($55.8 \pm 22.3\%$) but saw no significant change at 140 mmHg (see Fig. 3A). In addition to

being down-regulated at 100 mmHg, GM-CSF (Fig. 4A) was also down-regulated at 140 and 170 mmHg. However, there was no significant difference between experimental groups.

Under pulsatile conditions, which simulate in-vivo conditions more closely than static conditions, MCP-1 expression was down-regulated by $60.0 \pm 16.6\%$ and $55.1 \pm 16.2\%$ at 100 ± 20 and 170 ± 20 mmHg, respectively, relative to atmospheric controls (see Fig. 1B). OPN expression (see Fig. 2B) was significantly down-regulated at 140 ± 20 and 170 ± 20 mmHg. These data suggest that this response was inversely proportional to pressure magnitude; regression analysis of the results produced an R^2 value of 0.934. In contrast, VCAM-1 expression (Fig. 3B) was significantly up-regulated at 140 ± 20 and 170 ± 20 mmHg relative to atmospheric controls ($94.8 \pm 32.7\%$ and $141 \pm 39.3\%$, respectively). This increase in gene expression was also determined to be proportional to the pressure magnitude, with regression analysis calculating an R^2 value of 0.98. GM-CSF was significantly down-regulated at all cyclic pressures tested, relative to static controls (Fig. 4B). No significant difference between experi-

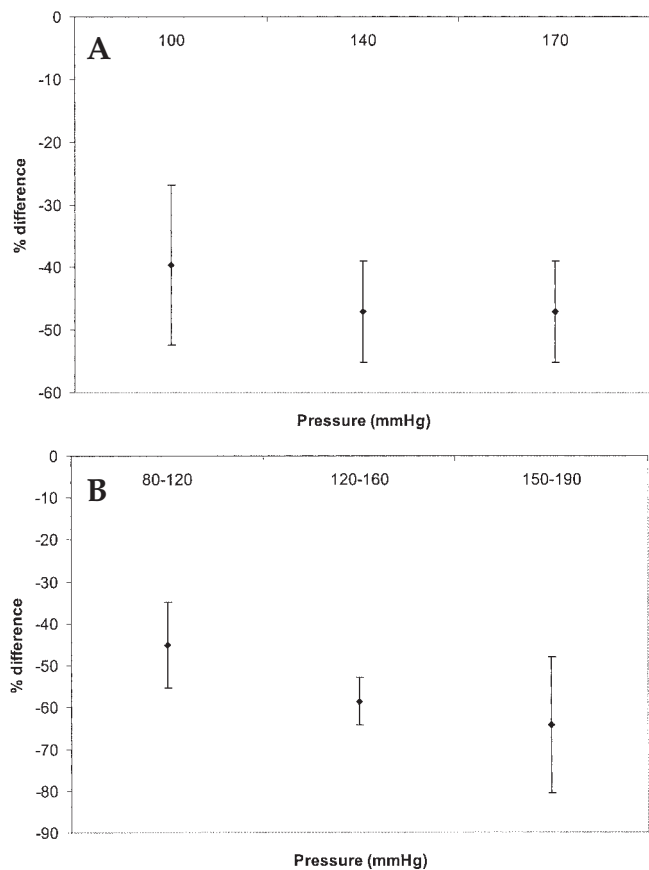


Figure 4: Expression of GM-CSF in porcine aortic valve interstitial cells exposed to static (A) and cyclic (B) pressure, relative to static controls. Conditions as Figure 1.

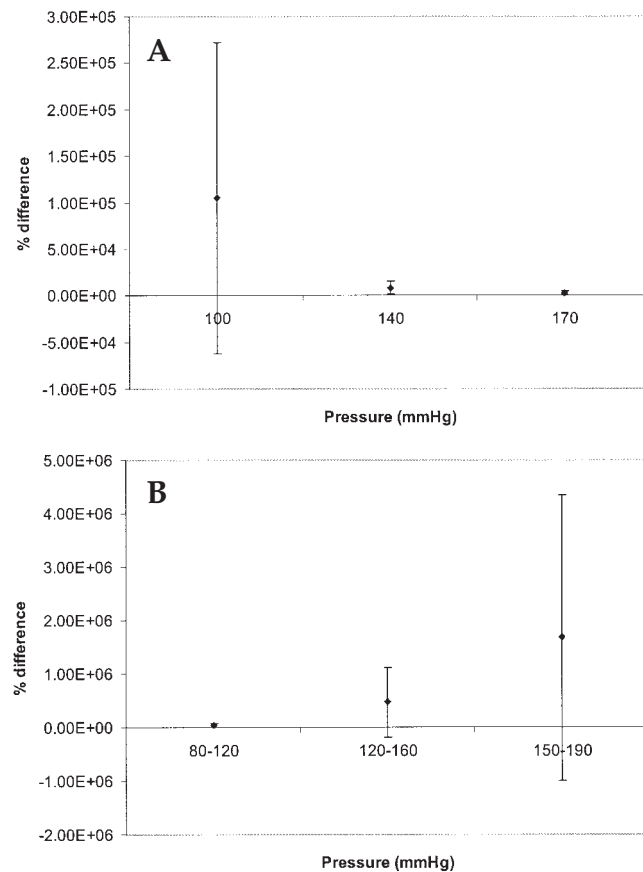


Figure 5: Expression of PAI-1 in porcine aortic valve interstitial cells exposed to static (A) and cyclic (B) pressure, relative to static controls. Conditions as Figure 1.

mental groups was evident, based on the 95% CI. The mean values for PAI-1 expression (Fig. 5B) showed an increase with pressure magnitude but, after calculating the 95% CI, this was not considered to be statistically significant.

Discussion

Until recently, aortic valve disease was believed to be a passive disease associated with rheumatic fever or ageing. However, a growing body of epidemiological evidence strongly supports the hypothesis that cardiovascular risk factors are involved in the cellular mechanisms that lead to aortic sclerosis and stenosis (3,5,12-14,26). While the results of these studies are compelling, few investigations have been made to understand the cell biology of aortic valve disease. In contrast, the cellular mechanisms leading to atherosclerosis have been studied extensively. It is not sufficient to assume that valvular cells will respond in the same way as vascular cells, as many studies have shown distinct phenotypic and molecular differences between the two populations (19,27-29). To the present

authors' knowledge, this is the first in-vitro experimental study to examine the response of the valvular cells, at the gene expression level, to a stimulus associated with the pathogenesis of aortic valve disease.

The pattern of gene expression was different for static and pulsatile pressure, indicating that porcine aortic VICs show a selective response to particular types of physical stimuli. This finding is not unexpected, as previous studies have shown that cardiovascular cells respond differently to stationary and dynamic signals (30,31). The greatest differences were in OPN and VCAM-1 mRNA expression. OPN was consistently up-regulated under all static pressure regimens, but was down-regulated at 140 and 170 mmHg mean cyclic pressure. In contrast, VCAM-1 was significantly down-regulated at 170 mmHg static pressure but showed a dose-dependent increase in expression in response to cyclic pressure. Although valvular cells are not exposed to static pressure under in-vivo conditions, this condition may provide insight into the potential pathway that leads to the generation of pro-inflammatory gene expression. One possible mechanism is the generation of reactive oxygen species

(ROS). Oxidative stress, which plays an important role in the pathogenesis of atherosclerosis, is mediated by a variety of ROS, which include superoxide, H₂O₂ and hydroxyl radical (32). They appear to participate in the inflammatory response by activation of transcription factors such as nuclear factor-kappa B, which can induce the transcription of a variety of redox-sensitive genes, including those encoding for cellular adhesion molecules (33-35). These genes enhance monocyte chemotaxis and adhesion and play an integral role in the development of aortic stenosis. The dynamic component is an important feature in oxidant stimulus, as steady forces do not induce oxidant stress (31).

There was no clear relationship between pressure (either static or cyclic) and the expression of MCP-1, GM-CSF, or PAI-1. There are two possible explanations for this; the first is that expression of these genes is not regulated by pressure. In addition to compressive forces, the aortic valve is exposed to an array of dynamic forces that include shear stress, flexion, and tension. Under hypertensive conditions, the magnitude of these other forces will be altered in addition to a change in the compressive force. Thus, although expression of MCP-1, GM-CSF and PAI-1 is not regulated by compression it may be responsive to tension. Additionally, when these forces are combined, they may have a synergistic effect on gene expression. For example, PAI-1 expression at 170 ± 20 mmHg was not significantly changed, but if cells were exposed to both compression and tension a significant up-regulation may be detected. Therefore, an investigation of alternative mechanical forces is required, as is the development of systems that can expose cells to combined forces. The second explanation for the lack of response of MCP-1, GM-CSF and PAI-1 to pressure is that the time interval was insufficient. The duration of these experiments was only 2 h, and this was relatively short. Other studies have examined in-vitro changes in gene expression for periods ranging from 1.5 h (36) to 13 days (37) using cardiovascular cells. The purpose of the present study was to examine the immediate effect of elevated pressure; however, the expression of GM-CSF and PAI-1 may become significant at later time points and would be expected in chronic disease conditions. This is plausible because the proteins translated from these mRNAs are identified in the latter stages of inflammation. Additionally, the high level of variability in PAI-1 expression at 150-190 mmHg suggests heterogeneity in the cell population, which could be the result of a delayed response of cells, with some cells showing increased expression while others were unchanged after 2 h.

The most compelling evidence that elevated cyclic pressure is involved in inflammation is the significant VCAM-1 up-regulation under conditions simulating

stage 1 and 2 hypertension, but not at normal physiological conditions. VCAM-1 is an essential protein in the progression of inflammation and is not detected in normal autopsy aortic valve leaflets. However, the protein is expressed in tricuspid and bicuspid non-rheumatic degenerative valves (38). This is the first time that VCAM-1 mRNA has been detected in non-diseased heart valve cells, even though the expression levels were very low. It is also the first time that a mechanism has been identified that accounts for the expression of VCAM-1 in diseased valves.

The OPN mRNA down-regulation under increased cyclic pressure is intriguing. OPN has been identified as an adhesin which is up-regulated during vascular remodeling and in diseases such as atherosclerosis and restenosis (39). OPN is also present in both heavily and minimally calcified aortic valves, but is absent from non-calcified purely regurgitant or normal aortic valves. In addition, OPN co-localizes with valvular calcific deposits, and macrophages have been identified in its vicinity (40). However, despite the strong association between OPN and calcification, phosphorylated OPN inhibits mineralization, suggesting that it has a protective role in the formation of ectopic calcification (41-44). In the context of the present study, OPN down-regulation may indicate that it is not involved in, or is not necessary for, inflammation. Furthermore, as OPN is down-regulated the tissue will become more susceptible to ectopic calcification. The ensuing calcification could be the result of activation of the VICs, which proliferate, migrate and change to an osteoblast-like phenotype.

The initial event that occurs in aortic valve stenosis, and atherosclerosis, is endothelial dysfunction (45). One limitation of the present study was that the endothelium was absent. Notably, endothelia on the aortic side of the valve express antioxidant genes and lack differential expression of pro-inflammatory genes, suggesting a protective role against inflammation and lesion development (46). Furthermore, endothelial dysfunction reduces the availability of nitric oxide, which has the potential to affect the interstitial cells by contributing to the potential for migration and proliferation, and increasing the inflammatory response (22). Therefore, if the endothelium is damaged or absent, the onset of a pro-inflammatory state is more likely to occur. This also suggests that pressure alone may not be sufficient to induce an inflammatory response in the VICs, but may accelerate lesion development if it is preceded by endothelial dysfunction. Further study is required to determine the response of endothelial cells to mechanical forces associated with hypertension, and how interstitial cell function may be regulated by the endothelium.

In conclusion, this is the first study to examine the effect of elevated pressure on differential pro-inflammatory gene expression in aortic valve interstitial cells. Compressive forces are potent stimuli for VCAM-1 up-regulation, and provide an association between hypertension and a pro-inflammatory state within the aortic valve. Elevated pressure results in the down-regulation of OPN, which may render the tissue susceptible to ectopic calcification. Regulation of the chemotactic genes MCP-1 and GM-CSF is not correlated to a change in pressure. It is unlikely that elevated cyclic pressure alone is sufficient to initiate the formation of aortic valve lesions but may accelerate the process following endothelial injury.

Acknowledgements

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Meeting discussion

MR. ADRIAN CHESTER (London, UK): Would your conclusions have changed if you had expressed your data relative to the physiologically stimulated cells rather than to cells that are quiescent, since under normal conditions the valve is subject to pressure - in other words, if you had used normotensive pressure against your class I and class II hypertensives?

DR. JAMES N. WARNOCK (Mississippi State, Mississippi, USA): I don't think the conclusions would change. If you look at our data - certainly for VCAM-1 and for osteopontin - under normal conditions there was no significant difference between gene expression for the samples and the atmospheric controls. However, there was a significant difference - certainly with the VCAM1 - at hypertensive conditions relative to normotensive conditions.