The pathomechanism of human myxomatous valvular degeneration at the mechanical and cellular level

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The purpose of this study was to explore the pathomechanism of human myxomatous valve degeneration by investigating changes in the phenotype of valvar cells, the metabolism of the extracellular matrix and their mechanical properties. Mitral valve specimens were harvested from patients who had undergone valve replacement, and divided into two groups: patients with a myxomatous mitral valve and a control group. Histological investigation showed that the morphology of the extracellular matrix was looser and less coordinated in myxomatous valves than in controls. α-SMA (α-smooth muscle actin) and Vimentin were positive and DNA (deoxyribonucleic acid) assay of leaflets and expression of SMemb (embryonic smooth muscle myosin heavy chain), MMP-13 (matrix Metalloproteinase-13), MMP-1 mRNA (messenger Ribonucleic Acid) of the myxomatous valves were increased while the hydroxyproline content, expression of TIMP-1 (tissue inhibitor of metalloproteinase-1) mRNA and mechanical properties were decreased compared with controls. Compared to the quiescent interstitial cells in non-myxomatous valves, interstitial cells in myxomatous valves exhibit myofibroblast activation and express excessive levels of matrix metalloproteinases. The balance between MMP/TIMP was disrupted. We conclude that over-activation of VICS (Valvar interstitial cells) and the imbalance of MMP/TIMP could be important features of the pathomechanism of myxomatous mitral valve degeneration.

Keywords
Myxomatous mitral valve; Degeneration; Valvar interstitial cell; Extracellular matrix; Mechanical property

1. Introduction
Due to continuous improvements in global medical care over many decades, the incidence of rheumatic valvar heart disease has been decreasing year by year. In contrast, myxomatous degenerative heart valve disease has gradually become a serious cardiovascular disease that affects the quality of life and life expectancy of the elderly worldwide [1–3]. The exact cause of myxomatous degenerative valve disease is still unknown. At present, research on this disease has focused on morphology, while the study of its pathological mechanism at the mechanical and cellular level has received little attention. The aim of this study was to explore the mechanism of valvar degenerative pathological changes by detecting changes in the phenotype of valvar interstitial cells, extracellular matrix (ECM) metabolism, valve structure, and mechanical properties, and therefore provide a theoretical basis for the prevention and treatment of myxomatous degenerative valve disease in the future.

2. Materials and methods

2.1 Valve acquisition, grouping and processing
A total 26 of mitral valve specimens (anterior leaflet) were selected from patients who underwent valve replacement surgery, which were then divided into two groups: A myxomatous mitral valve group (hereafter referred to as diseased, n = 20, specimens were obtained from patients with myxomatous degenerative mitral valve which could not be reconstructed, 12 males and 8 females aged 70.3 ± 7.06 years) and a non-myxomatous mitral valve group (hereafter referred to as control, n = 6, specimens were obtained from replaced prolapsed mitral valves where the mitral chordae tendineae had undergone acute mechanical rupture and failed to be reconstructed, with no evidence of pathological change, 4 males and 2 females aged 68.5 ± 5.89 years). There was no significant difference in gender and age between the two groups (P > 0.05). In order to ensure that every specimen of the diseased group represented a myxomatous degenerative valve, the following diseases were excluded: rheumatic heart disease, endocarditis, calcific degeneration as well as any uncertain diagnosis of myxomatous degeneration. Valve specimens of both groups were confirmed by postoperative pathological examination. All analyses were conducted by the pathologist who is an expert in the study of valvar heart diseases and blinded to the study group allocations. The specimens were immediately stored in cold phosphate buffer solution (PBS) solution containing antibiotics (penicillin 100
2.2.6.1 Extraction of the total RNA

Valve fragments were placed in an autoclavable mortar and soaked in DEPC, 1 mL of Trizol was added, the fragments were then ground down and transferred to an Eppendorf tube. After standing at room temperature for 5 minutes, 0.2 mL of chloroform was added to the sample and shaken vigorously for 15 seconds, then placed on ice for 5 minutes. Following centrifugation (14000 r/min) at 4 °C for 15 minutes, the upper aqueous phase was transferred to another Eppendorf tube. 500 μL of isopropanol was added to the sample, mixed and placed on ice for 10 minutes, followed by centrifugation (14000 r/min) at 4 °C for 10 minutes. The supernatant was discarded, and the precipitate was added to 1 mL of pre-cooled 75% ethanol, and after shaking was washed thoroughly. Centrifugation (14000 r/min) was completed at 4 °C for 5 minutes, and the supernatant was discarded. The precipitate was dried at room temperature for 5–10 minutes and dissolved in 20 μL of enzyme-free water, then stored in a −70 °C deep cryogenic refrigerator. A small amount of the above sample was used to detect the optical density (OD) value of total RNA to confirm the concentration and purity of RNA by means of an ultraviolet spectrophotometer (A260/A280). Required OD_{260nm}/OD_{280nm} is 1.8 or more and 2.0% agarose gel electrophoresis showed clear 18 s and 28 s bands, which indicated that RNA had been extracted successfully.

2.2.6.2 Reverse transcription

Reverse transcription was carried out according to the instructions of the reverse transcription kit (Fermentas) and samples after reverse transcription were stored in a −20 °C refrigerator.

2.2.6.3 Amplification of the target gene

Primer designs are shown in Table 1 and the PCR reaction system is shown in Table 2. Conditions for PCR amplification were: an initial denaturation at 95 °C for 5 minutes, denaturation at 94 °C for 45 seconds, annealing temperatures
Table 1. RT-PCR detection of SMemb, MMP-13, MMP-1 and TIMP-1 primer sequences.

<table>
<thead>
<tr>
<th>Primers (human)</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Product Length (bp)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMemb(human)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper primer</td>
<td>5′-CCGCCCAAGAAGTGACAATG-3′</td>
<td>21</td>
<td>376</td>
<td>64.2 °C</td>
</tr>
<tr>
<td>Down primer</td>
<td>5′-TGGAGTTTACGCCGAGATGC-3′</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-13(human)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper primer</td>
<td>5′-TGGCTGCCTCTCTTCTTCT-3′</td>
<td>18</td>
<td>235</td>
<td>49.3 °C</td>
</tr>
<tr>
<td>Down primer</td>
<td>5′-TCAAGTTTCCACGTACCT-3′</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1(human)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper primer</td>
<td>5′-GGAAGAATGAGGAGGCAATG-3′</td>
<td>21</td>
<td>523</td>
<td>57.4 °C</td>
</tr>
<tr>
<td>Down primer</td>
<td>5′-ATGAGCCGAAACAGATG-3′</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-1(human)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper primer</td>
<td>5′-AATTCCGACCTCCTGATCAG-3′</td>
<td>20</td>
<td>195</td>
<td>54.5 °C</td>
</tr>
<tr>
<td>Down primer</td>
<td>5′-GTTGTGGACCTGGAAGAT-3′</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tublin (reference gene)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper primer</td>
<td>5′-CTCATCACAGGCAAGGAT-3′</td>
<td>21</td>
<td>410</td>
<td>55 °C</td>
</tr>
<tr>
<td>Down primer</td>
<td>5′-TTAAGGTAAGTGTAGGTGGG-3′</td>
<td>21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

were set according to gene primers (see Table 1), annealing time of 45 seconds, extending at 72 °C for 90 seconds, a total of 33 cycles, second extending at 72 °C for 10 minutes and finally annealed at 4 °C.

Table 2. 50 µL RT-PCR reaction system.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× PCR buffer (Mg²⁺ Plus)*</td>
<td>5 µL</td>
</tr>
<tr>
<td>dNTP mixture (2.5 mM each)</td>
<td>4 µL</td>
</tr>
<tr>
<td>sense primer (10 pmol/µL)</td>
<td>2 µL</td>
</tr>
<tr>
<td>antisense primer (10 pmol/µL)</td>
<td>2 µL</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/µL)</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>dH₂O (RNase-free)</td>
<td>34.75 µL</td>
</tr>
<tr>
<td>cDNA</td>
<td>3 µL</td>
</tr>
<tr>
<td>total volume</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

*10 × PCR buffer (Mg²⁺ Plus): Tris-HCL (PH 8.3) 100 mM; KCl 500 mM; MgCl₂ 15 mM.

2.2.6.4 Gel electrophoresis

5 µL of final products of the target gene and reference gene were added into new Eppendorf tubes and mixed with 1 µL of 6× loaded buffer. Agarose gel electrophoresis was executed with the stain SYBR Green 1 and electrophoresis buffer of 0.5× TBE for 30 minutes at 100 volts of constant voltage. Bands were observed under ultraviolet light, and photographs were taken rapidly by the one-time imaging system of Polai. Computer software (Bandscan 5.0) analyzed the ratio of the gray value of the target gene to the gray value of the reference gene, which was the relative content of the target gene.

2.2.7 Statistical analysis

Statistical data are presented as standard deviations from the mean. Unpaired Student t-test was used to compare means of two experimental groups, and statistical significance was set at the 0.05 level. Statistical analyses were performed with SPSS ver. 23.0 (SPSS Inc., Chicago, IL, USA).

3. Result

3.1 Histology

Histological examination of non-myxomatous valve leaflets demonstrated that cells were distributed in all three layers, of an integrated and compact three-tier structure (a fibrous layer, a sponge layer, and a ventricular layer) which was interconnected by dense fibrous tissue. The diseased valve leaflets showed a slight increase in number of cells. The three-tier structure was still intact. However, fibrous tissue was loose and disordered, and the sponge layer was deeper with more voids (Fig. 1).

3.2 Immunofluorescence

Immunofluorescence for α-SMA was negative or weakly positive in the control group but positive in the diseased group, while Vimentin staining was positive in both groups (Figs. 2, 3).

3.3 DNA content and hydroxyproline content

A comparison between groups showed that the DNA content of the diseased group was higher than that of the control group, and the hydroxyproline content was lower than that of the control group (Table 3).

Table 3. DNA content and hydroxyproline content.

<table>
<thead>
<tr>
<th></th>
<th>DNA content (µg/mg)</th>
<th>Hydroxyproline content (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseased</td>
<td>0.120 ± 0.009*</td>
<td>4.20 ± 0.33*</td>
</tr>
<tr>
<td>Control</td>
<td>0.094 ± 0.010</td>
<td>5.21 ± 0.25</td>
</tr>
</tbody>
</table>

* P < 0.05.

3.4 Mechanical performance

Compared with the control group, the maximum load and maximum stress of the diseased group decreased, while the maximum strain and elastic modulus increased (Table 4).
Fig. 1. Histologic examination of valve leaflets. (A) Control group: Cells were distributed in all three layers, of an integrated and compact three-tier structure (fibrous layer, sponge layer, and ventricular layer) which was interconnected by dense fibrous tissue. (B) Diseased group: The number of cells increased slightly. The three-tier structure was still intact, however, fibrous tissue was loose and disordered, and the sponge layer was broader with more voids (A and B: hematoxylin and eosin; original magnification ×20).

Fig. 2. Immunofluorescent staining for α-SMA in the (A) control group and in the (B) diseased group.

Fig. 3. Immunofluorescent staining for Vimentin in the (A) control group and in the (B) diseased group.
3.5 Relative mRNA expression of SMemb, MMP-13, MMP-1 and TIMP-1

The relative content of the target genes—SMemb, MMP-13, MMP-1 and TIMP-1—and the internal reference in the diseased group and the control group are shown in Table 5. The expression of SMemb and MMP-13 are indicators that reflect the cell phenotype; they were hardly detected in the control group, but highly expressed in the diseased group. The expression of MMP-1 and TIMP-1 mRNA reflect general metabolism in the ECM. Compared with the control group, the expression of MMP-1 mRNA in the diseased group was increased, but the expression of TIMP-1 mRNA decreased. The gel images of the final products of SMemb, MMP-13, MMP-1 and TIMP-1 mRNA detected by RT-PCR are shown in Fig. 4.

Table 5. The ratio of the gray value of the target gene and the internal reference in the diseased group and the control group.

<table>
<thead>
<tr>
<th></th>
<th>SMemb</th>
<th>MMP-13</th>
<th>MMP-1</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseased</td>
<td>0.55 ± 0.07*</td>
<td>0.47 ± 0.06*</td>
<td>0.71 ± 0.08*</td>
<td>0.70 ± 0.05*</td>
</tr>
<tr>
<td>Control</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.27 ± 0.04</td>
<td>0.88 ± 0.06</td>
</tr>
</tbody>
</table>

*P < 0.05.

4. Discussion

VICs are the predominant cells responsible for the synthesis of extracellular matrix and expression of matrix-degrading enzymes and their inhibitors, which mediate matrix remodeling [4–7]. VICs have been demonstrated to exhibit cell phenotypes in two different states under different conditions, namely fibroblast-like phenotype and myofibroblast-like phenotype [3, 8]. In normal adult valves, VICs mainly manifest as a relatively quiescent fibroblast-like phenotype, while in infancy they exhibit an activated myofibroblast-like phenotype, both of which express Vimentin, but the latter is characterised by expression of α-SMA, SMemb and MMP-13 (collagenase-3) [9, 10]. Some scholars have concluded that activated VICs are also present in pathological cardiac valves, allogeneic valves after transplantation and tissue-engineered valves in vitro [11, 12].

The synthesis and secretion of matrix-degrading enzymes and their inhibitors are conducted by the activation of VICs. Matrix metalloproteinases (MMPs) are the most important enzymes that degrade ECM and can degrade almost all constituents of ECM [13, 14]. TIMP is a natural inhibitor of MMPs [15, 16]. As a pair, TIMP and MMP maintain a dynamic balance, and play an important role in regulating the homeostasis of valve ECM [17–19].
Cardiac valves require cells with differing mechanical properties to work together in concert; their strength is closely related to valve structure and chemical composition [20]. The normal heart valve includes a three-tier structure consisting of ventricular layer, spongiosa and fibrous layer, each having a different major constituent (in order: elastin, glycosaminoglycan, and collagen). Different components play differing mechanical roles in the movement of the valve, ensuring normal opening and closing of the valve throughout the cardiac cycle. Elastin permits radial stretch of the leaflets, which allows the valves to extend during diastole and shorten when the heart contracts; glycosaminoglycan shears and cushions impact transmitted from the ventricular layer and the fibrous layer; collagen fibers impart strength and stiffness to the valve, which maintains closure during systole. Clearly, the close relationship of composition-structure-function dictates that changes in the microscopic composition of the valve can lead to changes in the macrostructure, which in turn affects the mechanical properties of the valve and the maintenance of normal function.

The results of this study show that the VICs of non-myxomatous valves demonstrate a relatively static fibroblast-like phenotype, which may be related to the low metabolic rate of relatively normal valve tissue. Positive expression of α-SMA, SMemb and Vimentin was found in the VICs of the diseased group, presenting characteristics of a myofibroblast-like phenotype, which suggests increased metabolic activity with increased DNA content indicating activated VIC proliferation. RT-PCR analysis showed that the expression of MMP-1 mRNA in activated VICs was enhanced, while expression of TIMP-1 mRNA was weakened, suggesting loss of the dynamic balance between MMP/TIMP. We hypothesise that this imbalance could lead to the dysregulation of extracellular matrix metabolism in the diseased valve, which in turn results in a decrease in valve collagen content and structural instability. The hydroxyproline content test showed that the collagen content of the diseased group was decreased, and histological examination confirmed that fibrous tissue of the diseased group was loose and disordered, which supports this hypothesis. Changes in valve composition and tissue structure directly result in changes in the mechanical properties of the valve. The mechanical performance test of this study showed that the maximum load and maximum stress of the diseased valve decreased, while the maximum strain and elastic modulus increased, that is, its strength decreased while its elasticity increased. The former may be caused by an increase in distance between collagen fibers in the diseased group, a more random architectural orientation, and the decrease in fiber per unit area; while the latter is related to the widening of the sponge layer and the increase in water content of the network structure [21]. Changes in the mechanical properties of myxomatous degenerative valves are proposed to cause clinical mitral valve prolapse and lead to valvular insufficiency.

In summary, systemic blood flow impacts the valve leading to degeneration over time, which institutes mechanical stress changes in the valves. VICs are activated and then proliferate, increasing the expression of Vimentin, α-SMA, SMemb, and MMP-13, and activating extracellular matrix remodeling. This system allows the body to adapt to changes in the internal environment. However, in patients with myxomatous degenerative valves, VICs are over-activated and the expression of MMP/TIMP is imbalanced. This causes the dysregulation of ECM metabolism, decreased collagen content of the valve, and a change in structural hierarchy, which ultimately leads to a deterioration in the biomechanical properties of the valve. This study elucidates the profile of human myxomatous valvular degeneration and demonstrates the pathogenesis of myxomatous degenerative valve disease. Further studies should concentrate on whether overactivation of VICs are implicated in other blood fluid rheology related pathologies, such as hypertension and atherosclerosis, in addition to the role of genetic factors. Inhibition of overactivation of VICs might be one way to retard the development and progression of myxomatous valvular degeneration. We hope this work provides a theoretical basis for the prevention and treatment of myxomatous degenerative valve disease in the future.

5. Conclusions

Compared to the quiescent interstitial cells in non-myxomatous valves, interstitial cells in myxomatous valves exhibit myofibroblast activation and express excessive levels of matrix metalloproteinases. The homeostatic balance between MMP/TIMP is disrupted. We conclude that overactivation of VICs and the imbalance of MMP/TIMP could be important features of the pathomechanism of myxomatous mitral valve degeneration.

Author contributions

ND and MZ designed the research study. CH and QW performed the research. HX and HH provided help and advice on the mechanical and RT-PCR experiments. JS analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients involved in this study gave their informed consent. Institutional review board approval of our hospital was obtained for this study (IRB file number: 2020-36).

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Conflict of interest

The authors declare no conflict of interest.

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