Chymase Inhibition Prevents Fibronectin and Myofibrillar Loss and Improves Cardiomyocyte Function and LV Torsion Angle in Dogs With Isolated Mitral Regurgitation

Betty Pat, PhD; Yuanwen Chen, MD, PhD; Cheryl Killingsworth, DVM, PhD; James D. Gladden, BS; Ke Shi, MD; Junying Zheng, PhD; Pamela C. Powell, MS; Greg Walcott, MD, PhD; Mustafa I. Ahmed, MD; Himanshu Gupta, MD; Ravi Desai, MD; Chih-Chang Wei, PhD; Naoki Hase, MS; Tsunefumi Kobayashi, PhD; Abdelkarim Sabri, PhD; Henk Granzier, PhD; Thomas Denney, PhD; Michael Tillson, DVM; A. Ray Dillon, DVM; Ahsan Husain, PhD; Louis J. Dell’Italia, MD

Background—The left ventricular (LV) dilatation of isolated mitral regurgitation (MR) is associated with an increase in chymase and a decrease in interstitial collagen and extracellular matrix. In addition to profibrotic effects, chymase has significant antifibrotic actions because it activates matrix metalloproteinases and kallikrein and degrades fibronectin. Thus, we hypothesize that chymase inhibitor (CI) will attenuate extracellular matrix loss and LV remodeling in MR.

Methods and Results—We studied dogs with 4 months of untreated MR (MR; n=9) or MR treated with CI (MR+CI; n=8). Cine MRI demonstrated a >40% increase in LV end-diastolic volume in both groups, consistent with a failure of CI to improve a 25% decrease in interstitial collagen in MR. However, LV cardiomyocyte fractional shortening was decreased in MR versus normal dogs (3.71±0.24% versus 4.81±0.31%; P<0.05) and normalized in MR+CI dogs (4.85±0.44%). MRI with tissue tagging demonstrated an increase in LV torsion angle in MR+CI versus MR dogs. CI normalized the significant decrease in fibronectin and FAK phosphorylation and prevented cardiomyocyte myofibrillar degeneration in MR dogs. In addition, total titin and its stiffer isoform were increased in the LV epicardium and paralleled the changes in fibronectin and FAK phosphorylation in MR+CI dogs.

Conclusions—These results suggest that chymase disrupts cell surface–fibronectin connections and FAK phosphorylation that can adversely affect cardiomyocyte myofibrillar structure and function. The greater effect of CI on epicardial versus endocardial titin and noncollagen cell surface proteins may be responsible for the increase in torsion angle in chronic MR. (Circulation. 2010;122:1488-1495.)

Key Words: cardiac volume ■ collagen ■ heart contractility ■ heart failure ■ mitral valve

Chronic isolated mitral regurgitation (MR) in the dog is characterized by left ventricular (LV) dilatation, loss of interstitial collagen and noncollagen extracellular matrix (ECM) proteins, and progressive cardiomyocyte elongation and dysfunction.1-4 In both the acute (2 to 4 weeks) and chronic (4 and 6 months) MR dog, there is a significant decrease in LV interstitial collagen.1-3 In addition, there is downregulation of multiple noncollagen ECM scaffolding genes and transforming growth factor-β (TGFβ) signaling,4 suggesting that LV dilatation is due to collagen loss and an overall decrease in ECM supporting structure.

Clinical Perspective on p 1495

Isolated MR is associated with increased LV mast cell infiltration and degranulation,1,2 resulting in release of chymase, a proteolytic enzyme that is increased at both early and late stages of MR.3 Chymase mediates the majority of angiotensin II production in humans5 and MR dogs.6 However, angiotensin II type 1 receptor blockade does not attenuate collagen loss or prevent eccentric LV or cardiomyocyte remodeling in MR dogs.7 In addition to its angiotensin II-forming capacity, chymase directly activates matrix met-
allopain to collagen.12,13 and degrades fibronectin,10 resulting in cell death through the loss of cell-ECM connections.11 In addition, chymase activates kallikrein,12 thereby increasing bradykinin, which reduces fibronectin and collagen I and III messenger RNA expression in adult cardiac fibroblasts.13

Chymase has been linked to fibrosis through its angiotensin II–forming capacity. However, in the pure stretch of MR, the persistent elevation of mast cell chymase may contribute to interstitial collagen loss and the decrease in ECM, resulting in adverse LV and cardiomyocyte remodeling in MR. We hypothesize that blockade of chymase will prevent disruption of the ECM, resulting in improved LV remodeling and cardiomyocyte function in chronic MR.

Methods
Experimental Procedures
Mitrval valve regurgitation was induced at Auburn University College of Veterinary Medicine in mongrel dogs of either sex (19 to 26 kg) by chordal rupture as previously described.1–4,6,7 Dogs were randomly assigned to 2 groups: 4 months of untreated MR (n=9; male=5, female=4) or 4 months of MR treated with chymase inhibitor (CI) (4-{1-[4-methylbenzo(6) thiophen-3-yl]methyl}benzimidazole-2-yhio)butanoic acid; Teijin Pharma Ltd, Tokyo, Japan, at a dose of 100 mg/kg PO twice daily; n=8; male=2, female=6) started 24 hours after MR induction. The effective oral dose of CI was determined by demonstrating that pretreatment with CI (oral dose, 100 mg/kg twice daily) significantly attenuated an increase in interstitial fluid chymase activity during ischemia/reperfusion of the left anterior descending artery in the open chest dog using in vivo cardiac microdialysis to measure interstitial fluid chymase activity.14 Hemodynamic data were collected at baseline and after 4 months of MR. Dogs were transferred to the University of Alabama at Birmingham for euthanasia. Drug was withheld on the day of the terminal experiments. A third cohort of normal dogs (n=13; male=7, female=6) were analyzed by MRI before death and were used for cardiomyocyte isolation and tissue morphometry. All dogs in this study were between 2 and 4 years of age. This study was approved by the Animal Services committees at University of Alabama at Birmingham and Auburn University.

Terminal Study: Instrumentation
Animals were maintained at a deep plane of general anesthesia using 1% to 2% isoflurane in 100% oxygen. The heart was arrested with KCl, quickly excised, and placed in ice-cold Krebs solution, and the coronary arteries were flushed with the same solution. LV sections were flash-frozen in liquid nitrogen or perfusion fixed with 3% paraformaldehyde.

Isolated Cardiomyocyte Studies
Cardiomyocytes were isolated from the tissue by recirculating perfusion buffer supplemented with 2 mg/mL collagenase type II (Invitrogen, Carlsbad, Calif) as previously described.3 The [Ca2+]i, measured with the fluorescent indicator fluo 3-acetoxymethyl ester (Molecular Probes, Eugene, Ore), and the contractile activity (cell fractional shortening) of field-stimulated (5-millisecond square pulses with constant voltage at ≥20% above threshold and 1000-millisecond cycle length) cardiomyocytes was measured with a video edge detector (Crescent Electronics, Sandy, Utah) as previously described.3

Cine MRI and Tissue Tagging
MRI was performed on a 1.5-T GE Signa Horizon (Milwaukee, Wis) instrument optimized for cardiac application.

Cine MRI
The steady-state free precession technique was used to measure absolute LV volumes and ejection fraction. The shortest repetition and echo times were used (typical values, 4.5 and 1.9 milliseconds, respectively). Slice thickness was 10 mm with a zero interslice gap. True temporal resolution was 25 to 30 milliseconds in all cases. LV volumes were calculated from summed serial endocardial and epicardial contours as previously described.4 Left atrial volumes were calculated with a standard area-length method from 2- and 4-chamber views.

LV Time-Volume Curves
The LV volume at each time frame was computed, and an LV volume-time curve was constructed and differentiated with respect to time. End diastole was defined as the maximum-volume time frame, and end systole was defined as the minimum-volume time frame. Early diastole was defined as the first half of the diastolic interval. The early diastolic filling rate was defined as the maximum derivative (mL/s) and corrected for end-diastolic volume.15

Early Mitral Annular Velocity
The mitral annular velocity was computed by tracking the mitral annulus in a 4-chamber view from user-defined landmark points at end diastole and end systole with a nonrigid registration algorithm. A mitral annular displacement curve was constructed and differentiated with respect to time to obtain a mitral annular velocity curve.15

Tagged Cine MRI
A grid-tagged, fast-gradient-echo, cine sequence was used with a repetition time of 8.4 milliseconds and an echo time of 4.8 milliseconds on identical slice prescription as above. True temporal resolution was ±50 milliseconds. Tag line spacing in the grid was 7×7 mm. Two-dimensional torsion was measured by tracking a circular mesh of points in a basal and an apical slice. The mesh was identified the first time through the use of user-defined contours and tracked through the remaining imaged phases with improved harmonic-phase tracking.16 Normalized torsion, T, was computed from the rotation angle, ϕ, in the basal and apical slices by the following formula17: \[ T = \frac{(\sigma_{\text{apex}} - \sigma_{\text{base}})(\sigma_{\text{apex}} + \sigma_{\text{base}})}{2L}, \] where ρ is the epicardial radius and L is the distance between the basal and apical slices.

Collagen Analysis
Intersitial collagen was quantified with picric acid Sirius red F3BA in epicardial and endocardial sections as previously reported.4 Total LV endocardial collagen was identified by the colorimetric hydroxyproline method as previously described.4

Assessment of Cardiomyocyte Myofibrillar Degeneration
Hematoxylin and eosin–stained sections were assessed for myofibrillar degeneration with light microscopy. Longitudinally oriented tissue was graded on a scale of 1 to 5, with 1=20%, 2=20% to 40%, 3=40% to 60%, 4=60% to 80%, and 5=80% of cardiomyocyte degeneration per high-powered field (×40 objective, ×1600 total magnification) in 20 to 30 fields. All analyses were performed in a blinded manner.

Western Blot
LV midwall tissue was dounced in radioimmunoprecipitation assay buffer containing EDTA, protease, and phosphatase cocktail inhibitors (all from Pierce Biotechnology, Inc, Rockford, Ill). Lysates (10 to 40 μg) were separated on a 4% to 20% gradient Bis/Tris gel (Invitrogen) by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with antibodies to laminin (1:500; Abcam, Cambridge, UK), fibronecin (1:1000; Calbiochem, La Jolla, Calif), phospho-FAK (Tyr937) (1:200; Millipore, Billerica, Mass), FAK (1:200; BD Transduction Laboratories, San Jose, Calif) or β-actin (1:4000; Abcam) diluted in Blotto B (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) for 1 hour (room temperature) or overnight (4°C) and then with appropriate horseradish peroxidase–conjugated secondary antibodies (1:2000 to 1:5000). Bands were visualized by enhanced chemiluminescence (Pierce), scanned, and quantified in Scion.
**Titin Gel Electrophoresis**

LV tissue lysates were separated on 1% to 2% vertical SDS-agarose gels and stained with Coomassie blue. Wet gels were scanned and analyzed with 1-dimensional scan software (Scanalytics, Dayton, Ohio). The integrated optical densities of N2BA titin, N2B titin, total titin (N2BA plus N2B), and myosin heavy chain were determined as a function of the volume of the solubilized protein sample that was loaded (a range of volumes were loaded on each gel). The slope of the linear range of the relation between integrated optical density and loaded volume was obtained for each protein. This slope was normalized to total protein.

**MMP-2 Activity Assay and Gel Zymography**

LV endocardial midwall tissue (50 to 100 mg) was homogenized in 50 mmol/L Tris-HCl, pH 7.6, 0.01% Triton X-100, 15 mmol/L NaCl, 0.5 mmol/L CaCl₂, and 20 μmol/L ZnCl₂ and centrifuged at 16 000 g (30 minutes at 4°C). Lysates were assayed with the Biotrack MMP-2 activity assay System (RPN 2631, Amersham Biosciences, Inc, Piscataway, NJ). MMP-2 activity was normalized to total protein. For gel zymography, lysates were mixed with an equal volume of 30% dimethyl sulfoxide (DMSO) and separated on a 10% (vol/vol) Tris-glycine polyacrylamide gel containing 0.1% (wt/vol) gelatin. Gels were developed and stained with Colloidal Blue Staining Kit according to the protocol for Novex Zymogram gels (Invitrogen). Dog serum served as a positive control for both MMP-2 and MMP-9.

**TGFβ1 Activity Assay**

LV endocardial tissue (50 to 100 mg) was homogenized (50 mmol/L HEPES, 50 mmol/L Na₃O₄P₂, 100 mmol/L NaF, 10 mmol/L EDTA, pH 7.4, plus complete protease cocktail inhibitor; Roche, Indianapolis, Ind) and centrifuged at 16 000 g (20 minutes at 4°C). Lysates were assayed with the TGFβ1 ELISA kit (R&D Systems, Minneapolis, Minn) according to the manufacturer’s protocol. TGFβ1 activity was normalized to total protein.

**Bradykinin Assay**

LV endocardium (100 to 200 mg) was homogenized in 10 mmol/L Tris-HCl, pH 7.5 (1:2), containing complete protease inhibitor cocktail (Roche) and centrifuged at 10 000 g (15 minutes at 4°C). The supernatant was acidified with an equal volume of 1% trifluoroacetic acid in water and loaded onto a purification C₁₈ Sep-Pak cartridge (Waters Chromatography Division, Milford, Mass). Sample was loaded and the cartridge was washed with 6 mL of 1% trifluoroacetic acid in water. Peptides were eluted with 3 mL trifluoroacetic acid/acetonitrile/water (0.4:60:39.6), lyophilized overnight, and then dissolved in 50 μL H₂O. Peptides were eluted with 3 mL trifluoroacetic acid in water. Peptides were eluted with 3 mL trifluoroacetic acid/acetonitrile/water (0.4:60:39.6), lyophilized overnight, and then reconstituted in sample buffer from the Bradykinin enzyme immunoassay kit (S-1135, Peninsula Laboratories, San Carlos, Calif) and measured according to the manufacturer’s protocol. Bradykinin levels were normalized to total tissue weight.

**Statistics**

Data are presented as mean±SEM. Statistical analysis was performed on all groups by 1-way ANOVA and the Holm-Sidak posthoc test for significance with SigmaStat version 3.5. Values of *P*<0.05 were considered significant. ANCOVA (adjusting for gender) was performed on torsion angle, fractional shortening, and titin expression, and there was no effect of gender on outcome.

## Results

**LV Morphometry and Hemodynamics**

MR and MR+CI dogs had a similar increase in ratio of LV to body weight without an increase in right ventricular mass (Table 1). Mean heart rate, cardiac output, mean arterial pressure, pulmonary arterial wedge pressure, and pulmonary and systemic vascular resistances did not differ from normal in MR and MR+CI dogs. The failure of CI to decrease LV mass in MR dogs, as previously reported with renin-angiotensin system blockade,1,7 may be due to the failure of CI to decrease mean arterial pressure or systemic vascular resistance.

### Table 1. LV Morphometry and Hemodynamics in Normal and MR Dogs

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>MR</th>
<th>MR+CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>21±1</td>
<td>23±1</td>
<td>22±1</td>
</tr>
<tr>
<td>LV/body weight, g/kg</td>
<td>3.9±0.2</td>
<td>4.6±0.2*</td>
<td>4.7±0.2*</td>
</tr>
<tr>
<td>RV/body weight, g/kg</td>
<td>1.6±0.1</td>
<td>1.7±0.1</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>Sample size, n</td>
<td>13</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>100±3</td>
<td>109±4</td>
<td>109±4</td>
</tr>
<tr>
<td>Cardiac output, L/min</td>
<td>3.6±0.2</td>
<td>3.3±0.2</td>
<td>3.5±0.3</td>
</tr>
<tr>
<td>LV end-systolic pressure, mm Hg</td>
<td>105±3</td>
<td>109±3</td>
<td>105±2</td>
</tr>
<tr>
<td>Systemic vascular resistance, dynes·cm⁻¹·s⁻¹</td>
<td>2011±122</td>
<td>2207±225</td>
<td>1942±206</td>
</tr>
<tr>
<td>Pulmonary artery mean pressure, mm Hg</td>
<td>10±1</td>
<td>11±1</td>
<td>12±1</td>
</tr>
<tr>
<td>Pulmonary artery wedge pressure, mm Hg</td>
<td>3±0.4</td>
<td>5±1</td>
<td>5±1</td>
</tr>
<tr>
<td>Pulmonary vascular resistance, dynes·cm⁻¹·s⁻¹</td>
<td>170±14</td>
<td>176±24</td>
<td>167±15</td>
</tr>
<tr>
<td>Sample size, n</td>
<td>17</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

RV indicates right ventricular. Baseline values represent hemodynamics in all MR dogs (n=17) before the induction of MR. Values are mean±SEM. *P*<0.05, significant difference from the normal group.

### Table 2. MRI-Derived Indices of LV Remodeling in Normal and 4-Month-MR Dogs

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>MR</th>
<th>MR+CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV end-diastolic volume, mL</td>
<td>58±3</td>
<td>83±10*</td>
<td>87±10*</td>
</tr>
<tr>
<td>LV end-systolic volume, mL</td>
<td>38±2</td>
<td>48±6</td>
<td>51±7</td>
</tr>
<tr>
<td>LV stroke volume, mL</td>
<td>20±2</td>
<td>35±5*</td>
<td>36±4*</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>34±2</td>
<td>44±2*</td>
<td>42±3*</td>
</tr>
<tr>
<td>LV end-diastolic dimension, cm</td>
<td>3.6±0.2</td>
<td>4.3±0.1*</td>
<td>4.3±0.2*</td>
</tr>
<tr>
<td>LV end-systolic dimension, cm</td>
<td>3.3±0.1</td>
<td>3.6±0.1*</td>
<td>3.6±0.2*</td>
</tr>
<tr>
<td>Left atrial volume, mL</td>
<td>144±1</td>
<td>340±75*</td>
<td>344±64*</td>
</tr>
<tr>
<td>LV early diastolic filling rate, mL/s</td>
<td>148±24</td>
<td>240±48</td>
<td>269±48</td>
</tr>
<tr>
<td>LV early diastolic filling rate, EDVs</td>
<td>2.5±0.4</td>
<td>2.9±0.5</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>Peak early mitral annular velocity, mm/s</td>
<td>30±3.5</td>
<td>30±12</td>
<td>52±14</td>
</tr>
<tr>
<td>Peak early mitral annular velocity, % LA length/s</td>
<td>53±6</td>
<td>48±20</td>
<td>81±22</td>
</tr>
<tr>
<td>Normalized peak LV systolic torsion angle, °</td>
<td>2.54±0.84</td>
<td>3.15±0.36</td>
<td>4.16±0.31*</td>
</tr>
<tr>
<td>Sample size, n</td>
<td>7</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

EDV indicates end-diastolic volume; LA, long axis. Values are mean±SEM. *P*<0.05, significant difference from normal group. †P*<0.05, significant difference from MR group.

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Image version 4.0.3.2 (Scion Corp, Frederick, Md), and normalized to β-actin.

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**Bradykinin Assay**

LV tissue lysates were separated on 1% to 2% vertical SDS-agarose gels and stained with Coomassie blue. Wet gels were scanned and analyzed with 1-dimensional scan software (Scanalytics, Dayton, Ohio). The integrated optical densities of N2BA titin, N2B titin, total titin (N2BA plus N2B), and myosin heavy chain were determined as a function of the volume of the solubilized protein sample that was loaded (a range of volumes were loaded on each gel). The slope of the linear range of the relation between integrated optical density and loaded volume was obtained for each protein. This slope was converted to integrated optical density per 1 mg tissue (samples were solubilized at a fixed ratio of tissue weight and volume of solubilization solution).

LV endocardial midwall tissue (50 to 100 mg) was homogenized in 50 mmol/L Tris-HCl, pH 7.6, 0.01% Triton X-100, 15 mmol/L NaCl, 0.5 mmol/L CaCl₂, and 20 μmol/L ZnCl₂ and centrifuged at 16 000 g (30 minutes at 4°C). Lysates were assayed with the Biotrack MMP-2 activity assay System (RPN 2631, Amersham Biosciences, Inc, Piscataway, NJ). MMP-2 activity was normalized to total protein. For gel zymography, lysates were mixed with an equal volume of Tris-glycine SDS sample buffer (2X; Novex, Invitrogen) and separated on a 10% (vol/vol) Tris-glycine polyacrylamide gel containing 0.1% (wt/vol) gelatin. Gels were developed and stained with Colloidal Blue Staining Kit according to the protocol for Novex Zymogram gels (Invitrogen). Dog serum served as a positive control for both MMP-2 and MMP-9.

LV endocardial tissue (50 to 100 mg) was homogenized (50 mmol/L HEPES, 50 mmol/L Na₃O₄P₂, 100 mmol/L NaF, 10 mmol/L EDTA, pH 7.4, plus complete protease cocktail inhibitor; Roche, Indianapolis, Ind) and centrifuged at 16 000 g (20 minutes at 4°C). Lysates were assayed with the TGFβ1 ELISA kit (R&D Systems, Minneapolis, Minn) according to the manufacturer’s protocol. TGFβ1 activity was normalized to total protein.

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LV Remodeling and Function

LV end-diastolic volume and dimension were increased >40% in MR and MR+CI versus normal dogs (Table 2). In both MR groups, there was at least a 2-fold increase in LV stroke volume and left atrial volume in the absence of any other valvular disease. These findings, coupled with significant MR by analysis of cine MRI in each dog, documented a significant amount of regurgitation in all MR dogs. MRI-derived indices of diastolic function, including early diastolic filling from time-volume curves and early diastolic mitral annular velocity, tended to be increased compared with normal in both MR and MR+CI dogs (Table 2). MRI tissue tagging-derived peak LV systolic torsion angle was increased in MR+CI versus MR and normal dogs (Table 2).

Effect of Chymase Inhibition on ECM, FAK, and Bradykinin

CI had no effect on the decrease in LV endocardial and epicardial collagen (Figure 1A) and LV endocardial collagen by hydroxyproline (Figure 1B) in MR dogs. However, MMP-2 and MMP-9 activity did not differ from normal in MR and MR+CI dogs (Figure 1C and 1E). TGFβ1 activity was significantly decreased in MR dogs but did not differ from normal in MR+CI dogs (Figure 1D). Laminin was

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**Figure 1.** Effect of chymase inhibition on collagen homeostasis. Quantification of interstitial collagen volume percent in picric acid Sirius red F3BA (PASR)-stained sections (A) and of total collagen by hydroxyproline in LV endocardium (B). Quantitative assay of MMP-2 activity (C) and TGFβ1 activity (D) and representative gel zymography demonstrating MMP-2 and -9 (E) and bradykinin levels (F) in LV endocardial tissue from normal, MR, and MR+CI hearts. Values are mean±SEM. *P<0.05 vs normal; #P<0.05 vs MR.

**Figure 2.** Effect of chymase inhibition on noncollagen ECM proteins. Representative Western blots of laminin and fibronectin protein in normal (n=7), MR (n=7), and MR+CI (n=8) LV endocardium and epicardium. Densitometric analysis of laminin and fibronectin protein expression normalized to β-actin. Values are mean±SEM. *P<0.05 vs normal; #P<0.05 vs MR.
significantly decreased in the LV endocardium but not epicardium of MR and MR+CI dogs (Figure 2), whereas fibronectin was decreased in the MR endocardium and preserved with CI. There was a significant decrease in LV endocardial FAK phosphorylation in MR dogs, which was normalized with CI (Figure 3). In contrast, LV epicardial fibronectin was increased in MR dogs and MR+CI dogs compared with normal. LV epicardial phospho-FAK did not differ between normal and MR dogs but was significantly increased in MR+CI dogs (Figure 3). There was a 12-fold elevation in LV bradykinin levels in MR versus normal dogs (P<0.05), which was normalized in MR+CI dogs (Figure 1F).

**Isolated Cardiomyocyte Function**

MR hearts had myofibrillar loss characterized by the appearance of perinuclear vacuoles and cellular debris (Figure 4B and 4D) versus normal hearts (Figure 4A). This was significantly improved in MR+CI hearts (Figure 4C and 4E). Furthermore, cardiomyocyte fractional shortening was significantly decreased in MR versus normal and normalized in MR+CI cardiomyocytes (Table 3). However, calcium transients were equally depressed in both MR and MR+CI cardiomyocytes versus normal cells.

**Effect of Chymase Inhibition on Cardiomyocyte Sarcomeric Titin**

There was a significant increase in total titin levels in the LV epicardium of MR+CI versus normal and MR dogs (Figure 5A and 5B). In addition, there was a significant increase in LV epicardial N2B titin isoform in MR+CI dogs versus normal dogs (Figure 5B). In contrast, there were no significant changes in LV endocardial titin (Figure 5C).

**Discussion**

In the present study, we demonstrate that chronic chymase inhibition does not prevent collagen loss or eccentric LV remodeling in the MR dog. However, chymase inhibition preserves LV fibronectin and FAK phosphorylation, which significantly attenuates myofibrillar degeneration and improves cardiomyocyte function and LV torsion in isolated MR.

In MR and MR+CI dogs, there are equivalent increases in LV end-diastolic volume, left atrial volume, and stroke volume, which is consistent with similar 25% LV interstitial collagen loss in both groups. This is not associated with an increase in MMP-2 or MMP-9 activity in either group. Previous studies in canine MR have demonstrated a 6-fold increase in MMP-9 activity 6 hours after MR induction,
which returns to normal after 14 days, suggesting a short-term rather than prolonged activation of MMPs. In our MR dogs, a decrease in TGFβ1 activity is not improved by chymase inhibition, which can explain the persistent decrease in LV collagen or laminin in MR+CI dogs. Taken together, the early loss of interstitial collagen in MR may be related to elevated MMP activity, whereas long-term collagen loss may be due to decreased ECM synthesis. This may explain the failure of CI to improve LV dilatation. Furthermore, other mast cell contents such as tumor necrosis factor-α, which can activate MMPs and has been shown to be upregulated in MR, may be unaffected by CI.

Chymase inhibition prevents the loss of LV endocardial fibronectin in MR, in keeping with its ability to directly degrade fibronectin. It is interesting that LV bradykinin in MR hearts is increased 12-fold and normalized by CI. The increase in LV bradykinin may be due to chymase activation of kallikrein. Bradykinin has been shown to reduce fibronectin messenger RNA and protein expression by adult cardiac fibroblasts, whereas knockdown of kallikrein results in increased fibronectin expression in kidney mesangial cells. Thus, it is tempting to speculate that chymase mediates fibronectin loss by direct degradation and by indirectly decreasing its synthesis through increased bradykinin formation in the MR heart.

Chymase degradation of fibronectin has also been shown to cause a decrease in FAK phosphorylation, resulting in smooth muscle cell death. We have previously demonstrated a decrease in FAK tyrosine phosphorylation and disruption of the focal adhesion complex in 2- and 4-week MR dogs. In the present study, there is a concordant decrease in fibronectin and FAK phosphorylation in the LV endocardium of 4-month MR dogs. However, there is no evidence of increased apoptosis by staining with terminal deoxynucleotidyl transferase dUTP nick-end labeling in acute MR or 4-month MR dogs. Interestingly, LV epicardial fibronectin is increased in both MR and MR+CI dogs, and this increase is associated with preserved or increased FAK phosphorylation, respectively. An intact focal adhesion complex is essential for cell growth, survival, and myofibril assembly. As opposed to the volume-overloaded MR heart, increased FAK phosphorylation has been documented in animal models of pressure overload. Interestingly, mice with selective inactivation of cardiomyocyte FAK demonstrate increased myofibrillar degeneration, elongation and thinning of cardiomyocytes, and eccentric LV remodeling and heart failure after 6 months. Indeed, there is increased myofibrillar degeneration in MR hearts in the presence of decreased fibronectin and FAK phosphorylation, all of which are improved by CI. Thus, the decrease in FAK phosphorylation in MR and myofibrillar loss may be initiated by chymase-mediated breakdown of fibronectin in the cardiac interstitium.

Previous studies have implicated myofibrillar loss in the pathophysiology of contractile dysfunction in isolated MR. In the present investigation, isolated cardiomyocyte function is significantly depressed in MR dogs and improved with CI without an improvement in intracellular calcium transients. These data suggest that improved cardiomyocyte contractile performance is associated with preservation of myofibrillar structure. In addition, β-receptor blockade has also been shown to improve cardiomyocyte function and myofibrillar density in the MR dog. In MR+CI dogs, cardiomyocyte

| Table 3. Isolated Cardiomyocyte Function in Normal and MR Dogs |
|---------|---------|---------|
| Fractional shortening, % | Normal | MR | MR+CI |
| Calcium transients, F0/Fmax | Normal | MR | MR+CI |
| Dogs (total cells sampled), n | 8 (91) | 8 (134) | 8 (125) |

Values are mean±SEM.

Figure 5. Cardiomyocyte sarcomeric titin in MR. LV tissue lysates were separated by vertical SDS–agarose gel electrophoresis in normal (n=7), MR (n=8), and MR+CI (n=8) dogs. A, Representative blot of total titin (N2BA and N2B isoforms) and the titin degradation product T2 in the epicardium. Bands are normalized to myosin heavy chain (MHC) for quantitative analysis of LV epicardium (B) and endocardium (C). Values are mean±SEM. *P<0.05 vs normal; #P<0.05 vs MR.
functional improvement is not accompanied by an increase in LV ejection fraction but rather demonstrates an increase in LV torsion angle that is greater than both normal and MR dogs.

One of the major myofibrillar proteins in the heart is the large sarcomeric protein titin, which exists as 2 isoforms, N2BA and N2B. Previous studies demonstrate a relationship between increased LV diastolic stiffness and total titin levels and a shift from the more compliant N2BA isoform to the stiffer N2B in animal models of heart failure and pressure overload and in patients with aortic stenosis. However, MRI-derived indices of diastolic function are not compromised in MR+CI dogs despite increases in LV epicardial N2B titin, most likely counterbalanced by the persistent decrease in interstitial collagen. There is emerging evidence that titin also affects systolic function by determining lattice spacing of myofilaments and calcium sensitivity. In the present study, LV endocardial total titin does not differ among normal, MR, and MR+CI dogs, whereas LV epicardial titin is increased with a shift to the stiffer N2B isoform in MR+CI dogs. We speculate that this gradient of titin expression from the endocardium to epicardium can explain the significant increase in LV torsion in MR+CI dogs.

Torsion is the wringing motion of the LV along its long axis during systole, induced by contracting myofibers that are aligned 180° from the endocardium to epicardium. Studies in the intact animal demonstrate that short-term increases in preload and contractility are associated with increased LV torsion. Studies in the mouse heart demonstrate a gradient of myosin phosphorylation from the endocardium to epicardium that facilitate torsion because the direction of torsion during systole follows the direction of the epicardial fibers. However, changes in myofiber orientation and function, as well as collagen and/or other matrix proteins, from the endocardium to epicardium may affect torsion. Taken together, myofibrillar preservation combined with increased fibronectin and titin N2B expression from the endocardium to epicardium in MR+CI dogs may indeed drive the higher torsion angle. Furthermore, FAK is located at the Z disk at the insertion of titin in the cardiomyocyte, and there is evidence that the N2B region of titin interacts with integrins and FAK. Future studies will elucidate the mechanism that causes a titin isoform shift in the myocardium and whether this is dependent on an intact fibronectin-FAK interaction.

The LV endocardium is subjected to higher wall stress than the epicardium during a hemodynamic load. Thus, it is not unexpected that there is a greater loss of laminin in LV endocardium compared with LV epicardium of MR hearts, which is also paralleled by greater decreases in LV endocardial fibronectin. Taken together, better structural integrity and function of the LV epicardium versus endocardium may account for the greater torsion angle in the MR+CI dogs. Indeed, it has been postulated that an increased LV torsion angle in patients with aortic stenosis may represent an important functional compensation for a decrease in LV endocardial blood flow and function in concentric LV hypertrophy.
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**CLINICAL PERSPECTIVE**

The volume overload of isolated mitral regurgitation currently has no recommended therapy that improves left ventricular function or delays the need for surgery. Numerous studies in animal models have demonstrated extracellular matrix loss, rather than accumulation, in the pathophysiology of progressive left ventricular remodeling in a pure volume overload. Thus, blockade of the renin-angiotensin system has not proved successful in a situation of volume overload, in which collagen loss and cardiomyocyte slippage may occur early in the course of left ventricular remodeling. Chymase, in addition to formation of angiotensin II, can activate matrix metalloproteinases and directly degrade matricellular proteins important not only in connections to collagen but also in the initiation of detrimental intracellular signaling processes. Thus, blockade of chymase may prove to be an important therapeutic target in a pure volume overload.
Chymase Inhibition Prevents Fibronectin and Myofibrillar Loss and Improves Cardiomyocyte Function and LV Torsion Angle in Dogs With Isolated Mitral Regurgitation


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