Myocardial Collagen Turnover in Hypertrophic Cardiomyopathy

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Background—Myocardial interstitial fibrosis is a characteristic of hypertrophic cardiomyopathy (HCM). This study evaluated the collagen turnover in HCM and its impact on left ventricular (LV) diastolic function.

Methods and Results—Thirty-six HCM patients and 14 sex- and age-matched controls were studied. Collagen turnover was assessed as follows. By radioimmunoassay, a byproduct of collagen III synthesis (PIIINP) and 3 peptides resulting from collagen I synthesis (PICP and PINP) and degradation (ICTP) were measured. By ELISA, matrix metalloproteinases (MMPs) were determined, as follows: active MMP-2; active MMP-9; and MMP-1 as active, free (as active MMP-1 plus its precursor), and total (as free MMP-1 plus MMP-1/tissue inhibitor complexes). Tissue inhibitor of metalloproteinases-1 (TIMP-1) was also assayed. All patients underwent echocardiography. The difference in duration between transmitral forward (A) and pulmonary venous retrograde (AR) waves (A–AR) was considered an estimate of passive diastolic function. Furthermore, restrictive or pseudonormal LV filling patterns were considered to identify patients with passive diastolic dysfunction. Patients had higher levels of PIIINP, ICTP, MMP-2, MMP-9, and total TIMP-1 than did controls. PIIINP was inversely related to LV end-diastolic diameter. A–AR was inversely related to PICP, PINP, and their differences with ICTP (estimates of collagen I buildup). Furthermore, A–AR was directly related to MMP-1 and MMP-2.

Conclusions—As compared with controls, collagen turnover is enhanced in HCM patients. As collagen I synthesis prevails over degradation and MMP-1 and MMP-2 are inhibited, passive diastolic dysfunction occurs in patients with HCM.

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Key Words: diastole ■ cardiomyopathy ■ collagen ■ peptides ■ metalloproteinases

Hypertrophic cardiomyopathy (HCM) is characterized by asymmetrical left ventricular (LV) hypertrophy, disarray, interstitial fibrosis, and increased arteriolar wall thickness. Interstitial fibrosis is quantitatively relevant in HCM; Shirani et al showed that in young patients with HCM and sudden death (not in the dilated phase of the disease), cardiac collagen content was 8-fold greater than in controls and 3-fold greater than in patients with systemic hypertension, a disease known to activate interstitial fibrosis. Such interstitial fibrosis was spread across the left ventricle in HCM, not only in hypertrophied areas.

Collagen accumulation, through an increase in chamber stiffness, is an important determinant of passive diastolic dysfunction in secondary LV hypertrophy. The present study was designed to evaluate myocardial collagen turnover and to assess its impact on diastolic function in patients with HCM.

Methods

Study Population
Thirty-six patients with HCM were enrolled into the study from the Federico II University of Naples outpatient clinic (27 men; mean age 35 ± 9, range 20 to 53 years). Patients were age and sex matched with 14 healthy volunteers (9 men; mean age 35 ± 11, range 21 to 52 years). The diagnosis of HCM was based on the echocardiographic evidence of hypertrophied, nondilated left ventricle without apparent cause, such as systemic hypertension, aortic stenosis, etc. Only patients ranging in age from 20 to 50 years for women and up to 55 years for men were included, as the bone metabolism of these patients is not likely to be in a remodeling phase (ie, growth and senile/postmenopausal osteoporosis). All patients were in sinus rhythm and had a good acoustic window to allow reliable quantitative echocardiography. At the time of enrollment, 9 patients were being treated with β blockers, 6 with verapamil, and 2 with a combination thereof; 2 patients took diuretics, and the remaining 17 patients were not being treated with drugs.

Exclusion criteria were all the conditions in which a physiological or pathological activation of collagen turnover occurs, both in patients and in controls. By history and physical examination, as well as routine tests, we ruled out pregnancy, thyroid diseases, inflammatory states, tumors, organic heart disease other than HCM, systemic hypertension, bone and rheumatic diseases, recent trauma (<2 weeks) or surgery (<6 months), diabetes mellitus, chronic liver diseases or alcohol abuse, kidney insufficiency, and pulmonary fibrosis. Furthermore, treatment with drugs known to influence collagen metabolism (amiodarone, ACE inhibitors, angiotensin-1 or...
aldosterone receptor antagonists, statins, glucocorticoids, and estrogens) constituted grounds for exclusion.

The Ethics Committee of Federico II University of Naples approved the study protocol, and informed consent was obtained from each patient before enrollment.

Serum Measurement of Indices of Collagen Metabolism

To assess collagen turnover, we measured serum levels of peptides released during collagen synthesis and degradation and of the enzymes involved in collagen degradation. Peripheral venous blood samples were centrifuged at 4°C. Aliquots of 250 μL of serum were immediately stored at −20°C until the assay.

During collagen synthesis, procollagen type I carboxy-terminal and amino-terminal propeptidases (PICP and PINP) and procollagen type III amino-terminal propeptide (PIIINP) are cleaved from procollagen I and III, respectively, and released into blood. Collagen is degraded by matrix metalloproteinases (MMPs), as follows: a collagenase (MMP-1) cleaves collagen I and releases into blood its carboxy-terminal telopeptide (ICTP).7 PICP, PINP, PIIINP, and ICTP were measured by radioimmunoassay using antisera kits (Orion Diagnostica, Espoo, Finland). Molar differences between PICP and ICTP and between PINP and ICTP were calculated as estimates of balance between collagen I synthesis and degradation.

Collagen degradation is initiated by MMP-1 and further completed by gelatinases MMP-2 and MMP-9. MMP-1 is blocked by its tissue inhibitor (TIMP-1) by forming MMP-1/TIMP-1 complexes.8 We measured MMPs and TIMP-1 by ELISA kits (Amersham Pharmacia Biotech). All measurements were made in duplicate and averaged.

The activity assays for MMP-1, MMP-2, and MMP-9 use the form of a detection enzyme, which is activated by the respective MMP; free MMP-1 was measured after the activation of the pro-form of MMP-1 and represents the sum of active and pro–MMP-1. A 2-site ELISA “sandwich” format assayed total MMP-1 and total TIMP-1; they both represent free and bound forms. Because of technical problems, PINP, MMPs, and total TIMP-1 were measured in only 28 patients and 10 controls.

Echocardiography

Each patient underwent M-mode, 2-dimensional, and Doppler echocardiographic study (Hewlett-Packard Sonos 5500). All echocardiographic data represent the mean of 3 measurements on different cardiac cycles.

M-mode LV end-diastolic diameter (LVEDD) was measured and normalized to body surface area (LVEDDi).9 Patients with LV dilatation (ie, LVEDDi >32 mm/m²)10 were excluded.

LV hypertrophy was assessed by measuring maximal wall thickness from the short-axis view in 4 segments at the mitral valve and papillary muscle levels. As an indicator of the extent of LV hypertrophy, the sum of maximal wall thickness at either level in each of the 4 ventricular segments11 was considered. As an additional estimate, we calculated the Wille score, a point system to score the degree and distribution of hypertrophy.12

By Doppler echocardiography, peak flow velocities at rapid filling (E) and at atrial contraction (A), their ratio (E/A), and E wave deceleration time (Edec) were calculated at the mitral level; peak systolic (S) and diastolic (D) flow velocities, their ratio (S/D), and retrograde flow velocity (AR) were obtained from the pulmonary vein. As an estimate of diastolic dysfunction, the difference in duration between transmirtal forward and pulmonary venous retrograde waves (A–Ar) was calculated, which is linearly related to LV end-diastolic pressure in HCM.13

The following 4 patterns of LV filling were considered: (1) normal (E/A between 1 and 2, Edec between 150 and 250 ms), (2) prolonged relaxation (ie, E/A <1 and Edec >250 ms), (3) pseudonormal pattern (ie, E/A between 1 and 2, Edec between 150 and 250 ms, and ≥2 abnormal pulmonary venous flow indexes), and (4) restrictive filling (ie, E/A ≥2 and Edec <150). The following measures from pulmonary vein flow were considered abnormal: S/D >1, S/(S+D) (ie, systolic filling fraction) <0.40, retrograde flow velocity >35 cm/s, and negative A–Ar.

LV outflow tract gradient was evaluated using continuous-wave Doppler signals and the simplified Bernoulli equation. Care was taken to distinguish the ejection velocity from the mitral regurgitation jet.14 Color-flow Doppler imaging was used for identifying and semiquantitatively estimating mitral regurgitation.15

Statistics

Data are expressed as mean±1 SD. Normal distribution of all continuous variables was tested using the 1-sample Kolmogorov-Smirnov test. All variables were normally distributed; parametric tests were therefore used. Unpaired Student t test and linear regression analysis were used when appropriate. Age and body mass index were related to some variables in control subjects; therefore, analysis of covariance and partial correlation coefficients were performed to rule out their effects on these variables. A probability value of <0.05 was considered significant. All statistical calculations were performed by SPSS for Windows (release 11.0).

Results

Collagen Turnover in Patients and Controls

The clinical and echocardiographic characteristics of the 36 HCM patients are shown in the Table. Patients with HCM, compared with healthy subjects, had increased values of PIIINP, ICTP, active MMP-2, active MMP-9, and total TIMP-1 (Figure 1). Total MMP-1 values were below the resolution of the assay in controls (in accordance with the literature accompanying the kit) and in HCM patients. PINP and PINP–ICTP were inversely related to body mass index (r =−0.74, P =0.01; and r =−0.87, P =0.01, respectively) in controls. Correlations involving these peptides were thus corrected for body mass index.

An inverse relationship between PICP and age has been reported in normal men, but not in women; our findings were similar in our control men, as follows: (r =−0.70, P =0.05 for PICP; r =−0.75, P =0.03 for ICTP). Total TIMP-1 concentration was directly related to age in the whole group of controls (r =0.73, P =0.02). Linear correlations of parameters that were age dependent in normal men were controlled for age, irrespective of sex, as the number of women was small in both controls and patients.

Relationships Among Markers of Collagen Turnover in HCM

The difference between PICP and ICTP was inversely related to free MMP-1 (r =−0.49, P =0.001).

Active MMP-1 was significantly related to active MMP-2 (r =0.50, P =0.02) and active MMP-9 (r =0.52, P =0.02). Moreover, total TIMP-1 and free MMP-1 were directly related to one another (r =0.53, P =0.004).

Serum Markers of Collagen Metabolism and LV Diastolic Function in HCM

Serum concentration of PIIINP was inversely related to LVEDD (r =−0.36, P =0.03). A–Ar was inversely related to PICP and PINP and to the difference between the former and ICTP (Figure 2). Furthermore, A–Ar was directly related to free MMP-1, active MMP-2 (Figure 3), and total TIMP-1.
(r=0.38, P=0.05); a nonsignificant trend was also observed for active MMP-1 (r=0.35, P=0.07).

We computed the partial correlation coefficient of \((A-Ar)\) controlling for age and found similar relationships for PICP (r=−0.33, P=0.05) and ICTP (r=−0.47, P=0.005), whereas the relationships to total TIMP-1 (r=0.29, P=0.16) no longer reached the significance level. We further calculated the partial correlation coefficient of \((A-Ar)\) and, respectively, PINP and PINP−ICTP controlling for body mass index, and found stronger relationships (r=−0.51, P=0.02; r=−0.48, P=0.03).

To confirm the impact of collagen turnover on diastolic dysfunction, we divided patients into 2 groups according to patterns of diastolic filling (normal plus abnormal relaxation versus pseudonormal plus restrictive filling, on the assumption that the second group identifies patients with passive diastolic dysfunction and increased LV end-diastolic pressure). Patients with pseudonormal or restrictive patterns had higher levels of PICP (P=0.007), ICTP (P=0.009), their difference (P=0.01), and total TIMP-1 (P=0.007). Because these patients were significantly younger than the other subgroup (32±8 versus 38±10 years, P=0.05) and because

### TABLE 1. Clinical and Echocardiographic Characteristics of HCM Patients

<table>
<thead>
<tr>
<th>Continuous Variables</th>
<th>Mean±SD</th>
<th>Range</th>
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<tr>
<td>Age (years)</td>
<td>34.8±9.4</td>
<td>20–53</td>
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<tr>
<td>Hypertrophy score index (mm)</td>
<td>61.5±14.8</td>
<td>40–109</td>
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<tr>
<td>Maximal wall thickness (mm)</td>
<td>20.2±5.5</td>
<td>13–36</td>
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<td>Wigle score</td>
<td>5.7±2.7</td>
<td>1–10</td>
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<td>End-diastolic dimension (mm)</td>
<td>43.3±5.3</td>
<td>35–59</td>
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<tr>
<td>End-diastolic dimension index (mm/m²)</td>
<td>23.3±2.9</td>
<td>17–29</td>
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<td>Fractional shortening (%)</td>
<td>44.0±7.7</td>
<td>27.5–60.5</td>
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<tr>
<td>Outflow gradient (mm Hg)</td>
<td>16.7±39.3</td>
<td>0–110</td>
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<tr>
<td>((A-Ar)) (ms)</td>
<td>3.8±38.7</td>
<td>−75–79</td>
</tr>
<tr>
<td>Left atrial diameter (mm)</td>
<td>41.9±6.1</td>
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<th>Categorical Variables</th>
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<td>I</td>
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<td>Outflow gradient ≥30 mm Hg at rest</td>
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<tr>
<td>Mitral regurgitation ≥2</td>
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<tr>
<td>((A-Ar) &lt;0)</td>
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<tr>
<td>Filling patterns (restrictive + pseudonormal)</td>
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<td>53</td>
</tr>
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</table>

**Figure 1.** Comparison between controls and patients with HCM of peptides derived from collagen III synthesis (PII-INP), and from collagen I synthesis (PICP and PINP) and degradation (ICTP) (upper panel), and MMP-1 (free and active forms), active MMP-2, active MMP-9, and their tissue inhibitor (TIMP-1) (lower panel).
PICP, ICTP, and TIMP-1 were influenced by age in controls, we computed the analysis of covariance correcting for age and found similar differences ($P=0.04$, $P=0.05$, $P=0.03$).

### Other Possible Determinants of Altered Cardiac Collagen Metabolism in HCM

We did not find any correlation between serum markers of collagen turnover and severity of LV hypertrophy assessed as maximal LV wall thickness, LV hypertrophy score index, and Wigle score. Furthermore, we did not find any relationship with the magnitude or presence of LV outflow gradient, or with the degree of mitral regurgitation.

### Discussion

Diastolic dysfunction is a common feature in HCM and a major determinant of symptoms of pulmonary congestion. Isovolumetric relaxation is an active phenomenon regulated by the interplay of several factors, among them calcium metabolism, adrenergic activation, ischemia, etc. Passive diastolic function, in contrast, is mainly determined by LV wall properties, namely hypertrophy and interstitial fibrosis.

We have shown that passive diastolic function is the most important determinant of exercise tolerance in HCM.

Recent studies in transgenic animals with HCM support the hypothesis that interstitial fibrosis is secondary to the impairment in contractility of the mutant proteins and the consequent activation of trophic factors. Moreover, these studies demonstrate the possibility of influencing by drugs the extracellular matrix in HCM. In patients with LV hypertrophy secondary to systemic hypertension, myocardial interstitial fibrosis decreases after treatment with drugs that interfere with the renin-angiotensin-aldosterone system. This has been shown by the same biochemical markers used herein, as well as by endomyocardial biopsies. It is therefore relevant to assess the impact of collagen turnover on LV anatomy and function even in patients with HCM, in view of possible pharmacological interventions, which would offer an important tool in the (as yet largely unsatisfactory) treatment of symptoms due to diastolic dysfunction.

Figure 2. Correlation between difference in duration of forward and retrograde A wave (A−A) and PICP and PINP (left panel) and differences between PICP−ICTP and PINP−ICTP (right panel) in patients with HCM. To enhance clarity, confidence intervals are not shown.

Figure 3. Correlation between difference in duration of forward and retrograde A wave (A−A) and free MMP-1 (left panel) and active MMP-2 (right panel) in patients with HCM. Curved lines on either side of straight, fitted lines represent 95% CI of the mean (solid inner lines) and single observations (dashed outer lines).
Collagen Turnover in Patients and Controls

Patients with HCM present with an increase in collagen III synthesis and a contemporary degradation of collagen I (Figure 1). This suggests a remodeling in the extracellular matrix composition, with a shift from collagen I to collagen III. Our data cannot provide an explanation for this finding; because collagen I is reported to be stiffer than collagen III, a compensatory mechanism to the increase in wall stiffness (due to hypertrophy, disarray, and interstitial fibrosis) could be hypothesized.

MMP-2 and MMP-9 were significantly higher in patients with HCM than in controls (Figure 1). This finding suggests an activation of the extracellular matrix in HCM. Total TIMP-1 levels are also increased in patients compared with controls, and a similar finding has been observed recently in patients with systemic hypertension. TIMP-1 is the inhibitor of MMPs, and its increase might be the consequence of a feedback regulation to compensate increased MMP activity and collagen I degradation.

In HCM, contractility is decreased at a cellular level as a result of the reduced motility of mutant contractile proteins; this impairment might trigger, as a compensatory mechanism, local release of trophic factors (such as tumor necrosis factor α, endothelin-1, transforming growth factor β1, and insulin-like growth factor 1), which activate the extracellular matrix, leading to the development of interstitial fibrosis and altering the scaffold on which myocytes align, thus causing disarray.

It is, however, unproven whether impaired contractility causes disarray or disarray is the primary feature of the disease from which the impairment in LV contractility partly derives.

Relationships Among Markers of Collagen Turnover in HCM

The linear relationships of active MMP-1 with active MMP-2 and active MMP-9 suggest a homogeneous trend in HCM of all of the MMPs that we have explored. The difference between PICP and ICTP, which indicates the balance between collagen I synthesis and degradation, correlates inversely with free MMP-1. In other words, collagen I builds up when collagenase is suppressed. A similar inverse relationship has been found with the MMP inhibitor, TIMP-1; we can speculate that this finding is the consequence of a regulatory mechanism, as follows: when the MMPs decrease, so does their inhibitor. This hypothesis is supported by the direct linear relationship between free MMP-1 and total TIMP-1, as well as by similar findings in different human and animal models.

Collagen Turnover and Diastolic Dysfunction

In our patients, serum concentration of PIIINP is higher in the presence of a small left ventricle, which represents an anatomical marker of passive diastolic dysfunction in HCM.

We assessed passive diastolic function by A−Ar, a parameter dependent on LV end-diastolic pressure in HCM. In a transgenic rabbit model of HCM, it has been shown that simvastatin causes regression of interstitial fibrosis on histology and a contemporary improvement in diastolic function, as assessed by this index; thus, it seems that A−Ar adequately describes passive diastolic dysfunction resulting from increased interstitial fibrosis in HCM.Interstitial fibrosis is known to affect mainly myocardial stiffness; in HCM, the process is diffuse and involves all ventricular walls that exhibit altered regional stiffness. It is conceivable then that a homogeneous impairment in myocardial stiffness results in a parallel impairment in chamber stiffness, as shown in secondary hypertrophy due to aortic stenosis.

The deterioration in passive diastolic function, as assessed by the decrease in A−Ar, is paralleled by an increase in collagen I synthesis, as PICP and PINP increase (Figure 2). Furthermore, our data show that, when collagenase MMP-1 and gelatinase MMP-2 activities decrease, passive diastolic function is impaired (Figure 3); these results complement one another, in that the observed increase in collagen I synthesis with a concurrent suppression of catabolic enzymes leads to diastolic dysfunction. Similar findings were observed in patients with systemic hypertension. Collagen I degradation, as assessed by ICTP, however, increases together with synthesis in our patients; hence, to evaluate the balance between synthesis and degradation, we computed the differences between the 2 peptides of synthesis (PICP and PINP) and ICTP; this can be done because, for each molecule of collagen that is synthesized or degraded, 1 molecule of such peptides is released. Such differences were inversely related to A−Ar (Figure 2); this indicates that, when the balance is shifted toward synthesis, collagen I accumulates and causes passive diastolic dysfunction.

Our interpretation is confirmed when a different, qualitative estimate of passive diastolic dysfunction (patterns of LV filling) is used. Patients with a pseudonormal or restrictive filling pattern (ie, those with elevated LV filling pressures) had increased collagen I synthesis as PICP was significantly increased and showed signs of collagen I accumulation as PICP−ICTP was significantly increased.

Collagen Metabolism and LV Hypertrophy

In our study, no correlations between serum markers of collagen turnover and various scoring systems to assess LV hypertrophy were found. This finding is not surprising, in light of the autopsic observation by Shirani et al, who found no statistically significant relationship between collagen content and septal thickness. Varnava et al, in a morphological study, found a weak but significant relationship between fibrosis and heart weight. However, within each heart, correlations between regional thickness and fibrosis were inconsistent, suggesting that the relationship between wall thickness and interstitial fibrosis remains elusive in HCM. In addition, our approach analyzes collagen turnover instead of collagen content; one might speculate that collagen turnover is elevated during the development of LV hypertrophy and not when hypertrophy is in a steady state, as is the case in our adult patients.

Collagen Metabolism and LV Obstruction

No relationships between the serum markers of collagen turnover and the magnitude or the presence of LV outflow gradient were found. This suggests that in HCM the mechanical load due to the outflow gradient is not sufficient to cause
an activation of extracellular matrix as in secondary hypertrophy. It is reasonable to think that this occurs because of the dynamic nature of obstruction in HCM, which varies as a consequence of hemodynamic and functional states.

Limitations of the Study

No cardiac biopsies were performed in this study. Microscopic examination of cardiac biopsies is the most reliable method for measuring myocardial fibrosis, but this approach was not indicated in our patients; therefore, we used a reliable biochemical method already used by other groups in other cardiac diseases. It has been shown in secondary hypertrophy that PICP concentration correlates with myocardial collagen content assessed by myocardial biopsies. For the same lack of clinical indication, we did not perform cardiac catheterization to assess diastolic function.

Furthermore, because none of the markers we used is heart specific, we selected our study population very carefully to exclude other conditions that affect collagen turnover; one could hypothesize, however, that patients with severe symptoms were inactive, leading to bone catabolism as a source of the collagen degradation peptide ICTP. This is unlikely, as all of our patients had no or only modest exercise limitation, and none were inactive.

Total MMP-1 concentration was below the resolution of the assay in patients and controls, so we could not calculate the levels of TIMP-1/MMP-1 complexes (as the difference between total MMP-1 and free MMP-1) and, consequently, of free TIMP-1 (as the difference between total TIMP-1 and TIMP-1/MMP-1 complexes). This assessment could have given us a better picture of collagen metabolism.

Acknowledgments

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References

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