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Matrix Metalloprotease Activity Is Enhanced in the Compensated but Not in the Decompensated Phase of Pressure Overload Hypertrophy

Rossana Tozzi, Giuseppina Palladini, Silvia Fallarini, Rosanna Nano, Chiara Gatti, Cristina Presotto, Antonio Schiavone, Rosella Micheletti, Patrizia Ferrari, Roberto Fogari, and Stefano Perlini

Background: During the transition of pressure overload hypertrophy (POH) to heart failure (HF) there is intense interstitial cardiac remodeling, characterized by a complex balance between collagen deposition and degradation by matrix metalloproteases (MMPs). This study was aimed at investigating the process of cardiac remodeling during the different phases of the transition of POH to HF.

Methods: Guinea pigs underwent thoracic descending aortic banding or sham operation. Twelve weeks after surgery, left-ventricular (LV) end-diastolic internal dimension and ventricular systolic pressure were measured by combined M-mode echocardiography and micromanometer catheterization. The MMP activity, tissue-specific MMP inhibitors (TIMPs), and collagen fraction were evaluated in LV tissue samples by zymography, ELISA, and computer-aided analysis, respectively.

Results: Banded animals were divided by lung weight values into either compensated left-ventricular hypertrophy (LVH) or HF groups, as compared with sham-operated controls. All HF animals exhibited a restrictive pattern of Doppler transmitral inflow, indicative of diastolic dysfunction, and developed lung congestion. Compensated LVH was associated with increased MMP-2 activity, which was blunted after transition to HF, at a time when TIMP-2 levels and collagen deposition were increased.

Conclusions: The cardiac remodeling process that accompanies the development of POH is a phase-dependent process associated with progressive deterioration of cardiac function. Am J Hypertens 2007;20:663–669

Key Words: Extracellular matrix, fibrosis, interstitial remodeling, matrix metalloproteinases, pressure overload hypertrophy.

The development of hypertensive left-ventricular hypertrophy (LVH) and its transition to heart failure (HF) are associated with intense interstitial cardiac remodeling and progressive diastolic dysfunction, an important cause of clinical HF.[1] Left-ventricular (LV) dilation, a common feature of HF-associated cardiac remodeling, appears to play a causal role in the evolution of LV dysfunction. However, the precise molecular mechanisms mediating LV chamber dilation in the setting of hypertensive heart disease are still being elucidated.[2] In both human and animal studies, it has been reported that alterations in the collagen structure and composition occur within the myocardium, which may in turn influence LV geometry and function. Myocardial collagen fibrils ensure structural integrity of the adjoining myocytes, provide the means by which myocyte shortening is translated into overall LV pump function, and are essential for maintaining the alignment of the myofibrils within the myocyte through a collagen–integrin–cytoskeletal myofibril relation. Disruption or discontinuity within the fibrillar extracellular matrix (ECM) network will result in loss of the normal structural support, thereby leading to abnormal stress and strain patterns imposed on the myocyte fascicles during the cardiac cycle. This may in turn result in a

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From the Clinica Medica II, Fondazione IRCCS San Matteo, University of Pavia (RT, GP, CG, RF, SP), Pavia; Department of Animal Biology University of Pavia and IGM-CNR Department of Histochemistry and Cytochemistry (SF, RN), Pavia; and Prassis Sigma-Tau, Settimo Milanese (CP, AS, RM, PF), Milan, Italy.

Address correspondence and reprint requests to Dr. Stefano Perlini, Clinica Medica II, Fondazione IRCCS San Matteo, P.le Golgi 2, 27100 Pavia, Italy; e-mail: sperlini@unipv.it

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process that has been described as myocyte "slippage,"[3] associated with progressive LV chamber dilation, as well as with systolic and diastolic dysfunction. A key role in ECM remodeling and in LV dilation is played by a family of collagenolytic enzymes, known as matrix metalloproteinases (MMPs), that degrade several ECM components such as collagen, gelatin, elastin, and fibronectin.[4,5] The MMPs are finely controlled by specific tissue inhibitors (TIMPs) that prevent excessive and disproportionate matrix degradation. Both MMPs and TIMPs are involved in the regulation of ECM turnover of a variety of tissues, including the arterial wall and the myocardium.[6] In particular, gelatinases (MMP-2, MMP-9) may contribute to LV remodeling by altering both the basement membrane, which plays an important role in regulating molecular traffic around cardiomyocytes, vascular permeability, and LV interstitial hydration,[7] and the collagen fibers (struts) that connect each myocyte to the adjacent cells, thus preventing cell slippage.[3] Therefore, MMPs are very likely involved in the pathogenesis of vascular and cardiac hypertrophy.[8] Although it has been shown that structural changes induced by MMP activation are accompanied by alterations in LV function,[9] little is known on the dynamic changes in MMP activity and in TIMP concentration that might take place in the myocardial interstitium during the different phases of hypertensive heart disease.

The aim of this work was therefore to test the hypothesis that the interstitial remodeling process is not uniform during the different phases of the development of pressure overload hypertrophy (POH) and its progression to overt HF. In vivo pressure and echo-derived dimensions were contrasted to ex vivo evaluation of collagen deposition (fibrosis) and MMP-induced degradation in animals with either compensated LVH or already in overt HF.

Methods

Experimental Design

The experiments were performed on 31 five-week-old male outbred Guinea pigs (350 to 400 g; Charles River, Calco, Italy). Animals were housed under controlled environmental conditions, with food and water ad libitum. All procedures involving animals and their care were conducted in conformity with the "Guide of the Care and Use of Laboratory Animals" (NIH publication no. 86-23, revised 1985). To induce experimental hypertension, guinea pigs underwent thoracic descending aortic banding ($n = 14$) or sham operation ($n = 17$)[10] under ketamine–xylazine (100 mg/kg and 5 mg/kg, intraperitoneally) anesthesia. A silk suture was placed around the descending thoracic aorta and a 23-gauge needle. After securing the suture, the needle was removed, thereby creating a partial aortic constriction. Sham-operated animals underwent the same procedure without tightening the suture.

Echocardiographic and Hemodynamic Assessment

Twelve weeks after surgery, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) to perform an echocardiographic study with an ESAOTE sector scanner (Caris model 7200; Esaote SpA, Genova, Italy) equipped with a 7.5-MHz transducer. The LV end-diastolic (EDD) and end-systolic dimensions (ESD) were obtained from two-dimensional directed M-mode tracings in the parasternal short axis view at the level of the papillary muscles.[11–13] Transmitral Doppler flow velocity was measured at the tip of the valve leaflets. After a 24-h recovery, animals were anesthetized with urethane (1.5 g/kg, intraperitoneally), and a 2F Millar cathether (Millar Instruments, Houston, TX) coupled with a Gould amplifier (model 1 3-4615-50; Gould Inc., Cleveland, OH) was inserted into the left ventricle through the right carotid artery. Recordings of the LV pressure signal were displayed on a Gould (RS 3800) chart recorder, to allow measurement of LV peak systolic and end-diastolic pressures. Animals were then killed for subsequent analyses.

Tissue Sources

After sacrifice the heart was quickly excised and placed in cold (4°C) buffer (30 mmol/L histidine, 250 mmol/L sucrose, 2 mmol/L EDTA, pH 7.2). The atria, right ventricle, and the left ventricle were separated and weighted. Left and right lungs were also quickly excised and weighted. All weights were indexed to body weight. The LV tissue samples were then frozen in liquid nitrogen and stored at −80°C until subsequent analyses.

Experimental Groups

According to the lung weight index, banded animals were divided into either a compensated LVH group (cLVH), that is, animals in whom lung weight index was within the normal range observed in the sham-operated controls (reference upper limit: mean value + 2 standard deviations), or an HF group, that is, animals in whom transition to HF had already taken place as shown by a clear-cut increase in lung weight index, a marker of pulmonary congestion.

Matrix Metalloproteinase Extraction

After being washed in ice-cold saline, LV myocardial samples were homogenized in an ice-cold extraction buffer (1:10 wt/vol) containing cacodylic acid (10 mmol/L), NaCl (150 mmol/L), $ZnCl_2$ (1 mmol/L), $CaCl_2$ (20 mmol/L), NaN_3 (1.5 mmo/L), Triton-X 100 0.01% vol/vol (pH 5.0). The homogenate was then centrifuged (5 min at 10,000 rpm) and supernatant protein concentration was measured with the colorimetric Lowry method.[14] Samples were stored at −20°C before use.

Matrix Metalloproteinase Zymography

To detect MMP lytic activity, the myocardial extracts were normalized to a final concentration of 400 μg/mL in sample loading buffer (0.25 mol/L Tris-HCl, 4% sucrose wt/vol, 10% SDS wt/vol, and 0.1% bromophenol blue wt/vol, pH 6.8). After dilution, samples were loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin or collagen (type III denatured), under nonreducing conditions.[15,16] Gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), at 4°C in a running buffer. After the run, gels were washed twice in 2.5% Triton X-100 for 30 min, rinsed in water, and incubated for 18 h at 37°C in 50 mmol/L Tris-HCl, $CaCl_2$ 5 mmol/L, NaN_3 0.02% wt/vol, pH 8. To reveal zones of lysis, the gels were stained with Coomassie blue R-250 for 30 min and destained by changing the solution for 60 min, to be subsequently dried and analyzed. To detect MMP-1 and MMP-3 activity, the samples were activated by incubation at 37°C for 15 min in Tris-HCl 25 mmol/L containing trypsin 50 ng/mg extract.[17,18] The reaction was stopped by placing the samples in ice for 5 min. Zones of enzymatic activity were indicated by negative staining, as areas of proteolysis appeared as clear bands against a blue background. In all zymograms, prestained molecular weight markers (Novex, San Diego, CA) and positive controls (proenzyme MMP-1, MMP-2, MMP-3, and MMP-9, purified from mammalian cells; Oncogene, San Diego, CA) were included.

The zymograms were analyzed by a densitometer (GS 710 Densitomer; Bio-Rad, Hercules, CA) and data were expressed as optical density (OD), reported to 1 mg/mL protein content.

Tissue-Specific Matrix Metalloproteinase Inhibitor Assessment

The TIMP-1 and TIMP-2 assays were performed by commercial ELISA kits (Biotrak; Amersham Pharmacia Biotech, Buckinghamshire, England) that detect total TIMP-1 or TIMP-2 (ie, both free and complexed with MMPs). The TIMP concentrations were expressed as nanograms per milliliter reported to milligram per milliliter of proteins of each sample.

Collagen Evaluation and Cardiomyocyte Diameter

Histology was performed on frozen LV sections of sham and banded animals. Interstitial collagen quantification was performed on Masson's trichrome stained sections[19] by the use of a computer-based quantitative color image analysis system (Image Pro-Plus, Silver Spring, MD). The relative collagen content was expressed as a percentage of the total area of the myocardial tissue. For each animal, nine random high-power fields were chosen and averaged.[20] Using the same image analysis system, the minor diameter of a minimum of 30 cardiac myocytes from each section of each sample was identified and measured, as suggested by Rossi.[21]

Statistical Analysis

Results are expressed as mean values ± standard deviation or SEM. ANOVA was used to test the differences between the 3 experimental groups, followed by post hoc Dunnett test when appropriate. All statistical procedures were performed using the STATVIEW statistical software package (SAS Institute Inc., Cary, NC).

Results

Echocardiographic and Hemodynamic Data

The general features of the experimental groups are reported in Table 1, and the complete hemodynamic data are summarized in Table 2. Banded animals were divided by lung weight index into either cLVH ($n = 6$) or HF ($n = 8$) groups, as compared with sham-operated controls. At the end of the experiment the lung weight was much higher in HF than in cLVH animals, often associated with pleural effusions and other signs of HF. All groups were comparable in terms of body mass. As expected, aortic banding increased LV systolic pressure and LV weight, indicating the development of pressure overload LVH. Also, the right-ventricular weight index was increased in both cLVH and HF animals when compared with sham-operated controls.

Transmitral flow velocity was increased in both banded groups. However, all HF animals exhibited a restrictive

Table 1. General features of the experimental groups

	Sham (n = 17)	cLVH (n = 6)	HF (n = 8)
Body weight (g)	808 ± 75	841 ± 91	845 ± 83
LV weight index (g/kg)	1.89 ± 0.07	2.52 ± 0.13*	2.64 ± 0.10*
RV weight index (g/kg)	0.51 ± 0.12	0.74 ± 0.18*	0.96 ± 0.20*
Atrial weight index (mg/kg)	441 ± 120	482 ± 125	493 ± 140
Lung weight index (g/kg)	4.18 ± 0.26	5.09 ± 0.46	8.84 ± 0.78*†

Body weight, and left-ventricular (LV), right-ventricular (RV), atrial, and lung weight indices in sham, compensated (cLVH), and heart failure (HF) animals, 12 weeks after aortic banding. Data are shown as mean value ± standard deviation.

* $P < .05$ *v* sham; † $P < .05$ *v* cLVH.

Table 2. Hemodynamic assessments

	Sham (n = 17)	cLVH (n = 6)	HF (n = 8)
Heart rate (beats/min)	202 ± 17	205 ± 16	209 ± 21
Systolic LVP (mm Hg)	68 ± 5	94 ± 6*	91 ± 8*
Diastolic LVP (mm Hg)	4 ± 2	5 ± 2	7 ± 2
LV ESD (mm)	6 ± 0.9	6.8 ± 1.3	7.3 ± 1.5
LV EDD (mm)	9.4 ± 0.9	10.5 ± 1.4	11.6 ± 1.6*
Early velocity (m/sec)	0.51 ± 0.10	0.69 ± 0.11*	0.71 ± 0.12*

Systolic LVP = left-ventricular systolic pressure; Diastolic LVP = left-ventricular diastolic pressure; LV EDD = left-ventricular end-diastolic diameter; LV ESD = left-ventricular end-systolic diameter.

Hemodynamic data in sham, compensated (cLVH), and heart failure (HF) animals, 12 weeks after aortic banding. Data are shown as mean value ± standard deviation.

* $P < .05$ *v* sham.

pattern of Doppler transmitral flow, indicative of severe diastolic dysfunction, whereas 50% of cLVH animals showed a normal pattern of Doppler transmitral flow (Fig. 1). When compared with the sham group, LV end-diastolic dimension was only significantly increased in HF, but not in cLVH, animals. No difference was observed between the experimental groups in terms of LV end-systolic dimension, heart rate, LV diastolic pressure, and atrial weight index.

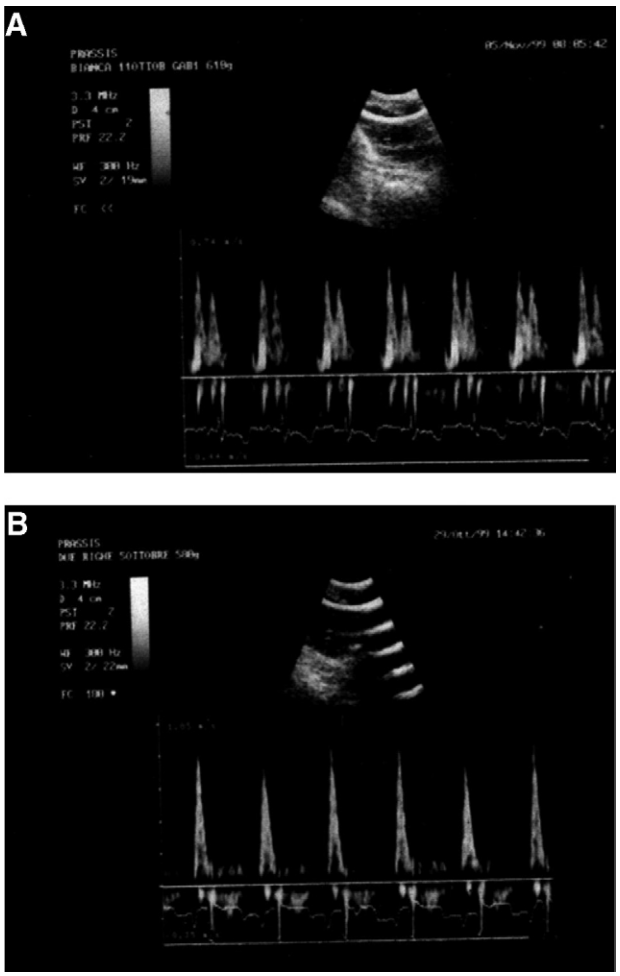

FIG. 1. Representative examples of the normal pattern of Doppler transmitral flow in a sham-operated animal (**A**) and of the restrictive pattern observed in the heart failure group (**B**). Of compensated animals 50% showed a normal pattern of Doppler transmitral flow.

Matrix Metalloproteinase Zymography

The extent of cardiac interstitial degradation was evaluated by analyzing gelatinase-A (MMP-2), gelatinase-B (MMP-9), collagenase (MMP-1), and stromelisin (MMP-3) zymography. An increase in MMP-2 lytic activity was observed in the cLVH group when compared with both HF and sham-operated animals (Fig. 2A). In contrast to MMP-2 lytic activity, which was detectable in all samples, MMP-1, MMP-3, and MMP-9 activity was not detectable.

Tissue-Specific Matrix Metalloproteinase Inhibitor Levels

Compared with the sham group, myocardial concentrations of the specific MMP-2 inhibitor TIMP-2 were increased in HF animals, but not in the cLVH group (Fig. 2B), whereas TIMP-1 levels were similar in the three experimental groups (sham: 1.90 ± 0.70 ng/mg per mg protein; cLVH: 1.80 ± 0.30 ng/mg per mg protein; HF: 2.10 ± 0.20 ng/mg per mg protein, P = not significant [NS]). The balance between MMP-2 activation and inhibition was assessed by examining the ratio between TIMP-2 levels and MMP-2 activity. The TIMP-2/MMP-2 ratio was much lower in cLVH than in HF animals (Fig. 2C), suggesting a shift of the activation–inhibition balance toward an activation of MMP-2 lytic activity in compensated hypertrophy when compared with overt HF.

Collagen Evaluation and Cardiomyocyte Diameter

Masson's trichrome staining was performed in all groups (Fig. 3). Collagen volume fraction was higher in HF animals than in sham-operated controls, with intermediate values in the cLVH group (Fig. 2D). No difference was observed in the mean minor diameter of cardiac myocytes between the compensated and the decompensated groups (23.02 ± 1.69 *v* 23.71 ± 3.48 μm, respectively; P = NS).

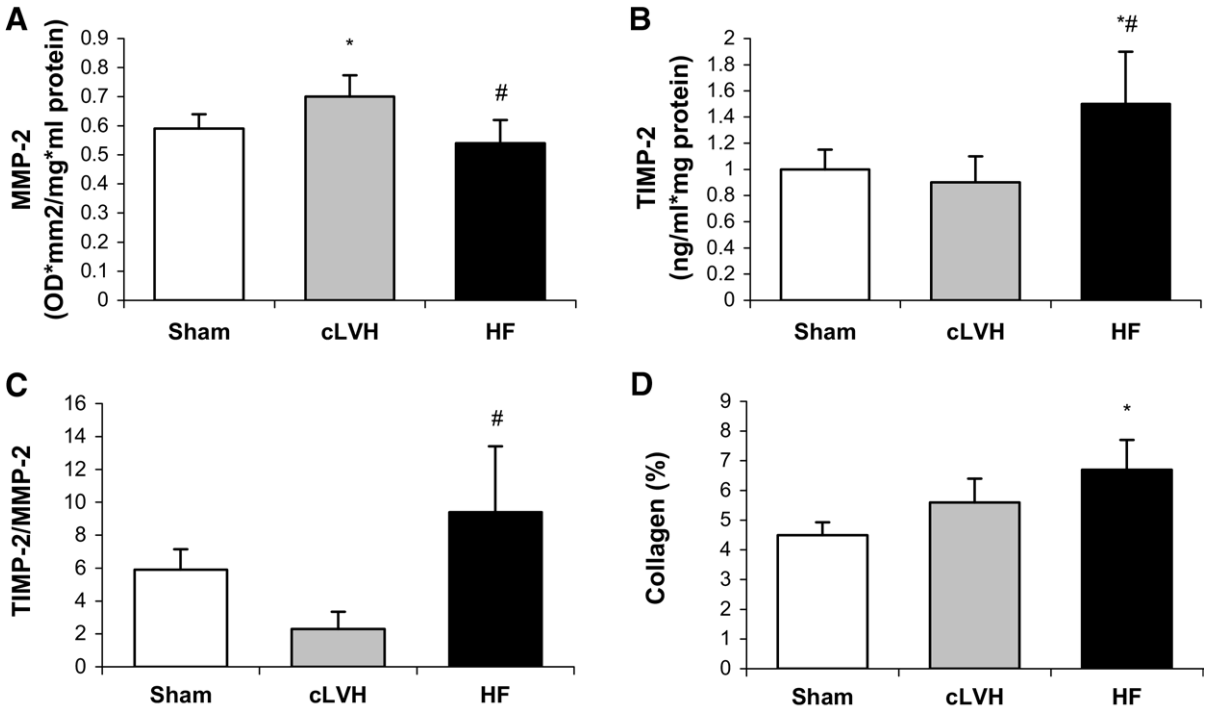

FIG. 2. Bar graphs of matrix metalloprotease-2 (MMP-2) activity (**A**), tissue-specific MMP inhibitor-2 (TIMP-2) concentration (**B**), TIMP-2/MMP-2 ratio (**C**), and relative collagen content (**D**), in the left-ventricle of sham (Sham), compensated left-ventricular hypertrophy (cLVH), and heart failure (HF) groups. The TIMP-2/MMP-2 ratio is expressed as ng/mL divided by densitometer units. Data are shown as mean value ± SEM. *$P < .05$ *v* sham; #$P < .05$ *v* cLVH.

Discussion

Left-ventricular hypertrophy secondary to chronic arterial hypertension and its transition to HF are associated with interstitial cardiac remodeling and diastolic dysfunction. Because it has been shown that MMPs have a predominant role in hydrolyzing ECM proteins, these enzymes have been proposed to mediate collagen degradation leading to LV dilation and, ultimately, to HF.[5] The aim of this work was to investigate cardiac interstitial remodeling during different phases of the development of LVH in an experimental model of POH, by evaluating the extent of both interstitial collagen deposition (reactive fibrosis, according to Brilla and Weber[22]) and degradation. The presence of lung congestion (taken as a marker of congestive HF) allowed to distinguish the animals with chronic pressure overload into two separate phases of the hypertrophic process, the former defined as *compensated* LVH, and the latter characterized by overt HF, that is, in a clearly *decompensated* stage of the experimental disease. The first phase was characterized by preserved cardiac function and evident interstitial degradation, whereas in the second phase all animals showed marked diastolic dysfunction, as indicated by a restrictive pattern of Doppler transmitral flow, lung congestion, end-diastolic chamber dilation, and reduced interstitial degradation associated with interstitial fibrosis. In cLVH, hypertrophy development was associated with increased MMP-2 lytic activity, which was blunted in the HF group together with a marked increase in TIMP-2 levels (Fig. 2). These changes in ECM degradation markers were paralleled by a significant increase in the interstitial collagen content, which was only evident in animals already in overt HF (Fig. 2). In the POH compensated stage, the increase in MMP activity may be induced by the alterations in the myocardial environment that take place in response to the hypertrophic stimulus, such as mechanical stress, neurohumoral activation, and release of inflammatory mediators and several signaling molecules, not counterbalanced by a concurrent increase in TIMP local concentration. The concurrent lack of interstitial collagen deposition in a situation expected to induce a profibrotic stimulus such as POH may be tentatively explained by this enhanced collagenolytic activity. Later in the natural history of pressure overload (at a time when the animals are in decompensated phase), MMP activity is blunted by a concomitant increase of TIMPs, associated with collagen deposition and progressive LV dilation. In fact, during this phase the TIMP/MMP ratio, a crude index of the inhibition/activation balance of the collagenolytic system, was increased, suggesting a shift toward a reduced rate of ECM degradation. Therefore, our results demonstrate that POH progression is associated with phase-dependent changes in MMP activity, TIMP abundance, and relative interstitial collagen content. Several animal model systems have demonstrated a time-dependent relationship between increased MMP expression and activity, extent of the myocardial remodeling process, and progression to heart failure.[3] In particular, Nagatomo et al[23] have demonstrated time-dependent changes in myocardial

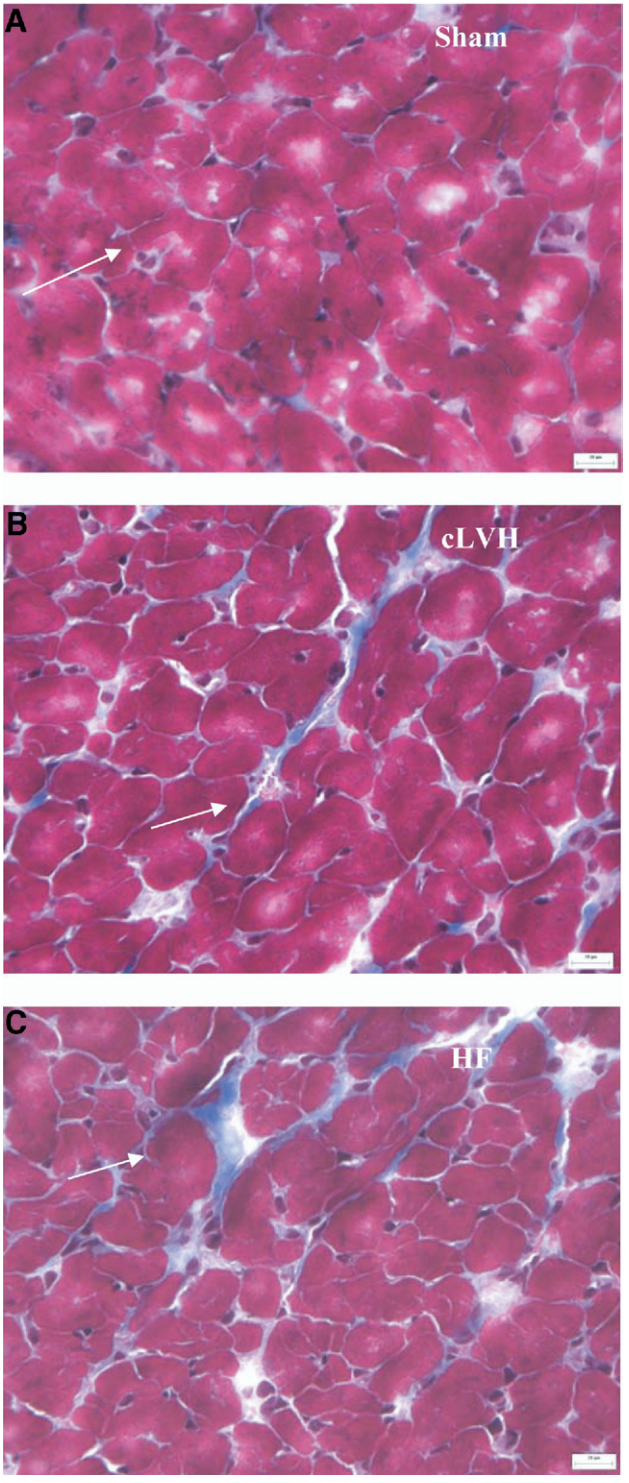

FIG. 3. Masson's staining of left-ventricular cryostatic sections in sham (**A**), compensated left-ventricular hypertrophy (cLVH) (**B**), and heart failure (HF) animals (**C**). Collagen fibers were stained in blue (arrow). In compensated animals collagen staining was intermediate in the two other groups (bar, 20 μm).

MMP levels after an acute and prolonged pressure or volume overload stimulus in a dog model. Acute pressure overload induced an increase in MMP activity. However, with prolonged pressure overload, MMP activity began to normalize and was accompanied by loss of inhibitory control. Our results extend these observations in a different experimental model and put them into the perspective of time changes that take place in the myocardial interstitium during the natural history of long-standing chronic pressure overload. When contrasting animals already in overt HF with animals still in a compensated stage of POH, we observed marked differences in terms of functional as well as structural indices associated with a different pattern of ECM remodeling. Although it is difficult to establish a direct cause and effect relationship, these data indicate a close association between the changes observed in the dynamics of collagen degradation and deposition and the progression of LVH to chamber dilation and overt HF. It is therefore possible to hypothesize that in the cLVH phase, pressure overload is accompanied by intense interstitial degradation, which at least partially counteracts (or prevents) the development of fibrosis and diastolic dysfunction. At a later stage of disease progression toward overt HF, increased TIMP concentration reduces MMP activity, concomitant with increased interstitial fibrosis, progressive diastolic dysfunction, and LV dilation.

Another issue that may be raised is the observation that chamber dilation did only take place in animals in whom transition to HF had already occurred. It has been reported that excessive MMP activation may lead to ECM scaffolding disruption and hence to loss of the structural integrity of the myocardium, leading to progressive myocyte slippage and chamber dilation especially under conditions of excessive wall stress, such as chronic pressure overload. The MMP activity increase may result in a reduction in fibrillar collagen cross-link formation leading to LV dilation, as reported by Gunja-Smith et al.[24] Moreover, Iwanaga et al[25] have demonstrated that MMP-2 activation may be one of the determining factors for LV enlargement and dysfunction in Dahl salt-sensitive rats. In our experiments, the process of ECM degradation induced by enhanced MMP activity might have made the interstitial space more prone to chamber dilation during long-standing pressure overload. This issue could be addressed by evaluating the collagen matrix ultrastructure, although this is beyond the scope of the present study. It is tempting to speculate that the MMP activity increase observed in the compensated phase may play a role in the disruption of ECM components, thereby facilitating the subsequent process of myocyte slippage and chamber dilation that becomes evident during transition to overt heart failure.

A possible limitation of our study is the use of Masson's staining to quantitatively evaluate the extent of interstitial collagen deposition. Although this might have led to underestimating the collagen content when compared with Sirius Red staining,[26] we believe that randomized readings of Masson's stained sections can reliably assess differences of relative collagen content between groups.

In conclusion, the present study demonstrated that en-

hanced MMP activation and intense interstitial degradation are evident in the compensated phase of POH. In contrast, after transition to overt HF has occurred, the MMP activity is decreased concomitant with an increase in TIMP concentration. These findings suggest an important role of MMP/TIMP balance during the progression of left ventricular hypertrophy to heart failure.

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