Abstract—Cathepsins are cysteine proteases that participate in various types of tissue remodeling. However, their expressions during myocardial remodeling have not been examined. In this study, we investigated their expressions in the left ventricular (LV) myocardium of rats and humans with hypertension-induced LV hypertrophy or heart failure (HF). Real-time PCR and immunoblot analysis revealed that the abundance of cathepsin S mRNA or protein in the LV tissues was greater in rats or humans with HF than in those with hypertrophy or in control subjects. Immunostaining showed that cathepsin S was localized predominantly to cardiac myocytes and coronary vascular smooth muscle cells, but also overlapped in part with macrophages. Elastic lamina fragmentations significantly increased in the LV intramyocardial coronary arteries of HF rats. The amount of elastolytic activity in the extract of the LV myocardium was markedly increased for HF rats compared with controls, and this activity was mostly because of cathepsin S. Although the amount of elastin mRNA was increased in the LV myocardium of HF rats, the area of interstitial elastin was not. The expression of interleukin 1β was increased in the LV myocardium of HF rats, and this cytokine was found to increase the expression and activity of cathepsin S in cultured neonatal cardiomyocytes. These results suggest that cathepsin S participates in pathological LV remodeling associated with hypertension-induced HF. This protease is, thus, a potential target for therapeutics aimed at preventing or reversing cardiac remodeling. (Hypertension. 2006; 48:979-987.)

Key Words: myocardial remodeling ■ hypertension ■ heart failure ■ cysteine proteases ■ cardiac myocytes
LV myocardium of DS rats and humans with or without hypertension-induced LV hypertrophy (H-LVH) or hypertension-induced HF (H-HF). We have also examined the production of Cats and Cyst C and its regulation in cultured neonatal rat cardiac myocytes (CMCs) and cardiac fibroblasts (CFCs).

**Methods**

**Animals**

Male inbred DS rats (Eisai, Tokyo, Japan) fed a diet containing 8% NaCl from 7 until 12 or 19 weeks of age were studied as models of H-LVH and H-HF (n=10 for each group), respectively.20 Rats maintained on a normal diet containing 0.3% NaCl served as age-matched controls (n=10 for each group). Rats were handled in accordance with the guidelines of Nagoya University School of Medicine. The animals were killed at the indicated ages by intraperitoneal injection of sodium pentobarbital (50 mg/kg of body mass). The LV-free wall was separated from the atria and right ventricle, weighed, and subjected to various analyses.

**Human Myocardial Biopsy**

The human portion of the study was approved by the Nagoya University School of Medicine Institutional Review Board. Humans endomyocardial biopsy specimens were obtained as described previously.21 All of the patients were hospitalized and diagnosed by established clinical, hemodynamic, echocardiographic, and cardiac catheterization criteria as H-LVH (n=8; 54.4 ± 13.7 years) or H-HF (n=8; 70.5 ± 6.5 years). Among H-HF patients, 2 were treated with calcium antagonist plus angiotensin-converting enzyme inhibitor, and 1 was treated with calcium antagonist plus angiotensin receptor blocker. The control subjects (n=7) found to have normal hearts (37.1 ± 13.7 years) had no history of cardiac disease. Informed consent was obtained from all of the subjects for use of the myocardial specimens in the present study.

**Quantitative RT-PCR Analysis**

Total RNA was extracted from tissue or cells, and the abundance of specific mRNAs was determined by reverse transcription and real-time PCR analysis as described previously.22 The sequences of primers and TaqMan probes specific for rat Cats S and K, Cyst C, types I and III collagen, fibronectin, interleukin (IL) 1β, and tumor necrosis factor (TNF)-α were described previously.8,15,23 The sequences of primers and probes specific for rat Cats B, G, L, and D and elastin, as well as human Cats S, and K and Cyst C, are shown in Table I (available online at http://hyper.ahajournals.org). The amount of each mRNA was normalized by the corresponding amount of GAPDH mRNA.

**Histology**

Van Gieson elastin staining and Picrosirius Red collagen staining were performed and analyzed for the amounts of the interstitial elastin and collagen as described previously.19,24 Elastin preservation in intramyocardial coronary arteries was graded as described previously.19 Immunostaining was performed as described.15 Negative controls were also performed with normal rabbit immunoglobulin (Vector) instead of primary antibodies.

**Elastase and Collagenase Assays, Immunoblot Assay, Cell Culture, and Immunocytofluorescence**

The detailed methods are given in the online supplement.

**Statistical Analysis**

Data were considered to be normally distributed and are presented as mean±SEM unless indicated otherwise. Differences were analyzed by Student t test or by ANOVA followed by Scheffe’s multiple comparison test. A P value of <0.05 was considered statistically significant.

**Results**

**DS Rats as Models of H-LVH and H-HF**

Consistent with previous findings,20,23,25 cardiac hypertrophy, as reflected by an increase in LV wall thickness and mass, was apparent in all of the rats at 12 weeks of age that had been fed the 8% NaCl diet (Table II). At 19 weeks of age, rats maintained on the 8% NaCl diet manifested HF, as reflected by a decrease in LV fractional shortening and an increase in LV end-diastolic diameter (Table II).

**Expression of Cats and Cyst C in the LV Myocardium During Remodeling**

Quantitative RT-PCR analysis revealed that the abundance of Cat S and K mRNAs was significantly increased in the LV myocardium of DS rats with H-LVH (+49 and +40%, respectively) or with H-HF (+359 and +135%, respectively) compared with the values for corresponding control rats (Figure 1A and 1B). Similarly, the amounts of Cat S and K mRNAs were increased in the LV myocardium of humans with H-LVH (+35 and +25%, respectively) or with H-HF (+158 and +81%, respectively), although only the increases in the H-HF patients were significant (Figure 2A and 2B). The abundance of Cyst C mRNA was significantly increased in the LV myocardium of H-LVH rats (+50%) and H-LVH patients (+68%) but not in that of rats or humans with H-HF (Figures 1C and 2C). Although the amount of Cat B mRNA was significantly increased in the LV myocardium of H-HF rats (94.5 ± 20.8 versus 50.2 ± 10.9; P<0.005), the abundance of Cat L, D, and G mRNAs did not differ between H-LVH or H-HF rats and their respective controls (data not shown).

Immunoblot analysis showed that the active forms of Cats S and K in the LV myocardium were 4.0- and 1.8-fold greater, respectively, in H-HF rats than in age-matched controls (Figure 3A and 3B). The amounts of these 2 proteins did not differ significantly between H-LVH rats and controls.
The amounts of Cats S and K also did not significantly change in the LV myocardium of rats maintained on the low-salt diet between 7 and 19 weeks of age (data not shown). The abundance of Cyst C in the LV myocardium did not differ significantly between H-HF and control rats (Figure 3C).

Immunohistochemical analysis revealed only a low level of expression of Cat S and K in the LV myocardium of control rats or humans (Figure 4). In contrast, the expression of Cat S was markedly increased throughout the myocardium of rats or humans with H-HF, with staining apparent in CMCs, vascular smooth muscle cells (SMCs), and dispersed macrophages (Figure 4; data not shown). Staining for Cat K was also increased in CMCs of rats and humans with H-HF. Staining for both Cats S and K showed regional and small increases in intensity in the LV myocardium of rats and humans with H-LVH (data not shown).

The intensity of staining for Cyst C in the LV myocardium did not seem to differ between rats or humans with H-HF and their respective controls (Figure 4).

Elastolytic and Collagenolytic Activities in the LV Myocardium During Remodeling

To test whether the Cats expressed in the LV myocardium are capable of degrading ECM components, we determined the total elastolytic and collagenolytic activities of myocardial extracts. When assayed under conditions optimal for CP activity, the elastolytic activity in the LV myocardium of H-HF rats was 3.8 times as great as that of age-matched controls (Figure 5A). The elastolytic activity was also increased in H-LVH rats but not to a significant extent (data not shown). LHVS (a specific inhibitor of Cat S), E64 (a specific inhibitor of CPs), and Cyst C inhibited the elastolytic activity of myocardial extracts from H-HF rats by...
71%, 80%, and 65%, respectively (Figure 5A). When assayed under conditions optimal for MMP activity, the elastolytic activity of myocardial extracts of all of the rats was much lower than that measured under acidic conditions (data not shown). The total collagenolytic activity in the LV myocardium of H-HF rats was significantly but only partially inhibited by LHVS or Cyst C (−28% and −32%, respectively; Figure 5B). The MMP inhibitor GM6001 inhibited the collagenolytic activity by 64% (Figure 5B), whereas the SP inhibitor phenylmethylsulfonyl fluoride had no significant effect (data not shown).

Figure 4. Immunohistochemical staining for Cats S and K and Cyst C in the LV myocardium of H-HF rats (A) and H-HF patients (B) and their respective controls. Arrowheads indicated vascular SMCs expressing Cat S. Bars, 25 μm.

Figure 5. Elastolytic (A) and collagenolytic (B) activities in the LV myocardium of H-HF and control rats (n=8 for each group). Elastolytic (acidic condition) and collagenolytic activities were assayed in the absence or presence of E64, LHVS, Cyst C, or GM6001 and are expressed in absorbance or fluorescence intensity units, respectively. E64 indicates a broad spectrum of CP inhibitor; LHVS, a specific Cat S inhibitor; Cyst C, endogenous CP inhibitor; GM6001, a broad spectrum of MMP inhibitor. Bovine spleen Cat S and human MMP-1 were also assayed as standards (▲). Data are mean±SEM. *P<0.01 vs 19W-C; †P<0.05, ‡P<0.01, vs H-HF without inhibitor.
Cats Expressions in Cultured CMCs

Immunofluorescence analysis revealed that, under serum-free conditions, neonatal rat CMCs expressed Cat S at only a low level (Figure 6A). Exposure of the cells to TNF-α/H9251 or IL-1β/H9252 for 24 hours, however, resulted in marked upregulation of the expression of Cat S. Consistent with these results, immunoblot analysis showed that the abundance of Cat S in cells treated with TNF-α or IL-1β was 3.4 and 2.2 times, respectively, that in control cells (Figure 6B). Furthermore, quantitative RT-PCR analysis revealed that TNF-α and IL-1β increased the abundance of Cat S mRNA by factors of 4.1 and 2.8, respectively (Figure 6C). These cytokines also increased the amounts of Cat K, B, and L mRNAs but to a markedly reduced extent compared with their effects on Cat S mRNA (data not shown). Neither transforming growth factor (TGF)-β1 nor aldosterone affected the abundance of Cat S mRNA in cultured cardiac CFCs (data not shown).

Measurement of elastolytic activity under conditions optimal for CP activity revealed that nonstimulated CMCs exhibited little such activity (data not shown). The activity present in cell lysates and the culture medium of CMCs stimulated with IL-1β was inhibited by 78% and 72%, respectively, by LHVS (Figure 6D).

Expression of IL-1β and TNF-α in the LV Myocardium of H-LVH and H-HF Rats

The amounts of IL-1β mRNA and protein in the LV myocardium were 3.2- and 2.3-fold greater, respectively, in H-HF rats than in controls (Figure 7A and 7B). In contrast, the expression of TNF-α at the mRNA and protein levels did not differ significantly between these 2 groups of rats (Figure 7C and 7D). The amounts of IL-1β or TNF-α mRNA (Figure 7A and 7C) or protein (data not shown) did differ between H-LVH and control rats.

Expression of ECM Components in the LV Myocardium of H-LVH and H-HF Rats

Quantitative RT-PCR analysis revealed that the amounts of mRNAs for fibronectin (+241 and +201%), type I collagen (+144 and +178%), type III collagen (+97 and +154%), and elastin (+98 and +129%) were significantly increased in the LV myocardium of H-LVH and H-HF rats, respectively (Table III). Consistent with these observations, staining with Sirius red showed that the interstitial collagen content of the LV myocardium was increased in H-LVH and H-HF rats by 201% and 180%, respectively (Figure 8A and 8B). In contrast, the interstitial elastin content of the LV myocardium, as revealed by staining with van Gieson’s solution, was significantly increased (+75%) only in H-LVH rats and normalized in H-HF rats (Figure 8C and 8D). Furthermore, intramyocardial coronary arteries showed an increase in the number of medial elastic lamina breaks in H-HF rats (Figure 8E and 8F).

Discussion

Our data have shown that: (1) Cat S and K are expressed at only a low level in the LV myocardium of control rats or normal humans, whereas the amounts of Cat S and K mRNAs and proteins are markedly increased in that of rats or humans with H-HF; (2) the expression of Cyst C at the mRNA or protein level does not differ significantly between rats or
humans with H-HF and corresponding controls; (3) the elastolytic activity of LV myocardial extracts is greatly increased in H-HF rats compared with controls, and this increase is mostly attributable to Cat S; (4) there is an unbalance between the collagen and elastin level in the LV myocardium of H-HF rats; and (5) Cat S is expressed at a low level in cultured neonatal rat CMCs under basal conditions, but the amounts of Cat S mRNA and protein in these cells are markedly increased in response to stimulation with IL-1β or TNF-α.

Although several proteases, known as SP4,5 and MMP2,3,6 systems, have been shown to participate in LV remodeling, evidence has suggested that other proteases also play a role in this process. It has recently been reported that Cat B was increased in patients with dilated cardiomyopathy and was shown to be a prominent protease involved in apoptosis, as well as degradation of myofibrillar proteins in myocardial infarction.26,27 Cat L was, thus, found to be important in the maintenance of heart structure and function, given that deficiency of this lysosomal peptidase resulted in the development of dilated cardiomyopathy.28,29 Several CPs are secreted from various cell types into the extracellular space, where they have the potential to express collagenolytic and elastolytic activities.12,15 We have now shown that changes in the expression of Cats at the mRNA and protein levels, as well as in Cat activity in the LV myocardium, accompany LV remodeling in rats and humans. Together, these various observations support the notion that Cats may participate in LV remodeling by mediating ECM degradation in cooperation with other proteases, such as MMPs and SPs.

Cats are synthesized as zymogens and undergo maturation to their enzymatically active forms during transport from the trans-Golgi network to the late endosome–lysosome compartment, although it is still unclear how pro-Cats mature in vivo.30 We have now shown that the amounts of Cat S mRNA and mature Cat S protein were significantly increased in the LV myocardium of H-HF rats. The amount of Cat S mRNA was also slightly increased in the myocardium of H-LVH rats, but this effect was not accompanied by an increase in the abundance of mature Cat S protein. Further studies investigating this issue should be very useful to increase our understanding on expression of Cat S mRNA and protein in the LV remodeling process.

The increase in the amount of elastolytic activity observed in the LV myocardium of H-HF rats was almost entirely because of CPs and, in particular, Cat S. This observation is consistent with our findings that the LV myocardium of rats or humans with H-HF contains increased amounts of Cat S, which is expressed by macrophages and vascular SMCs in addition to CMCs. Our observation that intramyocardial coronary arteries showed an increase in the number of medial elastic lamina breaks in H-HF rats provides indirect evidence that elastolytic activity is increased in coronary arteries, as well as in the myocardium, per se. Although the amount of elastin mRNA was increased in the LV myocardium of rats with H-LVH or H-HF, the interstitial elastin content was
significantly increased only in those with H-LVH, possibly because of the increased elastolytic activity in the LV myocardium of H-HF rats. Previous studies with animal models have demonstrated a cause–effect relation between changes in the levels of Cat S and elastin during pathological vascular remodeling. In vitro studies with SMCs or macrophages have suggested that the elastolytic activity of CPs is greater than that of SPs or MMPs. Furthermore, the elastase activity of such cells from Cat S–deficient mice is greatly reduced even after stimulation with inflammatory cytokines. Together, these findings suggest that Cat S is a major contributor to the degradation of elastin-rich ECM. The collagenolytic activity in LV tissue extracts of H-HF rats was only partially blocked by inhibitors of Cat S (LHVS and Cyst C), suggesting a limited contribution of this enzyme to such activity associated with LV remodeling. This finding is consistent with previous studies suggesting that MMPs play a greater role than do CPs or SPs in the collagen degradation associated with cardiovascular remodeling.

Neurohormones and proinflammatory cytokines regulate the production and activity of several proteases in neonatal or adult CMCs or CFCs. Stimulation of CFCs with TNF-α or IL-1β, for example, increases MMP expression and activity. Aldosterone increases MMP activity in cultured adult CMCs (but not in CFCs) through activation of the mineralocorticoid receptor. In addition, TNF-α and TGF-1 regulate the production and activity of various Cats in cultured SMCs, fibroblasts, and macrophages. We have also shown that, among various Cats examined, Cat S was upregulated specifically in cultured vascular SMCs by stimulation with TNF-α or IL-1β and that the active form of the enzyme accumulated at the cell surface in association with integrin αβ3. Our present study shows that IL-1β and TNF-α each increased the amounts of Cat S mRNA and protein in cultured neonatal rat CMCs, whereas TGF-1β and aldosterone were without effect. The abundance of IL-1β mRNA and protein was also increased in the LV myocardium of H-HF rats. These results indicate that such upregulation of IL-1β may contribute to the upregulation of Cat S in the LV myocardium of these animals.

Cyst C inhibits a spectrum of CPs, but its affinity is highest for Cat S. Unlike the endogenous tissue inhibitors of MMPs,
of which the expression is induced in parallel with MMPs during the progression to H-HF, the abundance of Cyst C mRNA and protein in the LV myocardium did not differ between rats or humans with H-HF and their respective controls. This work, in conjunction with previous studies, suggests that imbalance between proteases and relative endogenous inhibitors in the LV myocardium results in quantity and quality changes in the LV ECM components and their balances and translation from H-LVH to H-HF. In addition, Cyst C has been reported as more sensitive to detect renal insufficiency than creatinine in serum levels. Arimoto et al have reported recently that its serum level was elevated in the patients with mild-to-moderate HF. Further studies will be needed to clarify the source of Cyst C in the plasma and whether it is still functional or whether there is something altering its ability to function in the LV myocardium in animal and human HF. Deeper understanding of the importance of Cyst C participation in this setting may require further experiments using conditional genetic animal models.

Study Limitations and Perspectives

There are several limitations to the present study. First, we studied hypertension-induced HF in humans. It remains to be determined whether similar changes in Cat expression occur in human HF because of dilated cardiomyopathy or hypertrophic cardiomyopathy. Second, we have studied only a limited number of human samples. Although our study has demonstrated that the amounts of Cat S mRNA or protein in the LV myocardium are greater in patients with H-HF than in controls or those with H-LVH, the relation between Cat S expression and LV function was not examined. Despite these limitations, we believe that our experiments represent the first characterization of the expression of elastolytic Cats and their inhibitor Cyst C in the LVM and temporal changes during hypertensive-induced LV remodeling in DS rats, as well as humans, and their regulation in cultured CMCs. The present observations suggest that CPs, especially Cat S, may participate in pathological LV remodeling associated with HF in cooperation with other proteases, such as MMPs and SPs. This work raises numerous questions for further investigation and underscores the need to consider Cat S as a therapeutic target to prevent or reverse cardiac remodeling using genetic and pharmacological methods.

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Disclosures

None.

References


Elastolytic Cathepsin Induction/Activation System Exists in Myocardium and Is Upregulated in Hypertensive Heart Failure

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