TIMP-3 Deficiency Leads to Dilated Cardiomyopathy

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Background—Despite the mounting clinical burden of heart failure, the biomolecules that control myocardial tissue remodeling are poorly understood. TIMP-3 is an endogenous inhibitor of matrix metalloproteinases (MMPs) that has been found to be deficient in failing human myocardium. We hypothesized that TIMP-3 expression prevents maladaptive tissue remodeling in the heart, and accordingly, its deficiency in mice would alone be sufficient to trigger progressive cardiac remodeling and dysfunction similar to human heart failure.

Methods and Results—Mice with a targeted timp-3 deficiency were evaluated with aging and compared with age-matched wild-type littermates. Loss of timp-3 function triggered spontaneous LV dilatation, cardiomyocyte hypertrophy, and contractile dysfunction at 21 months of age consistent with human dilated cardiomyopathy. Its absence also resulted in interstitial matrix disruption with elevated MMP-9 activity, and activation of the proinflammatory tumor necrosis factor-α cytokine system, molecular hallmarks of human myocardial remodeling.

Conclusions—TIMP-3 deficiency disrupts matrix homeostasis and the balance of inflammatory mediators, eliciting the transition to cardiac dilation and dysfunction. Therapeutic restoration of myocardial TIMP-3 may provide a novel approach to limit cardiac remodeling and the progression to failure in patients with dilated cardiomyopathy. (Circulation. 2004;110:2401-2409.)

Key Words: remodeling ■ cardiomyopathy ■ metalloproteinases ■ inhibitors

Optimal cardiac structure and function are maintained by a tightly regulated myocardial microenvironment. In the healthy heart, the extracellular matrix (ECM) provides the structural framework for coordinated muscle cell contraction and also sequesters growth factors and cytokines, which interact with cell-surface molecules to dictate cell behavior and fate. The ECM thus assembles a dynamic microenvironment in which molecular cues converge to maintain tissue architecture by regulating myocyte orientation, elongation, hypertrophy, and apoptosis.1 In the failing heart, dysregulation of ECM biomolecules may play a central role in maladaptive myocardial remodeling and cardiac decompensation.

Tissue inhibitors of metalloproteinases (TIMPs) are important biological regulators of the cellular microenvironment.2 Classically known to inhibit the activity of matrix metalloproteinases (MMPs) that cleave ECM proteins,2 specific TIMPs also inhibit ADAM (a disintegrin and metalloproteinase) enzymes that shed inflammatory cytokines and their receptors.3 Clinical studies over the past decade document increased MMP activities associated with diseased hearts.4 Animal models of cardiovascular disease and transgenic mouse models further support a role for MMPs in cardiac remodeling.4–7 Similarly, clinical, experimental, and genetic approaches implicate the involvement of TIMPs in heart disease, and TIMP expression is selectively reduced in failing heart.8

Unique among TIMPs, TIMP-3 is ECM-bound, highly expressed in the heart, uniformly reduced in failing hearts, and a potent endogenous inhibitor of ADAMs implicated in cardiac disease.9–11 Notably, TIMP-3 is reduced in human and experimental heart failure in association with maladaptive myocardial remodeling.12 We hypothesized that TIMP-3 expression prevents maladaptive tissue remodeling in the heart; accordingly, its deficiency in mice would alone be sufficient to trigger progressive cardiac remodeling and dysfunction similar to human heart failure.

Methods

Experimental Animals

Timp-3−/− mice were generated as previously described13 and backcrossed 6 times into the C57/B16 strain. All animals were cared for in accordance with guidelines of the Canadian Council for Animal Care.
Left Ventricular Function
Echocardiography was performed by use of standard techniques after anesthesia with minimal concentrations of inhaled isoflurane. Three measurements were averaged for each parameter in every mouse. Ex vivo left ventricular (LV) function and passive pressure analysis were evaluated by use of a Langendorff perfusion apparatus as previously described.14

Geometric Mapping of the Left Ventricle
The LV volume was measured as previously described.14 LV volume was divided by the body weight to yield the indexed LV volume. Confocal Microscopy of Matrix Structure
The fibrillar collagen matrix in LV sections was stained by use of picrosirius histochemistry and imaged by use of a laser scanning confocal system (Zeiss LSM 510), obtaining an image stack of multiple slices through the 10-μm thickness of the myocardium. These were projected into a single image to assess the collagen % “volume” as previously described.12

Quantitative Taqman Real-Time PCR
Quantification of expression of hypotrophy, atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) was performed by use of the Applied Biosystems ABI prism 7700 sequence detection system (Taqman).15 Briefly, 1 μg of total RNA extracted from hearts was reverse-transcribed by use of random hexamers. Taqman reactions were carried out in 96-well plates by use of 0.5% cDNA, 12.5 μL of 2X Taqman universal polymerase chain reaction (PCR) master mix, 100 μmol/L probe, and 200 μmol/L of each primer and water to a final volume of 25 μL. 18S rRNA was used as an endogenous control. Primer sequences for murine TIMPs were as described15: for ANF, forward primer: 5′-GAGGGAGAAGATGCGGTAGA-3′; reverse primer: 5′-GCT-TCCTCAGTCTGCTACTA-3′; probe: 5′-FAM-TGAGGCTAT-GCCGCCGACGG-TAMRA-3′; and for β-MHC, forward primer: 5′-GTGCCAAGGCGCTGAATGAG-3′; reverse primer: 5′-GCAGACCGCTAGTCTGA-3′; probe: 5′-FAM-ATCTTGTGCTACCCAGCTC-3′).

TACE Immunoblotting and Activity
TNF-α converting enzyme (TACE) activity was estimated by the levels of soluble TNF-α and its receptor, p75, from coronary sinus effluent collected during isolated heart perfusion with 1 mL of antegrade PBS delivery. Immunoblotting was performed on LV protein extractions with a monoclonal antibody against mouse TACE activity was estimated by the Griess Reagent System (Promega Corp). Griess detection method for nitrite, a stable NO breakdown product, ELISA Assays
TNF-α and p75 levels were determined by use of an ELISA kit (R&D Systems). Nitric oxide levels were measured by use of the Griess detection method for nitrite, a stable NO breakdown product, using the Griess Reagent System (Promega Corp).

MMP Zymography
Levels of active MMP-2 and MMP-9 were estimated by gelatin zymography as described previously.16

Immunohistochemistry and Histology
LV sections were incubated with a monoclonal rat anti-mouse Mac3 antibody (PharMingen) diluted 1:50 for 1 hour at 37°C. The sections were washed and incubated in the secondary antibody, a biotin-conjugated rabbit anti-rat IgG. Macrophage staining was examined by light microscopy. Positive and negative controls were used. Additional sections were stained with hematoxylin-eosin and assessed for cardiac myocyte hypertrophy as described by others.17

Figure 1. RNA expression of timps in TIMP-3–deficient mouse. Taqman real-time quantitative PCR was used to assess expression of TIMP-1, -2, and -4. Values were normalized to 18S RNA. No differences were found between mice and wild-type littermates. Bars represent mean±SEM, *P<0.05 compared with TIMP-3−/− mice.

Statistical Analysis
Results are presented as mean±SEM unless otherwise specified. Comparison between groups was performed by one-way ANOVA. When significant, pairwise tests of individual group means were compared by use of the Student-Newman-Keuls test. LV function data were evaluated by ANCOVA. All statistical procedures were performed by use of the SAS software system (SAS Institute). Differences were considered significant at a value of P<0.05.

Results
We previously reported that mice in the mixed 129J and C57/Bl6 background develop pulmonary air space enlargement, resulting in premature morbidity.13 Backcrossing into the C57/Bl6 strain resulted in less severe pulmonary air space enlargement. Specifically, by 18 months of age, the mixed-background mice had a mean alveolar size of 81.0±4.0 μm in knockout mice versus 55.0±2.2 μm in mice (measured over 10 fields per mouse, n=5 mice per genotype, P<0.01 by 2-tailed t test), whereas the C57/Bl6 strain at 23 months of age had a mean alveolar size of 67.4±9.5 μm in knockout versus 47.8±2.9 μm in mice (n=3 mice per genotype, P=0.12 by 2-tailed t test). Notably, the knockout mice in the mixed background suffered from labored breathing at or before 18 months of age with subsequent morbidity, whereas the knockout animals in the C57/Bl6 strain lived well beyond 18 months and showed no signs of respiratory distress. Despite the absence of overt respiratory compromise, the morphometric changes found in the knockout mice constitute a significant increase in alveolar size (>40%) compared with other mouse models of emphysema.

We evaluated cardiac structure and function in mice with aging compared with age-matched and sex-balanced wild-type littermates. First, we assessed whether the expression of other TIMPs was altered in the heart as a compensatory mechanism in the absence of TIMP-3. Quantitative Taqman real-time PCR analyses showed that expression of TIMP-1, -2, and -4 was not different in null and control cardiac tissue (Figure 1), indicating that soluble TIMPs (TIMP-1, -2, and -4) did not compensate for the absence of ECM-bound TIMP-3.

Timp-3 Deficiency Impairs LV Function
We next studied ex vivo LV function in isolation by use of Langendorff heart perfusion. Cardiac function was compara-
ble in mature \textit{timp-3}−/− and wild-type littermates (mean age, 13 ± 1 months, Figure 2). However, with aging, we detected profound systolic and diastolic dysfunction in \textit{timp-3}−/− mice compared with their age-matched wild-type littermates (mean age, 21 ± 1 months). Specifically, significant reductions in LV peak systolic pressure, developed pressure, and positive dP/dt indicated impaired ventricular contractility, whereas reduced negative dP/dt and increased tau suggested delayed ventricular relaxation.

We evaluated in vivo LV function by echocardiography. Fractional shortening was significantly diminished in older \textit{timp-3}−/− mice, suggesting poor ventricular contractility (Figure 3A). Reduced fractional shortening was also observed in mature \textit{timp-3}−/− mice (mean age, 16 ± 2 months), although the difference was not statistically significant ($P=0.06$, Figure 3A). Altogether, in vivo echocardiography and ex vivo Langendorff measurements showed that a chronic absence of myocardial TIMP-3 compromised cardiac performance and was associated with features of cardiac failure.

**Timp-3 Deficiency Alters Cardiac Structure**

By use of echocardiography, the mean LV end-diastolic dimension (LVEDD) indexed to body weight was significantly higher in older \textit{timp-3}−/− mice compared with age-matched wild-type littermates (Figure 3B). Body weight was reduced in \textit{timp-3}−/− mice compared with age-matched wild-type littermates (echocardiography group, 21.5 ± 1.1 versus 25.9 ± 1.3 g, $P=0.02$). Computerized planimetry of heart sections also revealed that indexed LV volume was significantly increased in \textit{timp-3}−/− mice at 21 ± 1 months of age compared with age-matched wild-type controls (Figure 3, C and D). Heart-to–body weight ratio was higher in 21-month-old \textit{timp-3}−/− hearts compared with age-matched wild-type hearts (7.9 ± 0.5 versus 5.8 ± 0.8 mg/g, $P=0.001$), accompanied by increased cardiomyocyte cross-sectional area in these mice (1303 ± 357 versus 506 ± 135 square pixels, $P=0.001$), suggesting cardiomyocyte hypertrophy. Accordingly, the relative expression pattern of molecular markers of hypertrophy (ANF, β-MHC) as determined by Taqman real-time PCR was elevated in aged \textit{timp-3}−/− mice (Figure 4). Cardiomyocyte hypertrophy was evident at 16 months of age, corresponding with the onset of cardiac dysfunction and LV dilatation (Figure 4).

However, LV wall thickness was not different between the 2 groups, as detected by echocardiography (Figure 3E).

Given that TIMP-3−/− mice are prone to pulmonary air-space disease,\textsuperscript{13} we also assessed right ventricular (RV) structure and function. By echocardiography, there was no evidence of paradoxical septal motion to indicate RV dysfunction in any of the \textit{timp-3}−/− mice assessed ($n=16$). Similarly, the RV mass–to–body weight ratio was not significantly different in \textit{timp-3}−/− versus wild-type mice at 21 ± 1 months of age (0.51 ± 0.2 versus 0.44 ± 0.1 mg/g, $P=0.12$).

We determined the mechanical properties of the LV by measuring pressure-volume characteristics of arrested hearts (Figure 3F). The LV was more distensible in \textit{timp-3}−/− mice, indicating a loss of structural support. These data collectively demonstrate that \textit{timp-3}−/− mice exhibit features of human dilated cardiomyopathy, including chamber dilatation, cellular hypertrophy without myocardial wall thickening, and impaired contractile performance.
Maladaptive Matrix Remodeling With Loss of TIMP-3

Examination of myocardial collagen by confocal microscopy showed a significant loss of perimysial collagen fibers in *timp-3*/−/− hearts (Figure 5, A and B). A striking reduction in the fiber connectivity of the collagen network was evident at higher magnification (Figure 5C). Quantification of collagen volume by computer image analysis showed markedly reduced collagen content in older *timp-3*/−/− mice (Figure 5D).

Activities of MMP-2 and MMP-9, 2 widely implicated MMPs in heart diseases, were assessed by gelatin zymography (Figure 6A). Higher levels of active MMP-9 were observed in *timp-3*/−/− hearts, whereas wild-type controls showed predominantly pro-MMP-9. MMP-2 activity remained unaltered. Despite increased MMP-9 activity, MMP-9 protein levels were not significantly different in dilated *timp-3*/−/− hearts (open bars, *n*=8) compared with age-matched wild-type littermates (filled bars, *n*=5). LV of *timp-3*/−/− mice (*n*=9) was more distensible compared with age-matched wild-type littermates (*n*=11), indicating altered myocardial material properties and a loss of structural support. Bars represent mean±SEM. *P*<0.05. T3−/− indicates *timp-3*/−/− mice.

Activation of Inflammatory Cytokines in the Absence of TIMP-3

Among unique properties of TIMP-3 is the ability to inhibit ADAM17/TACE, which processes TNF-α and its receptors. Levels of the soluble TNF-α receptor II (p75) have been shown to reflect TACE activity. We measured levels of TNF-α, p75, and nitric oxide in the coronary sinus effluent of freshly isolated hearts (mean age, 22±1 months) collected on antegrade coronary perfusion. Shedding of p75 and soluble TNF-α from the heart was increased in *timp-3*/−/− mice compared with age-matched wild-type controls (filled bars, *n*=5). LV of *timp-3*/−/− mice (*n*=9) was more distensible compared with age-matched wild-type littermates (*n*=11), indicating altered myocardial material properties and a loss of structural support. Bars represent mean±SEM. *P*<0.05. T3−/− indicates *timp-3*/−/− mice.
abundance of TACE protein was similar between the 2 groups (data not shown), suggesting that elevated TACE activity in *timp-3*−/− mice was likely due to the absence of TIMP-3.

Inflammatory cells such as macrophages are an important source of both TNF-α and MMP production in the failing heart. Macrophage infiltration in the LV was assessed by Mac3 staining. Perivascular inflammation was observed in both TIMP-3−/− deficient and wild-type mice, although positive macrophage staining was infrequent (<0.1%) and was not different between groups (Figure 4).

**Discussion**

Cardiac remodeling determines the clinical progression of heart failure and is emerging as a therapeutic target in heart failure of all causes. As such, uncovering the genes and biomolecules that regulate cardiac remodeling will aid in the development of novel clinical therapies. Despite the observation that TIMP-3 is reduced in human and experimental heart failure in association with maladaptive matrix remodeling, a role for TIMP-3 in maintaining cardiac structure and function has never been established. By use of mutant mice, we determined that deficient TIMP-3 expression leads to...
cardiomyocyte hypertrophy, LV dilation, and cardiac dysfunction without antecedent myocardial injury. We also observed profound ECM disruption with elevated active MMP-9 in TIMP-3–deficient hearts and activation of the TNF-α/H3 system, hallmarks of myocardial remodeling in human heart failure. These mechanisms may originate from the unique capacity of TIMP-3 to inhibit both MMP and ADAM metalloproteinases, which are key factors in regulating ECM integrity and the bioactivation of inflammatory cytokines (Figure 7).

We previously reported that timp-3 deletion in mice (129J/C57Bl/6 mixed strain) results in premature morbidity and mortality in association with severe pulmonary air space enlargement.13 Those mice also exhibited labored breathing and decreased gas exchange efficiency.13 Mice used in the present study were of C57Bl/6 background. These mice show a less severe histological pulmonary defect and no sign of labored breathing with a normal life span, despite our recent observation that cultured TIMP-3–deficient embryonic lung buds exhibit decreased bronchiolar branching compared with

Figure 5. Content and spatial architecture of fibrillar collagen network. Perimysial collagen fibers showed disorganization and thinning in face of timp-3 deficiency in A, longitudinal, and B, cross-sectional views. C, Fiber connectivity of fibrillar collagen network was strikingly reduced in timp-3–/- hearts. D, Quantification of collagen volume fraction in 10-μm projected confocal stacks by use of computer image analysis showed markedly reduced collagen in older timp-3–deficient mice (open bars, n=4) compared with age-matched wild-type littermates (filled bars, n=4). *P=0.001. Bar=50 μm in A (×400), 10 μm in B and C (×600).
wild-type mice. However, because cor pulmonale can influence LV function because of bulging of the interventricular septum during diastole, we measured LV performance independent of the lungs and the right ventricle in an isolated Langendorff preparation. LV dysfunction was observed by both echocardiography and Langendorff assessments. Collectively, these data suggest that LV dysfunction in TIMP-3–deficient mice is not a consequence of pulmonary disease, although its contribution cannot be completely ruled out.

The content and organization of the fibrillar collagen matrix is believed to influence myocardial tissue properties, chamber geometry, and contractile function. Accordingly, the loss of matrix content and spatial architecture in *timp-3*−/− mice was associated with increased ventricular distensibility, chamber dilatation, and systolic and diastolic cardiac dysfunction. The fragmented fibrillar collagen network in these mice resembles the disrupted matrix of patients with dilated cardiomyopathy and can disrupt the physical connections between myocytes and adjacent matrix, resulting in a poorly coordinated contraction.

Myocardial tissue remodeling in the failing human heart is associated with elevated activity of TNF-α. Shedding of TNF-α is mediated predominantly by TACE (ADAM17), and TACE activity is strongly associated with ventricular dilatation and cardiac dysfunction in human heart failure. TNF-α receptor II, p75, can be released in soluble form by TACE or on binding to TNF-α. Compared with TNF-α, soluble p75 level has a longer half-life and is easily detectable and thus a preferred measure of overall TACE bioactivity. Soluble cardiac p75 and TNF-α levels were significantly increased in *timp-3*−/− mice compared with age-matched wild-type littermates and mature TACE abundance (*P*=0.4) were similar between groups. *P*=0.05. Bars represent mean±SEM.

Figure 6. MMP and cytokine dysregulation with loss of *timp-3*. A, Gelatin zymogram indicating increased MMP-9 activation with loss of *timp-3*, whereas MMP-2 activity was unaltered. B and C, MMP-9 protein levels were not significantly different in *timp-3*−/− hearts (n=4; *P*=0.2). D, In heart effluent, soluble p75 and TNF-α were elevated >2-fold in *timp-3*−/− hearts (n=4) compared with age-matched wild-type littermates (n=3, *P*=0.03 and *P*=0.05, respectively). Nitric oxide release was also elevated (*P*=0.06). E, Representative immunoblot for TACE in extracts from LV. By densitometry, pro-TACE abundance (*P*=0.4) and mature TACE abundance (*P*=0.6) were similar between groups. *P*=0.05.

Bars represent mean±SEM.

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peutic restoration of deficient myocardial TIMP-3 by gene or cell transfer may serve as a first step toward a novel approach to limit cardiac remodeling in patients predisposed to congestive heart failure. In summary, our study provides an important proof-of-concept that deficient TIMP-3 expression may contribute directly to maladaptive cardiac remodeling and the transition to heart failure through matrix degradation and cytokine activation. These novel observations in the timp-3-deficient mouse may aid in the development of effective antiremodeling strategies for the growing number of patients at risk of heart failure.

Acknowledgments
Funding was provided by Canadian Institute for Health Research (CIHR) grants to Dr Khokha and Dr Li. Dr Fedak was the recipient of the Vivien Thomas Young Investigator Award, American Heart Association, 2002. Dr Fedak is a Research Fellow of the CIHR and the Heart and Stroke Foundation of Canada.

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Figure 7. Proposed mechanism for cardiac remodeling and dysfunction with loss of TIMP-3. A, Cardiomyocytes surrounded by a rich network of interstitial matrix providing structural support and a dynamic microenvironment for cell signaling, maintaining normal cardiac geometry and function. B, Loss of TIMP-3 leaves TNF-α cytokine system unchecked, resulting in apoptosis and increased expression of MMP-9. Deficiency of TIMP-3 in interstitial space also promotes excessive MMP activity, resulting in matrix degradation. C, Chronic MMP-mediated matrix remodeling reduces structural support for heart cells, resulting in myocyte slippage and mural realignment, wall thinning, ventricular dilatation, and a poorly coordinated contraction, eliciting transition to cardiac failure.
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_Circulation._ 2004;110:2401-2409; originally published online July 19, 2004;
doi: 10.1161/01.CIR.0000134959.83967.2D
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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