Lack of Tissue Inhibitor of Metalloproteinases 2 Leads to Exacerbated Left Ventricular Dysfunction and Adverse Extracellular Matrix Remodeling in Response to Biomechanical Stress

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Background—Remodeling of the extracellular matrix (ECM) is a key aspect of myocardial response to biomechanical stress and heart failure. Tissue inhibitors of metalloproteinases (TIMPs) regulate the ECM turnover through negative regulation of matrix metalloproteinases (MMPs), which degrade the ECM structural proteins. Tissue inhibitor of metalloproteinases 2 is unique among TIMPs in activating pro-MMP2 in addition to inhibiting a number of MMPs. Given this dual role of TIMP2, we investigated whether TIMP2 serves a critical role in heart disease.

Methods and Results—Pressure overload by transverse aortic constriction (TAC) in 8-week-old male mice resulted in greater left ventricular hypertrophy, fibrosis, dilation, and dysfunction in TIMP2−/− mice compared with wild-type mice at 2 weeks and 5 weeks post-TAC. Despite lack of MMP2 activation, total collagenase activity and specific membrane type MMP activity were greater in TIMP2−/− mice. Loss of TIMP2 resulted in a marked reduction of integrin β1D levels and compromised focal adhesion kinase phosphorylation, resulting in impaired adhesion of cardiomyocytes to ECM proteins, laminin, and fibronectin. Nonuniform ECM remodeling in TIMP2−/− mice revealed degraded network structure as well as excess fibrillar deposition. Greater fibrosis in TIMP2−/− mice compared with wild-type TAC hearts was due to higher levels of SPARC (secreted protein acidic and rich in cysteine) and posttranslational stabilization of collagen fibers rather than increased collagen synthesis. Inhibition of MMPs including membrane type MMP significantly reduced left ventricular dilation and dysfunction, hypertrophy, and fibrosis in TIMP2−/− mice.

Conclusions—Lack of TIMP2 leads to exacerbated cardiac dysfunction and remodeling after pressure overload because of excess activity of membrane type MMP and loss of integrin β1D, leading to nonuniform ECM remodeling and impaired myocyte–ECM interaction. (Circulation. 2011;124:00-00.)

Key Words: tissue inhibitor of metalloproteinases membrane-type matrix metalloproteinases fibrosis hypertrophy

Increased afterload leading to excess biomechanical stress is a common cause of left ventricular (LV) remodeling, which leads to cardiac dysfunction and eventual heart failure.1,2 The extracellular matrix (ECM) is a dynamic structure, and its turnover is a physiological process that takes place in all organs. Adverse remodeling of the myocardial ECM, brought about by dysregulation in its turnover, is a key component of pressure overload–induced cardiomyopathy. This results in excess degradation and disruption of the ECM network structure or accumulation of ECM proteins and formation of fibrotic lesions. Myocardial fibrosis is also a well-known cause of diastolic dysfunction and diastolic heart failure.3,4 Matrix metalloproteinases (MMPs) degrade ECM proteins, whereas their proteolytic activity is kept in check by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). An imbalance in the function of MMPs and TIMPs occurs in heart disease, leading to adverse ECM remodeling, and TIMPs are emerging as critical regulators of this process.5–13

Among the 4 TIMPs,14 TIMP2 has the unique property of activating MMP2 through formation of a trimolecular complex with pro-MMP2 and membrane type 1 (MT1) MMP.15

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This function of TIMP2 is in addition to its ability to inhibit a number of MMPs. Tissue inhibitor of metalloproteinasises 2 levels are increased in the hearts of patients with aortic valvular stenosis16 and patients with pressure-overloaded cardiomyopathy.17 However, whether this rise in TIMP2 is a compensatory attempt by the heart to inhibit the elevated MMP activities or further contributes to disease progression by promoting activation of MMP2 remains to be determined. We investigated the role of TIMP2 in cardiac response to a pressure-overload model of heart failure. Loss of TIMP2 resulted in adverse remodeling of the ECM, whereas MMP inhibition prevented these unfavorable outcomes.

Methods
Detailed Methods are provided in the online-only Data Supplement.

In Vivo Pressure Overload by Transverse Aortic Constriction
Wild-type (WT) and TIMP2-deficient (TIMP2−/−)18 mice in C57BL/6 background underwent transverse aortic constriction (TAC) at 8 weeks of age to generate pressure overload as before.19 The sham-operated animals underwent the same procedure without the constriction of aorta. At 2 or 5 weeks postsurgery, hearts and lungs were excised from either genotype and formalin fixed or flash frozen in liquid nitrogen. All animal experiments were carried out in accordance with Canadian Council on Animal Care Guidelines and regulations of the Animal Care and Use Committee at the University of Alberta, as well as the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cardiac Functional Analysis by Echocardiography, Tissue Doppler Imaging, and Transmittral Doppler Imaging
Systolic and diastolic cardiac function was determined noninvasively by transthoracic echocardiography of anesthetized mice (0.75% isoflurane) using a Vevo 770 high-resolution imaging system equipped with a 20-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada) as before.11,12,20

In Vivo MMP Inhibitor Treatment
Mice received a broad-spectrum MMP-inhibitor, PD166793 (Pfizer Inc, 30 mg/Kg/d) by daily gavage11 1 day before TAC surgery and through the course of the study.

Protein Analysis, Western Blotting, Gelatin Zymography, Collagenase and MT1-MMP Activity Assays
Gelatin zymography was performed to detect pro and active MMP2 and MMP9 as before.11,12 Western blot analysis was performed to detect TIMP1, TIMP2, TIMP3 and TIMP4 as before,11,12 integrin β1D (Millipore),21 phospho-FAK (Tyr397, Millipore) and total FAK (Santa Cruz). In all Western blots, Poronex staining of the PVDF membrane was used as the loading control. Total collagenase activity was measured using fluorescent-based activity assays from EnzCheck (Molecular Probes) as before.11,12,24 HPRT (hypoxanthine-guanine phosphoribosyltransferase-1) was used as the internal control. Taqman primers and probe used to measure the expression of integrin β1 was purchased from ABInstruments (Mm01253230_m1), and for procollagen I-α1 and procollagen III-α1 type II as reported previously.15

RNA Expression Analysis
Total RNA was extracted using TriZol Reagent (Invitrogen), and mRNA expression analysis was performed by TaqMan RT-PCR as before.12,24 HPRT (hypoxanthine-guanine phosphoribosyltransferase-1) was used as the internal control. Taqman primers and probe used to measure the expression of integrin β1 was purchased from ABInstruments (Mm01253230_m1), and for procollagen I-α1 and procollagen III-α1 type II as reported previously.15

In Vitro Adhesion Assay
Adhesion of adult cardiomyocytes to ECM proteins were assessed by using an in situ CytoSelect Cell Adhesion Assay (ECM Array, Colorimetric Format, Cell Biolabs Inc) according the manufacturer’s instructions as before.19

Scanning Electron Microscopy
Scanning electron microscopy (SEM) was performed as before.9 Briefly, 2 to 3 mm pieces of the LV were fixed (2.5% glutaraldehyde and 2% paraformaldehyde), dehydrated and air-dried overnight, mounted on SEM stubs, sputter coated with Au/Pd, and imaged using Phillips Scanning Electron Microscope (FEI Company, Model: XL30).

Histological Analysis
Freshly excised hearts were arrested in diastole with 1 mol/L KCl, fixed in 10% buffered formalin, paraffin-embedded and processed for trichrome and picrosirious red staining as before.25 Myocyte cross-sectional areas were measured by tracing the cross section of the myocytes in trichrome-stained sections using Image Proplus software.26 Fluorescent staining for integrin β1 was performed on OCT-frozen tissue as described.

Statistical Analysis
Reported averaged values are Mean ± SEM. Statistical analysis was performed using the IBM SPSS Statistics 19 software. One-way ANOVA was followed by multiple-comparison testing (Student-Newman-Keuls test) if the results attained statistical significance (P<0.05). Pair-wise comparisons were made between the WT and TIMP2−/− sham, 2 week-TAC, and 5 week-TAC groups using the Student t test. For the data shown in Figure 1, Figure 2C, Figure 4B through D, Figure 5, Figure 6, Figure 7A through B, and online-only Data Supplement Figure III, we first confirmed that the data were normally distributed (Shapiro-Wilk Statistic; P<0.05), and then performed statistical analyses as noted above. Statistical significance was recognized at P<0.05.

Results
TIMP2 is Critical in Cardiac Response to Pressure Overload
We first investigated how cardiac TIMPs change in response to pressure overload (TAC). We found that, although TIMP1, TIMP3, and TIMP4 remained unaltered, TIMP2 protein levels increased significantly after 2 weeks and 5 weeks of TAC (Figure 1). To determine the precise role of TIMP2 in pressure overload-induced heart disease, we subjected mice lacking TIMP2 to pressure overload by TAC. After 2 weeks and 5 weeks, TIMP2−/−TAC hearts were larger than the parallel WT-TAC hearts (Figure 2Ai) with greater myocardial hypertrophy as determined by larger LV weight-to-tibial length ratio (Figure 2Aii) and cardiomyocyte cross-sectional area (Figure 2Bi and ii) compared to WT-TAC group. TIMP2−/−TAC hearts also exhibited exacerbated adverse pathological remodeling as detected by a larger increase in mRNA expression of molecular markers of cardiac disease, BNP, βMHC and α-skeletal actin (Figure 2Ci through iii). These results show that TIMP2 is upregulated in a pressure-overload state, and that loss of TIMP2 exacerbates the adverse pathological myocardial remodeling.

TIMP2-Deficient Mice Exhibit Exacerbated LV Dysfunction Post-TAC
Echocardiographic analysis of cardiac function revealed accelerated LV dilation and systolic dysfunction in TIMP2−/−TAC mice at 2 weeks post-TAC as evident from B-mode
(Figure 3Ai) and M-mode images (Figure 3Aii) showing reduced ejection fraction (EF), greater LV end-diastolic diameter (LVEDD), and reduced velocity of circumferential shortening (VCFc) compared with WT-TAC mice (Figure 3Bi through iii). The E /E' ratio, a measure LV filling pressure, was markedly elevated in TIMP2 /H11002 /H11002 -TAC mice (Figure 3C, Di, and online-only Data Supplement Table I). The left atrium (LA), and LA-to–body weight ratio (LA/BW) was enlarged by 2-fold in TIMP2 /H11002 /H11002 -TAC hearts (Figure 3Dii, online-only Data Supplement Figure I, and online-only Data Supplement Table I) further indicating worsened systolic dysfunction in these mice. The systolic dysfunction in TIMP2 /H11002 /H11002 -TAC mice exacerbated further by 5 weeks compared with the parallel WT-TAC group resulting in further elevations in E/E' and a restricted transmitral filling pattern (online-only Data Supplement Table I). These data indicate that TIMP2 is essential for an optimal adaptive functional response to increased biomechanical stress in the heart.

Lack of TIMP2 Leads to Excess Myocardial Fibrosis
Pressure overload triggered interstitial fibrosis in TIMP2 /H11002 -TAC, but not in WT mice, at 2 weeks, which became more severe and more prevalent by 5 weeks (Figure 4A). The more severe fibrosis in TIMP2 /H11002 -TAC hearts was not due to the increased expression of collagen type-I or type-III, the predominant components of fibrillar ECM, which were elevated similarly in both genotypes (Figure 4Bii and 4Biii). We found that levels of SPARC (secreted protein acidic and rich in cysteine), a protein that mediates extracellular stabilization of collagen fibers,27,28 were significantly elevated in TIMP2 /H11002 -TAC compared with WT-TAC hearts (Figure 4C). In addition, the greater myocardial fibrosis in TIMP2 /H11002 -TAC hearts was associated with higher expression of TIMP1, a well-known marker of fibrosis,24,29,30 which increased progressively and to a markedly greater extent in TIMP2 /H11002 -TAC compared with WT hearts post-TAC (Figure 4D). Interestingly, the increase in TIMP1 mRNA levels did not result in a similar rise in its protein level (Figure 1) indicating a dissociation between transcription and translation of this molecule. Hence, the increased myocardial fibrosis in TIMP2 /H11002 -TAC hearts is due to post-translational stabilization of collagen fibers rather than just the increased collagen production.

Figure 1. Pressure overload impacts each TIMP differently. Representative Western blot (i) and averaged protein quantification (ii) of TIMP1, TIMP2, TIMP3, and TIMP4 after 2 weeks (A) or 5 weeks (B) of sham operation or post-TAC. TAC indicates transverse aortic constriction; TIMP, tissue inhibitor of metalloproteinases; and A.U., arbitrary units. n=6/group. *P<0.05 compared with sham.
Detailed analysis of the ECM structure by scanning electron microscopy (SEM) revealed a remarkable degree of organization of the fibrillar ECM network in sham-operated hearts (Figure 4Ei through iv). By 2 weeks post-TAC, this fibrillar arrangement started to become distorted in the WT hearts (Figure 4Eii and iii), whereas TIMP2-TAC hearts showed a very severe and nonuniform disorganization of the ECM. Although degraded ECM was detected in some areas (Figure 4Ev), other areas showed severe thickening of the ECM fibrillar structure (Figure 4Evi). Clearly, loss of TIMP2 led to both quantitative and qualitative alterations in the ECM network structure.

Collagenase Activity is Augmented in TIMP2−/−-TAC Hearts
Consistent with the critical role of TIMP2 in conversion of pro-MMP2 to active MMP2, we found elevated levels of active MMP2 (64kDa) in WT, but not in TIMP2−/− hearts post-TAC, whereas MMP9 levels increased similarly in both genotypes (Figure 5A). Expression analysis of MMPs revealed that the mRNA levels of MMP2 and MMP9 were similar between genotypes at 2 weeks post-TAC, whereas MMP2 mRNA increased in TIMP2−/−-TAC hearts at 5 weeks, although no active MMP2 was detected in these hearts at any time point. Although expression of MMP8 and MMP12 were too low to be detected, MMP13 was elevated in TIMP2−/−-TAC hearts at 2 weeks but not at 5 weeks (online-only Data Supplement Figure III). Total collagenase activity was greater in TIMP2−/−-TAC compared with WT-TAC hearts (Figure 5B). Activity of MT1-MMP, a membrane-type MMP and a major collagenase that is potentially inhibited by TIMP2, was markedly greater in TIMP2−/−-TAC compared with WT-TAC at 2 weeks post-TAC (Figure 5C), although MT1-MMP protein or mRNA levels remained unaltered at this time (online-only Data Supplement Figure IIID and IV). The rise in MT1-MMP mRNA at 5 weeks post-TAC could contribute to further deterioration of cardiac structure and function in TIMP2−/− mice at later time points (online-only Data Supplement Figure IIID). These results show that TIMP2 is a critical negative regulator of collagenase activity, particularly MT1-MMP activity in the heart in a pressure-overload state.

Cell-ECM Adhesion is Impaired in TIMP2−/−-TAC Hearts
The severe LV dilation and reduced systolic function with increased collagenase activity in TIMP2−/−-TAC mice lead...
us to investigate if TIMP2-deficiency impacts the cell-ECM interaction. The cardiomyocyte-ECM connections are primarily mediated by integrins.33–35 Immunostaining (Figure 6A) and Western blot analysis on the membrane protein fraction (Figure 6B) showed elevated integrin \( \alpha/1D \) levels post-TAC in WT, but not in TIMP2\(^{-/-}\) hearts, whereas its mRNA levels increased similarly between the two genotypes (Figure 6C). Integrins act as biomechanical sensors and convert mechanical stress, to biochemical signals.35,36 One of the well-known intracellular signaling pathways activated by integrin \( \beta_1 \) is phosphorylation of focal adhesion kinase (FAK).37–39 Consistent with reduced integrin \( \beta_1 \) levels in the TIMP2\(^{-/-}\)-TAC cardiomyocyte membranes, p-FAK levels were significantly lower in these hearts compared with WT-TAC hearts (Figure 6D). In vitro cell-adhesion assay further revealed that, whereas post-TAC cardiomyocytes from either genotype showed similar impairment in adhesion to collagen type I, there was a greater impairment of cell adhesion to laminin.
and fibronectin by TIMP2−/−-TAC cardiomyocytes (Figure 6E). This indicates that loss of TIMP2 leads to selective loss of integrin β1D resulting in reduced FAK phosphorylation, and thereby compromising cardiomyocyte adhesion to the ECM.

**Inhibition of MMPs Ameliorated Myocardial Hypertrophy and Fibrosis, and LV Dysfunction in TIMP2−/−-TAC Mice**

In order to examine if absence of TIMP2 and the resulting uncontrolled proteolytic activities underlie the worsened LV dilation and dysfunction following pressure overload in these mice, we treated TIMP2−/−-TAC mice with the MMP inhibitor (MMPi) PD166793, which has also been demonstrated to efficiently inhibit the membrane type MMP, MT1-MMP.22,23 Total collagenase activity (Figure 7A) and MT1-MMP activity (Figure 7B) were markedly reduced in MMPi-treated TIMP2−/−-TAC hearts up to 5 weeks post-TAC, which was accompanied by a reduction in mRNA levels of MT1-MMP, MMP2, and MMP9, but not MMP13 (online-only Data Supplement Figure III). This suppression of MMP activity...
was accompanied by lack of LV hypertrophy (Figure 7Ci and ii) and interstitial fibrosis (Figure 7D). In addition, at 5 weeks post-TAC, MMPi-treated TIMP2/H11002/H11002 mice showed no systolic dysfunction, as indicated by preserved LV contractility (EF and VCFc) and lack of LV dilation (LVEDD) (Figure 8Ai and ii), as well as a marked reduction in LV filling pressure estimated by E/E’ ratio, normalized LA size and restoration of a normal transmitral filling pattern (Figure 8Bi through iii, and online-only Data Supplement Figure I). As such, early and persistent suppression of the increased MMP activity in the TIMP2-null setting rescued the pathological cardiac remodeling and systolic dysfunction.

Discussion

Excessive biomechanical stress from hypertension or aortic valve stenosis remains an important cause of systolic and diastolic failure.2 The mechanical stress exerted on the heart by pressure overload is transmitted to the ECM and the cell-ECM connections. Hence, the integrity of the ECM and its interaction with the cardiomyocytes is critical in optimal cardiac response to pressure overload. In this study we report that TIMP2-deficient mice exhibit early and severe dysfunction, as indicated by preserved LV contractility (EF and VCFc) and lack of LV dilation (LVEDD) (Figure 8Ai and ii), as well as a marked reduction in LV filling pressure estimated by E/E’ ratio, normalized LA size and restoration of a normal transmitral filling pattern (Figure 8Bi through iii, and online-only Data Supplement Figure I). As such, early and persistent suppression of the increased MMP activity in the TIMP2-null setting rescued the pathological cardiac remodeling and systolic dysfunction.

Tissue inhibitor of metalloproteases 2–deficient mice exhibited elevated collagenase activity despite lack of MMP2 activation. A, Gelatin zymography (i), band intensity for MMP9 (ii), pro-MMP2 (iii), and active MMP2 (iv) in WT and TIMP2^{-/-} hearts at 2 weeks postsham or TAC. Total collagenase activity (B) and specific MT1-MMP activity (C) in WT and TIMP2^{-/-} hearts post-TAC/sham. Coomassie blue staining was used as loading control. n=5 per group per genotype. *P<0.05 compared with sham; ‡P<0.05 compared with WT-TAC. WT indicates wild type; sh, sham; TAC, transverse aortic constriction; MMP, matrix metalloprotease; A.U., arbitrary units; and MT1, membrane type 1.

The worsened LV dilation and dysfunction in TIMP2^{-/-} mice following pressure overload was accompanied by a greater increase in total collagenase activity, particularly MT1-MMP compared with parallel WT hearts. Among the MMPs identified to contribute to different cardiomyopathies,9,13,45 MT1-MMP has a much greater triple-helical peptidase activity than other interstitial collagenases, such as MMP1 or MMP13,31,32 and targets a number of other ECM proteins.34,46–50 In fact, transmembrane-deletion mutant (soluble) MT1-MMP also possesses potent proteolytic activity toward a large number of ECM molecules.51 In addition, MT1-MMP also activates other collagenases, such as pro-MMP13,52 thereby further contributing to the proteolytic activities in a tissue. A direct interaction between the c-terminal domain of TIMP2 and the catalytic domain of MT1-MMP indicates that

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TIMP2 could act as a highly specific inhibitor of MT1-MMP.\textsuperscript{53} Important inhibitory function of TIMP2 against MT1-MMP has been reported in myocardial infarction.\textsuperscript{12} MT1-MMP has also been linked to myocardial fibrosis, because cardiac-specific overexpression of MT1-MMP resulted in myocardial fibrosis.\textsuperscript{23,54} Myocardial fibrosis contributes to cardiac dysfunction in different types of heart disease. It is a characteristic feature of pressure overloaded cardiomyopathy, where it leads to myocardial stiffness and diastolic dysfunction,\textsuperscript{3,55} whereas in myocardial infarction, replacement fibrosis secondary to myocyte loss is associated mainly with systolic dysfunction.\textsuperscript{56} MT1-MMP also promotes invasion of fibroblasts, the collagen-producing cells, through its focal collag enolytic activity.\textsuperscript{37} We found that TIMP2\textsuperscript{−/−}-TAC mice exhibit excess myocardial fibrosis, associated with elevated SPARC levels resulting in stabilization of the collagen fibers, while the rise in collagen production was similar between genotypes post-TAC. MT1-MMP has been shown to trigger myocardial fibrosis by increasing the expression of collagens.\textsuperscript{23,54} MT1-MMP activates pro-MMP2 through forming a trimolecular complex with TIMP2.\textsuperscript{15} MMP2 has also been shown to trigger fibrosis through releasing the ECM-bound latent transforming growth factor-\(\beta\) (TGF\(\beta\)), thereby inducing
collagen synthesis. Consistently, cardiac overexpression of MMP2 led to severe myocardial fibrosis. As such, an increase in MT1-MMP activity is inevitably associated with elevated MMP2 activation, unless when TIMP2 is lacking. Hence, the TIMP2/−/− mice provide an exciting model to examine the outcomes of MT1-MMP activation in the absence of MMP2 activation. Our study provides evidence for a novel profibrotic role for MT1-MMP through post-translational stabilization of the collagen fibers rather than increased production of collagens.

The biomechanical stress exerted on the heart is transmitted to the ECM. Integrins are cell-surface receptors that mediate ECM adhesion and convert the extracellular biomechanical stress to intracellular biochemical signaling. Integrin β1D levels increase during the compensatory phase of cardiac response to pressure overload. In contrast, in TIMP2−/− hearts, protein levels of integrin β1D did not increase following pressure overload despite a comparable increase in integrin β1D mRNA levels. Consistently, TIMP2−/−-TAC cardiomyocytes showed impaired adhesion to fibronectin, a basement membrane protein that cross-links with the integrins, and to laminin, the molecule that connects the ECM to the integrins. Tissue inhibitor of metalloproteinase 2 has been reported to regulate integrin β1 expression in skeletal muscle. Although we found that TIMP2 deficiency did not alter integrin β1 mRNA expression in the heart post-TAC, the reduced integrin β1 protein levels suggest its proteolytic degradation in TIMP2−/−-TAC hearts, which correlates with the higher collagenase and MT1-MMP activities in these hearts. Membrane type 1-MMP is a strong candidate among MMPs to mediate degradation of the integrin structure because it colocalizes to the cell membrane in proximity to the ECM-binding integrins. However, contribution of soluble MMPs such as MMP13 cannot be excluded. Consistent with our finding of exacerbated cardiac hypertrophy and fibrosis in TIMP2−/−-TAC mice, cardiac-specific deletion of integrin β1 resulted in cardiac fibrosis and heart failure, as well as intolerance to pressure overload, whereas loss of FAK signaling in the heart led to worsening of hypertrophy and fibrosis after pressure overload. The impaired cardiomyocyte-ECM connections that are caused by reduced levels
of integrin-β1D in the TIMP2−/−-TAC mice exacerbate the dilated cardiomyopathy in these mice. Our study provides a novel role for TIMP2 in the link between cardiomyocyte-ECM interaction and posttranslational regulation of integrin-β1 in the heart.

We further demonstrate that inhibition of the upregulated MMPs including MT1-MMP9,11,66 reduced the myocardial fibrosis and hypertrophy, which culminated in a marked improvement in the systolic dysfunction in TIMP2−/−-TAC mice. Hence the negative outcomes of TIMP2 deficiency are in fact due to its lack of MMP-inhibitory function and the resulting uncontrolled proteolytic activities in disease. For this reason, the rise in TIMP2 levels after pressure overload, as also observed in patients,16,17 is likely an adaptive response to dampen the elevated proteolytic activities that occur in heart disease.

Our study provides a novel insight into the role of TIMP2 in pressure-overloaded cardiomyopathy and the molecular mechanisms for fibrosis, ECM integrity, and cardiomyocyte-ECM interaction. In addition, this study completes the analysis of the role of each TIMP in cardiac recovery from pressure overload. Unlike TIMP140 and TIMP4,41 which do not impact cardiac response to pressure overload, TIMP3 deficiency severely exacerbates this process.9,24 Therefore, TIMP2 and TIMP3 have emerged as key players in pressure-overloaded cardiomyopathy, although they each function through a different mechanism. Confirmation of these findings in humans is essential in order to explore the possible interspecies differences in the function of TIMP2 and to facilitate potential therapeutic approaches based on replenishing TIMP2 and/or TIMP3 in heart disease.

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Disclaimers

None.

References


**CLINICAL PERSPECTIVE**

Tissue inhibitor of metalloproteinases 2 (TIMP2) is one of the TIMPs that is required for regulating the structural remodeling of the cardiac extracellular matrix. Tissue inhibitor of metalloproteinases 2 is the only TIMP with the unique property of activating matrix metalloproteinases 2 (MMP2) in addition to inhibiting a number of MMPs. Tissue inhibitor of metalloproteinases 2 levels are increased in the hearts of patients with aortic valvular stenosis and patients with pressure-overloaded cardiomyopathy. However, whether this rise in TIMP2 is detrimental (by activating MMP2) or beneficial (through inhibiting MMPs) to the diseased heart is not clear. Our study demonstrates that lack of TIMP2 severely compromises cardiac response to biomechanical stress through adverse structural remodeling and impaired myocyte–extracellular matrix interaction, leading to worsening of left ventricular dilation and dysfunction. On the basis of our study, the rise in TIMP2 in the hearts of patients with pressure-overloaded cardiomyopathy is a compensatory response to overcome the elevated proteolytic activities in these diseased hearts, although it may not reach a sufficiently high level to prevent the disease progression. For this reason, overexpression of TIMP2 could serve as a potential therapy in pressure-overloaded cardiomyopathy.
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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

In vivo pressure overload by transverse aortic constriction (TAC)

Wild-type (WT) and TIMP2-deficient (TIMP2\(^{-/-}\))\(^1\) mice in C57BL/6 background were bred in our animal facility at University of Alberta. Eight-week old male WT and TIMP2\(^{-/-}\) mice underwent transverse aortic constriction (TAC) to generate pressure overload as before.\(^2\) Briefly, mice were anesthetized with 1.5 ketamine (100mg/kg) and xylaxine (10 mg/kg). A horizontal skin incision of 1 cm was made at the level of second intercostal space. A 6-0 silk suture was passed under the aortic arch. A bent 27-gauge needle was then placed next to the aortic arch and the suture was snugly tied around the needle and the aorta, between the left carotid artery and the brachiocephalic trunk. The needle was quickly removed allowing the suture to constrict the aorta. The incision was closed in layers and the mice were allowed to recover on a warming pad until they were fully awake. Mice received penicilline and buprenorphine for the first 24 hours. The sham animals underwent the same procedure without the constriction of aorta.

At 2 weeks or 5 weeks post-surgery, sham and TAC mice from either genotype were lethally anesthetized, heart and lung tissue were excised and formalin-fixed or flash-frozen in liquid nitrogen and stored in -80\(^{\circ}\)C until being used for experiments. All animal experiments were carried out in accordance with Canadian Council on Animal Care Guidelines and regulations of Animal Care and Use Committee at University of Alberta, as well as the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.
Cardiac functional analysis by echocardiography, tissue Doppler imaging (TDI) and transmitral Doppler (TMD) imaging

Systolic and diastolic cardiac function was determined by transthoracic echocardiography, non-invasively using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada) as before. Mice were anesthetized with 0.75% isoflurane for the duration of the recordings. M-mode images were obtained for measurements of LV wall thickness (LVWT), LV end-diastolic diameter (LVEDD), and LV end-systolic diameter (LVESD) (measures of LV dilation). LV ejection fraction (EF) was calculated using the following equation: \( \text{EF(\%)} = \frac{\text{LVEDV}-\text{LDESV}}{\text{LVEDV}} \times 100 \) as a measure of systolic function.

Diastolic function was assessed using pulsed-wave Doppler imaging of the transmitral filling pattern with the early transmitral filling wave (E-wave) followed by the late filling wave due to atrial contraction (A-wave). Isovolumetric relaxation time (IVRT) was calculated as the time from closure of the aortic valve to initiation of the E-wave. The deceleration time of the E-wave deceleration time (DT) was determined by measuring the time needed for the down-slope of the peak of the E-wave to reach the baseline while the rate of E-wave deceleration rate (EWDR) was calculated as the E-wave divided by the DT. Tissue Doppler imaging (TDI) represents a novel and validated technique to assess systolic and diastolic function with a reduction in E’ and an elevation in E/E’ being considered valid markers of elevated LV filling pressure and diastolic dysfunction. TDI was made at the inferolateral region in the radial short axis at the base of the LV with the assessment of early diastolic (E’) and late diastolic (A’).
myocardial velocities. The maximal anteroposterior LA diameter was measured by M-mode in the parasternal long-axis view and used as LA size.

**In vivo MMPI treatment**

Mice received a broad-spectrum MMP-inhibitor, PD166793 (Pfizer Inc, 30mg/Kg/day) by daily gavage\(^4\) one day before TAC surgery and through the course of the study. Systolic and diastolic cardiac functions were assessed at 2 weeks and 5 weeks post-TAC.

**Protein analysis, Western blotting, Gelatin zymography, collagenase and MT1-MMP activity assays**

Total protein was extracted from frozen tissue by mechanical homogenization in EDTA-free RIPA extraction buffer and quantified using BioRad DC protein assay. Gelatin zymography was performed to detect pro- and active MMP2 and MMP9 as before.\(^4,5\) Western blot analysis was performed to detect TIMP1, TIMP2, TIMP3 and TIMP4 as before.\(^4,5\) Integrin β1D (Millipore),\(^6\) phospho-FAK (Tyr397, Millipore) and total FAK (Santa Cruz). In all Western blots, Ponceau staining of the membrane was used as the loading control.

For *in vitro* activity assays LV myocardial protein was extracted using cyobuster protein extraction reagent (Invitrogen). Total collagenase activities were measured using fluorescent-based activity assays from EnzCheck (Molecular Probes) as before.\(^4,5\) MT1-MMP activity was measured as described\(^7,8\) using a specific MT1-MMP fluorogenic substrate (Calbiochem). LV myocardial protein extracts were incubated in the presence of the MT1-MMP substrate (37°C), and excitation/emission (328/400nm, Spectramax M5 microplate reader) were recorded over 5
hours. The negative controls did not include the MT1-MMP substrate or protein extract. Fluorescence readings were converted to MT1-MMP activity by using a recombinant active MT1-MMP construct (MT1-MMP catalytic Domain, Calbiochem) in a parallel set of reactions.

**RNA expression analysis**

Total RNA was extracted using TriZol Reagent (Invitrogen) and mRNA expression analysis was performed by TaqMan RT-PCR as before. HPRT (hypoxanthine-guanine phosphoribosyltransferase-1) was used as the internal control. Taqman primers and probe used to measure the expression of integrin β1 was purchased from ABInstruments (Mm01253230_m1), and for pro-collagen I-α1 and pro-collagen III-α1 type II as reported previously.

**In vitro adhesion assay**

Adhesion of adult cardiomyocytes to ECM proteins were assessed by using an in situ CytoSelect Cell Adhesion Assay (ECM Array, Colorimetric Format, Cell Biolabs, Inc) according the manufacturer’s instructions as before. Cardiomyocytes were isolated from mice of either genotype at 2 weeks after sham or TAC–operation and were plated (20,000 cells/well) in wells coated with different ECM proteins. After 1hr of incubation (2% CO2, 37°C), cells were washed with PBS to remove the non-adherent cells prior to adding Cell Stain solution. The excess staining solution was washed with PBS, and extraction solution was added to redissolve the dye. The solution was transferred to a fresh plate and optical density (OD) was recorded at 560 nm.
Scanning Electron microscopy

Scanning electron microscopy (SEM) was performed as before. Briefly, 2-3mm pieces of the LV were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Phosphate Buffer for 48 hrs at room temperature. Samples were washed in 0.1M Phosphate Buffer (3X15 min), followed by quick distilled water rinse. Dehydration was carried out by a series of ethanol washes. Hexamethyldisilazane (HMDS) was used (instead of critical point drying) for tissue preparation. Samples were air dried overnight, mounted on SEM stubs, sputter coated with Au/Pd, and imaged using Phillips Scanning Electron Microscope (FEI Company, Model: XL30).

Histological analysis

Freshly excised hearts were arrested in diastole with 1M KCl, fixed in 10% buffered formalin, paraffin-embedded and processed for trichrome and picrosirious red staining as before. Myocyte cross-sectional areas were measured by tracing the cross section of the myocytes in trichrome-stained sections using Image Pro-plus software.

Fluorescent staining for integrin-β1 was performed on OCT-frozen tissue as described. Briefly, transverse sections (20 μm) were direct mounted onto gelatin coated slides, and after being hydrated and blocked, sections were incubated with rabbit α-human integrin β1 primary antibody (1:500, AB 1952; Millipore, Billerica, MA), and Cy3-conjugated goat α-rabbit IgG secondary antibody (1:500, Jackson ImmunoResearch, West Grove, PA). Slides were cover slipped with Citifluor (Electron Microscopy Sciences; Hatfield, PA), immunoreactivity was visualized with a Nikon Eclipse C1confocal microscope (MicroVideo Instruments; Avon, MA),
and images were acquired with identical settings for all samples. Specificity of labeling was verified by exclusion of primary antibody and use of control IgG.
Supplemental Table 1 – Echocardiographic assessment of systolic and diastolic function in WT and TIMP2\(^{-/-}\) mice after sham, 2 weeks or 5 weeks after transverse aortic constriction (TAC), and after MMPI-treatment at 2 weeks and 5 weeks post-TAC. LVEDD= LV end diastolic diameter; LVESD=LV end systolic diameter; LVFS=LV fractional shortening; LVEF=LV ejection fraction; VCFc= Velocity of circumferential shortening corrected for heart rate; LVPWT=LV Posterior wall thickness. E-wave= early transmitral inflow velocity; A-wave=transmitral inflow velocity due to atrial contraction; DT=E-wave deceleration time; EWDR=E-wave deceleration rate (E-wave/DT); E’=early tissue Doppler velocity; LA=Left atrium; BW=body weight. *p<0.05 compared to sham, ‡ p<0.05 compared to WT-TAC. § p<0.5 compared to TIMP2\(^{-/-}\)-TAC.
Supplemental Figure 1 – Echocardiographic images of the left atrium (LA) by B-mode (i) and M-mode (ii) imaging in WT and TIMP2−/− mice after 2 weeks of sham or TAC (A). B) Left atrial images or after 5 weeks of TAC in WT, TIMP2−/− and TIMP2−/−+MMPi mice. The double-headed arrow shows the diastolic atrial size.
Supplemental Figure 2 – Lung water weight (wet weight – dry weight) in WT and TIMP2/-mice after sham or TAC, and in TIMP2/-+MMPi after 2 weeks or 5 weeks of TAC. n=8/group/genotype. * p<0.05 compared to sham, ‡p<0.05 compared to WT-TAC, § p<0.05 compared to TIMP2/--TAC
**Supplemental Figure 3.** Relative mRNA expression of myocardial MMPs sham-operated WT and TIMP2−/− heart, 2 weeks and 5 weeks post-TAC. Myocardial MMP expression for TIMP2−/− mice treated with MMP inhibitor (MMPi) for 5 weeks post-TAC is shown in black bar (n=8/group/genotype). R.E.= relative expression. * p<0.05 compared to corresponding sham; ‡ p<0.05 compared to corresponding WT-TAC; § p<0.05 compared to corresponding TIMP2−/−-TAC.
Supplemental Figure 4 – Representative Western blot (A) and averaged MT1-MMP protein levels (B) on membrane protein fraction from WT and TIMP2−/− mice after sham or TAC. n=4/sham, 6/TAC/genotype. AU=arbitrary units.
REFERENCES


