Cardiac matrix metalloproteinase-2 expression independently induces marked ventricular remodeling and systolic dysfunction

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Cardiac matrix metalloproteinase-2 expression independently induces marked ventricular remodeling and systolic dysfunction. Am J Physiol Heart Circ Physiol 292: H1847–H1860, 2007. First published December 8, 2006; doi:10.1152/ajpheart.00434.2006.—Although enhanced cardiac matrix metalloproteinase (MMP)-2 synthesis has been associated with ventricular remodeling and failure, whether MMP-2 expression is a direct mediator of this process is unknown. We generated transgenic mice expressing active MMP-2 driven by the α-myosin heavy chain promoter. At 4 mo MMP-2 transgenic hearts demonstrated expression of the MMP-2 transgene, myocyte hypertrophy, breakdown of Z-band registration, lysis of myofilaments, disruption of sarcomere and mitochondrial architecture, and cardiac fibroblast proliferation. Hearts from 8-mo-old transgenic mice displayed extensive myocyte disorganization and dropout with replacement fibrosis and perivascular fibrosis. Older transgenic mice also exhibited a massive increase in cardiac MMP-2 expression, representing recruitment of endogenous MMP-2 synthesis, with associated expression of MMP-9 and membrane type 1 MMP. Increases in diastolic [control (C) 33 ± 3 vs. MMP 51 ± 12 μl; P = 0.003] and systolic ([C 7 ± 2 vs. MMP 28 ± 14 μl; P = 0.003]) left ventricular (LV) volumes and relatively preserved stroke volume ([C 26 ± 4 vs. MMP 23 ± 3 μl; P = 0.16]) resulted in markedly decreased LV ejection fraction (C 78 ± 7% vs. MMP 48 ± 16%; P = 0.0006). Markedly impaired systolic function in the MMP transgenic mice was demonstrated in the reduced preload-adjusted maximal power (C 240 ± 84 vs. MMP 78 ± 49 mW/μl2; P = 0.0003) and decreased end-systolic pressure-volume relation ([C 7.5 ± 1.5 vs. MMP 4.7 ± 2.0; P = 0.016]). Expression of active MMP-2 is sufficient to induce severe ventricular remodeling and systolic dysfunction in the absence of superimposed injury.

heart failure; fibrosis; mitochondria; sarcomere

THE PAST DECADE HAS WITNESSED an increasing interest in the interactions of cardiomyocytes with the extracellular matrix, particularly with regard to the development of cardiac failure and fibrosis. Modulation of the cardiac extracellular matrix by various members of the large matrix metalloproteinase (MMP) gene family has provided important mechanistic insights into the evolution of left ventricular failure in the setting of both ischemic and nonischemic disease (10, 11, 20, 29, 49, 51). The MMP gene family includes >20 discrete members, including the interstitial collagenases (e.g., MMP-1 and –13), the gelatinases (MMP-2, –9), and membrane-associated enzymes, including membrane type 1(MT1)-MMP (60). MMP-2 has been the focus of considerable interest, as previous studies in animal models of heart failure, including the salt-sensitive hypertensive rat (19, 43), mitral regurgitation in the dog (52), and experimental myocardial infarction in the rat (41), have all shown excessive activation of MMP-2 as heart failure progressed. Inhibition of MMP-2 activation with nonspecific agents, including an angiotensin-converting enzyme inhibitor or curcumin, was associated with prevention or improvement of left ventricular dysfunction and remodeling in chronic heart failure models (8, 43). In acute studies, nonspecific MMP-2 inhibition with doxycycline or o-phenanthroline improved hemodynamic function after ischemia-reperfusion injury and prevented left ventricular remodeling several weeks later (59, 62). In a mouse model, targeted deletion of the MMP-2 gene attenuated the frequency of left ventricular rupture and late remodeling after experimental myocardial infarction (18).

Recent clinical studies demonstrate an association between circulating levels of MMP-2 and cardiac dysfunction in patients with hypertrophic cardiomyopathy who exhibit systolic dysfunction (39), with ischemic cardiomyopathy (58), and with congestive heart failure (4, 64). Elevated circulating MMP-2 levels are associated with left ventricular remodeling after myocardial infarction (34) and also predict poor outcome in patients with congestive heart failure (15). Despite this substantial experimental and clinical evidence for a central role of MMP-2 in cardiac dysfunction, the question remained whether MMP-2 alone, in the absence of superimposed injury, is sufficient to trigger the pathophysiological processes characteristic of ventricular remodeling and contractile failure. To answer this question we generated transgenic mice with cardipecific expression of constitutively active MMP-2. We find that cardiac MMP-2 transgenic mice, in the absence of additional experimental manipulation, develop profound systolic dysfunction, fibrosis, and cardiomyocyte dropout, thereby demonstrating that MMP-2 is a direct determinant, as opposed to an associated factor, of the development of ventricular remodeling and failure.

MATERIALS AND METHODS

The investigation was approved by the Animal Care Subcommittee of the San Francisco Veterans Affairs Medical Center and conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, Revised 1996). Generation of cardiac-specific MMP-2 transgenic mice. Neonatal rat cardiac fibroblasts were isolated and cultured as reported previ-
ously (6). The MMP-2 cDNA was prepared by RT-PCR from poly(A) RNA isolated by standard methodology. The murine and rat cDNAs for MMP-2 share 100% homology within the coding region (31). The MMP-2 cDNA was cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA) and Val107 in the prodomain was mutated to Gly107, generating a constitutively active MMP-2 enzyme (56). Validation of the enzymatic activity of this construct was confirmed by subcloning the prodomain-mutated MMP-2 into the expression plasmid pcDNA3.1 (Invitrogen), followed by transient transfection of COS-7 cells. Serum-free conditioned medium was harvested after 48 h, clarified by centrifugation at 10,000 × g for 10 min, and analyzed by gelatin zymography as described previously (31). MMP-2 activity was determined with a biotinylated gelatinase substrate kit according to the manufacturer’s instructions (Chemicon). Latent recombinant human MMP-2 was expressed and purified as reported for MMP-9 (36).

The prodomain-mutated MMP-2 cassette was recovered from the TOPO-TA plasmid with primers encoding a Kozak consensus sequence and the c-myc epitope tag (E42QKLIQDEDL) at the COOH terminus. The MMP-2/c-myc expression cassette was isolated by PCR to add Sall and HindIII restriction sites, followed by cloning into these restriction sites in the α-myosin heavy chain promoter vector (the kind gift of Dr. Jeffrey Robbins, Children’s Hospital Research Foundation, Cincinnati, Ohio). The assembled construct was excised by NotI digestion, purified, and microinjected into 129Sv × CD1 background fertilized pronuclei. Transgenic mice were identified by PCR of tail genomic DNA using a 5′ primer overlapping the α-myosin heavy chain promoter and a 3′ primer for MMP-2. Southern blot analysis was performed with a 32P-labeled PCR product. Four founders were obtained, and three lines (712, 713, and 729, with 4–6 insert copies and single integration sites) were carried in the CD-1 background as heterozygotes. Each line manifested a virtually identical phenotype, and data primarily from lines 712 and 713 are shown in this report.

Transgene expression. Hearts were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.5% CHAPS, and 0.5% sodium deoxycholate, plus a protease inhibitor cocktail] with 25 strokes of a loose-fitting Dounce pestle. The homogenate was incubated on ice for 15 min and pelleted at 700 × g for 6 for 20 min. Cardiac extracts (200 μg protein/sample) were incubated overnight at 4°C with 100 μl of gelatin-Sepharose beads (Sigma-Aldrich, St. Louis, MO) in 500 μl of 50 mM Tris-HCl (pH 7.4) to affinity absorb MMP-2. Thereafter the beads were washed three times in binding buffer, followed by elution in an equal volume of 2× SDS-PAGE sample buffer. Western blots were performed as reported previously (3). In brief, left ventricles from wild-type and MMP-2 transgenic mice at 4 and 8 mo of age (n = 6 for each study group) were rinsed with calcium- and magnesium-free phosphate-buffered saline (PBS) at 4°C containing 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1× Complete Mini-protease inhibitor cocktail (Roche). Fifty milligrams of finely diced ventricle was homogenized in 0.75 ml of hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1× Complete Mini-protease inhibitor cocktail] with 25 strokes of a loose-fitting Dounce pestle. The homogenate was incubated on ice for 15 min and pelleted at 700 g for 5 min at 4°C. Pelleted nuclei were washed twice in the hypotonic buffer, and the purity of the preparation was confirmed by inspection under phase-contrast microscopy. Nuclear extracts were prepared according to Dignam et al. (12). KCl (1 M) buffer was used for extraction, followed by dialysis overnight at 4°C in 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM dithiothreitol, and 0.2 mM PMSF. Protein concentrations were determined with the bicinchoninic acid assay (Pierce).

Measurement of cardiac nuclear JunB and FosB content. AP-1 transcription factors JunB and FosB were quantitatively assessed with a solid-phase ELISA kit (TransAM, Active Motif). In brief, 10 μg of nuclear extract protein was incubated in a 96-well format with an immobilized oligonucleotide encoding the consensus AP-1 binding motif (5′-TGAGTCA-3′), washed, and incubated with anti-JunB or anti-FosB murine monoclonal antibodies according to the manufacturer’s instructions. Thereafter, the wells were washed, incubated with HRP-conjugated goat anti-mouse IgG, and developed with tetramethylbenzidine-soluble HRP substrate. Data are expressed as absorbance at 450 nm compared with control wells incubated with nuclear extract without immobilized oligonucleotide. Specificity of nuclear protein binding was confirmed by coincubation with a 10-fold molar excess of soluble oligonucleotide.

Histology. Mice were anesthetized with ketamine-xylazine and arrested in diastole with 50 mM KCl. The hearts were then perfused for 30 min at 20 mmHg pressure with ice-cold buffered 4% paraformaldehyde, followed by paraffin embedding. Five-micrometer sections of each study group were stained with hematoxylin and eosin, Masson’s trichrome, or Picrosirius red by standard methodology. For electron microscopy, hearts were perfused with ice-cold PBS and blocks of the left ventricular free wall were fixed in modifed Karnovsky solution. Ultrathin

Western blot analysis. Clarified ventricular protein lysates (25 μg/sample) were electrophoresed, transferred, and probed with murine monoclonal anti-MMP-2 (Ab-3, Oncogene), rabbit anti-MMP-9 (Chemicon), murine monoclonal anti-MT1-MMP (Ab-4, Oncogene), or murine monoclonal anti-MMP-13 (D-17, Santa Cruz Biotechnology). The secondary antibodies were HRP-conjugated F(ab)2 goat anti-mouse IgG or goat anti-rabbit IgG (Zymed), followed by development with ECL reagent. For Western blot analysis of troponin I and β-actin, ventricular extracts were prepared from liquid nitrogen-frozen powder according to Wang et al. (62), and 25 μg of extract was used for each sample. Murine monoclonal anti-troponin I (clone 8L7) was obtained from Spectral Diagnostics (Toronto, ON, Canada). Murine monoclonal anti-β-actin was obtained from Sigma-Aldrich. Secondary antibodies and development were as detailed above. All Western blots were performed on a minimum of six ventricular lysates from each line and transgenic mice at 4 and 8 mo of age. For quantitation of troponin I abundance, 10 ventricular extracts at 4 and 8 mo were prepared from wild-type and transgenic mice. Scanning laser densitometry was used to quantify the relative abundance of the MMP and troponin I proteins.

Troponin I-MMP-2 interaction. Ventricular extracts were prepared from liquid nitrogen-frozen powder according to Wang et al. (62). Ventricular extracts (300 μg) in 150 μl of 50 mM Tris-HCl (pH 7.4)-0.1% Triton X-100 were tumbled overnight at 4°C with 1 μg of monoclonal anti-troponin I (clone 8L7). Thereafter, 80 μl of protein G-coupled Sepharose beads (Sigma-Aldrich) was added and tumbled at 4°C for 6 h. Bound protein was eluted by adding 30 μl of Tris-glycine SDS sample buffer (Invitrogen) and analyzed by gelatin zymography. The negative control was handled in an identical manner but with the exception that the anti-troponin I antibody was not included in the incubation mixture.

Isolation of cardiac nuclear extracts. Ventricular nuclear extracts were prepared as reported previously (3). In brief, left ventricles from wild-type and MMP-2 transgenic mice at 4 and 8 mo of age (n = 6 for each study group) were rinsed with calcium- and magnesium-free phosphate-buffered saline (PBS) at 4°C containing 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1× Complete Mini-protease inhibitor cocktail (Roche). Fifty milligrams of finely diced ventricle was homogenized in 0.75 ml of hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1× Complete Mini-protease inhibitor cocktail] with 25 strokes of a loose-fitting Dounce pestle. The homogenate was incubated on ice for 15 min and pelleted at 700 g for 5 min at 4°C. Pelleted nuclei were washed twice in the hypotonic buffer, and the purity of the preparation was confirmed by inspection under phase-contrast microscopy. Nuclear extracts were prepared according to Dignam et al. (12). KCl (1 M) buffer was used for extraction, followed by dialysis overnight at 4°C in 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM dithiothreitol, and 0.2 mM PMSF. Protein concentrations were determined with the bicinchoninic acid assay (Pierce).
sections were stained with lead citrate-uranyl acetate by standard methodology.

Left ventricular morphometry was performed on circumferential sections (2 mm) stained with Picosirisius red according to Junqueira et al. (22), and sections were examined under polarized light. Mid-left ventricular cross sections \((n = 6\) ventricles per study group) were divided into six equal sections and digitized, and the collagen volumes were determined with ImageJ (National Institutes of Health). Interstitial collagen volumes were determined according to Brilla et al. (7).

Perivascular collagen areas (PVCAcs) were determined according to Karam et al. (23). Perivascular collagen was measured on digitized images separately around each coronary artery (diameter \(> 10 \mu m\)) visible in each analyzed field (8–10 midventricular cross section fields for each studied left ventricle; \(n = 6\) ventricles per study group).

Cardiomyocyte cross-sectional areas were determined according to Akishita et al. (1), with ImageJ analysis of digitized images. Suitable cross sections were defined as having nearly circular capillary profiles and cardiomyocyte nuclei. Fifteen to twenty cardiomyocyte cross sections were measured on each mid-left ventricular cross section \((n = 6\) ventricles per study group).

**RESULTS**

**Hemodynamic analyses.** Systolic blood pressure and heart rate were measured with a noninvasive computerized tail cuff system (BP-2000; Visitech Systems, Apex, NC). Mice were trained for a few days, and recordings were made on the next 2 days, with at least 18 of 20 successful readings each day.

Transhoracic echocardiography was performed on conscious mice with a 15-MHz linear array transducer and a commercially available imaging system (Acuson Sequoia c256, Acuson, Siemens, Mountain View, CA).

For pressure-volume (PV) studies MMP-2 transgenic mice (12 mo) and age-matched littermate controls were induced with isoflurane, intubated, and mechanically ventilated with a volume-control mouse ventilator. Anesthesia was maintained at appropriate levels by checking reflex responses as well as hemodynamic perturbations and adjusting the inhaled isoflurane dose (0.25–1.0%). All studies with anesthetized mice were performed on a heating pad at 37°C. After bilateral cervical vagotomy, the left internal jugular vein and the right common carotid artery were cannulated with an infusion catheter and a 1.4-Fr PV conductance catheter (Millar Instruments, model SPR-839), respectively. Aortic root pressures were measured, and the PV catheter was advanced in a retrograde fashion into the left ventricle. Baseline left ventricular PV loops were acquired, and then the inferior vena cava was isolated via a subdiaphragmatic abdominal incision. After hemodynamic stabilization, PV loops with inferior vena cava occlusion were performed in triplicate. Subsequently, parallel conductance was estimated with a 10-µl injection of 15% saline solution and the catheter was volume calibrated with heparinized blood-filled calibration cuvettes. The mice were killed, and the left and right ventricular weights were measured.

All hemodynamic results are expressed as means ± SD; \(P\) values were calculated with a 2-tailed \(t\)-test. The data were analyzed with PVAN software (version 3.2; Millar Instruments), and statistical comparisons were performed with JMP (version 5.1; SAS Institute).

**RESULTS**

**Generation and validation of cardiac-specific transgenic mice expressing MMP-2.** The transgenic construct consisted of an expression cassette for constitutively active MMP-2, achieved through mutation of the promoderm, coupled to a c-myc epitope tag to distinguish transgenic from native MMP-2 protein (Fig. 1a). Expression was driven by the \(\alpha\)-myosin heavy chain promoter. Confirmation of the enzymatic activity of the transgenic expression cassette was provided by transient transfection of COS-7 cells. As shown in Fig. 1b, gelatin zymography of transfected COS-7 conditioned medium revealed a gelatinolytic band of 68 kDa, which is the same relative molecular mass observed with recombinant MMP-2. A direct MMP-2 enzymatic assay confirmed the enzymatic activity of the prodomain-mutated MMP-2 protein (Fig. 1c).

Four transgenic founders were identified (Fig. 1d), and three lines (712, 713, and 729) carried as heterozygotes were characterized. Each transgenic line displayed a virtually identical phenotype, and data are detailed in this report primarily from lines 712 and 713. We note that transgenic progeny from all three lines were in the expected Mendelian distribution and there were no cardiac structural or histological defects in 4-wk-old mice. Western blots of ventricular extracts confirmed MMP-2/c-myc transgenic protein expression in all three transgenic lines (Fig. 1e). Cardiomyocyte-specific MMP-2 transgene expression was confirmed by immunohistochemical (IHC) staining for the c-myc epitope tag (Fig. 1f), which revealed a uniformly strong signal. Fluorescent in situ zymography, using a quenched gelatin substrate, confirmed that transgenic MMP-2 was enzymatically active. As shown in Fig. 1fC, wild-type ventricles did not demonstrate in situ enzymatic activity, while in the transgenics (Fig. 1fD) there was clear evidence for enzymatic activity in the cardiomyocytes. Inclusion of a specific cyclic peptide MMP-2 inhibitor (CTTHWGFTLCGG, 25 µmol/l; Ref. 9) eliminated fluorescent in situ activity (not shown).

**Transgenic MMP-2 induces multiple alterations in cardiac cell structure.** The hearts from 4-mo-old MMP-2 transgenic mice and age-matched littermate controls were examined by light and electron microscopy. The results of these studies are summarized in Fig. 2. At the light microscopic level, hematoxylin and eosin-stained sections of the left ventricular free wall demonstrated cardiomyocyte hypertrophy, early disorganization, and small foci of cellular dropout without evidence for inflammatory cell infiltration (Fig. 2a, cf. A and B). Cardiomyocyte cross-sectional area was significantly increased in the 4-mo-old MMP-2 transgenic mice (wild type 188 ± 14 µm² vs. 238 ± 12 µm²; \(P < 0.05, n = 6\) ventricles per study group).

At the transmission electron microscopic level, the age-matched littermate controls revealed intact cardiomyocyte cellular structure with well-registered Z-banding patterns and intact myofibrils and mitochondria (Fig. 2b, A). In contrast, the MMP-2 transgenic mice displayed major abnormalities, particularly of the myofibrils and mitochondria (Fig. 2b, B). There was extensive loss of myofibrils and morphological nuclear changes consistent with cardiomyocyte hypertrophy. In addition, the mitochondria were pleiomorphic, and many had poorly defined cristae and matrices. In addition to the alterations in the cardiomyocytes, there was evidence for cardiac fibroblast proliferation (Fig. 2b, C and D), with the acquisition of the ultrastructural features of an activated, synthetic phenotype, as evidenced by abundant rough endoplasmic reticulum and secretory vesicles. Furthermore, the cardiac fibroblasts were surrounded by bands of organized fibrillar collagen, consistent with increased cardiac extracellular matrix synthesis. At a higher level of magnification (Fig. 3), areas of myofibril rarefaction and lysis were evident (Fig. 3B), which was accompanied by proliferation of T tubules and frequent lipid droplet inclusions. Ultrastructural changes in the mitochondria were very evident at this level of magnification, with...
widespread heterogeneity in mitochondrial volumes and loss of structural definition, particularly of the cristae (Fig. 3D).

Cellular localization of transgenic MMP-2 protein interaction with troponin I. Wang et al. (62) have reported an intracellular colocalization of MMP-2 protein with sarcomeric troponin I following acute ischemia-reperfusion injury. To more precisely define the cellular localization of the c-myc epitope-tagged MMP-2 transgenic protein, we performed additional IHC studies using an enhanced DAB staining for the epitope, followed by examination using Nomarski interference microscopy. As demonstrated in Fig. 4a, left ventricular cross sections have abundant pericellular deposition of the IHC reaction product, consistent with extracellular transport of the transgenic MMP-2 protein, while no reaction product is seen in the wild-type controls (cf. Fig. 4a, A and B). Ventricular sections cut along the longitudinal axis of the cardiomyocytes show significant intracellular IHC staining for the c-myc epitope tag (Fig. 4a, C). At higher-power examination using Nomarski interference microscopy, the intracellular transgenic MMP-2 protein is localized in a regularly spaced banding pattern consistent with a sarcomeric localization (Fig. 4a, D).

To confirm the impressions gained by IHC staining, we performed troponin I pulldowns of ventricular lysates, followed by gelatin zymography to detect MMP-2. As detailed in Fig. 4b, there is a specific physical association of the intracellular MMP-2 protein with troponin I. While minimally detectable MMP-2 was recovered with the troponin I pulldown in ventricular lysates from wild-type animals, there was readily detectable MMP-2 in the pulldowns from the transgenic mice. Most recovered MMP-2 was 66 kDa in size, consistent with enzymatic activation through prodomain processing.

Cardiac MMP-2 expression leads to marked ventricular remodeling. Left ventricular free wall histological sections were examined at 4 and 8 mo in MMP-2 transgenic mice and age-matched littermate controls. The results of these analyses are summarized in Fig. 5. There was a significant increase in the interstitial collagen volume fractions of the MMP-2 transgenics when assessed at 4 mo (wild type 3.8 ± 0.2% vs. transgenic 8.2 ± 1.2%; \( P < 0.05, n = 6 \) ventricles per study group; cf. Fig. 4a, A and B). By 8 mo the interstitial collagen volume fractions of the left ventricular free wall had increased to 14.6 ± 2.1% in the MMP-2 transgenic mice, compared with...
6.5 ± 1.8% in the age-matched controls (P < 0.05; n = 6 ventricles per study group). There were also extensive areas of replacement fibrosis in areas of cardiomyocyte dropout (Fig. 5a, D). As detailed in Fig. 5b, there was an increase in extent of perivascular fibrosis in the MMP-2 transgenic mice at 8 mo compared with the age-matched controls (cf. Fig. 5a, A vs. B). The apparent increase in perivascular fibrosis in the MMP-2 transgenic mice was quantified by measuring PVCA. At 8 mo, wild-type left ventricles had a PVCA of 0.08 ± 0.02 mm², while the MMP-2 transgenic left ventricles had a PVCA of 0.18 ± 0.02 mm² (P < 0.05; n = 6 ventricles for each study group). The perivascular fibrosis was associated with a mononuclear cell infiltrate not seen in the age-matched wild-type controls (cf. Fig. 5a, C and D).

**Transgenic MMP-2 induces downstream expression of endogenous MMP-2 and -9 and MT1-MMP.** Quantitative Western blot analyses were performed on ventricular lysates from wild-type and MMP-2 transgenic mice at 4 and 8 mo of age (n = 6 ventricles for each study group). Quantitation was provided by scanning laser densitometry. Representative Western blots
showing pairwise alignments of three wild-type and three transgenic lysates at 4 and 8 mo are shown in Fig. 6, a and b. As expected, the c-myc epitope tag on the transgenic MMP-2 was readily detected in ventricular lysates from 4-mo-old mice (Fig. 6a). By 8 mo the level of transgene expression, as determined by scanning laser densitometry, had increased by 2.2 ± 0.4-fold (P < 0.05; n = 6 for each group). Wild-type ventricular lysates contained low levels of MMP-2, which was increased 3.9 ± 0.2-fold (P < 0.05; n = 6 for each group) in the 4-mo-old transgenic mice. There was no significant difference in endogenous MMP-2 expression between 4 and 8 mo in the wild-type ventricular lysates (8 mo 1.2 ± 0.1-fold compared with 4-mo lysates; P > 0.05, n = 6 for each group). In contrast, there was a massive 22.3 ± 2.5-fold increase in MMP-2 expression in the 8-mo transgenic ventricular lysates compared with the 4-mo transgenic ventricular lysates (P < 0.01; n = 6 for each group). As there was only a modest increase in the expression levels of the c-myc-epitope-tagged transgenic MMP-2, this large increase must represent recruitment of endogenous MMP-2 gene expression.

MMP-9, MT1-MMP, and MMP-13 were not detected in wild-type or MMP-2 transgenic ventricular lysates at 4 mo. At 8 mo these MMPs were detectable in the order MMP-9 > MT1-MMP ≫ MMP-13. MMP-9 in the ventricular lysates of the MMP-2 transgenic mice at 8 mo is most probably derived from the prominent perivascular infiltration of mononuclear cells observed at this time.

The net levels of MMP activity in the heart may be regulated by changes in tissue inhibitor of metalloproteinase (TIMP)-1-4 expression. We performed Western blot analysis of left ventricular free wall lysates using specific antibodies, the results of which are summarized in Fig. 6b. The figure shows representative Western blots for three wild-type and three MMP-2 transgenics at 4 and 8 mo of age. For quantitation, the Western blots were scanned by laser densitometry (n = 6 for each group). There were no significant differences in TIMP-1-4 expression levels between the wild-type and MMP-2 transgenic mice at 4 mo of age. The levels of TIMP-1-4 were also not significantly changed in the 8-mo-old wild-type mice compared with the 4-mo-old wild-type mice [TIMP-1 8 mo vs. TIMP-1 4 mo: 1.2 ± 0.2-fold (P > 0.05), TIMP-2 8 mo vs. TIMP-2 4 mo: 1.1 ± 0.1-fold (P > 0.05), TIMP-3 8 mo vs. TIMP-3 4 mo 0.85 ± 0.2-fold (P > 0.05), and TIMP-4 8 mo vs. TIMP-4 4 mo 0.9 ± 0.2-fold (P > 0.05); n = 6 for each study group].

There were modest, but significant, decreases in the 8-mo MMP-2 transgenics in the abundance of TIMP-1 (0.72 ± 0.16-fold of wild-type controls; P < 0.05), TIMP-3 (0.61 ± 0.17-fold of wild-type controls; P < 0.05), and TIMP-4 (0.74 ± 0.12-fold of wild-type controls; P < 0.05) (n = 6 for each study group). There was no significant difference in the abundance of TIMP-2 in the 8-mo MMP-2 transgenics compared with the wild-type controls (1.1 ± 0.1-fold; P > 0.05, n = 6 for each study group).
Wang et al. (62) have reported that an intracellular form of MMP-2 can cleave troponin I and thereby contribute to myocardial dysfunction following ischemia-reperfusion injury. To determine whether chronic expression of MMP-2 exerted a similar effect, the abundance of troponin I was determined by Western blot analysis of wild-type and MMP-2 transgenic ventricular lysates at 4 and 8 mo. These data are summarized in Fig. 6c. There were no significant differences in troponin I abundance at 4 mo between the wild type and MMP-2 transgenics. At 8 mo, troponin I abundance had significantly decreased by a mean of 18% compared with the age-matched controls, but there was substantial biological variation among the sample set (SD = 9%; P < 0.05, n = 10).

Transgenic MMP-2 induces amplifying transcriptional networks: induction of endogenous MMP-2. We recently reported (6) that in vitro MMP-2 transcription by hypoxic cardiomyocytes is mediated by specific components of the AP-1 transcription factor family, including JunB and FosB. These studies were extended to an analysis of MMP-2 transcriptional regulation within the context of ischemia-reperfusion injury using the ex vivo Langendorff preparation (3). These studies demonstrated that ischemia-reperfusion injury rapidly induced MMP-2 gene transcription and translation that was mediated by the specific binding of JunB-FosB heterodimers to the endogenous MMP-2 promoter. To determine whether a similar transcriptional regulatory mechanism was responsible for the massive increases in endogenous MMP-2 expression, we isolated ventricular nuclear proteins from 4- and 8-mo-old wild-type and MMP-2 transgenic mice. The abundance of the JunB and FosB transcription factors in the nuclear protein preparations was quantitatively assessed with an ELISA-based oligonucleotide binding method. The results of these studies are
summarized in Fig. 7. The relative abundance of the JunB and FosB transcription factors was not significantly different in nuclear protein preparations of 4-mo-old wild-type and MMP-2 transgenic mice. The abundance of the JunB and FosB transcription factors remained unchanged in the 8-mo-old wild-type mice. In contrast, there was a nearly fourfold increase in the abundance of the FosB transcription factor in nuclear protein preparations from the 8-mo-old transgenic mice, while JunB levels were statistically unchanged. These patterns are consistent with those obtained with ischemia-reperfusion injury of the heart, in which basal transcription of the MMP-2 gene is driven by JunB-JunB homodimer occupancy of the AP-1 binding site in the MMP-2 promoter, while induced MMP-2 transcription is driven by preferential occupancy with the transcriptionally more potent JunB-FosB heterodimers (3).

Morphological and hemodynamic assessment of MMP-2 cardiac transgenics. Through 8 mo of age MMP-2 transgenic mice showed normal behavior and general appearance. Trans-thoracic echocardiograms in unanesthetized MMP-2 transgenic mice performed at 4 mo (n = 10) and 6 mo (n = 6) were unremarkable, with normal left ventricular ejection fraction and fractional shortening. Ex vivo Langendorff studies performed at 6 mo of age in 13 mice showed normal values for basal left ventricular developed pressure and left ventricular end-diastolic pressure and no differences compared with wild-type littersmates. At 8 mo, body weight, the ratio of heart weight to body weight, systolic blood pressure, and heart rate as measured in vivo (n = 15) continued to be normal and showed no significant differences between MMP-2 transgenic and age-matched wild-type littersmates (61).

Hearts from wild-type and MMP-2 transgenic mice at 12 mo were perfusion fixed in diastole and stained with Masson trichrome (Fig. 8a). Cross sections at the midventricular plane revealed marked biventricular dilation in the MMP-2 transgenics compared with the wild-type controls (cf. Fig. 8a, A vs. B). Figure 8 summarizes in vivo hemodynamic assessment of left ventricular volume measurements, in which the MMP-2 transgenics have significantly increased left ventricular end-diastolic volumes and increased end-systolic volumes, with a diminished ejection fraction.

The comprehensive results of the in vivo hemodynamic assessment are summarized in Table 1. Consistent with the ventricular cross sections shown in Fig. 8, there was a considerable increase in both left and right ventricular dimensions without an increase in left or right ventricular mass or left ventricular mass-to-body weight ratios (Table 1). Baseline heart rate and systolic pressure were similar in the 12-mo-old wild-type and MMP-2 transgenic mice, but there was a trend toward an increase in left ventricular end-diastolic pressure that did not reach statistical significance. Significant ventricular enlargement was evidenced by a >50% increase in diastolic volume and a fourfold increase in systolic left ventricular volumes. Although stroke volume was relatively preserved, the left ventricular ejection fraction was markedly decreased from a mean 79% in the wild types to 48% in the MMP-2 transgenics. Markedly impaired systolic function in the MMP-2 transgenics was demonstrated by a 68% decrease in preload-adjusted maximal power and a significant decrease in the end-systolic pressure-volume relation (Table 1). In addition to the
Fig. 6. Western blot analysis of MMP, tissue inhibitor of metalloproteinase (TIMP) and troponin I expression. **a**: Left ventricular extracts from wild type (WT) and MMP-2 cardiac transgenics (TG) were prepared at 4 and 8 mo of age, and levels of c-myc epitope-tagged transgenic MMP-2, endogeneous MMP-2, MMP-9, membrane type 1 (MT1)-MMP, and MMP-13 were determined by Western blot analysis. **b**: TIMP-1-4 expression assessed at 4 and 8 mo. For **a** and **b**, see RESULTS for quantitative assessment of MMP and TIMP expression levels. **c**: Quantitative assessment of left ventricular troponin I levels in wild-type and MMP-2 transgenic mice at 4 and 8 mo. Data are expressed as means ± SD; n = 10 ventricles/group (*P < 0.05).
elevated LV end-diastolic pressure, the mildly decreased peak $-dP/dt$ and prolonged $\tau$ are evidence for an element of diastolic dysfunction in the MMP-2 transgenic mice, where $-dP/dt$ is the minimum first derivative of LV pressure decline with respect to time and $\tau$ is the time constant of LV pressure decline. The absence of compensatory hypertrophy in the context of significant ventricular dilation is consistent with a profound cardiomyopathy and markedly increased ventricular wall stresses.

**Table 1. Invasive hemodynamics**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 7)</th>
<th>MMP-2 Transgenic (n = 8)</th>
<th>t-Test P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>45.8 ± 2.9</td>
<td>44.3 ± 2.9</td>
<td>0.30</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>125 ± 10</td>
<td>120 ± 16</td>
<td>0.44</td>
</tr>
<tr>
<td>RV free wall mass, mg</td>
<td>37 ± 6</td>
<td>35 ± 3</td>
<td>0.58</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>612 ± 44</td>
<td>599 ± 85</td>
<td>0.69</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>110 ± 17</td>
<td>106 ± 10</td>
<td>0.62</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>4 ± 3</td>
<td>8 ± 4</td>
<td>0.07</td>
</tr>
<tr>
<td>LV end-diastolic volume, μl</td>
<td>33 ± 3</td>
<td>51 ± 12</td>
<td>0.003</td>
</tr>
<tr>
<td>LV end-systolic volume, μl</td>
<td>7 ± 2</td>
<td>28 ± 14</td>
<td>0.003</td>
</tr>
<tr>
<td>LV stroke volume, μl</td>
<td>26 ± 4</td>
<td>23 ± 3</td>
<td>0.16</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>78 ± 7</td>
<td>48 ± 16</td>
<td>0.0006</td>
</tr>
<tr>
<td>LV $+dP/dt$, mmHg/s</td>
<td>11,100 ± 2,000</td>
<td>11,000 ± 1,900</td>
<td>0.94</td>
</tr>
<tr>
<td>LV $-dP/dt$, mmHg/s</td>
<td>$-9,600 ± 1,900$</td>
<td>$-8,500 ± 1,800$</td>
<td>0.23</td>
</tr>
<tr>
<td>$\tau$ (logistic), ms</td>
<td>4.0 ± 1.0</td>
<td>6.0 ± 1.8</td>
<td>0.02</td>
</tr>
<tr>
<td>Preload adjusted maximal power, mW/μl²</td>
<td>240 ± 84</td>
<td>78 ± 49</td>
<td>0.0003</td>
</tr>
<tr>
<td>End-systolic pressure-volume relation</td>
<td>7.5 ± 1.5</td>
<td>4.7 ± 2.0</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Values are means ± SD. MMP, matrix metalloproteinase; LV, left ventricular; RV, right ventricular. $-dP/dt$, maximum first derivative of LV pressure rise with respect to time; $+dP/dt$, minimum first derivative of LV pressure decline with respect to time.

**DISCUSSION**

The MMP-2 cardiac transgenic model provides unequivocal pathophysiological evidence for a primary role of MMP-2 in the evolution of pleiotropic cardiac structural and functional...
abnormalities in the absence of superimposed injury. Significant cardiomyocyte structural alterations, including hypertrophy and focal areas of cellular dropout, were observed at 4 mo in the absence of functional deficits. The ultrastructure of the MMP-2 transgens at 4 mo demonstrated multiple abnormalities, including myofilament lysis, hypertrophy of T tubules, and extensive mitochondrial abnormalities. In addition, there was ultrastructural evidence for intrinsic cardiac fibroblast activation and proliferation, with deposition of interstitial collagens. This pattern of ultrastructural features has been described in animal models of dilated cardiomyopathy (40) and in patients with dilated or hypertrophic cardiomyopathy (27, 45). Furthermore, mitochondrial abnormalities similar to those observed in the cardiac MMP-2 transgenic mice have been described in these conditions, with associated mutations in mitochondrial DNA (5, 28). Taken together, the ultrastructural changes in the cardiac MMP-2 transgenic mice are most consistent with a primary cardiomyopathy phenotype. Support for this conclusion is provided by our recent observation (61) that isolated right ventricular trabeculae from MMP-2 cardiac transgenics exhibited impaired contractile force and diminished responses to inotropic stimulation. The contractile force of skinned trabeculae from MMP-2 cardiac transgenics was also diminished, consistent with a primary myofilament contraction defect.

Concurrent with the ultrastructural studies, there were significant increases in the interstitial collagen volume fraction in 4-mo-old transgenic mice compared with age-matched littermate controls. Extensive replacement fibrosis was evident by 8 mo in the MMP-2 transgenic mice, consistent with ongoing cardiomyocyte cellular dropout. Although at first glance one would anticipate that increased MMP-2 expression should result in a decreased level of cardiac interstitial collagen, our findings in the MMP-2 transgenic mouse closely resemble analyses of cardiac tissue from patients with idiopathic dilated cardiomyopathy, in which large increases in gelatinase activity were associated with increased total collagen content (17). Similarly, Matsusaka et al. (35) noted increased cardiac collagen content in mice with intact levels of MMP-2, as opposed to MMP-2-knockout mice, in the setting of TNF-α-induced cardiomyopathy. Formal enzymatic analyses have demonstrated that interstitial collagens are not efficiently cleaved by MMP-2 under physiologically relevant conditions (47, 55), and the accumulation of interstitial collagens in the MMP-2 transgenic mouse is consistent with these observations.

There was also extensive perivascular fibrosis and inflammatory cell infiltration in the transgenic mice at 8 mo. This pattern of perivascular fibrosis and inflammation has been ascribed to aldosterone-mediated oxidative stress, with associated inflammatory cell infiltration within the setting of the failing heart (25, 42, 54). Inflammatory cell infiltration has also been attributed to release of monocyte chemoattractant protein-1 in the hypertrophic and failing heart (48). MMP-2 may directly contribute to macrophage infiltration, because MMP-2-derived degradation peptides from laminin and fibronectin directly induce macrophage migration (33).

Despite these extensive morphological changes evident in light and electron microscopic analysis, serial noninvasive studies, including echocardiography and measurements of heart rate, blood pressure, and heart weight-to-body weight ratios, remained normal through 8 mo of age in the MMP-2 transgenic mice. Thus these mice exhibited hemodynamic compensation in the basal state, although, as noted above, the response of isolated right ventricular trabeculae from these mice to inotropic stimulation was impaired (61). However, by 12 mo of age an increased mortality in the MMP-2 transgenic mice was evident, as were manifestations of overt congestive heart failure, including reduced activity, ascites, and cyanosis. These findings are in accord with the invasive hemodynamic measures in intact animals summarized in Table 1, which are indicative of an advanced cardiomyopathy.

Western blot analysis of MMP-2 transgenic hearts at 4 mo demonstrated the expected increased expression of MMP-2 derived from the transgene, with no evident expression of MMP-9, MT1-MMP, or MMP-13. By 8 mo in the transgenics there was a dramatic increase in endogenous (i.e., non-transgene derived) MMP-2 expression. MMP-2 gene expression and translation are induced by multiple factors or conditions present in the failing heart. For example, we recently demonstrated (6) that hypoxia alone is sufficient to induce MMP-2 transcription and translation in cultured cardiac fibroblasts and cardiomyocytes. Furthermore, endothelin-1, interleukin-1β, and angiotensin II, all of which are elevated in the failing heart, additively increased MMP-2 transcription and translation in vitro (6). Oxidative stress is also a potent stimulus for MMP-2 synthesis in cultured cardiac fibroblasts (50). As shown in this study, initial expression of relatively small amounts of active MMP-2 results in the engagement of amplification loops associated with massive upregulation of endogenous MMP-2 expression in the 8-mo-old transgenic mice. This likely represents activation of discrete transcriptional regulatory networks operative on the MMP-2 promoter, as demonstrated by the time-dependent increase of the AP-1 component FosB in the ventricular nuclear extracts of 8- as opposed to 4-mo-old transgenic mice. FosB forms a potent transactivation complex with JunB characterized by prolonged DNA binding interactions and increased transcriptional activity (3, 6). This progressive amplification process is the likely explanation for the eventual failure of adaptation that characterized the eventual development of severe systolic dysfunction.

MMP-9 was not detected in ventricular lysates of MMP-2 transgenic mice at 4 mo but was significantly induced in the transgenic mice by 8 mo. The increase in cardiac MMP-9 expression is consistent with the ventricular and perivascular infiltration of inflammatory cells described above. While the rodent interstitial collagenase MMP-13 was only minimally induced by 8 mo in the MMP-2 transgenic mice, MT1-MMP was readily detected. Expression of MT1-MMP at this time could contribute to activation of intrinsic MMP-2. The patterns of MMP-2, -9, and -13 and MT1-MMP expression in the MMP-2 transgenic mice closely resemble those described in a rat hypertensive heart failure model, in which hypertensive remodeling preceded left ventricular dilatation (43). While enhanced MMP-9 transcription is most likely attributable to cardiac expression of inflammatory cytokines such as TNF-α, the enhanced synthesis of MT1-MMP may very well be driven by calcineurin-nuclear factor of activated T cells (NFAT) signaling, which participates in pathological cardiac hypertrophy (63). We recently demonstrated (2) that the transcriptional regulation of MT1-MMP is unique among members of the MMP gene family and is driven by a specific NFAT isoform, c1.
Cardiac tissue expresses all four defined isoforms of TIMP, and considerable attention has been given to the impact of relative MMP and TIMP stoichiometries on the net balance of proteolytic activity during ventricular remodeling and dilatation [reviewed by Fedak et al. (13)]. While we did not observe any significant changes in TIMP-2 protein expression levels at 8 mo in the MMP-2 transgenic mice, modest decreases in the protein expression levels of TIMP-1, -3, and -4 were observed. Although not large, these decreases, coupled with the very large increase in MMP levels at 8 mo, are consistent with a substantial increase in net MMP activity, which has been described during the transition from hypertrophy to heart failure in hypertensive rats (19).

What are the mechanisms underlying the effects of MMP-2 on myocardial function? As reviewed recently by Janssens and Lijnen (21), most studies of MMP effects on myocardial function have used specific MMP-2-, MMP-9-, or TIMP-knockout mice. Other than the present report, only one study has examined the effects of cardiac-specific expression of a defined MMP molecule. In this study Kim et al. (24) showed that cardiac-specific expression of the interstitial collagenase MMP-1 produced a net loss of cardiac interstitial collagen with deterioration of both systolic and diastolic function, leading to the suggestion that this functional phenotype occurs as a consequence of disrupted cardiomyocyte-extracellular matrix coupling. The phenotype of the MMP-1 cardiac transgenic mouse differs significantly from the phenotype of the MMP-2 transgenic mice described in this report, in which a major reduction in systolic function was observed with greatly increased interstitial collagen volumes. The documented substrate spectrum of this enzyme is remarkably broad and includes a large number of non-extracellular matrix protein substrates (57). For example, MMP-2 cleaves big endothelin-1 to generate a potent vasoconstrictor (14). The peptide adrenomedullin is associated with cardiac hypertrophy in rats (38), and MMP-2 was recently shown to cleave adrenomedullin in vitro and in vivo, thereby generating a vasoconstrictor molecule (32). Novel cardiac intracellular substrates for MMP-2 have also been described, including poly(ADP-ribose) polymerase and myosin light chain after ischemia-reperfusion injury (26, 44).

As detailed in this study, we did detect the transgenic MMP-2 protein in both the extracellular (pericellular) and intracellular spaces. As the MMP-2 transgenic cDNA expression cassette included a normal secretory sequence the extracellular transport of the protein is to be expected. Intracellular transgenic MMP-2 is likely the consequence of reinternalization of secreted protein, which has been described for MMP-1, MMP-2, and MT1-MMP (16, 30, 46). Intracellular cleavage of nuclear lamin A/C by MMP-1 has been associated with resistance to apoptosis, while intracellular cleavage of centromeric components by MT1-MMP has been associated with chromosomal instability (16, 30). These unconventional (i.e., non-extracellular matrix degrading) activities of the MMPs were recently reviewed by Strongin (53).

The significance of potential troponin I cleavage in the MMP-2 transgenic mice is unclear, as we did not observe significant differences in troponin I content of ventricular lysates at 4 mo, a time in which there were major ultrastructural and morphological changes in both cardiomyocytes and fibroblasts. Troponin I content was modestly and variably decreased in the MMP-2 transgenic mice at 8 mo, which may be a consequence of the dramatic increase in total MMP-2 synthesis at this time. Thus it is difficult to conclude that the primary mode of MMP-2 action relates to troponin I degradation within the context of chronic, as opposed to acute ischemia-reperfusion-mediated MMP-2 expression.

In summary, this is to our knowledge the first report demonstrating that cardiac-specific expression of active MMP-2, without superimposed injury, leads to a heart failure phenotype associated with myocyte dropout, mitochondrial disruption, and severe ventricular remodeling associated with major depression of primary systolic function. These observations are consistent with clinical reports associating MMP-2 activity with various forms of human heart failure and strongly support the hypothesis that MMP-2 has a primary, or causative, role in this disorder. Thus our data suggest that inhibition of MMP-2 activity could represent a therapeutic approach for the treatment of chronic heart failure or the prevention of ventricular remodeling. The transgenic model we have described in this report represents a unique genetic template for studies directed toward these goals.

REFERENCES


14. Fernandez-Patron C, Radomski MW, Davidge ST.

15. Junqueira LC, Bignolas G, Brentani RR.


20. Iwanaga Y, Aoyama T, Kihara Y, Onozawa Y, Yoneda T, Sasayama


