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Osteopontin-Stimulated Expression of Matrix Metalloprotease-9 Causes Cardiomyopathy in the mdx Model of Duchenne Muscular Dystrophy

Saurabh Dahiya,* Srikanth Givvimani,† Shephali Bhatnagar,* Natia Qipshidze,† Suresh C. Tyagi, † and Ashok Kumar*†

Duchenne muscular dystrophy (DMD) is a lethal inherited disease of skeletal muscle resulting from a mutation in the dystrophin gene. A vast majority of patients with DMD also develop cardiomyopathy, and 10–15% of patients die from cardiac failure (1). Furthermore, >90% of patients with the milder Becker muscular dystrophy and female DMD carriers show cardiac involvement (2–5). A murine model of DMD, the mdx mouse, also lacks dystrophin because of a nonsense point mutation in exon 23 of the dystrophin gene (6). Hearts of mdx mice share many features of the DMD cardiomyopathy (7–9). Similar to patients with DMD, mdx mice experience a progressive development of cardiac defects, although the pathology is milder at a young age. However, by the age of 42 wk, mdx mice display several features of cardiomyopathy such as echocardiogram abnormalities, impaired conduction, arrhythmias, autonomic dysfunction, and reduced left ventricular function (8, 10, 11). In addition, mdx mouse hearts experience progressive accumulation of connective tissues, suggesting that fibrosis is also responsible for some features of cardiomyopathy in these mice (9). However, the pathophysiologic mechanisms leading to cardiac dysfunction and fibrosis in DMD remain poorly understood.

Matrix metalloproteinases (MMPs) are a family of zinc-containing, calcium-dependent proteases that have an important role in extracellular matrix remodeling, inflammation, fibrosis, and activation of various latent cytokines and cell adhesion molecules in both physiologic and pathologic conditions (12). MMPs are synthesized as secreted or transmembrane proenzymes and processed to an active enzyme by the removal of an N-terminal propeptide (12, 13). The proteolytic activity of MMPs is tightly controlled by their interaction with endogenous tissue inhibitors of MMPs (TIMPs), which inhibit enzymatic activity of MMPs (12–14). There are four known TIMPs (i.e., TIMP 1–4), each of which binds at a different rate of interaction and affinity to a target MMP (14). Recent reports suggest that the expression and activity of several MMPs are dysregulated in dystrophic muscle of mdx mice (a mouse model of DMD) (20–22). Our prior work has described that inhibition of MMP-9 (also known as gelatinase B) reduces several features of skeletal muscle pathology in mdx mice (23). Similar to MMP-9, the levels of MMP-2 (gelatinase A) have also been found to be significantly upregulated in myofibers of mdx mice (23, 24). Miyazaki et al. (22) reported recently that genetic deletion of MMP-2 inhibits fiber growth and angiogenesis in skeletal muscle of mdx mice, suggesting that MMP-2 is a positive regulator of...
regenerating fibers growth in mdx mice. However, it remains unknown how the expression of various MMPs is affected in cardiac muscle of mdx mice. Because loss of dystrophin influences skeletal muscle regeneration and cardiac function with only partially overlapping mechanisms (25), it is essential to investigate the role of various dysregulated MMPs in both cardiac and skeletal muscle in dystrophic models. Furthermore, the biochemical mechanisms leading to altered expression of various MMPs in models of DMD remain to be investigated.

Osteopontin (OPN), also known as early T cell activation 1, was originally discovered as an inducible marker of transformation of epithelial cells. It is a secreted, integrin-binding matrix-phosphorylated glycoprotein involved in a number of cell functions, including cell adhesion and migration, inflammation, angiogenesis, tissue remodeling, and tumor development (26). Published reports suggest that OPN can induce the expression of number of inflammatory molecules, including MMP-9 in some cell types (27, 28). A recent study has demonstrated that protein levels of OPN are significantly increased in cardiomyopathy and dystrophic muscle of mdx mice (29). Interestingly, both OPN and MMP-9 have been found to be the major mediators of fibrosis in skeletal and cardiac muscle of mdx mice (29). More importantly, genetic ablation of OPN in mdx mice significantly attenuated fibrosis in skeletal and cardiac muscle of mdx mice (29). Interestingly, both OPN and MMP-9 have been found to be the major mediators of fibrosis in skeletal and cardiac muscle of mdx mice (29). We hypothesized that increased levels of OPN cause inducible expression of MMP-9, leading to cardiac fibrosis and dysfunction in mdx mice.

In this study we used genetic mouse models, we have investigated the role of MMP-9 in association with cardiac dysfunction in mdx mice. Our experiments demonstrate that the levels of MMP-9 are increased in the hearts of mdx mice. Genetic deletion of MMP-9 significantly reduced several features of cardiomyopathy, including fibrosis, and led to improved heart function in mdx mice. This study also demonstrates that elevated levels of osteopontin, a recently identified mediator of fibrosis in mdx mice (29), contributed significantly to the increased expression of MMP-9 in both skeletal and cardiac muscle of mdx mice.

Materials and Methods

Animals

Mdx (strain: C57BL/10ScSn DMDtm1Tvu) mice and Mmp9-knockout (strain: FVB. Cg-Mmp9−/−m2J/Jm2J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mmp9-knockout mice were first crossed with C57BL10ScSn mice for seven generations and then with mdx mice to generate littermate wild type (WT), mdx/Mmp9+/+ and mdx/Mmp9−/−. All genotypes were determined by PCR analysis from tail DNA as described (23). Mice were housed under conventional conditions with constant temperature and humidity and fed a standard diet. To study the effects of OPN on the expression of MMP-9, 3-week-old WT mice were given a single retroorbital injection of recombinant mouse OPN protein (R&D Systems, Minneapolis, MN) at a dose of 100 µg/kg body weight. Mice were euthanized after 12 h. To investigate the role of OPN in the expression of MMP-9, 3-week-old mdx mice were treated with i.p. injections of either 200 µg anti-OPN (R&D Systems) or 200 µg/ml isotype control (BioLegend, San Diego, CA) every third day for 10 d (total of three injections). Twenty-four hours after the final injection, the mice were euthanized, and heart and skeletal muscles were isolated for biochemical analysis. All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Louisville and conformed to the American Physiological Society’s Guiding Principles in the Care and Use of Animals.

Histologic and immunohistochemical analyses

Cardiac tissues were removed, frozen in isopentane cooled in liquid nitrogen, and sectioned in a microtome cryostat. For the assessment of tissue morphology, 5-µm-thick transverse sections of each muscle were stained with H&E, and staining was visualized on a microscope (Eclipse TE 2000-U), a digital camera (Digital Sight DS-F1), and NIS Elements BR 3.00 software (all from Nikon). The images were stored as JPEG files, and Zimage levels were equally adjusted using Photoshop CS2 software (Adobe). The extent of fibrosis in transverse cryosections of heart was determined using Mason’s Trichrome staining kit following a protocol suggested by the manufacturer (Richard-Allan Scientific). Area under fibrosis or necrosis in cardiac section was quantified using MetaMorph Image analysis software (version 4.5). For the detection of macrophages in heart cryosections, anti-F4/80 (dilution 1:100; clone C1A3-1, AbD Serotec) was used in conjunction with the VECASTAIN ABC staining kit (Vector) with 3,3’-diaminobenzidine substrate according to the manufacturer’s protocol. Number of F4/80-positive cells was measured using a method as previously described (23). A necrotic area in the heart was transverse cryosections of heart was identified by immunostaining with C3-labeled goat anti-mouse IgG (1:3000, Invitrogen) as described (23).

Gelatin zymography

To determine MMP-9 activity, we performed zymography using a similar protocol described previously (25). Cardiac (left ventricle [LV]) muscle extracts were prepared in nonreducing lysis buffer (50 mm Tris-Chl [pH 8.0], 200 mm NaCl, 50 mm NaF, 0.3% [IPG] curve); MMP protease inhibitor mixture). An equal amount of protein (80 µg per sample) was separated on 8% SDS-PAGE containing 1 mg/ml gelatin B (Fisher Scientific) under nonreducing conditions. Gels were washed in 2.5% Triton X-100 for 1 h at room temperature followed by incubation in reaction buffer (50 mm Tris-HCl [pH 8.0], 50 mm NaCl, 5 mm CaCl2 and 0.02% sodium azide) for 48 h at 37°C. To visualize gelatinolytic bands, gels were stained with Coomassie brilliant blue dye at room temperature followed by destaining buffer (10% methanol and 10% acetic acid in distilled water). The gels were photographed for determination of gelatinolytic activity.

Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) for individual genes was performed using an ABI Prism 7300 sequence detection system (Applied Biosystems) using a method as described previously (31). The first-strand cDNA reaction (0.5 µl) was subjected to real-time PCR amplification using gene-specific primers. The primers were designed according to ABI primer express instructions using Vector NTI software and were purchased from Sigma-Genosys (Spring, TX). The sequences of the primers used are as follows: MMP-2: 5’-ACA GCC GAC GAC CTC AGG GT-3’ (forward) and 5’-CAG CAC AGG AGC CAG AGC AC-3’ (reverse); MMP-3: 5’- GTG TGT GGT TGT GGT CTC ATC CTA-3’ (forward) and 5’-CGG GGT CCT CTT GTG TCT CTC-3’ (reverse); MMP-9: 5’- AAT CCG TGT AGC C-3’ (forward) and 5’-TTG AGC TTC GGG ATT CCA AT-3’ (reverse); MMP-14: 5’-ATT TGA TGA GGT TTG CCA-3’ (forward) and 5’-TCG GAA TCA TAA GAG GTG-3’ (reverse); Coll1α: 5’-TCA AGA TGG TCC CCC TGG AC-3’ (forward) and 5’-CCT TGC CTT CAC CCG CG-3’ (reverse); Coll4α: 5’-AAC TGG ACT CCA ATC TTC CG-3’ (forward) and 5’-TTA CCC TTC AGT CTA-3’ (reverse); Acta2: 5’-AAC TGG ACT CCA ATC TTC CG-3’ (forward) and 5’-TTA CCC TTC AGT CTA-3’ (reverse); Col3a1: 5’-ACA AGG TCC TTC TCA GTC GAG CAG GAA TCA-3’ (forward); TIMP-1: 5’-TTG GTT CAT CTC TGG CTA GCA T-3’ (forward) and 5’-GAT ATC TGC GGC ATT TCC CAC A-3’ (reverse); TIMP-2: 5’-GTC TCT TCA TGG TCT GGT GGG-3’ (forward) and 5’-GAT GAC GAG GAC TGA TCT TGC-3’ (reverse); TIMP-3: 5’-CAG ATG AAG ATG TAC CAG GGC TCT TCC-3’ (forward) and 5’-AAAG GGC TGT AGC GAC GAT TAA T-3’ (reverse); CD68: 5’-TTA TCA TGG TCT TGG CTA TCA C-3’ (forward) and 5’-ATC AGA GAA ATG TAC CAG GGC TCT TCC-3’ (reverse); IL-1β: 5’-CTC GAG GAT GAA GAA TCT TCG G-3’ (reverse); TNF-α: 5’-GAC TCA TCC AGG ACG AGG TAA-3’ (forward) and 5’-AGA TCC TCT ATC GGG AGA-3’ (reverse); β-actin: 5’-CAG GCA AAT TGG CTC ACA-3’ (forward) and 5’-GTC TGC TAC ACA AAG AGG-3’ (reverse).

Real-time PCR assays were performed in 25-µl reactions, consisting of 2× (12.5 µl) Brilliant SYBR Green QPCR Master Mix (Stratagene), 400 nmol/L primers (0.5 µl each from the stock), 11 µl of water, and 0.5 µl of template. The thermal conditions consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 60°C for 1 min, and, for a final step, a melting curve of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. All reactions were performed in triplicate to reduce variation. The data were analyzed using SDS software version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using β-actin as an endogenous control, and the normalized values were subjected to a 2−ΔΔCt formula to calculate the fold change between the control and experimental groups. The formula and its derivations were obtained from the ABI Prism 7900 sequence detection system user guide.

Western blot

Heart tissues were isolated from mice, washed extensively with PBS and LV muscle was homogenized in lysis buffer A (50 mm Tris-Chl [pH 8.0], 200 mM NaCl, 50 mM NaF, 1 mM dithiothreitol, 1 mM sodium orthovanadate). The extent of fibrosis in transverse cryosections of heart was...
date, 0.3% IGEPAL, and protease inhibitor mixture). Approximately 100 μg protein was resolved on each lane on 8–12% SDS-PAGE, electrophoresed onto nitrocellulose membrane, and probed using anti–MMP-9 (0.1 μg/ml; catalog no. AF909; R&D Systems), anti-phospho p44/p42 (dilution 1:1000; catalog no. 9101; Cell Signaling), anti-p44/p42 (dilution 1:1000; catalog no. 9102, Cell Signaling), anti–phospho-JNK1/2 (dilution 1:1000; catalog no. 9251; Cell signaling), anti-JNK1/2 (0.2 μg/ml; catalog no. sc-474; Santa Cruz Biotechnology), anti-phospho p38 (dilution 1:1000; catalog no. 9216; Cell Signaling), anti-p38 (dilution 1:1000; catalog no. 9212; Cell Signaling), anti-phospho Akt (dilution 1:1000; catalog no. 9271; Cell Signaling), anti-Akt (dilution 1:1000; catalog no. 9272; Cell Signaling), and anti-tubulin (dilution 1:3000; catalog no. 9214; Cell Signaling) and detected by chemiluminescence. Gel images were quantified using ImageJ software from the National Institutes of Health.

**Echocardiography**

Transtracheal echocardiography was performed on mice as described previously (32, 33). A Hewlett-Packard Sonos 7770 echocardiographic system equipped with a 15-MHz shallow-focus 15-6L phased-array transducer was used for measurements of LV function. The mice were sedated with 2.2.2 tribromoethanol (TBE, Sigma T48 402; 240 μg/kg body weight) and the chest was shaved. The transducer probe was placed on the left hemithorax of the mice in the partial left decubitus position. Two-dimensionally targeted M-mode echocardiograms were obtained from a short-axis view of the LV at or just below the tip of the mitral–valve leaflet. LV size and the thickness of LV wall were measured. Only M-mode echocardiography with well-defined continuous interfaces of the septum and posterior wall were collected. For quantification of LV lengths and wall thickness, LV short- and long-axis loops and LV two-dimensional echocardiography image-guided M-mode tracings at the level that yielded the largest diastolic dimension were digitally recorded. LV dimensions at diastole and systole (LVDD and LVIDd, respectively) were measured and averaged. Fractional shortening (FS) was calculated as (LVDD – LVIDd) / LVDD ×100%.

**Statistical analyses**

Results are expressed as mean ± SD. Statistical analysis used Student t test or ANOVA to compare quantitative data populations with normal distribution and equal variance. A p value < 0.05 was considered statistically significant unless otherwise specified.

**Results**

**MMP-9 levels are increased in hearts of 12 mo old mdx mice**

Because mdx mice develop significant cardiomyopathy by the age of 1 y (8), we first measured the levels of MMP-2 and MMP-9 protein in LV muscle of 1-y-old mdx mice using gelatin zymography and Western blot techniques. As shown in Fig. 1A, the levels of MMP-9 were dramatically increased in the LV of mdx mice compared with WT mice. However, there was no significant difference in the levels of MMP-2 between WT and mdx mice (Fig. 1A). Because we were interested in investigating the role of MMP-9 in cardiac pathology in mdx mice, in the same experiment, we also evaluated the levels of MMP-9 protein in LV of mdx/Mmp9−/− mice. As depicted in Fig. 1A, MMP-9 was undetectable in mdx/Mmp9−/− mice, confirming ablation of MMP-9 in these mice. Deletion of Mmp9 gene did not have any major effect on MMP-2 activity in the hearts of mdx/Mmp9−/− mice (Fig. 1A). Western blotting of protein extracts further confirmed increased levels of MMP-9 in heart of mdx/Mmp9−/+ mice copared with WT mice and MMP-9 was undetectable in mdx/Mmp9−/− mice (Fig. 1B).

**Genetic ablation of MMP-9 reduces cardiac injury in mdx mice**

To understand the role of MMP-9 in cardiac abnormalities, we first performed H&E staining on transverse cryosections of cardiac tissues isolated from 1-y-old littermate WT, mdx/Mmp9+/+, and mdx/Mmp9−/− mice. Examination of H&E-stained sections revealed the presence of patches of a necrotic area containing cellular infiltrates in LV muscle of mdx/Mmp9+/+ mice (Fig. 2A). In contrast, hearts of mdx/Mmp9−/− mice showed significantly reduced necrotic areas and cellular infiltrate in the LV (Fig. 2A, 2B).

**Ablation of MMP-9 inhibits accretion of macrophages and expression of inflammatory cytokines in cardiac muscle of mdx mice**

Accumulating evidence indicates that inflammation contributes significantly to the striated muscle pathogenesis in both DMD patient and mdx mice (35). Because MMP-9 is a major mediator of extracellular matrix breakdown and inflammatory response (12), we investigated whether the inhibition of MMP-9 affects the infiltration of macrophages and the expression of proinflammatory cytokines in cardiac muscle of mdx mice. Immunostaining of a cardiac section with anti-F4/80 (a marker for macrophages) showed that the concentration of macrophages was considerably higher in mdx/Mmp9+/+ mice compared with WT mice (Fig. 3A, 3B). However, the number of macrophages was considerably reduced in cardiac muscle of mdx/Mmp9+/− mice compared with mdx/Mmp9+/+ mice (Fig. 3A, 3B). Furthermore, mRNA level of CD68, a cell surface marker for macrophages, was significantly lower in the LV wall of mdx/Mmp9+/− mice compared with mdx/Mmp9+/+ mice (Fig. 3C), further suggesting that the inhibition of MMP-9 reduces the accretion of macrophages in the heart of mdx mice.

Increased amounts of proinflammatory cytokines such as TNF-α and IL-1β have been reported previously in the dystrophic muscle
of mdx mice (36). We next investigated whether MMP-9 affects the expression of TNF-α and IL-1β in cardiac muscle of mdx mice. As shown in Fig. 3D, mRNA levels of IL-1β were significantly higher in the heart of mdx/Mmp9+/+ mice compared with WT littermates. However, the mRNA levels of IL-1β were found to be significantly reduced in the cardiac muscle of mdx/Mmp9−/− compared with mdx/Mmp9+/+ mice (Fig. 3D). Interestingly, we noticed that the mRNA levels of TNF-α were significantly reduced in the heart of mdx/Mmp9+/+ mice compared with WT mice and there was no significant difference between mdx/Mmp9+/+ and mdx/Mmp9−/− mice at the age of 12 mo (Fig. 3D). These results suggest that the expression of IL-1β and TNF-α is differentially affected in heart of mdx mice and MMP-9 causes increased expression of only IL-1β.

**Genetic deletion of MMP-9 attenuates age-related collagen deposition in heart of mdx mice**

Cardiac fibrosis, defined as the excessive accumulation of extracellular matrix such as collagens, is a major pathologic feature of patients with DMD, causing systolic and diastolic dysfunctions and conduction defects in the heart (37). Because MMP-9 has been found to exacerbate fibrosis in many disease states (12, 38–40), we next examined whether the inhibition of MMP-9 can reduce cardiac fibrosis in mdx mice. Staining of cardiac muscle sections with anti-F4/80 using a method as described in Materials and Methods. The representative photomicrographs demonstrate that inhibition of MMP-9 reduces the concentration of macrophages in cardiac muscle of mdx mice. Scale bars, 50 μm. n = 6 in each group.

**FIGURE 2.** Ablation of MMP-9 reduces cardiac injury in mdx mice. A, Frozen cross-sections made from heart (at the center of ventricles) of 1-y-old WT, mdx/Mmp9+/+ and mdx/Mmp9−/− mice were stained with H&E. The data demonstrate that the deletion of Mmp9 gene in mdx mice improves cardiac structure. Arrows indicate a damaged or necrotic area. Scale bars, 50 μm. B, Quantification of necrotic area in H&E-stained images. *p < 0.01, values significantly different from WT mice; #p < 0.01, values significantly different from mdx/Mmp9+/+ mice. C, Representative photomicrographs of cardiac sections stained with Cy3-labeled goat anti-mouse IgG demonstrating that cardiac injury is considerably reduced in mdx/Mmp9−/− mice compared with mdx/Mmp9+/+ mice. Scale bars, 50 μm. n = 6 in each group.

**FIGURE 3.** Role of MMP-9 in the accumulation of macrophages and expression of inflammatory cytokines in heart of mdx mice. A, Frozen heart sections prepared from 1-y-old WT, mdx/Mmp9+/+ and Mdx/Mmp9−/− mice were immunostained with anti-F4/80 using a method as described in Materials and Methods. The representative photomicrographs demonstrate that inhibition of MMP-9 reduces the concentration of macrophages in cardiac muscle of mdx mice. Scale bars, 50 μm. B, Quantification of number of F4/80-positive cells in heart sections. *p < 0.01, values significantly different from WT mice; #p < 0.01, values significantly different from mdx/Mmp9+/+ mice. C, The mRNA levels of CD68 in the heart of WT, mdx/Mmp9+/+ and mdx/Mmp9−/− mice measured by QRT-PCR assay. D, Fold changes in mRNA levels of TNF-α and IL-1β in the heart of WT, mdx/Mmp9+/+, and mdx/Mmp9−/− mice. n = 6 in each group. *p < 0.01, values significantly different from WT mice; †p < 0.01, values significantly different from mdx/Mmp9+/+ mice.
Masson trichrome staining showed that the level of collagens (blue color) was significantly reduced in 1-y-old mdx/Mmp9+/− compared with littermate mdx/Mmp9+/+ mice (Fig. 4A). Reduced fibrosis in the heart of mdx/Mmp9+/− mice was also confirmed by quantification of an area stained positive for collagens in multiple sections (Fig. 4B). We also measured mRNA levels of collagen I and III with QRT-PCR. As shown in Fig. 4C, transcript levels of Col1a1 and Col3a1 were significantly reduced in the cardiac muscle of mdx/Mmp9+/− mice compared with mdx/Mmp9+/+ mice. The mRNA levels of collagen I (i.e., Col1a1) were also reduced in mdx/Mmp9+/−, but they were not statistically different from mdx/Mmp9+/+ mice (Fig. 4C). These results indicate that MMP-9 mediates the fibrotic response in the heart of mdx mice.

**Ablation of MMP-9 improves LV function in 12-mo-old mdx mice**

Because inhibition of MMP-9 reduced cardiac injury and fibrosis, we next sought to determine whether deletion of MMP-9 can also improve heart function in mdx mice. LV function was assessed by echocardiography in littermate WT, mdx/Mmp9+/+, and mdx/Mmp9+/+ mice at the age of 12 mo. Cardiac parameters were normal in WT mice (Fig. 5). However, in mdx mice, an increase in LV internal diameter during diastole (LVIDd) and LV posterior wall thickness during diastole (LVPWd) with concomitant decrease in FS, the typical features of age-related cardiomyopathy in the mdx mouse model (8), were noticeable (Fig. 5A–D). Interestingly, LVIDd and LVPWd were found to be significantly reduced in mdx/Mmp9+/− mice (Fig. 5B, 5C). Furthermore, ablation of MMP-9 significantly improved FS in mdx mice (Fig. 5D). These results suggest that MMP-9 is involved in cardiac dysfunction in mdx mice.

**Effect of deletion of MMP-9 on the expression of other MMPs and TIMPs in the hearts of mdx mice**

Accumulating evidence suggests that there is a cooperative interaction between various MMPs to promote effective tissue degradation in various conditions, including muscular dystrophy (12, 41). However, the activity of MMPs can be affected by the levels of TIMPs, which directly bind and inhibit MMPs (12). We investigated whether the ablation of MMP-9 affects the expression levels of other MMPs or TIMPs in the hearts of mdx mice. QRT-PCR analysis showed that the mRNA levels of MMP-2, MMP-3, MMP-10, MMP-12, TIMP-2, and TIMP-3 were significantly reduced in the heart of mdx/Mmp9+/+ mice compared with WT littermates (Fig. 6). Interestingly, the mRNA levels of MMP-3 and MMP-12 (but not MMP-2, MMP-10, TIMP-2, or TIMP-3) were found to be significantly reduced in the heart of mdx/Mmp9+/− mice compared with mdx/Mmp9+/+ mice (Fig. 6). In contrast, there was no significant difference in mRNA levels of MMP-14 or TIMP-1 between WT, mdx/Mmp9+/+, and mdx/Mmp9+/− mice (data not shown). These results suggest that in addition to MMP-9, the expression of several other MMPs is dysregulated in the heart of mdx and MMP-9 contributes to the increased expression of at least MMP-3 and MMP-12.

**Ablation of MMP-9 reduces the phosphorylation of ERK1/2 and Akt in hearts of mdx mice**

Published reports suggest that the loss of dystrophin leads to several signaling defects in skeletal and cardiac muscle of mdx mice (42). We investigated whether MMP-9 affects the activation of specific MAPKs and Akt kinase in cardiac muscle of mdx mice. Protein extracts prepared from LV of 1-y-old littermate WT, mdx/Mmp9+/+, and mdx/Mmp9+/− mice were immunoblotted using Ab that detects phosphorylated or total forms of various MAPKs or Akt. As shown in Fig. 7A, the phosphorylation (but not total protein levels) of ERK1/2 and Akt kinase were found to be considerably increased in LV of mdx/Mmp9+/+ mice compared with WT. Interestingly, ablation of MMP-9 significantly reduced phosphorylation of both ERK1/2 and Akt in the hearts of mdx mice (Fig. 7A, 7B). In contrast, there was no significant difference in the total or phosphorylated forms of JNK1/2 and p38 MAPK between WT, mdx/Mmp9+/+, and mdx/Mmp9+/− mice (Fig. 7A).

**OPN augments the levels of MMP-9 in cardiac and skeletal muscle of mdx mice**

Because OPN levels are increased in the serum of mdx mice (29) and OPN is known to stimulate the expression of MMP-9 (27, 28), we next sought to determine whether OPN contributes to the increased levels of MMP-9 in skeletal and cardiac muscles of mdx mice. We performed two sets of experiments. In the first set, 3-wk-old WT mice were given a retroorbital injection of recombinant OPN protein or saline alone. After 12 h, skeletal and cardiac muscles were isolated and used to measure the levels of MMP-9 protein. Interestingly, treatment with OPN considerably increased the protein levels of MMP-9 in both heart (Fig. 8A) and tibial anterior (TA) muscle (Fig. 8B). In the second set of experiments,
MMP-9 IN CARDIAC INFLAMMATION AND FIBROSIS IN DMD

FIGURE 5. Effects of ablation of MMP-9 on cardiac function in mdx mice. A, M-mode echocardiograms of 1-y-old WT, mdx/Mmp9+/+, and Mdx/Mmp9−/− mice obtained with two-dimensional echocardiography from short-axis midventricle view of hearts. Arrows represent LVPWd. Fold change in LVIDd (B) and LVPWd (C) values between WT, mdx/Mmp9+/+, and Mdx/Mmp9−/− mice. D, FS gave higher values for mdx/Mmp9−/− mice compared with littermate mdx/Mmp9+/+, providing an evidence of improvement in heart function after inhibition of MMP-9 in mdx mice. n = 6 in each group. *p < 0.01, values significantly different from WT mice; †p < 0.01, values significantly different from mdx/Mmp9+/+ mice.

FIGURE 6. Role of MMP-9 on the expression of various MMPs and TIMPs in heart of 1-y-old mdx mice. Total mRNA was isolated from the heart of 1-y-old WT, mdx/Mmp9+/+, and mdx/Mmp9−/− mice, and the mRNA levels of various MMPs and TIMPs were measured by QRT-PCR. n = 4 in each group. *p < 0.01, values significantly different from WT mice; †p < 0.01, values significantly different from mdx/Mmp9+/+ mice.

mdx mice (4 wk old) were given i.p. injection of OPN-neutralizing Ab. The mice were then sacrificed, and amounts of MMP-9 protein in cardiac and skeletal muscle were determined by Western blot. Interestingly, treatment with OPN-neutralizing Ab significantly reduced the levels of MMP-9 in both TA (Fig. 8C and cardiac (Fig. 8D) muscle of mdx mice. These results suggest that the increased levels of OPN contribute to the expression of MMP-9 in striated muscle of mdx mice.

Discussion

Cardiomyopathy, a major pathologic feature in DMD, refers to a condition in which the heart is abnormally enlarged or dilated and thickened or stiffened (43, 44). As a result, the heart’s ability to pump blood is weakened. It is increasingly clear that cytoskeletal defects produce cardiomyopathy through the combination of both structural and biochemical mechanisms (43, 45, 46). In patients with DMD, the loss of functional dystrophin protein results in several pathologic changes, including myocyte necrosis, inflammation, fibrosis, tachycardia, and impaired contractile properties leading to heart failure and mortality (2–5). However, the biochemical mechanisms leading to cardiomyopathy in DMD remain poorly understood.

MMPs have a critical role in extracellular matrix turnover in both physiologic and pathologic remodeling (12, 41, 47). MMP-9 is one of the major MMPs expressed in the heart of both humans and animal models. Increased myocardial expression and activity of MMP-9 have been observed in a variety of experimental myocardial injuries, such as the permanent coronary artery occlusion model in rodents (48, 49) and the reperfusion injury in porcine models (50, 51). Elevated levels of MMP-9 have also been observed in the failing human heart, suggesting a possible role of MMP-9 in cardiomyopathy (52, 53). Moreover, targeted deletion of MMP-9 in mice has been found to attenuate coronary artery ligation-induced LV enlargement and accumulation of collagens, further implicating a role for MMP-9 in cardiac remodeling after ischemic injury (54). This study provides experimental evidence that the levels of MMP-9 are also elevated in the mdx model of DMD and that MMP-9 contributes to cardiac abnormalities in mdx mice.

Inflammatory response that includes accretion of immune cells, such as macrophages and neutrophils, contributes significantly to the disease progression in patients with DMD (46, 55–57). Whereas the major function of these cells is to remove the damaged tissue through phagocytosis, persistent activation of these phagocytes also causes striated muscle necrosis in DMD models (58, 59). These cells are also the major source of a variety of inflammatory cytokines, chemokines, cell adhesion molecules, and matrix degrading enzymes, the increased expression of which leads to cardiac dysfunction. Indeed, depletion of inflammatory immune cells has been found to ameliorate the pathogenesis in models of DMD (59–62). The role of inflammation in DMD pathology is also evident by the findings that prednisone, which has therapeutic value in DMD, functions by reducing inflammation (35). Importantly, MMP-9 is one of the major mediators of inflammatory response, degradation of extracellular matrix, and fibrosis (12, 15–19). Furthermore, MMP-9 is also known to cause proteolytic processing of a number of inflammatory cytokines, chemokines, and cell adhesion molecules (12). Recently it was reported that MMP-9 can directly cleave OPN in biologically active fragments (63). Among others, TGF-β is one of the most important molecules, which gets activated by MMP-9 (64). Elevated levels of TGF-β have been observed in the circulation of DMD patients and animal models, which positively correlate with interstitial fibrosis in skeletal and cardiac muscle (12, 64). Indeed, we have previously reported that the inhibition of MMP-9 reduces the levels of active TGF-β in the dystrophic muscle of mdx mice.
Therefore, one of the potential mechanisms by which elevated levels of MMP-9 cause cardiac fibrosis in mdx mice could be through proteolytic activation of TGF-β. Moreover, our results demonstrating that the ablation of MMP-9 reduces the accretion of macrophages and expression of inflammatory cytokines (Fig. 3) are consistent with other published reports suggesting an important role of MMP-9 in regulation of these responses in other pathologic conditions (12, 15–19).

Accumulating evidence suggests that there is a cooperative interaction between various MMPs, and many tissue degenerative conditions involve the increased expression and activation of multiple MMPs (23). The members of the MMP family often activate each other, such as membrane type 1 MMP activates MMP-2 or MMP-13 and MMP-3 activates MMP-9 (12, 13, 65). In contrast, the activity of MMPs can be inhibited by increased expression of various TIMPs. We have found that the transcript levels of several other MMPs and TIMPs are also significantly upregulated in the heart of mdx mice (Fig. 6). Moreover, we noticed that the ablation of MMP-9 significantly reduced the mRNA levels of MMP-3 and MMP-12 in the heart of mdx mice (Fig. 6). Interestingly, increased expression of MMP-3 has been suggested as an independent predictor of cardiac failure and death in patients with dilated cardiomyopathy (66). Similarly, MMP-12 is a positive regulator of vascular smooth cells proliferation and atherosclerotic plaque development (67–69). These findings indicate that MMP-9 might also be exacerbating cardiomyopathy in mdx mice through augmenting the expression or activation of other MMPs.

In addition to mechanical instability, loss of dystrophin protein leads to the aberrant activation of a number of cell signaling pathways in skeletal and cardiac muscle (42). Previous studies, including those by us, have reported the increased activation of many signaling proteins (e.g., ERK1/2, JNK1, p38MAPK, and Akt kinase) and transcription factors (e.g., NF-κB and AP-1) in skeletal and cardiac muscles of dystrophic mice (36, 42, 70–75). More recent studies have shown that proper regulation of some of these signaling pathways has enormous potential to attenuate disease progression in DMD (42, 76). Furthermore, it is also evident that different signaling proteins are activated at different stages of disease progression in cardiac and skeletal muscle of mdx and other models of muscular dystrophy (reviewed in 42). Our experiments have shown that MMP-9 causes the activation of ERK1/2 and Akt in heart of 1-y-old mdx mice (Fig. 7). Although the physiologic significance of the activation of ERK1/2 and Akt

FIGURE 7. Effect of ablation of MMP-9 on the activation of MAPKs and Akt in heart of 1-y-old mdx mice. A, Protein extracts prepared from heart of 1-y-old WT, mdx/Mmp9+/+, and mdx/Mmp9−/− mice, were immunoblotted using Ab against phosphorylated or total ERK1/2, JNK1/2, p38MAPK, and Akt protein. The representative immunoblots demonstrate that the level of phosphorylation of ERK1/2 and Akt was reduced in LV of mdx/mmp9−/− mice compared with mdx/Mmp9+/+ mice. B, Densitometric quantification of immunoblots. n = 4 in each group. *p < 0.05, values significantly different from mdx/Mmp9+/+ mice; #p < 0.05, values significantly different from mdx/Mmp9+/+ mice.

FIGURE 8. Involvement of OPN in expression of MMP-9 in heart and skeletal muscle of mdx mice. Three-week-old WT mice were given a single retroorbital injection of OPN protein (100 μg/kg body weight). After 12 h, the levels of MMP-9 protein in LV and TA muscle were measured by Western blot. Representative immunoblots and quantification of bands (bar diagrams) demonstrate that OPN increases the levels of MMP-9 protein in (A) heart and (B) TA muscle of mice. *p < 0.05, values significantly different from mice treated with PBS alone. Three- to four-week-old mdx mice were given three i.p. injections of anti-OPN or isotype control every third day for a total of 10 d. LV and TA muscles isolated were used to measure the level of MMP-9 protein by Western blot. The data demonstrate that treatment with OPN-neutralizing Ab significantly reduces the levels of MMP-9 protein in both (C) heart and (D) TA muscle. n = 3 in each group. *p < 0.05, values significantly different from mdx mice treated with isotype control.
kinase remain unknown, previous studies have shown that increased activation of these signaling proteins can cause muscle hypertrophy (77). It is thus possible that MMP-9 causes cardiac hypertrophy in mdx mice through the activation of these signaling proteins. Our results are also in agreement with a previously published report demonstrating that genetic ablation of MMP-9 prevents cardiac hypertrophy in a model of heart failure (54). Intriguingly, ERK1/2 and Akt kinase are also involved in inducible expression of MMP-9 in response to specific stimuli (78, 79), suggesting that there may be a positive feedback loop in which ERK1/2 and Akt promote MMP-9 expression and vice versa in the heart of mdx mice. However, it is also possible that the reduced activation of ERK1/2 and Akt is a result of reduced cardiomyopathy in MMP-9-deficient mdx mice.

Although the role of MMP-9 in dystrophinopathy is increasingly clear, the mechanisms leading to the increased levels of MMP-9 remain less understood. It was recently reported that muscle biopsy specimens from patients with DMD and dystrophic muscle of mdx mice have elevated levels of OPN (29). Because the deletion of either OPN (29) or MMP-9 (Fig. 4) considerably reduced fibrosis in cardiac muscle, we tested the hypothesis that OPN might be one of the potential stimuli to augment the expression of MMP-9 in cardiac and skeletal muscle of mdx mice. Our experiments demonstrating that the administration of OPN enhance the expression of MMP-9 in normal mice (Fig. 8A, BB) and anti-OPN reduces the levels of MMP-9 in mdx mice (Fig. 8C, SD), suggest a potential link between these two molecules in the pathogenesis of mdx mice. It is also noteworthy that, whereas the inhibition of MMP-9 has been found to reduce the severity of disease progression in animal models of many tissue degenerative disorders, there is still no clinically approved drug that specifically blocks the activity of MMP-9. Broad-spectrum MMP inhibitory drugs developed earlier failed in various clinical trials because of a musculoskeletal syndrome (41). Whereas more specific inhibitors of MMPs are under development in various pharmaceutical companies for clinical use, a potential alternative approach to inhibiting MMP-9 activity could be via targeting the molecules, such as OPN, which induces the expression of MMP-9 in particular disease models.

In summary, this demonstrates the efficacy of MMP-9 inhibition to improve cardiomyopathy in mdx mice and suggests that MMP-9 can serve as an important molecular target for treatment of cardiomyopathy in patients with DMD. More investigations are required to evaluate the effects of inhibition of MMP-9 by multiple approaches and in higher-model organisms before considering MMP-9 as a therapeutic target for patients with DMD.

Disclosures
The authors have no financial conflicts of interest.

References


