The Role of MMP-2 on Collagen Degradation in Skeletal Muscle after Functional Overload in Adult Mice

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Abstract

The extracellular matrix (ECM) is essential in transmitting forces, protecting tissues from injury, and regenerating injured tissues. The ECM is primarily composed of collagen, thus the ability to breakdown and synthesize collagen molecules are instrumental in muscle fiber integrity. Studies have demonstrated that matrix metalloproteinases (MMPs) play a major role in the maintenance and remodeling of the ECM with MMP-2 being a prime component of this process. However, the functional role of MMP-2 in skeletal muscle remains largely unknown. The purpose of this study was to determine the role of MMP-2 in ECM deposition, specifically collagen IV degradation, in MMP-2 KO and WT mice after 0-day and 2-, 4-, and 8-weeks of functional overload (FO) of the plantaris muscle. Results showed that following FO, MMP-2 KO mice expressed a higher intensity of collagen IV in comparison to the WT group. This study demonstrates that MMP-2 activity does aid in ECM remodeling following increased load on skeletal muscle.

Introduction

Skeletal muscle is one of the most dynamic and plastic tissues in the human body that contains a cellular composition and architecture designed to support mobility and facilitate human function (1). In humans, 40% of total body weight and 50-70% of all body proteins is skeletal muscle (1). In response to exercise, injury, and disease, skeletal muscle will undergo changes in order to maintain muscle function (2). The cellular and molecular processes of muscle...
plasticity is still not completely understood; thus, further research is needed to increase our understanding of the cellular and molecular factors that aid in mediating muscle plasticity. Previous studies have focused on the skeletal muscle extracellular matrix (ECM) and its role in regulating muscle plasticity. Skeletal muscle contains numerous fibers that are held together by the ECM (3). The ECM is primarily composed of collagen (dominated by collagen IV) and its functional role is to transmit forces, protect tissues from injury, and regenerate injured tissues (3). The ability to break down and synthesize new collagen molecules in a tightly controlled and organized fashion is critical to maintain optimized force transmission and reflects the high degree of adaptability in ECM tissue (3). Excessive accumulation of ECM components, especially collagens, either due to excessive ECM production, alteration in ECM-degrading activities, or a combination of both, is defined as fibrosis (4). Skeletal muscle fibrosis impairs muscle function, negatively affects muscle regeneration after injury, and increases muscle susceptibility to re-injury, and is seen as a major cause of muscle weakness (4). A pathological accumulation of collagen is a common phenotype among many different types of musculoskeletal disease states, in which the balance between collagen production and degradation becomes dysregulated (3). Understanding mechanisms that degrade muscle fibrosis will help advance our knowledge of the events that occur in dystrophic muscle diseases and will aid in developing innovative anti-fibrotic therapies to reverse fibrosis in such pathologic conditions (4).

Degradation of collagen represents the remodeling of the connective tissue during the mechanical loading of fibroblasts and ECM structures (5). Collagens can be degraded prior to or after the secretion from the cell and this process is initiated extracellularly by matrix metalloproteinases (MMPs) (5). MMPs are a family of enzymes that are viewed as key
regulatory molecules in the formation, remodeling, and degradation of ECM components in both physiological and pathological processes in many tissues (6). In skeletal muscle, MMPs play a crucial role in the homeostasis and maintenance of myofiber functional integrity by breaking down the ECM and regulating skeletal muscle cell migration, differentiation, and regeneration (6). MMP-2 appears to be one of the most important MMPs associated with the function and dysfunction of skeletal muscle appearance (5). By regulating the integrity and composition of the ECM in skeletal muscle, MMP-2 plays an essential role in myofiber proliferation and differentiation, fiber healing after injury, and maintenance of the surrounding connective tissue (5). A previous study that assessed the activity of MMP-2 and MMP-9 in reperfusion injury following skeletal muscle ischemia found that upregulation of both MMPs occurred in a rat model of skeletal muscle lower limb occlusion (7). The increase in MMP-2 and MMP-9 activity correlated with a reduction in type IV collagen, which indicated that more tissue damage occurred (7). Similarly, another study that examined whether MMP-2 is associated with degenerative changes in annulus fibrosus (AF) of intervertebral disc (IVD) degeneration rats found that MMP-2 KO rats profoundly compromised their inability to remodel collagen matrices (8). These results suggested that in the absence of MMP-2, collagen remodeling is significantly impaired, affirming the role MMP-2 plays in collagen turnover and structural alteration in AF (8). Although these studies have shown that MMP-2 may play a role in collagen degradation, little studies have been done on understanding its role under increased mechanical loading on skeletal muscle adaptation.

Therefore, the purpose of this study was to determine the role of MMP-2 in ECM deposition, specifically collagen IV degradation, in MMP-2 KO and WT mice after 0-day and 2-, 4-, and 8-weeks of functional overload (FO). It was hypothesized that the MMP-2 KO mice
would have an increased collagen surrounding the skeletal muscle fibers compared to the WT after FO. This study should provide further insight on the cellular and molecular mechanisms involved in muscle plasticity, which may lead to new interventions and treatment therapies for muscle-related disorders.

**Materials and Methods**

*Antibodies*

The primary antibody used for immunohistochemistry was rabbit polyclonal antibody to collagen IV IgG from AbCam (Cambridge, Mass., USA). The secondary antibody used for immunohistochemistry was goat anti-rabbit conjugated to peroxidase IgG (Vector Laboratories, Burlingame, Calif., USA). The DAB substrate kit for peroxidase was from Vector Laboratories.

*Collagen IV Immunohistochemistry*

A portion of the plantaris muscle was cut from the muscle mid-belly and mounted vertically on cork blocks using a mounting media. Frozen muscle cross-sections (10μm) were then taken from the mid-belly of the plantaris from wild type (WT) and MMP-2 knockout (KO) mice using a Leica cryostat (CM 1950) (Leica, Buffalo Grove, IL) and mounted on slides. All slides were stored at -20° C until immunohistochemical staining (9).

Frozen muscle sections were removed from the freezer and thawed until room temperature. A hydrophobic circle was drawn around the muscle sections using a PAP pen (Ted Pella, Inc, Redding, CA). The sections were placed in a humidifier chamber and incubated in blocking solution (5 ml PBS, 0.005 g nonfat dry milk, 0.005g bovine serum albumin, 50 μl of 10% Triton X-100) at room temperature for 1 hr. Blocking solution was removed and the samples were incubated with the primary antibody solution at room temperature for 1 hr. The
primary antibody solution consisted of anti-collagen IV in rabbit IgG at 1:50 dilution in blocking solution. Following incubation, the primary antibody slides were rinsed 3x5 min in PBS, and then incubated with the secondary antibody for 1 hr at room temperature. The secondary antibody solution was goat anti-rabbit conjugated to peroxidase IgG at 1:50 in blocking solution. Following 3x5 min rinses in PBS, the slides were then developed with 1,1'-diaminobenzidine (DAB) substrate for 5 min at room temperature. Another 3x5 min rinse in PBS was performed and slides were then placed in a Coplin jar and washed 3 times with DiH₂O, followed by a 2x2 min wash in DiH₂O. Slides were then immersed in 75% EtOH for 7 min, followed by 95% EtOH for 7 min, and then 100% EtOH for 7 mins. The slides were then immersed for 2 min in CitriSolv, followed by a second container of CitriSolv until mounted. Slides were mounted with a cover slip using permount. Sections of MMP-2 KO and WT mice were always stained together in pairs to avoid large or systematic differences in staining intensity between genotypes (9).

Image Analyses

Threshold analysis was used to determine collagen-staining quantity. Images were taken using the Motic USB 5.0 camera (Motic 5.0 M pixel USB 3.0P, and Motic Images plus 3.0 software, Motic China Group Co.). Images were captured using a 10X lens objective for each section of the muscle. Images were then opened on Image J64 (National Institutes of Health, USA) and COL4a1/ECM percent area analysis protocol was conducted. The void space corrected area fraction was used to determine the collagen content in each WT and MMP-2 KO muscle section.

Results

Type IV Collagen Staining
Figure 1a shows the plantaris muscle sections from WT and MMP-2 KO mice at each time point of immunohistochemically stained type IV collagen. There was no obvious difference in the 0-day FO mice between groups. However, threshold analysis revealed that in the 2-, 4-, and 8-wk MMP-2 KO mice, collagen IV intensity was greater than the WT group. In 0-day FO WT mice, the mean area of collagen staining was 19.85 ± 22.73% compared to 19.81 ± 10.17% in the MMP-2 KO mice (Fig.1b). 2-wk FO WT mice contained 33.41 ± 20.67% type IV collagen while MMP-2 KO mice had 37.69 ± 2.73% (Fig. 1b). The 4-wk FO WT mice had a mean type IV collagen of 29.01 ± 32.44%, while the 4-wk FO MMP-2 KO mice had a mean of 39.88 ± 10.68% (Fig. 1b). The 8-wk FO WT mice had a mean type IV collagen of 18.86 ± 15.43%, while the 8-wk FO MMP-2 KO mice had a mean of 20.01 ± 10.62% type IV collagen (Fig. 1b).
Figure 1a. Immunohistochemical collagen staining of the plantaris muscle from WT (left column) and MMP-2 KO (right column) mice.
Figure 1b. Mean type IV collagen staining quantification by threshold image analysis in the plantaris muscle of WT and MMP-2 KO mice. Values are means ± SEM for n=2 mice at each time point.

Discussion

The cellular and molecular processes that contribute to muscle plasticity in skeletal muscle are still not completely understood. Therefore, the aim of this study was to determine the role of MMP-2 on collagen IV degradation in the ECM in WT and MMP-2 KO mice after 0-day and 2-, 4-, and 8-weeks FO. Results showed that in the 0-day FO mice, there was no obvious difference in collagen IV intensity between the WT and MMP-2 KO mice (Fig 1b). Previous studies have shown that after skeletal muscle is stretched or injured, synthesis of nitric oxide (NO) directly activates MMP-2 (10). The activation of MMP-2 allows for the release of hepatocyte growth factor (HGF) from the ECM, so that HGF can bind to the c-met receptor for satellite cell activation (10). This supposedly leads to muscle fiber growth and repair by regulating the integrity and composition of ECM in skeletal muscle (10). Therefore, the similarity in collagen IV intensity between the 0-day WT and MMP-2 KO mice may have been due to the lack of a stimulus for NO production such as exercise, injury, or disease (10).

Results also showed that in agreement with our hypothesis, there was a trend of increased collagen content in the 2-, 4-, and 8-week FO MMP-2 KO compared to WT mice (Fig. 1b). Since mice underwent FO on the plantaris muscle, increased collagen intensity may be due to how the muscle is used. Rullman et al. (11) suggested that while MMP-2 is expressed in skeletal muscle, it is not rendered active until muscle undergoes physical exercise such as occurs with injury, exercise, or disease. Consistent with these findings, a study conducted by Zhang et al. (12) found that MMP-2 KO mice who underwent 2-wk FO on the plantaris muscle demonstrated an increase
in gene expression for both MMP-2, as well as key ECM components including type IV collagen, fibronectin, and laminin. MMP-2 KO mice also showed significantly less hypertrophy and ECM remodeling compared to the WT group (12). Therefore, MMP-2 plays a critical role in collagen degradation during ECM remodeling and it is likely that exercise or injury is needed to activate MMP-2 in skeletal muscle.

Limitations to this study include a small sample size for a total of 16 mice as there were only 2 mice in each WT and MMP-2 KO group at each time point. The validity of these results may have been influenced due to the small sample size, since no statistical analyses were run to determine the significant difference between the WT and MMP-2 KO mice. Also, there are limited studies that assess the role of MMP-2 on collagen degradation in a FO model; thus, interpretation of the data is difficult as there are very few studies available for comparison.

In conclusion, we found that the absence of MMP-2 resulted in increased collagen IV content compared to the WT counterparts following FO. This finding suggests that MMP-2 plays a critical role in collagen IV breakdown under muscle hypertrophying conditions via ECM remodeling. Further research is needed for understanding the cellular and molecular processes that contribute to skeletal muscle plasticity.
References


