Expression Of Intracellular MMP-2 After Muscle Injury Does Not Increase With Aging

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Introduction: Skeletal muscle atrophy is a common complication following muscle disuse, long-term immobilization, or nerve damage. We are currently focusing on the role of aging in injury-induced muscle atrophy and its relation to intracellular matrix metalloprotease-2 (MMP-2), an MMP-2 isoform lacking an N-terminus signal peptide. Previous research in our lab has shown that upregulation of MMP-2 is linked to degradation of extracellular matrix and subsequent loss of muscle integrity. We initially hypothesized that aging would increase intracellular MMP-2 expression by activating an alternative promoter of the MMP-2 gene, leading to severe muscle atrophy and damage.

Methods: The F8-del transgenic mouse strain was engineered by our collaborator Dr. David Lovett. The F8-del cassette consists of the rat MMP-2 promoter, the first two exons, and first intron of the MMP-2 gene, driving the β-galactosidase reporter gene. To model muscle injury, a unilateral Achilles tendonectomy was performed on the right hind limb of each mouse. No surgery was performed on the contralateral leg and served as an internal control. Mice were under isofluorane anesthesia for the duration of the procedure. All procedures performed have been approved by an institutional review board for animal research.

Beta-galactosidase Assay: β-gal levels were used as a proxy for MMP-2 promoter activity. A chemiluminescent β-gal assay (Clontech) was used to quantitatively measure expression of MMP-2 in tissue. Left-right gastrocnemius, brain, heart, liver, lungs, and kidneys from 24 month old (n=7) and 3 month old mice (n=10) were harvested 2 weeks after injury and homogenized in Reporter Lysis Buffer. 25 µL of tissue homogenate were aliquoted onto a 96-well plate and incubated at room temperature with 200 µL β-gal Substrate and Reaction Buffer (Clontech). Optical density (OD) was measured in a BioTek plate reader after 1 hour of incubation. β-gal concentration was extrapolated from the OD and normalized to the total protein concentration of each sample as determined by BCA assay (Pierce).

Real-Time PCR (RT-PCR): Left-right gastrocnemius from 4-month F8-del mice (n=8) were harvested one week after injury and bisected longitudinally. Half of each muscle was homogenized in 500 µL of TriZol Reagent (Invitrogen) for total RNA extraction. The entire right gastrocnemius was harvested and processed for this group. RNA was converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was run using Light Cycler 480 SYBRGreen kit (Roche) with primers for the F5 region of the intracellular MMP-2 gene, full-length MMP-2, and GAPDH as a housekeeping gene. Expression of i-MMP-2 and MMP-2 was also compared between aged 18 month (n=3) and young 4 month old (n=3) F8-del mice that did not receive an injury; half of the right gastroc was used for RT-PCR.

Immunohistochemistry (IHC): The other half of the left-left gastrocnemius from the young F8-del mice was mounted onto cork discs with 10% tragacanth gum. The tissue mounts were flash-frozen in liquid nitrogen-cooled isopentane and sectioned at 12 µm. For the uninjured F8-del mice, half of the right gastroc was fixed in 4% paraformaldehyde overnight and then dehydrated in a series of ethanol and xylene baths overnight. Samples were embedded in paraffin and sectioned on a microtome in 5 µm sections. Antigen unmasking was performed with sodium citrate buffer (pH=6.0) in 900 C water bath for
Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol. Slides were then blocked with rabbit serum (Vectastain Elite) for 20 minutes and incubated with goat anti-mouse i-MMP-2 primary antibody overnight. After washing, slides were incubated with biotinylated anti-goat secondary antibody for 30 minutes. Slides were developed with Vectastain ABC reagent (streptavidin conjugated peroxidase), visualized with DAB substrate, and counterstained with Mayer’s hematoxylin. IHC was also performed for full length MMP-2 on the same set of samples, with an incubation time of 30 minutes for the MMP-2 primary antibody.

**Results:** Average β-gal levels were compared for the organs and muscles of old (n=4) and young (n=6) mice. The brain, liver, kidney, and gastrocnemius of old mice showed significantly higher β-gal levels than those of young mice (p<0.05). The highest levels were seen in the kidney (787.9 +/- 453.0 µg/mL and 66.2 +/- 27.4 for old and young respectively), brain (617.5 +/- 181.3 and 304.3 +/- 65.4), and gastrocnemius (162.8 +/- 56.5 and 102.2 +/- 47.4). There was no significant difference between groups for β-gal activity in the heart (110.3 +/- 42.5 and 63.4 +/- 25.5) and lungs (137.0 +/- 94.8 and 152.0 +/- 92.0).

There was a 1.3 fold increase in F5 i-MMP-2 expression in the injured legs of the young F8-del mice (n=8) compared to uninjured contralateral leg and a 5.3 fold increase in expression of the full-length MMP-2. Between the uninjured aged and young mice (n=3 each), there was no significant change in the expression of the F5 i-MMP-2 and MMP-2 genes. IHC staining for i-MMP-2 and full-length MMP-2 in tissue showed no difference in staining intensities in aged mice compared to younger controls.

**Discussion:** Our results indicate that there is little correlation between intracellular MMP-2 and aging in injury-induced atrophy. Even though promoter activity is seen to be increased in older mice after an injury, the IHC stains show that i-MMP-2 and MMP-2 are seen in similar levels in tissues of aged and young mice. The seven day end point after injury may be past the point of highest MMP-2 expression, with the expression returning to baseline levels by the time of harvest. Real-time PCR results also showed an increase in transcription of both forms of MMP-2 in the injured legs of young mice, while in the uninjured model, older mice had reduced expression of MMP-2 compared to younger controls. This reduced baseline of MMP-2 expression in older mice could be attributed to the higher growth rate of the younger mice and therefore a higher overall gene expression. We conclude that i-MMP-2 does not play a larger role in muscle injury with increasing age. Future experiments will utilize chromatin histoinmunoprecipitation (CHIP) against AP-1 family transcription factors to verify if an alternative i-MMP-2 promoter is being transcribed after an injury.

**Significance:** An individual’s age is not necessarily a factor in the effectiveness of a treatment for injury-induced muscle atrophy that involves inhibiting MMP-2 and i-MMP-2.
Figure 1: MMP-2 transcription activity in young vs. old F8-del mice as measured by β-galactosidase reporter

Figure 2: Expression of i-MMP-2 and full-length MMP-2 after tendonectomy
IHC Staining of MMP-2 and i-MMP-2 in Young vs. Old Mice

Figure 3: Expression of i-MMP-2 and full-length MMP-2 in aged vs. young mice