Knockdown of Collagen V during the Inflammatory Healing Phase Significantly Affects Quasi-Static Tendon Mechanics

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Introduction

Collagen V is a quantitatively minor component of collagen fibrils with major regulatory roles throughout tendon healing. Our established murine model of collagen V haploinsufficiency demonstrated diminished recovery of mechanical properties and altered fibril morphology following tendon injury; which supports the important modulatory role of collagen V in tendon injury repair.[1] However, these studies utilized conventional mouse models of collagen V deletion and therefore the lack of collagen V during development and maturation *and* the effect on the injury response is confounding. Thus, the isolated role of collagen V at defined phases of tendon healing following injury remains unknown. Therefore, the objective of this study was to elucidate the specific mechanistic regulatory role(s) of collagen V in the late inflammatory and remodeling responses of tendon healing in a normal matrix using inducible collagen V null and heterozygous models. We hypothesize that decreased collagen V during the inflammatory and remodeling phases will result in significantly decreased dose-dependent tendon mechanical properties during both phases.

Methods

Animal Surgery

Adult male wild-type (WT) (n = 15), Col5a1$^{flox/+}$ (n = 45), and Col5a1$^{flox/flox}$ (n = 45) mice with a tamoxifen (TM) inducible Cre were utilized for this study (IACUC approved). Bilateral partial width, full thickness patellar tendon injury was performed on the Col5a1$^{flox/+}$ and Col5a1$^{flox/flox}$ mice at maturity (120 days) under sterile conditions as described.[2] Cre-induced excision of the conditional alleles of the transgenic mice was performed at 5 days following surgery during the late inflammatory phase (TM5) and 21 days following surgery during the remodeling phase (TM21) via two consecutive daily IP injections of tamoxifen (2mg/40g body weight). The TM5 mice were sacrificed at 3 and 6 weeks post-injury and the TM21 mice were sacrificed 6 weeks post injury (n = 15/genotype/timepoint). The WT uninjured control mice were administered TM doses (3 days of 4mg/40g body weight) at 120 days old and were sacrificed 30 days later. The patellar-tendon-tibia complexes were harvested and prepared for uniaxial mechanical testing.

Mechanical Testing

The tendons were subjected to viscoelastic mechanical testing, which consisted of 10 cycles of preconditioning and stress relaxations at 3%, 4%, and 5% strain. Each stress relaxation was followed by 10 cycles of frequency sweeps, quasi-static ramp to failure, and 5% stress relaxation. The ramp to failure data was used to determine stiffness, modulus, maximum load, and maximum stress. Percent relaxation was quantified for each percent strain level.

Statistics

One-way ANOVAs with Bonferroni correction post-hoc tests were performed to compare the WT uninjured and injured controls to the injured Col5a1$^{flox/+}$ (HET) and Col5a1$^{flox/flox}$ (NULL) tendons at each Cre-induction and healing time point (TM5 at 3 weeks, TM5 at 6 weeks, TM21 at 6 weeks) to define the specific effect of collagen V at each healing phase. Significance was set at $p \leq 0.05$ and trends were set at $p \leq 0.1$.

Results

Cross-sectional area and stiffness of the injured tendons were significantly increased compared to the uninjured WT tendons at both induction and healing time points. The injured WT tendons trended towards increased cross-sectional area compared to HET tendons at TM5 induction after 3 weeks of healing, but the opposite trend was observed between the injured WT and HET at TM5 and TM21 induction after 6wks (data not shown). The injured WT tendons were significantly stiffer than the injured HET and NULL tendons at TM5 after 3 and 6 weeks of healing (Fig. 1A & 1B), but these differences were not seen with TM21 at 6 weeks (data not shown). Max load of the uninjured WT was significantly greater than the injured WT tendons at TM5 after 3 weeks of healing, injured NULL tendons at TM5 after 3 and 6 weeks of healing, and injured HET tendons at TM5 and TM21 after 6 weeks of healing (Fig. 2). Injured WT tendons also had increased max load compared to injured HET tendons at TM5 after 3 (Fig. 2A) and 6 weeks of healing (Fig. 2B). Decreasing trends in max load were

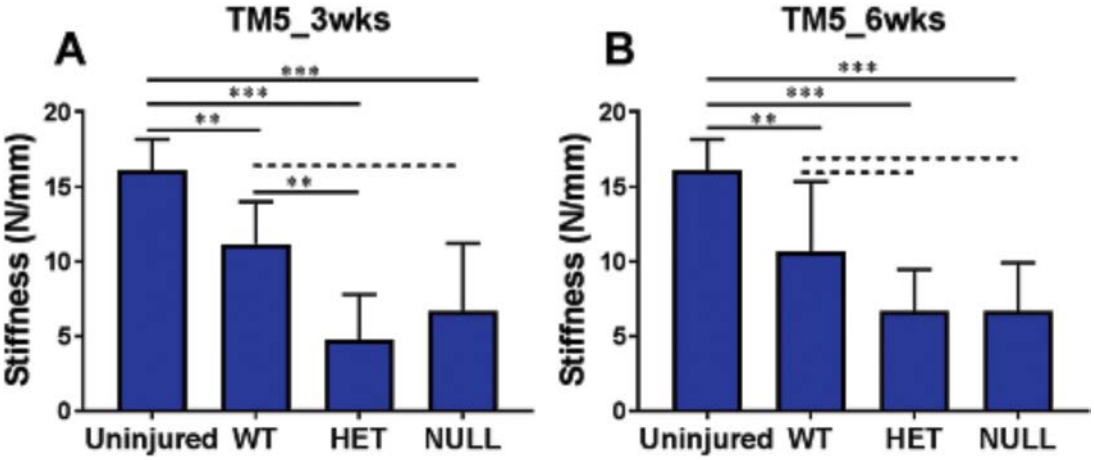

Figure 1. Stiffness. Uninjured tendons exhibited increased stiffness compared to injured tendon and injured WT tendons were stiffer than the HET and NULL tendons when collagen V was knocked out of 5 days post-surgery (TM5) after **(A)** 3 and **(B)** 6 weeks of healing ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.01$.

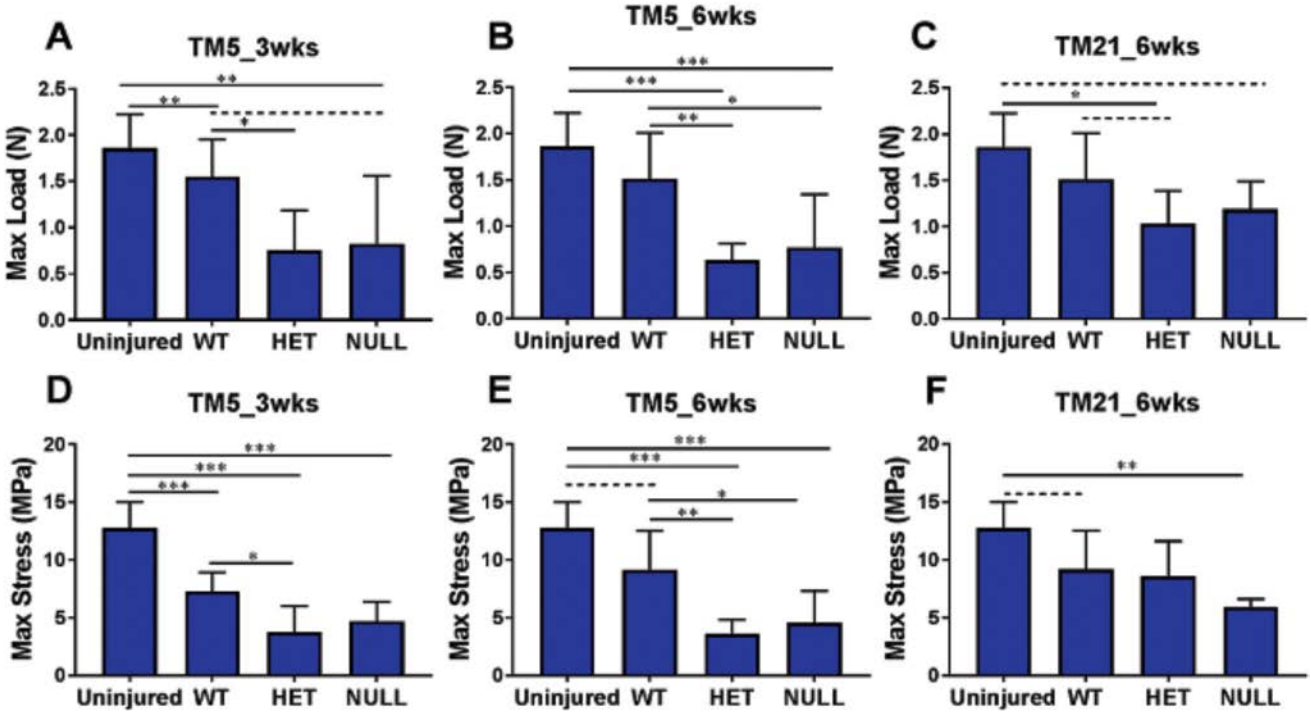

Figure 2. Max Load and Max Stress. Injured tendons exhibited decreased **(A-C)** max load and **(D-F)** mas stress compared to uninjured WT when collagen V was knocked ouut at 5 days post-surgery after **(A&D)** 3 and **(B&E)** 6 weeks of healing. These differences were lessened at the 21 day induction time point and 6 weeks of healing **(C&F)**. ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, ---: $p \leq 0.01$.

observed between injured WT and NULL at TM5 after 3 weeks of healing (Fig. 2A), and at TM21 after 6 weeks of healing between uninjured WT and injured NULL, and injured WT and HET tendons (Fig. 2C). Significant differences in max stress were observed between the uninjured WT tendons and injured tendons at TM 5 after 3 weeks, injured HET and NULL tendons after TM 5 after 6 weeks, and injured WT (trend) and NULL tendons at TM21 after 6 weeks (Fig. 2D-F). The injured WT tendons also exhibited increased max stress compared to the injured NULL at TM5 after 6 weeks and injured TM5-induced HET tendons at both healing time points (Fig. 2D & 3E). Stress relaxation of the injured tendons at 3 and 4% strain was increased compared to the uninjured WT tendons at both induction and healing time points (data not shown). Statistical differences in stress relaxation were observed with TM5 after 3 weeks of healing between injured WT and HET tendons at 4% (trend), injured WT and NULL tendons at 3% and 4% (data not shown). Stress relaxation at 5% strain was increased for the injured HET and NULL tendons compared to the uninjured WT at TM 5 and TM21 (trend) independent of healing time, and decreased for the injured WT tendons compared to the injured HET and NULL tendon at TM5 after 3 weeks (data not shown). No differences were observed between the injured HET and NULL tendons across all parameters, induction, and healing time points.

Discussion

This study investigates the mechanistic regulatory role(s) of collagen V in the late inflammatory and remodeling phases of tendon healing in a normal matrix using inducible

collagen V null and heterozygous models. Overall, the injured tendons exhibited significantly altered material and structural properties independent of genotype. Contrary to our hypothesis, these differences were not allele dose-dependent as no differences were observed between the HET and NULL tendons. This is contrary to the dose-dependent response observed in a previous study where collagen V knockdown induced at the time of surgery resulted in trending differences in cross-sectional area and max stress between injured HET and NULL tendons. This demonstrates that the degree of collagen V deficiency does not have a significant effect on the healing response in the late inflammatory and remodeling phases. Interestingly, knocking down collagen V during the late inflammatory phase resulted in substantial deficits in tendon mechanics, and this effect was not as pronounced when collagen V was altered during the remodeling phase. This observation confirms the direct correlation between collagen V production and tendon inflammation as concluded in a study where collagen V was substantially increased in chronically inflamed connective tissue and supports the unique binding and connecting role of collagen V during the inflammatory process. Further investigation is required to elucidate the mechanistic role of collagen V at the gene and protein level and to define the pathologic and functional significance of collagen V.

Significance

This study demonstrates the role collagen V on tendon repair at defined healing phases and provides mechanistic insights toward understanding the healing response of a normally developed tendon in a collagen deficient healing environment.

Acknowledgements

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