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Controlled treadmill exercise eliminates chondroid deposits and restores tensile properties in a new murine tendinopathy model

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ABSTRACT

Tendinopathy is a widespread and disabling condition characterized by collagen fiber disruption and accumulation of a glycosaminoglycan-rich chondroid matrix. Recent clinical reports have illustrated the potential of mechanical loading (exercise) therapies to successfully treat chronic tendinopathies. We have developed a new murine tendinopathy model which requires a single injection of TGF-β1 into the Achilles tendon midsubstance followed by normal cage activity for 2 weeks. At this time, tendon maximum stress showed a dramatic (66%) reduction relative to that of normal controls and this persisted at four weeks. Loss of material properties was accompanied by abundant chondroid cells within the tendon (closely resembling the changes observed in human samples obtained intra-operatively) and increased expression of *Acan, Col1a1, Col2a1, Col3a1, Fn1* and *Mmp3*. Mice subjected to two weeks of daily treadmill exercise following TGF-β1 injection showed a similar reduction in tendon material properties as the caged group. However, in mice subjected to 4 weeks of treadmill exercise, tendon maximum stress values were similar to those of naive controls. Tendons from the mice exercised for 4 weeks showed essentially no chondroid cells and the expression of *Acan, Col1a1, Col2a1, Col3a1,* and *Mmp3* was significantly reduced relative to the 4-week cage group. This technically simple murine tendinopathy model is highly amenable to detailed mechanistic and translational studies of the biomechanical and cell biological pathways, that could be targeted to enhance healing of tendinopathy.

1. Introduction

Since effective treatment options for human tendinopathies are limited (Maffulli et al., 2010), the development of reliable *in vivo* animal models can improve our understanding of the disease process, thereby facilitating identification of novel therapeutic strategies. Three primary criteria in assessing the utility of such models for future clinical translation are (1) a reduction of tendon mechanical properties, as occurs in human tendinopathy (Arya and Kulig, 2010), (2) histopathologic features mimicking those of the human disease and (3) the amenability of the model for physiotherapy or biologic intervention.

A consistent feature of human tendinopathy is the accumulation of a chondroid matrix within the tendon body, as previously demonstrated by biochemical analysis (Corps et al., 2012; Samiric et al., 2009), histopathology (Kannus and Jozsa, 1991; Khan et al., 1999), and immunohistochemistry (Scott et al., 2008). Importantly, it appears that the chondroid accumulation is an injury response, rather than an adaptation to compression (Ralphs et al., 1992; Vogel and Peters, 2005), since there is marked upregulation of aggrecan expression in individuals with painful Achilles tendinopathy (Corps et al., 2006; de Mos et al., 2009). Furthermore, using a rat supraspinatus overuse model, Attia et al. (2012) observed an increase in glycosaminoglycans (GAGs) in the tendon mid-portion following 8 weeks of downhill running. Hence, we hypothesize that strategies to remove GAG deposits from tendons, or retard their formation, represent clinically relevant therapies.

There is increasing evidence that eccentric loading protocols can effectively treat human tendinopathies (Ohberg et al., 2004; Silbernagel et al., 2011; van der Plas et al., 2012; Visnes and Bahr, 2007). Animal studies (Eliasson et al., 2012; Gelberman et al., 1986; Palmes et al., 2002) have also demonstrated benefits of mechanical stimulation for tendon healing. While results from both clinical and bench studies highlight the importance of mechanobiologic stimulation, very little is known regarding the mechanisms through which this may occur (Sussmilch-Leitch et al., 2012).

The objectives of the current study were firstly, to develop a non-surgical murine tendinopathy model characterized by chondroid

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accumulation and loss of tensile properties, and secondly, to determine the efficacy of controlled mechanical loading to reverse progression of tendinopathy. We utilized TGF-β1 injection to induce tendinopathy, as this growth factor has both been demonstrated to stimulate chondrogenesis in numerous tissue and cell culture models (Shintani and Hunziker, 2007; Diederichs et al., 2012; Lorda-Diez et al., 2009; Morales, 1991) and is a critical biological factor translating mechanical overuse injury of tendon cells into a biological response (Maeda et al., 2011). We hypothesized that firstly, a single injection of TGF-β1 into the Achilles tendon results in tendinopathic changes which mimic human pathology and secondly that tendinopathic mice subjected to controlled exercise exhibit a reduction in chondroid deposits and a restoration of tensile properties.

2. Methods

2.1. Human studies

Intraoperative tendon specimens (IRB ♯11122301) were obtained from the proximal origin of the extensor carpi radialis brevis (ECRB) and distal origin of the biceps brachii tendons from patients undergoing surgical debridement for painful tendinopathy.

2.2. Induction of murine tendinopathy

C57Bl6 male mice (12 weeks old) were injected (28 G needle) into the mid-portion of the right Achilles tendon with 100 ng active TGF-β1 (PeproTech Inc) in 6 μL of 0.1%(v/w) BSA in saline. The study design included naïve (uninjured) controls, an acute response group (48 h post-injection), and mice which received cage or treadmill activity (Fig. 1).

2.3. Mechanical stimulation

At 24 h post-injection, mice began uphill (17°) treadmill running (Stoelting/Panlab) at 32 cm/s, 20 min/day, 5 days per week (Li et al., 2011) for 2 or 4 weeks.

2.4. Geometry and mechanical testing

The Achilles tendon–calcaneus complex was dissected and the calcaneus potted in methyl methacrylate. Tendon cross-sectional area (CSA) was measured using a precision caliper (for width) and a laser displacement sensor (for thickness), assuming a rectangular geometry (Wang et al., 2012). Material testing was conducted at a plantar flexion angle of 45° (Wang et al., 2006) with the specimen in an isotonic saline bath at 37 °C. Each tendon was preloaded to 0.05 N, followed by preconditioning (0.05–0.55 N at 0.1 N/s for 20 cycles), a five minute recovery in an unloaded state, a static stress relaxation test (5% strain at 2.5%/s, held for 600 s), and a load to failure test at 0.5%/s.

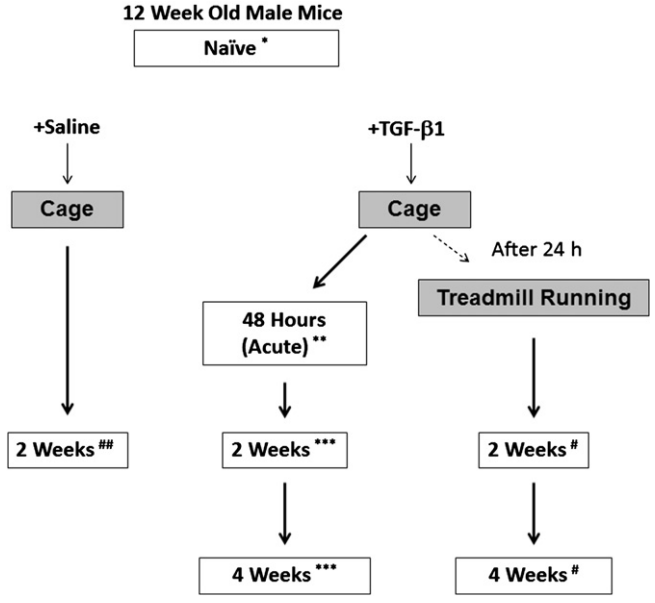

Fig. 1. Schematic of experimental design. * $n=10$ mice (20 tendons) for PCR, $n=5$ mice (8 tendons) for biomechanics, $n=3$ mice for histology; ** $n=10$ for PCR, $n=7$ for biomech, $n=3$ for histo; *** $n=10$ for PCR, $n=6$ for biomech, $n=3$ for histo; # $n=10$ for PCR, $n=5$ for biomech, $n=3$ for histo; ## $n=3$ for histo.

2.5. Histology and immunohistochemistry (IHC)

Following fixation, decalcification, and paraffin embedding, the Achilles tendon-bone complex was sectioned longitudinally and stained with Safranin O (Wang et al., 2012). The number of cells per 350×300 μm^2 field was counted using ImageJ (NIH), for each of four stained images per tendon specimen, by two investigators blinded to the treatment group. For IHC, deparaffinized sections were incubated with the following primary antibodies (10 μg/ml) overnight at 4 °C: high molecular weight aggrecan core protein (anti-DLS, Plaas et al., 2007), ADAMTS5 (anti-KNG, Plaas et al., 2007), collagen I (Abcam, ab-34710) and collagen III (Abcam, ab-7778). Sections were counter-stained with methyl green.

2.6. Quantitative PCR (QPCR)

The tendon proper (i.e. excluding calcaneal insertion and proximal myotendinous junction) was dissected fresh and placed in RNALater™ (Qiagen) at −20 °C. For RNA isolation, 20 tendons, pooled for analysis of each experimental group, were combined in liquid nitrogen, fragmented in a Bessman Tissue pulverizer, and extracted in 1 ml of Trizol™ (Life Technologies) by vortexing for 60 s. RNA was purified with an RNeasy Mini Kit™ (Qiagen) and yields of RNA were approximately 50 ng per tendon. cDNA was synthesized using the SuperScript First-Strand Synthesis Kit™ (Life Technologies) using 1 μg of RNA. All primers were from Life Technologies, Inc.: *Acan* (Mm00545794_m1); *Adamts5* (Mm01344180_m1); *Gapdh* (Mm99999915_g1); *Col1a1* (Mm00801666_g1); *Col2a1* (Mm01309565_m1); *Col3a1* (Mm00802331_m1); *Mmp3* (Mm00440295_m1); and *Fn1* (Mm01256744_m1). Amplifications were performed in triplicate with an Applied Biosystems 7300 Real-Time PCR System as follows: 50 °C, 2 min; 95 °C, 10 min; 95 °C, 15 s; 60 °C 1 min; repeated 39 times (Velasco et al., 2011). Data was processed as ΔCt (relative to *Gapdh*) for each gene at each time point, to provide relative transcript levels and fold-change was calculated as $2^{-\Delta\Delta Ct}$ relative to the ΔCt of the comparison group specified.

2.7. Statistical analysis

Biomechanical, cell counting, and gene expression results were compared across time points using one-way ANOVA with Tukey's post-hoc tests (SPSS 17, IBM, Armonk, NY). To test our study hypothesis, at each time point, an unpaired, two-tailed Student's *t*-test was used to compare data from the cage and treadmill groups.

3. Results

3.1. Striking histopathologic similarities between human and murine tendinopathy

Images from naive murine Achilles (Fig. 2A), 48 h following TGF-β1 injection (Fig. 2B), and normal human patellar tendon (Fig. 2I) exhibited the same, essentially GAG-free, linear organization of collagen fibers and cells. Typical images from TGF-β1 injected murine tendons illustrate that at both 2 (Fig. 2D) and 4 weeks (Figs. 2E and F), tendons showed pericellular and interfibrillar accumulation of GAG, an increase in chondrocyte-like cells, and a loss of parallel arrangement of collagen fibers in and around GAG-enriched areas. Of particular note, the development of these pathological features required the injection of TGF-β1, since injection of saline/BSA did not result in any marked changes in cell morphology or matrix appearance at 2 weeks (Fig. 2C). Histopathologic features of the affected 4-week murine tendons were also seen in tendinopathic human ECRB and biceps samples (Fig. 2G and H).

3.2. Decreased tensile properties following TGF-β1 injection and cage activity

At all times (48 h, 2 and 4 weeks) post-TGF-β1 injection, significant reductions in stiffness (~43%), maximum stress and