Introduction

Tendon is a dynamic tissue which transmits tensile loads between muscle and bone, thus allowing joint movement. It is populated by tenocytes, a specialized, mechanosensitive cell type organized into gap junction-linked arrays. Like osteoblasts, tenocytes are responsible for synthesizing and maintaining a biomechanically competent extracellular matrix, and for mounting an adaptive response to periods of increased loading (i.e., exercise). Following tendon injury, cartilage, bone and fat metaplasia are often observed, making the optimization of tenocyte differentiation an important clinical goal.

Scleraxis (SCX) is a DNA-binding transcription factor which is present in tendon from the condensation stage and persists into adulthood. SCX forms heterodimers with NFAT-C (Nuclear factor of activated T-cells, cytoplasmic) and directly regulates transcription of the gene for Type 1 collagen (Col1a1), which encodes the most abundant protein in tendon. Thus, SCX is a key regulator of tenocyte differentiation, and its expression in tenocytes is strongly induced by TGF-beta signaling. Tgfb and Scx knock-outs demonstrate significantly impaired formation of load-bearing tendons.

Mechanical signaling plays an important role in tendon physiology. In paralyzed embryos, tendon formation occurs but is markedly inhibited. During development and in adults, long-term exercise leads to stronger tendons, whereas immobility or injury leads to tendon weakening and metaplasia. Given its apparent role in regulating the tendon phenotype, Scx is a likely candidate for load-induced gene regulation. A previous study found that cyclic stretch increased the mRNA levels of Scx, tenascin C, and Types I and III collagen in rat bone marrow-derived mesenchymal stem cells, but the effect of varying mechanical parameters was not specifically examined. Zhang and Wang subjected rabbit tendon cells grown in grooved 2D cultures to 4% or 8% strain for 12 hours and found only modest increases in tendon-related genes (Scx was not examined), attesting to the need for further optimization of mechanical loading regimens.

Abstract

Following tendon injury, cartilage, bone and fat metaplasia are often observed, making the optimization of tenocyte differentiation an important clinical goal. In this study, we examined the effect of static and cyclic mechanical loading on the expression of genes which play a role in tenocyte differentiation and function, namely scleraxis (Scx) and Type I collagen (Col1a1), and determined the effect of varying mechanical parameters including (1) static vs dynamic load, (2) increasing strain magnitude, (3) inclusion of 10 s rest periods, and (4) increasing cycle number. Cyclic loading resulted in a greater increase of tenocyte gene expression than static loading over 3 weeks in culture. Increasing strain levels potentiated the induction of tenocyte genes. The insertion of a 10 s rest periods further enhanced tenocyte gene expression, as did increasing repetition numbers. These results suggest that mechanical signaling exerts an important influence on the expression of genes which play a role in determining the tendon phenotype. Further work is required to confirm and extend these findings in primary cells such as resident tendon progenitor/stem cells, in order to provide an improved understanding of biology from which optimized rehabilitation programs can be developed.

Keywords: Tenocyte, Mechanotransduction, Tendinopathy
In this study we examined the effect of three-dimensional static and cyclic mechanical loading on in vitro mRNA levels of key genes related to the tendon phenotype Scx and Type I collagen (Col1a1) in a mesenchymal cell line (C3H10T1/2 cells), and determined the effect of varying mechanical parameters including, (1) static vs dynamic load, (2) increasing strain magnitude, (3) inclusion of 10s rest periods, and (4) increasing cycle number.

Methods

Cell culture

A multi-potent mesenchymal cell line (C3H10T1/2) was obtained from the American Type Culture Collection. Originally derived from embryonic mouse limb bud cultures, this cell line is well characterized for its ability to undergo differentiation into adipocyte, chondrocyte and osteoblast lineages, however there are few published references to their ability to form tendon. Primary tenocytes were obtained from the tail tendons of adult (8-10 week old) heterozygous transgenic mice expressing green fluorescent protein (GFP) under the control of the Scx promoter. Cells were grown at 37°C in a CO2 incubator on standard Sarstedt (Montreal) tissue culture plates in DMEM containing 10% fetal bovine serum, 0.5 mM glutamine, 25 units/ml penicillin and 25 μg/ml streptomycin and passaged 1:5 when confluent using 0.25% trypsin-EDTA. Frozen cell stocks were maintained at -80°C.

Preparation of bioartificial tendons

Bioartificial tendons (BATs) were prepared to allow the application of dynamic mechanical loads to cells in a 3D collagen environment. Subconfluent cells were detached in 1 ml of trypsin for 10 minutes, diluted with Dulbecco’s Modified Eagle Medium containing 10% fetal bovine serum, passed through a 70 μm filter to exclude cell clumps, and counted with a hemocytometer. 5 x 10^6 cells were centrifuged at 300 rcf for 7 minutes and resuspended in 5 ml of Type I collagen solution consisting of 3.5 ml Purecol (Sigma-Aldrich, Oakville, Canada), 1 ml of 5 x DMEM, and 0.5 ml fetal bovine serum, brought to 7.4 pH with 0.1 N NaOH immediately prior to use. 150 μl of cell-collagen mixture was pipetted into each well of an untreated TissueTrain plate (FlexCell International, Hillsborough NC) according to the manufacturer’s instructions, yielding enough for four 6-well plates to be run in parallel for each experiment. The gel was allowed to set for 2 hours, and the resulting BATs were covered with 2 ml of Dulbecco’s Modified Eagle Medium containing either 1% or 10% fetal bovine serum and 0.25 mM ascorbic acid. In this system, cells develop static tension over the ensuing 48 h as they condense the surrounding matrix.

Mechanical loading regimes

After 48 hours, BATs were either maintained under static load conditions (i.e. no superimposed strain), or subjected to superimposed, cyclic mechanical strain in which single variables were altered in successive iterations (duration, strain level, rest insertion, cycle number). Studies were conducted with multiple replicates, and each study was conducted at least twice. For all studies, we used a frequency of 0.1 Hz in order to minimize the risk of detaching the BATs from their tethers, and because this is similar to the frequency used clinically in tendinopathy rehabilitation emphasizing slow, heavy loading. First, BATs were subjected to static load vs cyclic load (5%, 2 hrs/day) for 1, 2 or 3 weeks. Next, BATs were subjected to strains of varying magnitude (0, 2.5%, 5%, 7.5% or 10%, 2 hours/day for 2 weeks). We then examined the effect of inserting a 10s rest period, while keeping all other parameters constant. (10% strain, 100 cycles, daily for 2 weeks). Finally, the optimized protocol (10% strain with 10s rest periods) was varied by changing the number of repetitions (10, 100, 1000).

Microscopy and image analysis

BATs were removed from the CO2 incubator and scanned digitally at 1x magnification (EPSON 4990, Toronto) at the start and conclusion of the culture periods. BAT width was measured using the calibrated measuring tool of an imaging software program (Photoshop CS2, Adobe, San Jose CA). BATs were harvested for cryosectioning by detaching from their tethers with scissors, laid flat in a plastic cryomold, covered with Optimal cutting temperature medium (Thermo Scientific, Toronto), frozen on dry ice and stored at -80°C. Cryosections were cut at 10 microns, stained with haematoxylin and eosin, dehydrated and coverslipped with permanent mounting medium. Images were obtained with a digital camera (Retiga EXi, qImaging, Surrey, Canada) attached to a microscope (Carl Zeiss Canada, Toronto). Other BATs were examined in their entirety by detaching from their tethers, rinsing with phosphate buffered saline, fixing in 3.7% paraformaldehyde, permeabilizing in 0.1% Triton, blocking in 1% bovine serum albumin, and labeling with Alexa Fluor 488 phallolidin (Invitrogen, Burlington, Canada) (stains actin). BATs were then placed between two cover slips and examined using multiphoton excitation fluorescence and second harmonic generation as described previously. To confirm the expression pattern of GFP in adult transgenic mice, patellar tendons were dissected free, fixed for 1 hr in 2% paraformaldehyde in phosphate buffered saline and examined as above.

RNA isolation and qPCR

Total RNA was extracted from BATs at the indicated time or from musculoskeletal tissues of the adult CD-1 mice (8-10 weeks age), using the TRIzol reagent RNA extraction method according to manufacturer’s instructions (Gibco BRL, Life Technologies), after homogenizing tissue in a grinding mill (Mikrodisemembrator, Sartorius, Mississauga, Canada). RNA concentration and purity
were determined using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Toronto). cDNA was prepared from total RNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Gene expression was quantitated using qPCR on an ABI 7500 Fast system with TaqMan probe and primer sets. Expression levels were determined using the relative quantitation method with a standard curve. Gene expression was normalized to 18s rRNA abundance.

**Gene silencing**

Dharmacon© gene silencing reagents were obtained from Thermo Scientific, Toronto, Canada. Cells were trypsinized and replated at 150,000 cells per well of a 6 well plate overnight, then exposed to RNAi targeted against Scx, or against a random scrambled sequence for 24 h. Cells were then retrypsinized. A portion were held back to confirm gene knock-down, and the remainder were used to create BATs as above and the effect of knockdown on collagen remodeling and density determined after 48 hrs using second harmonic generation analysis.

**Statistical analysis**

To detect differences in gene expression, planned comparisons using the Mann-Whitney U test were conducted, with significance predetermined at p<0.05.

**Results**

**Confirmation of the importance of Scx expression in adult tendon**

Scx is a known requirement for embryonic tendon differentiation. We examined the expression of Scx in adult tendon and other closely related collagen-rich musculoskeletal structures (Figure 1A). Scx and Col1a1 expression were highest in tendon, compared to cartilage, bone, muscle, or meniscus. Other examined tissues such as lung or liver demonstrated negligible levels of Scx. In adult tendon, Scx expression was highest in load-bearing collagen fibre bundles. A population of vascular-associated cells in tendon also exhibited Scx expression, but this was barely detectable (Figure 1 C,D,E).

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**Figure 1. Scx expression in adult tendon.** (A) Tendon exhibited the highest expression of Scx among adult musculoskeletal tissues. The same pattern of expression was seen for Col1a1 (not shown); n=6 mice. (B) Spectral scanning confirmed the specificity of GFP emission profile from fluorescent cells in Scx-gfp transgenic tendons shown in C-E, confirming relative specificity of Scx in tenocytes. (C) Second harmonic generation microscopy demonstrates the contrast between collagen rich fibre bundles (F) and adjacent capillaries (V) which occasionally course through adult tendon tissue. (D) The GFP signal is strongest in the load-bearing collagen region, confirming this cell type as adult tenocytes. E. Merged image.
Knockdown of Scx expression in mouse adult tenocytes resulted in a reduced collagen density in bioartificial tendons, confirming the importance of Scx in maintenance of the tendon phenotype (Figure 2).

**Cyclic mechanical load potentiates, but is not required for, tenocyte differentiation in vitro**

Prior to applying cyclic mechanical loads to bioartificial tendons, we examined the influence of the Flexcell culture system on Scx expression. In this system, C3H10T1/2 cells remodel the initial collagen and develop static tension within the BAT if they are allowed to remain longitudinally tethered (Figure 3A). Interestingly, Scx expression increased dramatically over the first week in culture with static load alone (Figure 3B). The addition of cyclic load potentiated Scx expression (Figure 3C). Col1a1 expression showed a similar but smaller trend in response to cyclic load using this waveform, but it was not statistically significant (1.8±0.14 strained 3 weeks vs 1.42±0.18, p=0.107, Figure 3D).

**Strain dependence**

Next we examined whether increasing the applied strain level would lead to further gains in Scx and Col1a1 expression. Strains were applied daily for 2 weeks over a range of strain levels to C3H10T1/2 cells grown in the bioartificial tendon system. Increasing strain levels were associated with higher
levels of Scx and Col1a1, and a more highly differentiated morphology (Figure 4).

**Insertion of rest periods potentiates tenocyte differentiation**

We conducted a pilot study to examine whether the insertion of rest periods between each loading cycle has the potential to enhance tenocyte differentiation. C3H10T1/2 cells were grown in the bioartificial tendon system with loads applied daily as described in the Methods. The insertion of 10s rest periods appeared to potentiate the induction of Scleraxis and Col1a1 (Figure 5).

**Cycle number**

Finally, using the optimized protocol (10% strain, 10s rest periods) we examined the effect of increasing repetition numbers on Scx expression in C32H10T1/2 cells (Figure 6). There was a statistically significant increase in Scx expression with highest number of repetitions (p=0.028), but not with the lower repetition numbers. This suggests that a relatively small number of repetitions may be insufficient to induce tenocyte differentiation signaling, and that longer duration loading regimens may improve differentiation.

**Discussion**

Tendon is a dynamic tissue which, like muscle, is capable of sensing and responding to dynamic loading (reference Kjaer review from same issue). The cell types within tendon capable of responding to mechanical stimuli include tenocytes, mast cells, vascular cell types, and mesenchymal cells which are thought to reside perivascularly in the loose connective tissue linings. It has been suggested for many years that mesenchymal cells may be recruited into tendon tissue following injury, where they participate in the (limited) ability of tendon regeneration\(^2^0\). However, the role and niche of mesenchymal cells in tendon have not been firmly established\(^2^1\). While local recruitment of stem/progenitor cells may provide a source of new tenocytes to assist in the body’s attempt to regenerate the injured tendon, it may also result in the appearance of poorly differentiated cell types such as cartilage, fat, or bone – a common pathological finding in surgical biopsies of chronically painful tendons\(^6\). Based on the current results, it is conceivable that exercise following injury, as part of a rehabilitation program, could promote the correct differentiation of local stem/progenitor cells along the tenocyte lineage.
Figure 4. Effect of increasing strain on tenocyte differentiation. (A,B) Tendon-related genes were expressed at higher levels with increasing strain levels in C3H10T1/2 cells (n=4 per strain level). (C,D) The morphology of bioartificial tendons subjected to 0% and 10% strains are shown (haematoxylin and eosin). In C, note the presence of numerous poorly differentiated cells, identified according to their rounded morphology, evidence of cellular proliferation, and their surrounding loose, basophilic extracellular matrix. In D, nuclei appear more elongated.

Figure 5. Tenocyte differentiation in response to standard or rest-inserted loading applied daily for 2 weeks. The insertion of 10s rest periods appeared to potentiate the induction of Scx and Col1a1. C3H10T1/2 cells were seeded into BATs, allowed to rest for 48 hours, then cyclically loaded at 10% strain. The only difference between the strain-no rest and strain-rest conditions was the insertion of a 10s rest period in between each repetition (n=8 per condition).
In this study, we used Scx and Col1a1, as indicators of the tenocyte lineage (Figure 1). Scx was selected because Scx knock out mice demonstrate a profound deficit in the formation of load-bearing tendons\textsuperscript{14}. Other proposed tendon “marker” genes include tenomodulin, tenascin-C and mohawk. We confirmed in adult tenocytes that Scx was localized in load-bearing regions of tendon, and that Scx knock-down resulted in less highly organized three dimensional collagen matrix (Figure 2). However, we also observed a low level of Scx expression in cells associated with the tendons vascularity, hence Scx cannot be viewed as a completely specific tenocyte marker in adult mice. A further limitation of current tendon research is the lack of detailed knowledge of additional pathways which initiate and maintain the tenocyte phenotype. In a recent rat study of the supraspinatus tendon, Scx was not abundantly expressed, and its expression was not increased by overuse, whereas Col1a1 was (Attia et al, unpublished results). Thus, future work may be needed to identify other tenocyte markers for use in other species, particularly as there is no currently available mouse model of overuse tendinopathy reference Warden’s review, this issue\textsuperscript{22}.

The question of how to provide the optimal mechanical loading to promote tendon adaptation and tendon healing is of great importance to sports and vocational rehabilitation clinicians. Currently used rehabilitation regimes, e.g. the Alfredson program for Achilles tendinopathy and related variants, were developed based on concepts of muscle activity (e.g. eccentric loading)\textsuperscript{23,24}. However, the optimal conditions for tenocytes may not be identical to those for skeletal muscle\textsuperscript{1}. Certainly, higher strains showed the clearest benefit in promoting genes relevant to tenocyte differentiation compared with static or low-strain loading (Figure 4), but caused no change in a cartilage-related gene (Col2a1, data not shown). It will be important to design \textit{in vivo} experiments where varying tendon strains are prescribed and the resultant adaptation, or lack thereof, is monitored at the tissue and cellular levels. \textit{In vivo} tendon loading involves many variables other than strain level\textsuperscript{25}. Nonetheless, tracking tendon strains and loads during exercise may assist therapists in prescribing exercise regimes with greater accuracy, as well as providing a method to track patient responses to an exercise intervention over time.

This study is limited in that we relied on a mesenchymal cell line (C3H10T1/2) rather than primary tendon-derived progenitor cells\textsuperscript{26}. Currently there is no single cell surface marker for purifying tendon-derived progenitor cells, thus clonal expansion of single cells is perhaps the best currently available protocol for growing these cells\textsuperscript{26}. Zhang and Wang subjected culture-expanded tendon-derived cells to cyclic loading, but the identity of their cells was not confirmed. Thomopoulos et al. subjected mesenchymal stromal cells in 3D culture to cyclic or tensile load, and found that Scx, Col1a1 and cellular alignment were promoted by tensile loading, but not compressive, loading\textsuperscript{27}. Kuo and Tuan subjected human bone-marrow derived mesenchymal cells to cyclic loading in the same three-dimensional model system used here, however the mechanical loading parameter chosen was quite minimal (1% strain for 30 minutes per day at 1Hz). In this study, Scx levels declined over time in non-loaded groups but was maintained (but not increased) by cyclic loading. Thus, our study which employed higher, rest-inserted strain levels may have a greater potential to induce Scx and other tendon-related genes than previously examined protocols. An advantage of the study by Kuo and Tuan is that primary human cells were used. However, the relevance of bone-marrow derived MSCs in tendon healing is not established.

Most interestingly, the insertion of 10s rest periods between loading cycles potentiated tenocyte differentiation in the current experimental model (Figure 5). The mechanisms for this differential response to rest-inserted loading requires further work. It may result, as has been suggested for osteoblasts, from the kinetics of calcium signaling, a key upstream event in
mechanotransduction of both bone and tendon cells. There may be a possibility to further optimize current rehabilitation regimes by the inclusion of rest periods, or by the use of discrete loading periods, as opposed to single daily sessions of loading. These concepts require further research, but the current studies demonstrate the potential for this line of inquiry.

In conclusion, the current study confirms that mechanical loading can potentiate the induction of genes related to the tendon phenotype, and furthermore suggests that this strain-induced gene regulation is, at least in part, mediated by Sca. This biological mechanism may underlie some of the known beneficial effects of exercise during tendon repair and regeneration.

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References

