Mechanobiological Response of Tendon Stem Cells: Implications of Tendon Homeostasis and Pathogenesis of Tendinopathy

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ABSTRACT: Tendons are constantly subjected to mechanical loading in vivo. Recently, stem cells were identified in human, mouse, and rabbit tendons, but the mechanobiological responses of tendon stem cells (TSCs) are still undefined. Using an in vitro system capable of mimicking in vivo loading conditions, it was determined that mechanical stretching increased TSC proliferation in a stretching magnitude-dependent manner. Moreover, low mechanical stretching at 4% ("clamp-to-clamp" engineering strain) promoted differentiation of TSCs into tenocytes, whereas large stretching at 8% induced differentiation of some TSCs into adipogenic, chondrogenic, and osteogenic lineages, as indicated by upregulated expression of marker genes for adipocytes, chondrocytes, and osteocytes. Thus, low mechanical stretching may be beneficial to tendons by enabling differentiation of TSCs into tenocytes to maintain tendon homeostasis. However, large mechanical loading may be detrimental, as it directs differentiation of TSCs into non-tenocytes in tendons, thus resulting in lipid accumulation, mucoid formation, and tissue calcification, which are typical features of tendinopathy at later stages. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:639–643, 2010

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Tendons transmit muscular forces to bone and are constantly subjected to mechanical loading in vivo. As such, tendons are susceptible to pathological changes, known as tendinopathy. Tendinopathy is especially prevalent in both occupational and athletic settings that involve repetitive motions, indicating that excessive mechanical loading placed on tendons is a major contributor to the development of tendinopathy. Nevertheless, the precise pathogenic mechanisms of tendinopathy remain unclear. However, the typical histopathological features of tendinopathy have been identified, including accumulation of lipid cells, mucoid degeneration, tissue calcification, or some combination thereof.[1] These findings suggest that tendons contain cells with the potential to exhibit multi-phenotypes that differ from tenocytes, the resident cells in tendons, which express the fibroblast phenotype. Indeed, tendon stem/progenitor cells (TSCs) were recently identified from humans, mice, and rabbits, and TSCs were shown to have multi-differentiation potential. These stem cells can differentiate into non-tenocyte lineages such as adipocytes, chondrocytes, and osteocytes.[2,3]

Stem cells are able to perpetuate themselves through self-replication and differentiation, a process which generates mature cells of a particular tissue. Currently, most studies in stem cell research focus on isolation, identification, and characterization of stem cells. Few studies have investigated the effects of mechanical loading on stem cells, which is nevertheless essential for gaining a better understanding of the physiology and pathology of load-bearing tissues such as tendons.[4] Existing studies, however, have indicated that mechanical loading regulates stem cell proliferation and differentiation. For example, mechanical stretching stimulates proliferation of human bone marrow mesenchymal stem cells (BMSCs).[5] Fluid shear stress promotes the differentiation of embryonic stem cells into endothelial cell lineage,[6] and concurrent application of tensile and rotational loading to human and bovine BMSCs induces differentiation into ligament cells.[7]

As mechanical loading is an inherent part of tendon environment, and mechanobiological response of TSCs is completely undefined, we aimed to determine the effect of mechanical loading on TSCs. Our hypothesis was that mechanical stretching regulates TSC proliferation and differentiation in a stretching magnitude-dependent manner. To test this hypothesis, we used an in vitro model system, with which different levels of uniaxial mechanical stretching were applied to TSCs, and cell proliferation and differentiation were then examined.

MATERIALS AND METHODS

Isolation of Rabbit TSCs and Cell Culture

TSCs were isolated from the patellar and Achilles tendons of 15 New Zealand white rabbits (female, 4–6 months, 3–4 kg). The procedure for cell isolation was based on a previously published protocol.[2] In the procedure, tendon sheath and surrounding paratenon of patellar and Achilles tendons were removed, and the middle portion tissues were minced into fine pieces. They were then digested with a solution of collagenase type I and dispase. The suspensions were centrifuged to obtain cell pellets, which were subsequently re-suspended in DMEM (Lonza, Walkersville, MD) supplemented with 20% FBS (Atlanta Biologicals, Lawrenceville, GA). Next, the cell suspension was diluted with growth medium (DMEM + 20% FBS) to 1 cell/μl and then cultured in T25 flasks. After 8–10 days in culture, patellar TSCs (PTSCs) and Achilles TSCs (ATSCs) formed colonies on the culture surface of the flask. The cell colonies were sub-cultured in new flasks for up to two passages to obtain sufficient numbers of TSCs for cell stretching experiments.

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Cell Stretching Experiments

Considering that patellar and Achilles tendons are primarily subjected to uniaxial mechanical loading in vivo, we investigated the mechanobiological response of TSCs to cyclic uniaxial stretching using an in vitro system we have designed to study tendon mechanobiology. The stretching system consisted of a control unit, driving motor, and silicone dishes.[8] The custom-made silicone dishes were elastic, transparent, and nontoxic to cultured cells.[8] The culture surface of the dish was a 3×6 cm^2 area fabricated with microgrooves,[8] with a ridge and groove width of 10 μm and a depth of 3 μm. The microgrooves in each dish were oriented along the stretching axis. A total of 2.2×10^5 TSCs were plated in each silicone dish containing cell growth medium. The silicone dishes had been coated with 10 μg/ml of ProNectin-F (Sigma, St. Louis, MO) to promote cell attachement. After cells were plated in silicone dishes for 12 h, cyclic stretching of 4% or 8% at 0.5 Hz was applied to silicone dishes for 12 h. Control TSCs were cultured in the silicone dishes with the same culture medium, but without stretching. A total of six dishes for each loading condition (e.g., 4%) and control (i.e., no stretching), respectively, were used, and at least three cell stretching experiments were performed. Following mechanical stretching, cell morphology was examined by phase contrast microscopy. Cell proliferation was measured 3 days after stretching by counting cells with a hemocytometer; cell differentiation, however, was determined immediately after stretching. The procedures for determining cell differentiation involved RNA extraction and then quantitative real-time RT-PCR analysis (qRT-PCR) to determine expression of cell marker genes (collagen type I, collagen type II, PPARγ, Sox9, and Runx2).

It should be noted that the 4% and 8% stretches applied in cell stretching experiments are so called "clamp-to-clamp" strains, not cell strains "seen" by the cells, which vary from cell to cell and are much smaller than the "clamp-to-clamp" strains because of their incomplete transmission to silicone substrate and to cells. Using the same in vitro model system, our previous studies showed that 4% stretching does not induce inflammatory response of tendon cells in terms of PGE_2 production, whereas 8% does,[9] suggesting that the applied stretching magnitudes 4% and 8% may represent the range of strains that tendon cells may experience in vivo during normal and injurious activities, respectively.[10]

qRT-PCR for Gene Analysis

The specific marker gene expression of differentiated TSCs was determined using qRT-PCR. Total RNA was obtained by extraction using an RNeasy Mini Kit with an on-column DNase I digest (Qiagen, Chatsworth, CA). From total RNA, first-strand cDNA was then synthesized by reverse transcription with SuperScript II (Invitrogen, Carlsbad, CA). The following conditions for the cDNA synthesis were applied: 65°C for 5 min and cooling 1 min at 4°C, then 42°C for 50 min, 72°C for 15 min. The qRT-PCR was carried out in a Chromo 4 Detector (MJ Research, Waltham, MA) using QIAGEN QuantiTect SYBR Green PCR Kit (Qiagen). Rabbit-specific primers were used for collagen type I, collagen type II, PPARγ, Sox9, and Runx2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for an internal control. The forward and reverse primer sequences were designed according to published methods.[11,12] All primers were synthesized by Invitrogen.

Relative gene expression levels are expressed as $2^{-\Delta CT}$, where CT is the cycle threshold of each RNA sample from the stretched or nonstretched cells. ΔCT is defined as: $\Delta CT = (CT_{target}/CT_{GAPDH})_{differentiation} - (CT_{target}/CT_{GAPDH})_{control}$. At least three independent experiments were performed to determine the standard deviation (SD) of ΔCT.

Statistical Analysis

One-way analysis of variance (ANOVA) was used, followed by Fisher's PLSD post hoc test for multiple comparisons. Differences between two groups were considered significant when the *p*-value was less than 0.05.

RESULTS

In response to 4% or 8% mechanical stretching, TSCs became highly elongated and aligned along the microgrooves in the axis of stretching (Fig. 1A). In contrast, fewer cells on the nonstretched microgrooved surfaces were elongated, and many were not well spread out (Fig. 1B). Furthermore, compared to control cells, 4% stretching increased the number of PTSCs by 18.1%, whereas 8% stretching increased the number by 36.1% (Fig. 2A). The number of ATSCs increased 76.7% after 4% stretching and 193.3% after 8% stretching (Fig. 2B). Note that, however, without mechanical stretching, ATSCs proliferated much slower than PTSCs (Fig. 2).

In addition, application of 4% stretching to PTSCs and ATSCs significantly increased cellular expression of collagen type I gene, but not PPARγ (a marker for adipocytes), collagen type II and Sox9 (markers for chondrocytes), and Runx2 (marker for osteocytes) (Fig. 3A, B). However, application of 8% stretching to the cells significantly increased expression of all these genes, albeit at different levels (Fig. 3C, D).

DISCUSSION

Tendons are constantly subjected to mechanical loads, due to their essential role of transmitting muscular forces to bone to enable body movement. Because they are living tissues, tendons respond to mechanical loads by changing their metabolism, and with time this mechanobiological response leads to changes in their structural and mechanical properties.[10] Therefore, it is of great interest to understand how the newly discovered TSCs respond to mechanical loading, so that how these loading-induced physiological changes occur can be better understood. Using a novel in vitro system, patellar and Achilles TSCs were subjected to cyclic uniaxial stretching, mimicking in vivo mechanical loading on patellar and Achilles tendons. Both patellar

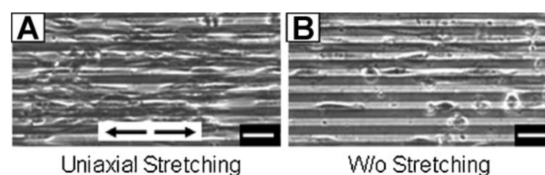

Figure 1. The application of cyclic uniaxial mechanical stretching to rabbit TSCs. (A) Morphology of stretched cells on the microgrooved surface (double arrow indicates stretching direction). (B) Morphology of TSCs on the nonstretched microgrooved surface. It is seen that more cells were present after stretching (A) compared to nonstretching control (B). (Bars = 30 μm.)

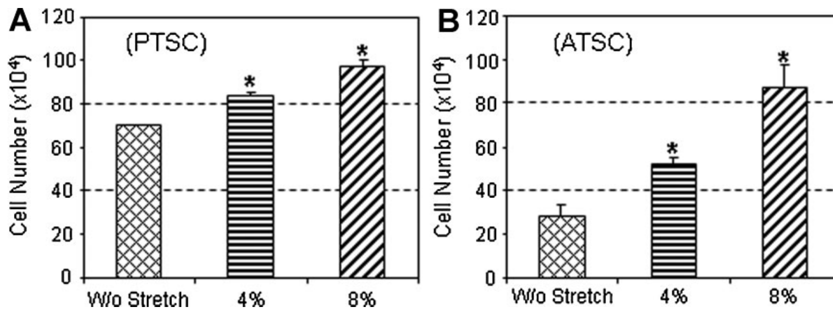

Figure 2. The effect of mechanical stretching on TSC proliferation. (A) Rabbit patellar TSCs (PTSCs); and (B) rabbit Achilles TSCs (ATSCs). With increased stretching magnitude, both PTSCs and ATSCs increased their proliferation. (* $p < 0.05$.)

and Achilles TSCs were found to increase their proliferation when exposed to cyclic mechanical stretching. Therefore, mechanical stretching of TSCs may increase the population size of the stem cells in vivo. However, the increased cell population due to loading-induced proliferation could include both self-renewed cells, a hallmark of stem cells, and differentiated progeny cells. There are two possibilities of TSC division in response to mechanical stretching: a) symmetric division, in which one TSC divides into two identical daughter cells; and b) asymmetric division, in which one TSC divides into two daughter cells, with one becoming the same TSC (self-renewal) while another becomes a progeny cell. Therefore, further study should determine the exact mechanical stretching conditions under which TSCs undergo symmetric division or asymmetric division. This will contribute to understanding the mechanisms of how mechanical loading maintains tendon homeostasis or causes tendon patho-physiology. In addition, the molecular mechanisms for such loading-induced effect on the type of TSC division should be investigated.

It is interesting to notice that ATSCs grew much slower than PTSCs without mechanical stretching (Fig. 2). In fact, we found that the doubling time of ATSCs was about 2.5 times longer than that of PTSCs. With mechanical stretching, however, the proliferation rate of ATSCs was much faster than that of PTSCs. This inherent difference in proliferative potential between PTSCs and ATSCs indicates that the characteristics of TSCs vary according to the tendon under study. A previous study also showed that the differentiation potential of mesenchymal stem cells, which include TSCs, depend on the tissue of origin.[13]

In addition to regulating TSC proliferation, mechanical stretching was shown for the first time in this study to alter TSC differentiation pathways. Stretching at 4% appeared to promote tenocyte differentiation, as the cells expressed high levels of collagen type I gene but not the gene markers for adipogenic, chondrogenic, and osteogenic lineage of cells (Fig. 3A, B). The equal enhancement of collagen type I gene expression between stretching magnitudes of 4% and 8% suggests that some TSCs differentiated towards tenocytes, and the size of

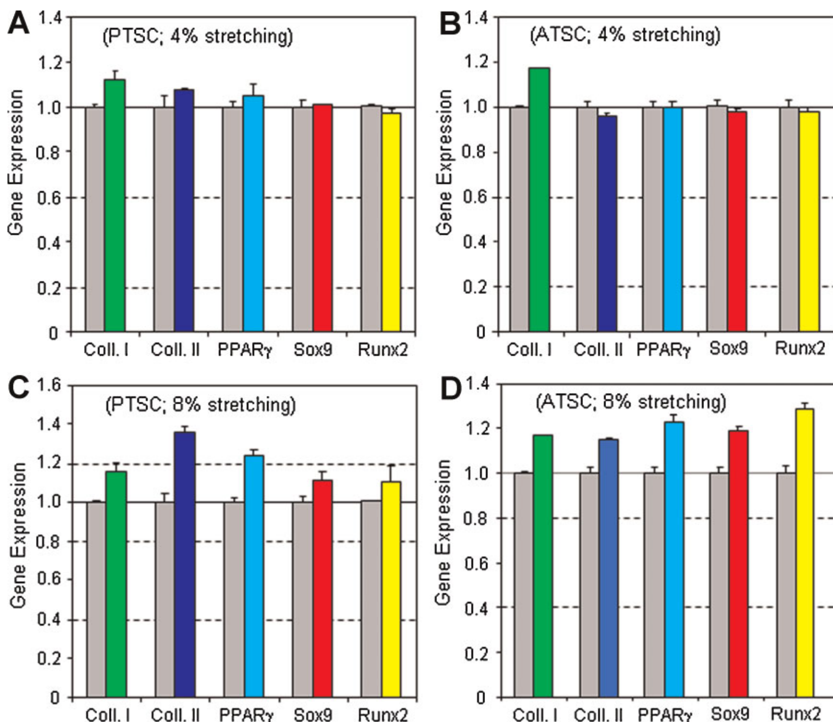

Figure 3. The effect of mechanical stretching on the expression of marker genes by rabbit PTSCs and ATSCs. Compared to nonstretched, control cells (gray columns), 4% stretching significantly increased expression of collagen type I gene ($p < 0.05$), but not other marker genes (A, B); however, 8% stretching significantly increased expression of all genes (C, D) ($p < 0.05$). The gene expression levels were normalized to GAPDH, obtained from at least three independent cell stretching experiments, and presented as $2^{(-\Delta CT)}$.

differentiated tenocyte populations under the two stretching conditions could be similar. Stretching at 8%, however, induced at least some TSCs to differentiate into adipogenic, chondrogenic, and osteogenic lineage of cells, as PPARγ, collagen type II, Sox9, and Runx2 genes, which are specific marker genes for adipocytes, chondrocytes, and osteocytes,[14] increased their expression. While the molecular mechanisms for stretching-modulated TSC differentiation require further study, this finding suggests that normal mechanical loading (or low mechanical stretching) may stimulate TSCs to undergo self-renewal and also differentiate into tenocytes. Therefore, appropriate mechanical loading of tendons in vivo, such as exercise, is beneficial for maintaining the pool of TSCs and increasing the number of tenocytes from stretching-induced TSC differentiation. These two mechanisms together provide an effective way for maintaining tendon homeostasis. On the other hand, large mechanical loading is detrimental, as it induces differentiation of TSCs towards non-tenocyte lineages of cells, such as adipocytes, chondrocytes, and osteocytes, or a combination of the three. Consequently, these cells in vivo may lead to lipid formation, mucoid degeneration, and tissue calcification, which are often seen in tendon lesions of tendinopathic patients.[1,15] The cellular mechanisms governing TSC differentiation to adipocytes, chondrocytes, and osteocytes under large mechanical stretching are unknown. One possibility is that there were multiple TSC populations, with each population differentiating towards adipocytes, chondrocytes, and osteocytes, respectively. A recent study showed that BMSCs contain both pre-osteoblastic and pre-adipocytic cell populations.[16] One interesting goal of further research is to identify the multi-cell populations in TSCs.

While the current study is the first to look into mechanobiological response of TSCs, it should be noted that TSCs used in this study were heterogeneous in the sense that these cells, like adult stem cells in culture, do not exhibit the same characteristics. Also, this study is limited in that only the effect of mechanical stretching magnitude on TSC proliferation and differentiation was examined. The results obtained from a wider range of loading conditions in future studies will define more precisely how different loading regimens influence TSC proliferation, self-renewal, and differentiation, thus helping to define the role of different mechanical loading conditions in tendon homeostasis and pathophysiology such as tendinopathy.

Another limitation of this study is that neither matrix proteins (e.g., collagen) nor interstitial fluid surrounding TSCs, as in vivo, were included in our in vitro model system. In future studies, three-dimensional matrix and interstitial fluid should be incorporated so that mechanobiological responses of TSCs can be more closely examined. In addition, it is known that when 4% substrate stretching is increased to 8%, the lateral compressive strain is increased proportionally; it is not known, however, whether increased tensile strain or compressive strain induces differentiation of TSCs into non-tenocytes, or cells with a phenotype different from tenocytes. Future studies are required to sort out the exact "strain signal" that is responsible for specific TSC differentiation.

In spite of these limitations, the finding that TSCs were capable of differentiating into non-tenocyte lineages of cells in response to large mechanical loading does suggest that mechanical loading, being an inherent part of the tendon environment, likely functions as a "niche factor"[4] regulating the function of TSCs. The finding also has an important implication in the study of tendinopathy. Although the development of tendinopathy likely involves multiple factors, such as genetics and aging,[17] chronic mechanical loading on the tendon is considered to be the major cause of tendon inflammation and degeneration.[10,18,19] However, the precise pathogenesis of tendinopathy is still up for debate. It is well recognized that adult stem cells function to repair and regenerate injured tissues. However, accumulating evidence has pointed to the prominent role of adult stem cells in tissue pathologies, such as tumorigenesis.[20] We therefore suspect that TSCs play a major pathogenic role in the development of tendinopathy. The discovery of TSCs in patellar and Achilles tendons, two tendons that are commonly susceptible to tendinopathy,[21–25] opens a new research avenue for the investigation of the precise pathogenic mechanisms responsible for the mechanical loading-induced development of tendinopathy.

Several in vitro studies have been performed to investigate the role of mechanical loading in the pathogenesis of tendinopathy.[9,26–29] These studies, however, mainly focused on the effect of mechanical loading, either in the form of biaxial stretching[26] or uniaxial stretching[9] on tendon fibroblasts instead of TSCs, as these cells were not identified until recently.[2,3] Future studies should look into the interactions between TSCs and tenocytes under various mechanical loading conditions.

In conclusion, this study shows for the first time, to our knowledge, that mechanical stretching increases TSC proliferation and alters TSC differentiation pathways, depending on the magnitude of stretching. Low mechanical stretching at 4% promoted differentiation of TSCs into tenocytes, whereas large stretching at 8% induced differentiation of a sub-population of TSCs into adipogenic, chondrogenic, and osteogenic lineages. Future studies should investigate the mechanotransduction mechanisms of altered TSC proliferation, including symmetric and asymmetric division, and differentiation pathways in response to different loading regimens.

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