High magnitude, *in vitro*, biaxial, cyclic tensile strain induces actin depolymerization in tendon cells

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Summary

***Background*: the cytoskeleton is a dynamic arrangement of actin filaments that maintain cell shape and are vital in mediating the mechanobiological response of the cell.**
***Methods*: to determine the cytoskeletal response to varying *in vitro*, biaxial stretch amplitudes, rat-tail tendon cells were paired into control and cyclically strained groups of 4.75, 9.5, or 12% strain at 1 Hz for 2 hours and the actin cytoskeleton stained. The cells were analyzed for actin staining intensity as a measure of relative depolymerization and for cell shape. Collagenase gene expression was measured in cells undergoing 12% cyclic strain at 1 Hz for 24 hours.**
***Results*: there was no significant difference in the degree of actin staining intensity between the control group and cells strained at either 4.75 or 9.5%. However, cells strained at 12% demonstrated a significant decrease in actin staining intensity (depolymerization) compared to control cells, increased collagenase expression by 81%, and a clear shift towards a more rounded cell shape.**
***Conclusion*: the results of this study demonstrate that the previously reported induction of collagenase activity associated with the application of high magnitude, *in vitro*, tensile strains may actually be a result of cytoskeletal depolymerization, which causes loss of tensional homeostasis and alteration of cell shape.**

KEY WORDS: actin intensity, cell shape, collagenase, mechanobiology, tendinopathy, under-stimulation.

Introduction

The ability of cells to respond to mechanical loads is central to the concept of mechanotransduction and the maintenance of tissue homeostasis[1]. In tendons and ligaments, the mechanobiological response of the cell is mediated, in large part, through the cytoskeleton, and alterations in the shape and structure of the cytoskeleton are known to influence a variety of changes in gene expression[2].
While the precise level of mechanobiological stimulation required to maintain tendon homeostasis is not currently known, it is very likely that an abnormal level of stimulation may have a role in the etiopathogenesis of tendinopathy[3,4]. Numerous investigators have suggested that over-stimulation of tendon cells, secondary to repetitive loading, results in a pattern of gene expression that can lead to tendinopathy[5-8]. The presence of increased levels of interstitial collagenase mRNA and protein in ligament and tendon cells is a well-known marker of tendinopathy[9]. Physiologically high (15%) strain magnitudes have been shown to up-regulate collagenase activity in ligament cells in monolayer culture[6]. In contrast, numerous studies have also shown that the loss of cytoskeletal tensional homeostasis, secondary to stress deprivation of tendon cells *in situ* (under-stimulation), can markedly stimulate interstitial collagenase mRNA expression and protein synthesis[10-14].
The reasons for these conflicting observations remain unclear. Several studies have shown that chemically or physically induced disruptions of the actin cytoskeleton can trigger an up-regulation of collagenase (mRNA) in fibroblasts[2,11-13,15]. Loss of matrix stress through the release of cell-seeded collagen gels has been shown to cause the loss of cytoskeletal organization and an up-regulation of collagenase gene expression[12,15]. Likewise, an increase in actin depolymerization has been demonstrated in chondrocyte/agarose constructs exposed to 15% compressive strains[16]. Therefore, it is possible that tendon cells that are exposed to high magnitudes of tensile strain in monolayer may alter their cytoskeletal structure. Consequently, the resulting increased actin depolymerization in response to either high strain magnitudes or stress deprivation may actually be the stimulus for the up-regulation of interstitial collagenase reported in these studies. We hypothesize that cells subjected to high strains in monolayer culture significantly alter their cytoskeletal structure resulting in increased actin depolymerization, loss of cytoskeletal homeostatic tension, and increase in collagenase expression.

Materials and methods

Exercise Protocol

Following Institutional Animal Care and Use approval and in accordance with the standards of the Muscle, Ligament, and Tendons Journal[17], tendon cells from rat tail tendons were expanded from explant cultures and utilized in the study. All cells were from passage 2 to 5. Rat-tail tendon cells were plated at 100,000 cells per well into 6 well collagen I coated BioFlex© plates (Flexcell International Corp., Burlington, NC). Cells were allowed to adhere for 24 hours prior to a cyclic strain protocol and maintained in DMEM media supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 0.2% gentamicin, and 7.5 g/L ascorbate. Plates were paired into control and the following equibiaxial, cyclically tensile strained (1 Hz) groups (n=18 wells per group): (1) 5% strain (2) 10% strain (3) 15% strain. The cells were cyclically strained using a Flexercell Strain Unit® with BioFlex Loading Stations (25mm) (Flexcell International Corp., Burlington, NC) for 2 hours at 1 Hz using a sinusoidal strain pattern from 0% strain to the corresponding actual maximum strains of 4.75, 9.5, and 12%.

Actin staining

The cells were fixed with 3.7% formaldehyde during the last 2 minutes of the cyclic strain protocol and then an additional 8 minutes with no strain for a total fixation time of 10 minutes. The fixative was removed and cells were washed with phosphate buffered saline (PBS). Cells were then incubated in 10% sucrose for 15 minutes after which the sucrose was removed and cells were rinsed with PBS. Cellular actin filaments were stained with rhodamine phalloidin (50 U/ml) solution (Invitrogen Corp., Carlsbad, CA) for 20 minutes. The stain was removed and cells were rinsed with PBS. The BioFlex© membranes were cut from the flexcell plates and mounted cells-up on a glass slide. Vecta-mount with DAPI (Vector Laboratories, Burlingame, CA) was added to each membrane and then overlaid with a #1 coverslip. Individual cells were photographed in the central portion of the membrane through the coverslip on a Zeiss Axiovert 200M microscope with a 40x objective (Zeiss International, Oberkochen, Germany).

Actin Intensity

The intensity of actin staining was used as an indicator of actin polymerization[16]. Each of the isolated cells photographed (n=420 per group) were measured for actin staining intensity using Scion Image (Scion Corporation, Frederick, MD). For each image, the background optical density mode was subtracted from the cell's mean optical density to obtain the cell's actin intensity value. The cell's actin intensity values range from 0 to 255 with 0 corresponding to intense actin staining (white) and 255 corresponding to no actin staining (black). The average actin intensity for each of the three cyclically strained groups was then compared to its control group using a t-test with Bonferroni correction and significance set at $p<0.0167$.

Cell Conformation

All of the photomicrographed cells in the paired 12% cyclic strain (n=489) and control groups (n=420) were additionally analyzed (Scion Image) to determine their cell conformation by measuring their length and width. For each cell, the conformation (ratio of cell length: width) was determined as the response variable. A cell conformation of 1 corresponded to a round cell while higher values corresponded to more elongate cells. Factors that could have affected cell conformation were treatment (cyclic strain *vs* control), plates (nested within treatments), and wells (nested within plates). The data were analyzed (SAS PROC MIXED) by means of a multi-nested ANOVA with p-values reported.

MMP-13 Gene Expression Measurements

Additional rat-tail tendon cells were plated and cultured in a similar manner as the previous exercise protocol. These cells were paired into control and cyclically loaded at 12% strain for 24 hours at 1 Hz. After this loading protocol, rat-tail tendon cells were harvested immediately in RNAlater™ (Qiagen, Valencia, CA) to arrest all cellular metabolism. Total RNA from these and control (untreated) cells were then isolated using the RNeasy Plus Mini Kit (Qiagen). Approximately 400 ng of RNA was then converted into cDNA using the Invitrogen SuperScript III Reverse Transcription system (Carlsbad, CA). Real Time Quantitative PCR was performed using the TaqMan® Gene Expression Assay from Applied Biosystems (ABI, Foster City, CA) in the ABI 7500 Fast System. Rat MMP-13 primers and TaqMan® probes (ID # Rn01448197_m1) were obtained from the TaqMan® Gene Expression Assay database at ABI (http://allgenes.com). The endogenous control used for all Q-PCR experiments was Eukaryotic 18s rRNA (ID # Hs99999901_s1). Results were analyzed using the Sequence Detection System software available from ABI. Every treatment was repeated six times and results were statistically evaluated using a t-test with significance set at $p<0.05$.

Results

There was no significant difference in the degree of actin staining intensity between the control group and cells strained at either 4.75 or 9.5% actual substrate strain (Tab. 1). However, cells strained at 12% actual substrate strain demonstrated a significant ($p<0.01$)

decrease in actin staining intensity when compared to control cells (Tab. 1). This would indicate a significant increase in actin depolymerization at this strain level. In addition, results of the cell conformation measurements and photomicrographs revealed a clear shift towards a more rounded cell shape in the 12% strain group (Figs. 1, 2).

The results of the cell conformation nested ANOVA are that there is significant variation among wells within plates (P=0.0002) and significant variation among plates (P<0.0001). However, regardless of well and plate variation, the mean conformation value for the control cells (4.17 +/- 0.09) was significantly greater (p<0.0001) than those cells undergoing 12% substrate strain (2.17 +/- 0.10) (mean +/- SEM). Gene expression analysis revealed that although not statistically significant (p=0.12), the average MMP-13 transcript levels in the cyclically strained cells (38 ± 21) were 81% higher than in the control cells (21 ± 2).

Table 1. Cellular Actin Staining Intensity Value (range 0-255 with 0 corresponding to intense staining and 255 corresponding to no staining).

	5% Strain	10% Strain	15% Strain
Control	154 ± 6	169 ± 10	155 ± 19
Cyclic Strain	163 ± 13	173 ± 6	191 ± 13
p-value	0.1560	0.3886	0.0009

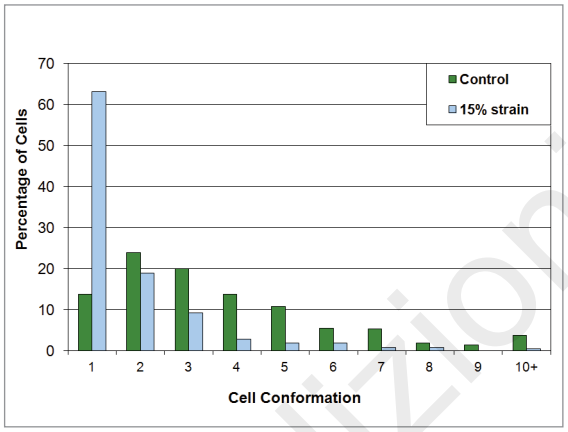

Figure 1. Histogram of percentage of cells within each cell conformation range for 15% strain as well as control cells. A cell conformation of 1 corresponds to a round cell while higher values correspond to more elongate cells.

Discussion

The results of this study demonstrate that high (12%) levels of *in vitro*, biaxial, substrate strain can significantly increase actin depolymerization, thereby altering the shape of tendon cells. However, exposure of tendon cells to lesser substrate strains (≤9.5%) caused no difference in their actin polymerization when compared to controls. This is in accord with a previous *in vitro* study in which 15% of applied cell strain to chondrocytes led to increased actin depolymerization[16]. It has been theorized that the increase in actin depolymerization seen in response to large substrate strains may actually decrease the cell modulus, enabling the cell to undergo large distortions without damage[16,18]. A previous monolayer study demonstrated that the cellular cytoskeleton, in response to transient stretch, fluidizes, or becomes depolymerized, with a prompt decrease in cell stiffness corresponding with the amplitude of the imposed stretch[19].

The actin cytoskeleton plays an important biomechanical function in determining cell-matrix interactions and regulation of the cellular response to mechanical stimuli[18]. In tendons and ligaments, the mechanobiological response of the cell is mediated, in large part, through the cytoskeleton, and alterations in the shape and structure of the cytoskeleton are known to influence a variety of gene expressions[1,2]. These alterations in cytoskeletal structure can also affect the mechanobiological response of the cells, as both changes in cell shape as well as the

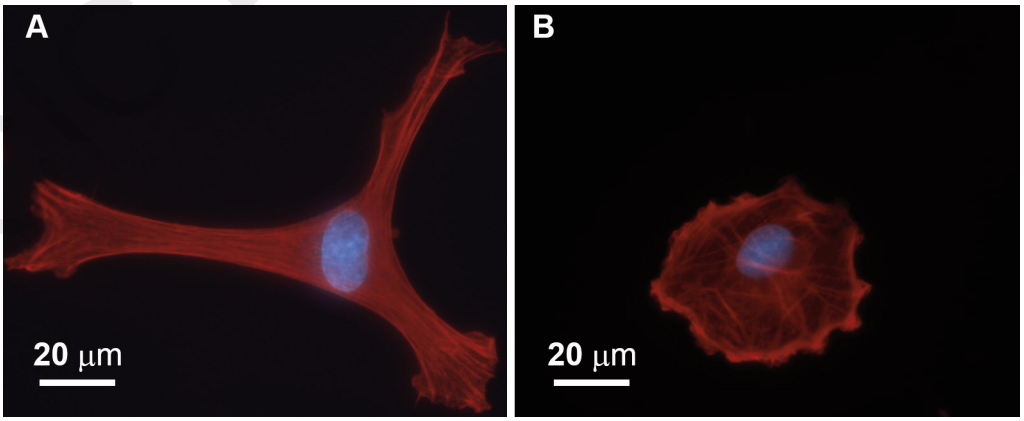

Figure 2. Representative photomicrographs of an elongate cell with intense fluorescence of actin stress fibers in the control group (A) and a round cell with less intense staining of the actin stress fibers in the 15% strain group (B).

depolymerization of the actin cytoskeleton have been shown to influence collagenase gene expression[2,11,15]. Tendon cells are also capable of actively contracting their extracellular matrix through an alpha smooth muscle actin based mechanism[12,20]. This contraction can re-establish the cytoskeletal tensional homeostasis of these tendon cells *in situ* by recalibrating their mechanobiological set point with respect to collagenase protein synthesis[20].

While not significantly different, the results of the current study demonstrate an increase in collagenase expression (81%) in cells undergoing 12% biaxial cyclic strain compared to controls. Previous *in vitro* studies have shown a threshold effect with similar small increases in collagenase with high strains and a minimal change in collagenase at lower strains[6,8]. In one such study, applied equibiaxial cyclic tensile strains of 15 and 10% resulted in a slight up-regulation (58%) or down regulation (30%) of collagenase content respectively[6]. Another *in vitro* study showed that uniaxial tensile cyclic strains of 8% increased the expression of collagenase by 145%, while 4% strain increased it by only 20%[8]. This threshold effect seen with the amount of applied strain and collagenase expression in previous studies coincides with the threshold level of actin depolymerization seen in the current study. While the cells in the current study were exposed to cyclic equibiaxial strain (Flexcell International Corp., Burlington, NC), it was recently demonstrated experimentally and computationally that the strain level is not uniform across the well[21]. Cells in the central portion of the well were shown to experience strains of up to 2% less than the mean value[21]. This heterogeneous strain field may explain the highly variable collagenase expression that resulted in a non-statistically significant comparison to controls in the current study.

Abnormal tendon cell stimulation has been suggested to play a role in the etiology of tendinopathy[3,4]. Several investigators have suggested that it is the mechanobiological response of the cells to repetitive overloading that initiates the degenerative cascade that leads to tendinopathy[6-8]. However, the use of high magnitude cyclic strain to elicit a relatively small catabolic gene expression in tendon and ligament cells cultured on artificial substrates may not be clinically relevant[5-7]. Although tendons may naturally undergo strains greater than 12%, these strains have been shown to lead to isolated collagen fibril damage[13,22]. In addition, tendon cell strain *in situ* has been shown to be significantly less than whole tendon strain[3]. Therefore, it is unlikely that such high levels of repetitive tendon cell strain could be reached and maintained *in vivo* without significant damage occurring within the extracellular matrix of the tendon.

In experimental studies, tendons have been loaded to induce isolated fibrillar damage, and these damaged fibrils have been shown to relax and lose their ability to transmit load[13,23]. This inability of the damaged fibrils to transmit load prevents them from maintaining a homeostatic mechanobiological stimulus to those cells associated with the damaged fibrils[13]. Consequently, alterations of cell-matrix interaction, secondary to isolated fibrillar damage, could result in a mechanobiological under-stimulation of tendon cells. This loss of homeostatic tension within the cytoskeleton due to loss of extracellular matrix tension has been documented in rat tail tendons, and results in the alteration of cell nuclear shape[10] and a significant increase in collagenase mRNA expression and protein synthesis[11,13]. Therefore, the exposure of cells to either high strain *in vitro* or loss of strain *in situ* results in cytoskeletal depolymerization, loss of cytoskeletal tensional homeostasis, and alteration of cell shape.

Although the exposure of cells to either strain or loss of strain may appear to result in initially similar outcomes, the difference in the response could be due based on temporal reasons. Cells exposed to high strains, that are non-damaging to the matrix, will recover their actin cytoskeletal stiffness within 200 second after loading[19]. Cells that are exposed to the loss of matrix tension however, lose their tensional homeostasis until the matrix tension can be restored which may take days[20], with loss of substrate elasticity inducing a higher level of collagenase gene expression[24]. The restoration of tensional homeostasis in lax tendons can occur through an actin-mediated cellular contraction of the extracellular matrix which may be an important mechanism in the recovery of matrix tension in tendons[25].

The results of the current study suggest that the up-regulation of collagenase associated with the application of high magnitude (>12%) tensile strains[6] may actually be a result of cytoskeletal depolymerization, loss of cytoskeletal tensional homeostasis, and alteration of cell shape. Because it is unlikely that cells *in situ* are ever exposed to such high tensile strains[3], the mechanism(s) responsible for the up-regulation of collagenase seen in repetitive use injuries[4] may not, in fact, be due to over-stimulation of cells but rather an under-stimulation of cells and subsequent loss of cytoskeletal tensional homeostasis secondary to alterations in the extracellular matrix ability to transfer loads[23].

Conflict of Interest

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

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