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MMP-1, IL-1β, and COX-2 mRNA Expression is Modulated by Static Load in Rabbit Flexor Tendons

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Abstract—Tendon cells respond to their mechanical environment by synthesizing and degrading the surrounding matrix. This study examined how expression of genes associated with tendon degeneration is affected by static loads. Forty flexor tendons from 10 New Zealand White rabbits were harvested and secured in a tissue loading system. A static load of 0, 2, 4, or 6 MPa was applied to tendons for 20 h. MMP-1, IL-1β, COX-2, GAPDH, and 18s mRNA expression was measured by qRT-PCR. MMP-1 expression in tendons loaded to 6 MPa was significantly increased 259% compared to tendons loaded to 4 MPa. Relative to a 0 MPa load, IL-1β expression was inhibited with load at 4 MPa (48%) while COX-2 expression was increased at 6 MPa (219%). A polynomial regression analysis found a significant positive correlation between creep and expression of MMP-1 ($R^2 = 0.53$, $p < 0.001$) and IL-1β ($R^2 = 0.55$, $p < 0.001$). The results of this study indicate that moderate load inhibits IL-1β and high load stimulates COX-2 relative to stress shielding. MMP-1 expression is up-regulated with high loads compared to moderate loads. The correlation between creep and expression suggests that the pathway for MMP-1 and IL-1β expression, leading eventually to tendon degeneration, may be regulated by the biomechanical factor creep.

Keywords—Mechanotransduction, Overuse injury, Organ culture, Tendon creep.

INTRODUCTION

Tendon overuse injuries are characterized by soreness, pain, and limited range of motion. These injuries are slow to heal, difficult to treat and often recurrent.[16] Tendon cells, like other connective tissue cells, respond to their mechanical environment by synthesizing and degrading their surrounding matrix.[6,18,35] A careful balance is required to ensure matrix degradation does not result in a reduction of strength below the functional demands of the tendon. Tendon overuse injuries are thought to develop when excessive loading leads to an accumulation of microdamage and elicits a maladaptive cellular response.[3]

Further research is necessary to determine what constitutes excessive loading and which genes may be involved in the maladaptive response. Previous cell culture models have demonstrated that mechanical loading up-regulates expression of collagenase (MMP-1), interlukin-1β (IL-1β), and cyclooxygenase-2 (COX-2).[32,40] Tsuzaki *et al.*,[32] found that 2 h of cyclic strain to 3.5% followed by an 18 h rest period led to the up-regulation of IL-1b and COX-2, but did not affect MMP-1. Yang *et al.*,[40] demonstrated that 4 h of 4% cyclic stretching decreased IL-1b induced expression of MMP-1 and COX-2 in human patellar tendon fibroblast. In addition, MMP-1 and COX-2 expression, induced by IL-1b, was further increased with 8% cyclic stretching. These studies indicate a strong relationship between mechanical stimulus applied to cells and the expression of genes associated with matrix remodeling.[15,28] It is difficult, however, to relate the strains applied to the cell surface substrate to those that cells would experience *in vivo*. In addition, cell culture models eliminate possible cell to cell and cell matrix interactions.

In vitro organ culture models are an appealing alternative, allowing precise control over various loading parameters while maintaining the interactions between cells and the surrounding matrix. Previous *in vitro* organ culture models have measured the effects of cyclic loading on factors such as morphology, cellularity, and collagen orientation[13] as well as DNA and protein synthesis.[7,30] These studies demonstrate that tendons under load maintain their viability and metabolic activity. Recent *in vitro* studies have examined the effects of mechanical load on gene expression in tendon explants. Lavagnino *et al.*[20] demonstrated that collagenase expression was inhibited with cyclic strain while Arnoczky *et al.*[4] found similar results with static loading. While these studies reveal a

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mechanotransduction pathway for MMP-1 expression, the loads applied in these studies were relatively low limiting their contribution to understanding overuse injuries.

In this study we examined the effects of sustained static loads applied to rabbit flexor tendons within a physiologic range,[22] on the expression of MMP-1, IL-1β, and COX-2. While overuse injuries are typically associated with repetitive forceful motions, static loads due to sustained awkward postures can also lead to injury.[9] Static loading presents fewer parameters to control compared to cyclic loading, such as loading rate, frequency, and duty cycle. Improving our understanding of the tendons response to static loads can help define the initial parameters for subsequent cyclic loading experiments.

We also examined the role creep may have on the expression of these genes. Mechanical tensile tests have found creep damage contributes significantly to tendon failure[37,39] and has been suggested as a possible mechanism in the development of overuse injuries.[12] Studying how static loads and creep affect the expression of these cytokines and proteases may improve our understanding of the etiology of tendon overuse injuries.

METHODS

Loading System

A custom built tendon loading system, described elsewhere,[5] was used to simultaneously apply static loads to four tendons. Briefly, the loading system consisted of six independent actuators each applying a tensional load to a single tendon. Tendons were held by two clamps. The position of the upper clamp was controlled by the actuator while the lower clamp was held stationary. Both clamps extended away from the actuator so the tendon could be fully submerged in media. A compact load cell, in series with the clamps, continuously recorded tension applied to the tendon. Labview software V6.0 was used to control the actuators and collect data.

Tendons

Four flexor digitorum profundus tendons, approximately 40 mm in length, were harvested from the hind paws of 10 New Zealand White rabbits under sterile conditions (40 tendons total). The rabbits were euthanized for a separate and unrelated study. Tendons were immediately placed in CO_2 Independent Media (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen, Carlsbad, CA), 1% antibiotic/antimicotic (Invitrogen, Carlsbad, CA) and 100 μg/mL of ascorbic acid (Sigma Aldrich, St. Louis, MO).

Loading

The cross-sectional area (CSA) of each tendon was measured with a load applied micrometer.[10] Briefly, tendons were fit into a slot 1.3 mm wide. A plunger measuring 8 mm long and 1.3 mm wide was pressed down on the tendon with a 50 g weight applying a constant pressure of 0.05 MPa for 30 s. CSA was calculated from the measured tendon thickness and the width of the slot. The fibrocartilage zone of the tendon (Region B/C according to Okuda *et al.*[26]) was secured in the proximal clamp and the distal end was secured to the distal clamp. Screws were tightened to 1 N-m. Tendons were preconditioned by loading from 1 to 2 MPa for 20 cycles. After preconditioning, tendons were statically loaded to 0 MPa (stress-shielded), 2, 4, or 6 MPa of static load for 20 h. This time period was chosen to allow enough time for possible creep damage to accumulate and elicited a cellular response.

Throughout loading, tendons were maintained submerged in supplemented media kept at 37 °C. Clamp position was measured every 30 s (Fig. 1). Gauge length was defined as the clamp-to-clamp tendon length under a 0.5 N load after preconditioning. Initial strain was defined as the strain at the beginning of static load, while final strain was the resulting strain after 20 h of static load. Creep was defined as the difference between initial and final strain. At the end of loading, tendons were released from the clamps and a 5 mm section of the tendon was cut, weighed, and snap frozen in liquid nitrogen. The 5 mm section was cut at

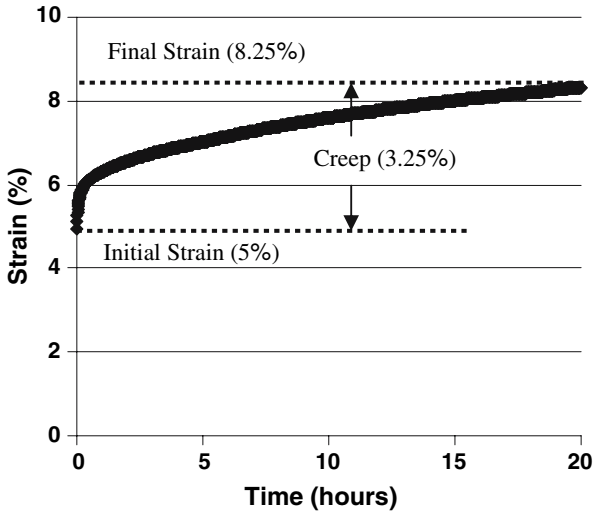

FIGURE 1. Typical creep curve for tendon loaded under a 6 MPa static load for 20 h. Initial strain, final strain, and creep are noted. Initial strain is measured after preconditioning.

least 3 mm away from either clamp to avoid possible clamp effects. Sections were stored at −70 °C until processed.

RNA Extractions

Total RNA was extracted from each tendon section using TRIzol Reagent (Invitrogen, Carlsbad, CA). After extraction, RNA was quantified using the Ribogreen assay (Invitrogen, Carlsbad, CA) and treated with Amplification grade DNase (Invitrogen, Carlsbad, CA). An aliquot of 500 ng was then reverse transcribed (Taqman Reverse Transcription Reagents, Applied Biosystems, Foster City, CA) to cDNA.

Real Time PCR

Expression levels of 18s, GAPDH, MMP-1, IL-1β, and COX-2 were quantified by Real Time PCR (ABI-Prism 7000 Sequence Detection System) using Applied Biosystem's SYBR Green master mix. Oligonucleotide sequences for the genes of interest were obtained from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Primer sets (Table 1) were designed from the sequences using Applied Biosystem's Primer Express software V2. Data was analyzed using the standard curve method (Applied Biosystems, ABI Prism 7700 SDS User Bulletin #2) with each 0 MPa loading sample selected as the calibrator.

GAPDH mRNA levels (mean (SD)) for tendons in the 0 MPa (100%) loading group were significantly greater (repeated measures analysis of variance (RMANOVA), $p < 0.05$) compared to tendons in the 4 (50% (23)) and 6 (59% (23)) MPa loading groups. A significant difference was also found between the 2 (94% (52)) and 4 MPa loading groups. No significant differences were found in 18s rRNA expression levels between any of the loading groups, therefore 18s was chosen as the internal control to normalize expression levels of the other genes of interest.

Statistics

Differences in means were compared by repeated measures ANOVA with a Tukey follow-up test. Gene expression values were log transformed to normalize their distribution before means were compared. Best-fit regression analysis was used to examine correlations between creep and gene expression. Stress shielded tendons were excluded from this analysis as they experienced no creep. All statistical analyses were performed with SAS (SAS Institute Inc., Cary, NC) and significance was taken as $p < 0.05$.

RESULTS

Loading

One tendon was discarded and not included in subsequent analysis because its strain curve showed creep was accelerating and failure was imminent. Mean initial strain increased significantly with each increase in load level (Fig. 2). Initial strains ranged between 1.5 and 8.9%. Increased static load also lead to greater creep strain with a significant difference between the 6 and 2 MPa loading groups ($p = 0.02$). Mean final strains are the sum of initial strains and creep (Fig. 2), and were significantly different between each loading group. Final strains ranged from 2.03 to 11.49%.

Gene Expression

MMP-1 expression was 259% greater in tendons exposed to a 6 MPa compared to tendons exposed to a 4 MPa ($p = 0.02$) (Fig. 3). A similar trend was found in expression of IL-1β (161%, $p = 0.09$) and COX-2 (158%, $p = 0.08$) between these two loading groups. Static load reduced IL-1β expression compared to

TABLE 1. Primer sequences used for real-time PCR analysis.

Genes	Gene name	Primer sequences (forward/reverse)	Accession number
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase	5′-GGATTTGGCCGCATTGG-3′ 5′-CAACATCCACTTTGCCAGAGTTAA-3′	L23961
MMP - I	Matrix Metalloproteinase -1	5′-AGGAGCCTTCCCAAGAGGAA-3′ 5′-CTTGTCTCTTGCATATCAGGATGATG-3′	M25663
COX-2	Cyclooxygenase - 2	5′-CACGCAGGTGGAGATGATCTAC-3′ 5′-TTCCTGGCCCACAGCAAA-3′	U97696
IL-1β	Interlukin 1β	5′-TCCAGACGAGGGCATCCA-3′ 5′-CTGCCGGAAGCTCTTGTTG-3′	D21835
18s	18s rRNA	5′-AGTGCGGGTCATAAGCTTGC-3′ 5′-GGTGTGTACAAAGGGCAGGG-3′	X00640

Abbreviations and gene names, primer sequences and Genebank accession number are presented.

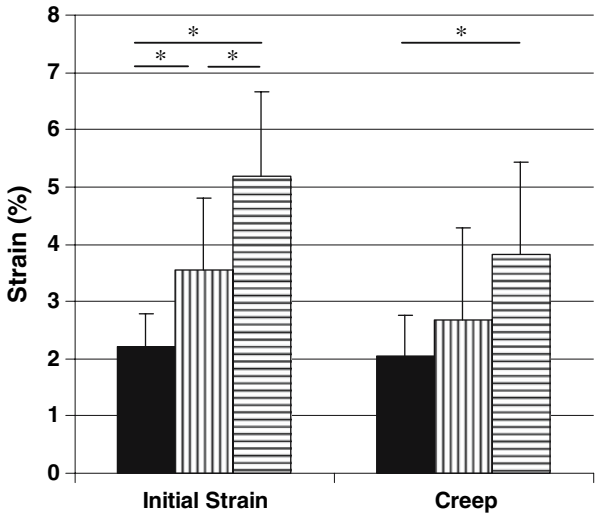

FIGURE 2. Mean (±SD) initial strain and creep in tendons statically loaded to 2 (solid), 4 (vertical lines), or 6 (horizontal lines) MPa for 20 h ($n = 39$, $*p < 0.05$).

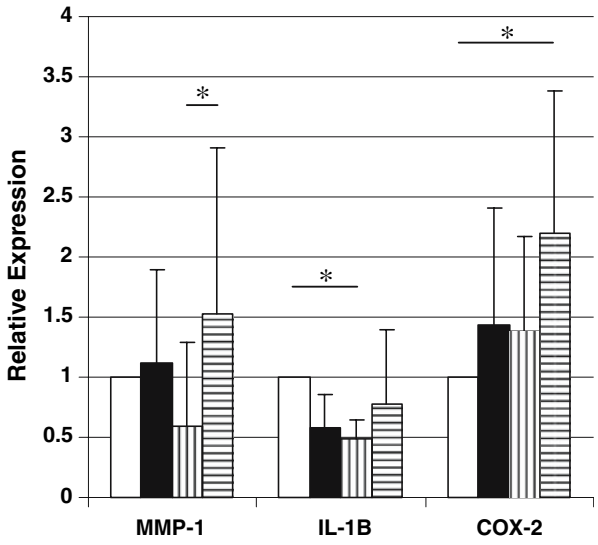

FIGURE 3. Bars represent geometric mean of expression levels in tendons statically loaded to 2 (solid), 4 (vertical lines), or 6 (horizontal lines) MPa relative to 0 (open) MPa. Error bars present coefficient of variability. MMP-1 expression increased 259% between the 4 and 6 MPa loading groups. Relative to stress shielding, IL-1β expression decreased 48% with a 4 MPa static load while COX-2 expression increased 219% with a 6 Mpa load ($n = 39$, $*p < 0.05$).

stress shielding with a 4 MPa load producing a decrease of more that 48% ($p < 0.01$). In contrast, static loading resulted in an increase in COX-2 expression, with a 6 MPa load leading to a 219% increase ($p < 0.01$) over stress-shielded tendons (Fig. 3).

A second order polynomial produced the best fit between creep and expression of MMP-1 ($R^2 = 0.53$, $p < 0.001$, 2nd order coefficient $p < 0.04$) and between creep and IL-1β ($R^2 = 0.55$, $p < 0.001$, 2nd order coefficient $p < 0.02$) (Fig. 4). No correlation was found between creep and expression of COX-2. Regression analysis between load and expression found no strong correlations ($R^2 < 0.17$) for any gene.

DISCUSSION

This study applied static loads to rabbit flexor tendons to examine the effects of increased loads on the expression of genes associated with tendon degeneration. Relative to stress shielded tendons, MMP-1 expression was not significantly changed, IL-1β expression was inhibited significantly at 4 MPa and COX-2 expression was up-regulated significantly at 6 MPa. A 6 MPa load increased MMP-1 expression relative to a 4 MPa load. Interestingly, increased MMP-1 and IL-1β expression correlated with greater creep strain.

Interlukin-1β (IL-1β) is a powerful cytokine involved in many signal transduction pathways and plays a central role in inflammatory and immune responses.[8] Stimulation of bone resorption by osteoclasts, synthesis of acute phase response proteins by hepatocytes and collagenase production in connective tissue are among a few of the pathways in which IL-1β is involved.[8]

This is the first study, to our knowledge, to report a decrease in IL-1β mRNA expression in response to static load. Il-1β is known to induce the expression of other inflammatory mediators including MMP-1, stromelysin, and COX-2.[2,33,40] *In vivo* models have shown stress shielding induces over-expression of IL-1β[34] while cell culture models have reported cyclic strain up-regulates its mRNA expression.[1,32] Uchida *et al.*[34] proposed the hypothesis that mechanical force, either by excess or absence may induce the secretion of cytokines, such as IL-1β, stimulating enzyme production which may lead to cleavage of the collagen matrix. The findings in this study support this hypothesis. IL-1β expression is greater in stress shielded tendons, relative to those exposed to moderate (4 MPa) loads. However, the inhibitory effect is not as great in tendons exposed to higher (6 MPa) loads.

Cyclooxygenase-2 (COX-2), the inducible form of cyclooxygenase, is induced by physical stimulation or inflammatory mediators.[11] Cyclooxygenases catalyze the conversion of arachidonic acid to prostoglandins, which are powerful mediators for pain and acute inflammation.[25] Prostoglandins are also thought to initiate repair and prolong the inflammatory reaction.[16]

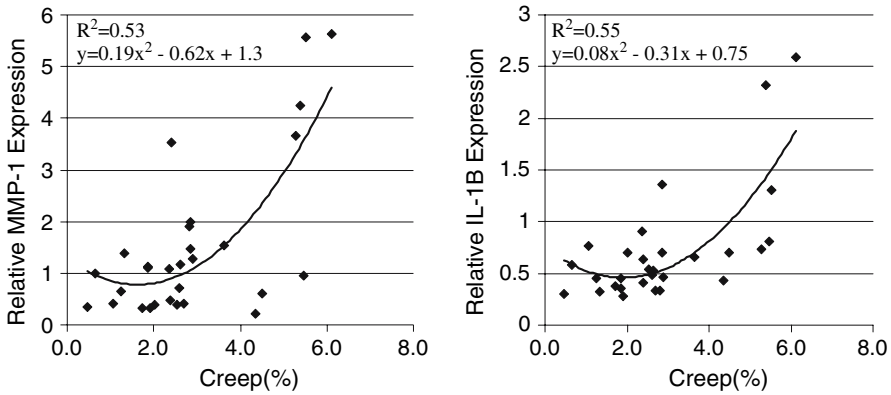

FIGURE 4. Polynomial regression fit between creep and the expression of MMP-1 mRNA (R^2 = 0.53) and between creep and the expression of IL-1β mRNA (R^2 = 0.55). Stress shielded tendons were excluded from this analysis as they experienced no creep. A significant ($p < 0.001$) correlation was found between creep and the expression of both genes.

Unlike IL-1β, no level of static loading decreased COX-2 expression. Recent *in vivo* animal studies have shown that tendon overuse[27] and rotator cuff tears[17] are associated with increased COX-2 expression. Cell culture studies have identified strain levels which elicit an increase in COX-2 mRNA expression.[32,36,40] Tsuzaki *et al.*[32] found that stretching tendon cells with 3.5% elongation at 1 Hz for 2 h increased COX-2 expression nearly 300% compared to un-stretched controls. Yang *et al.*[40] demonstrated that 4 and 8% cyclic stretching of human patellar fibroblasts stimulated COX-2 expression to 183 and 288% respectively, compared to that of non-stretched cells. The findings in this study demonstrate that extended static loads also stimulate COX-2 mRNA expression relative to unloaded controls.

Interstitial collagenase (MMP-1) belongs to the matrix metalloproteinase (MMP) family, a group of tightly regulated zinc dependent enzymes capable of degrading intact fibrilar collagen, proteoglycans, and other extracellular matrix components.[24] MMP's play a crucial role in the normal development, repair, and remodeling of connective tissues. The failure to properly regulate these enzymes can lead to improper or excess matrix degeneration and is thought to play a role in the development of pathological conditions.[24,28]

The inhibition of MMP-1 expression in connective tissue due to mechanical load has been well documented.[4,20,21] In our study, however, a static load of 2 or 4 MPa did not lead to a statistically significant decrease in MMP-1 expression. In an *in vitro* rat tail tendon study similar to ours, Arnoczky *et al.*[4] measured MMP-1 expression in rat tail tendons statically loaded to 0 (stress-shielded), 0.16, 0.77, 1.38, and 2.6 MPa for 24 h. They found expression to decrease in a dose dependent manner. However, even at the highest load of 2.6 Mpa, inhibition was incomplete, as seen in fresh controls. The authors suggest this may be due to an uneven distribution of load across the fibrils[14] with the center fibrils not carrying enough load to completely inhibit MMP-1 expression. It may be that in the larger rabbit tendon there may be more central fibers not experiencing much load. If so, this would explain the reduced MMP-1 inhibition with the 2 and 4 MPa loads.

With greater load (e.g., 6 MPa), however, instead of reducing MMP-1 mRNA expression, there was a significant increase in expression relative to a moderate 4 MPa load. These results are contrary to the findings by Arnozcky *et al.*[4] in which the highest load led to the lowest level of MMP-1 expression. The maximum load in their study was 2.6 MPa. Our study suggests that at higher stresses, static load may begin to up-regulate MMP-1 expression. In a recent study, Lavagnino *et al.*[19] applied an average point load of 41 MPa with a corresponding strain of 13.2% to rat tail tendon fascicles in order to induce fibrilar damage. *In situ* hybridization found an increase in interstitial collagenase (MMP-13) expression in the cells localized near the damaged fibrils. They proposed that the increase in expression was due to under-stimulation of the tendon cells as a result of the damaged fibrils no longer transferring load to the cells. Our study, however, exposed tendons to a maximum stress of 6 MPa resulting in average final strains of 9.0% which were well below the stress and strains applied by Lavagnino *et al.*[19] to produce partial tendon failure. In addition, our loading histories showed no sudden drop in stress with increased strain, a sign of fibril rupture. This indicates that high sustained static loads, below those necessary to produce sudden fibrilar damage, are capable of eliciting an increase in expression of MMP-1 compared to moderate static loads.

Creep damage[37,39] is a possible explanation for this behavior. While fibrilar damage may not occur with the initial load, it can result over time through creep.

The non-linear correlation between creep and expression of MMP-1 and IL-1β (Fig. 4) supports this hypothesis. The increase in expression may be due to the micro-structural changes associated with creep as a result of load. These changes include fiber recruitment, fiber sliding, and fibril rupture[29,31] all mechanisms that would alter the mechanical environment of the surrounding cells.

An unexpected result of our study was the significant differences found in GAPDH between load levels. While other *in vitro* studies have not found GAPDH to vary with loading conditions,[4,20,32] the proliferative state of cells has been found to affect its expression.[23] It is unclear whether this was the case in our study or if other factors led to the difference in GAPDH levels. These differences precluded the use of GAPDH as an internal control for our study and 18s was used instead.

A limitation of our study was that it only examined mRNA expression. Many proteins such as the matrix metalloproteinases are tightly regulated after expression.[24] Examination of protein levels and enzyme activity should be conducted to confirm the findings in this study. A second limitation was that strain was measured clamp-to-clamp while gene expression was measured at the mid-substance. Strain is known to be unevenly distributed in tendons loaded *in vitro*,[41] therefore further examination into the relationship between creep and gene expression should measure strain at the mid-substance if possible. A related issue is that the tendon CSA will change with creep and therefore the stress calculations will change. Since we did not measure CSA during loading we did not consider this change in our calculations. A third limitation of the study was the use of stress shielded tendons as controls. Stress shielding is known to affect gene expression,[4] and therefore is not an ideal "no treatment" control. Comparisons to fresh frozen samples however are also limited as culturing tendons under load introduces a number of confounding factors including excising the tissue, submersion in media, and clamping, all factors that may affect gene expression in addition to loading. Finally, this study examined the role of static load and the resulting creep on the expression of genes associated with overuse injuries. Another, and possibly more important, risk factor for overuse injuries are forceful repetitive loads as these contribute both creep and fatigue damage.[37–39] The results of this study should be used to inform the design of experiments which investigate the role of cyclic loads and its various parameters (peak, duty cycle *etc*.).

In conclusion, we find the expression of genes associated with tendon degeneration is modulated by extended periods of static loads within a physiologic range. Furthermore, expression of MMP-1 and IL-1β correlate with creep. High static loads and the resultant creep may be a pathway in the accumulation of microdamage and the expression of proteases and cytokines that lead to chronic tendon injury.

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