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Release of Pro-Inflammatory Cytokines from Muscle and Bone Causes Tenocyte Death in a Novel Rotator Cuff In Vitro Explant Culture Model

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Abstract

Tendinopathy is a significant clinical problem thought to be associated with altered mechanical loading. Explant culture models allow researchers to alter mechanical loading in a controlled *in vitro* environment while maintaining tenocytes in their native matrix. However, current models do not accurately represent commonly injured tendons, ignoring contributions of associated musculature and bone, as well as regional collagen structure. This study details the characterization of a mouse rotator cuff explant culture model, including bone, tendon, and muscle (BTM). Following harvest, BTM explants were maintained in stress-deprived culture for one week and tendon was then assessed for changes in cell viability, metabolism, matrix structure and content. Matrix turnover occurred throughout culture as manifested in both gene expression and biosynthesis, but this did not translate to net changes in total collagen or sulfated glycosaminoglycan content. Furthermore, tendon structure was not significantly altered throughout culture. However, we found significant cell death in BTM tendons after 3 days in culture, which we hypothesize is cytokine-induced. Using a targeted multiplex assay, we found high levels of pro-inflammatory cytokines released to the culture medium from muscle and bone, levels that did cause cell death in tendon-alone controls. Overall, this model presents an innovative approach to understanding rotator cuff injury and tenocyte mechanobiology in a clinically-relevant tendon structure. Our model can be a powerful tool to investigate how mechanical and biological stimuli can alter normal tendon health and lead to tendon degeneration, and may provide a testbed for therapeutics for tendon repair.

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Keywords

rotator cuff; explant; cytokines; tendon; mechanobiology; muscle; bone

Introduction:

Rotator cuff injuries are among the most prevalent and devastating musculoskeletal injuries affecting the aging population. Full-thickness tears in the rotator cuff tendons are reported to be prevalent in greater than 20% of the population over the age of 50^{1,2} and this prevalence increases even further with increasing age. This has a major impact on the aging and elderly populations, as tears in the rotator cuff can result in shoulder pain, loss of upper extremity function, and overall decreased independence.³ While the exact etiology of age-related rotator cuff degeneration or tendinopathy is still unknown, the most heavily agreed upon cause is overuse of the rotator cuff tendons.^{4,5} Many groups have sought to understand how mechanical force can lead to tendon degeneration through both *in vivo* and *in vitro* studies, using animal models as well as primary tenocyte, tendon stem cell (TSC) and tendon explant culture models.

Animal models have been used extensively to study the onset of tendinopathy through overuse exercise of the tendon itself,⁶ induced inflammation of the tendon and joint via injection of cytokines and drugs,^{7,8} and unloading of rotator cuff muscles via botox injection, immobilization or nerve transection.⁹⁻¹⁴ Animal models provide several advantages over other models, primarily the ability to manipulate mechanical forces externally without disrupting native tendon architecture or the junctions with muscle and bone, which are known to be critically important to tendon function.^{15,16} However, the environment of rotator cuff tendons *in vivo* is extremely complicated as there is cross-talk between the three tissue types in addition to contributions from the vascular, lymphatic, immune, and nervous systems. This can make mechanistic studies challenging, particularly when investigating outcomes of potential drug therapies or physiological processes like aging where there can be both local and systemic changes that may affect tendon homeostasis. Alternatively, cell-based studies are excellent at determining mechanisms by which tenocytes may respond to mechanical loading on a single-cell basis and how injured or diseased tenocytes may respond differently to treatments.¹⁷⁻¹⁹ However, cell-based studies lack the native three-dimensional extracellular matrix environment that tenocytes thrive in, and a number of studies have shown that this environment is critical to cell behavior in a variety of cell lines.^{20,21}

One solution to this limitation is *ex vivo* explant culture, where tissues are removed from the body and placed directly in culture medium. This technique allows cells to continue to live within their native environment while simultaneously allowing researchers to perturb cellular signaling or behavior in a controllable manner through *in vitro* culture. While explant culture models of tendon are less common than that of other tissues, several models have been used to identify key mechanisms in tenocyte mechanobiology. Many groups have focused specifically on the rodent tail fascicle models,²²⁻²⁷ which provide simplified tendon structure, the ability to use transgenic animals to alter matrix proteins, and a large quantity

of samples from a single animal. Although less common now, previous research has also explored large animal explant models, specifically from the cow, dog, rabbit and sheep,²⁸⁻³⁴ via biopsy or dissection of tendon segments for culture studies of explant metabolism. However, while these models have provided critical insight into some of the basic mechanisms of the tenocyte response to mechanical loading, they still do not accurately represent the structure or organization of the tendons that are most commonly injured in the clinic, which have large associated musculature, complicated regional collagen structure, and fibrocartilaginous insertions into bone, all of which contribute to the local tenocyte environment.

The purpose of this study was to develop a mouse rotator cuff explant culture model which includes the humeral head, supraspinatus tendon, and supraspinatus muscle with their native connections retained intact. In this first study, we developed a stress-deprived explant culture model and assessed cell viability, explant metabolism, protein synthesis, and tendon extracellular matrix composition and structure over the course of one week in culture. Based on previous studies in the rat tail fascicle models, we hypothesized that our bone-tendon-muscle (BTM) explant model would maintain collagen structure and tissue organization during this time period. We also hypothesized that cell death may be present in BTM tendons due to early release of proteins such as cytokines from the bone and muscle, and we investigated this further in the present study. For comparison, we also developed a mouse flexor tendon explant model which did not contain muscle or bone and has a simpler and more organized structure, similar to previously established rodent tail fascicle models.

Materials and Methods:

Tendon Harvest and Culture.

Tendons were harvested from approximately 125 C57/BL6J male mice at 4 months of age directly following sacrifice per an approved animal use protocol (MIT CAC #0815-097-18). Flexor digitorum longus tendons (FDL) were harvested from hind limbs as previously described³⁵ and a 10-millimeter segment from the intrasynovial portion of the tendon was excised for culture. For bone-tendon-muscle (BTM) explants, supraspinatus tendons were dissected free of soft tissue³⁶ and excised along with associated muscle, which was cut via sharp dissection from the supraspinatus fossa, and bone, which was cut via dissection scissors at the base of the humeral head. All explants were then washed in sterile 1x phosphate buffered saline (PBS) for 45 minutes and then placed into 24-well culture dishes (Corning Life Sciences, Tewksbury, MA) (Fig. 1). All explants were maintained in standard culture medium consisting of low glucose Dulbecco's Modified Eagle Media (1 g/L (Corning Life Sciences, Tewksbury, MA)) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Pittsburgh, PA), 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Sigma) for up to 7 days. For the purposes of this study, samples were cultured in the absence of any mechanical stimulus (stress-deprived culture); future studies will investigate the addition of mechanical loading to maintain tissue homeostasis.^{24,25} Medium was changed every two days for all cultures (Fig. 1).

Explant Viability and Metabolism.

Explants were assessed for cell viability via live/dead staining. Whole explants were incubated for 5-10 min in 1x PBS containing fluorescein diacetate (FDA; 4 µg/ml, Sigma) and propidium iodide (35 µg/ml, Sigma) for viable and non-viable cells, respectively. Tendons were then imaged using a FLUOVIEW FV3000 confocal laser scanning microscope (Olympus Scientific Solutions, Waltham, MA) at 10x magnification. FDL explants were imaged whole, but removal of the bone was necessary for visualization of the supraspinatus tendon during imaging. To ensure penetration into the interior of the explant, excess non-tendon soft tissue was removed prior to imaging, and internal tenocytes were identified by their organization in rows between collagen fibers. Confocal z-stacks were obtained for each sample, and composites were created by averaging by maximum intensity to produce a single image for quantification containing multiple layers of tissue. On average, stacks of images penetrated approximately 110 µm into the depth of the tissue (total thickness ~300-400µm), and this depth was consistent between groups and time points. Cell viability was quantified using Fiji³⁷ by counting the numbers of viable and non-viable cells via point selection of fluorescent maximum in images of viable and non-viable cells within a selected region of interest (where tendon was imaged). Live cell data are expressed as the percentage of total viable cells in the region of interest, and cell density is the total number of non-viable and viable cells within the region of interest. For control samples, explants were assessed for viability at harvest and then on every other day in culture (days 0, 1, 3, 5, and 7).

Explant metabolism was assessed via the oxidation-reduction conversion of the weakly fluorescent blue dye resazurin to the highly red fluorescent resorufin during a specified culture period.³⁸ Resazurin (25 mM, Fisher Scientific, Hampton, NH) diluted in 1x PBS was added to the culture medium at 10% v/v concentration and samples were incubated for 3 hours. Following incubation, culture medium was removed and fluorescence intensity was measured using a fluorescence plate reader at 530/590nm. It is important to note that this assay is representative of the entire BTM explant including bone, tendon and muscle as opposed to all other assays which were specific to tendon. Fluorescence intensity was normalized to daily control wells that contained resazurin media with no explants. Metabolism was assessed for control samples every other day in culture (days 1, 3, 5 and 7).

Biosynthesis and Biochemical Analyses

Synthesis of total protein and sulfated glycosaminoglycans (sGAG) was measured by incorporation of ³H-proline (5 µCi/mL) and ³⁵S-sulfate (20 µCi/mL), respectively (Perkin-Elmer, Norwalk, CT). Cell proliferation was also measured by incorporation of ³H-thymidine (1 µCi/mL, Perkin-Elmer), but in different experiments than those measuring ³H-proline incorporation. All radiolabels were added to the culture medium for 24 hours. When terminated, explants were washed in 1x PBS, weighed and digested with proteinase K (5mg/ml) (Roche, Indianapolis, MN) for 16-18h. Radiolabel incorporation was measured using a liquid scintillation counter (Perkin-Elmer). sGAG content and total collagen content were measured from the digested explants using the dimethylmethylene blue (DMMB)³⁹ and hydroxyproline (OHP) assays,⁴⁰ respectively, as described previously. All biochemical analyses were normalized to DNA content, which was measured using the PicoGreen dye-

binding assay.⁴¹ Biosynthesis and biochemical analyses were performed every other day in culture (days 1, 3, 5, and 7).

Gene Expression Analyses

FDL explants and supraspinatus tendons from BTM explants were harvested at days 1, 3, 5, and 7, flash frozen with liquid nitrogen, and stored at -80°C for analysis of gene expression as described previously.^{42,43} Samples were also obtained as day 0 control, which were flash frozen directly after dissection. For extraction of total RNA, samples were first pulverized and homogenized in TRIzol reagent, chloroform was added and then samples separated using phase-gel tubes (Qiagen, Chatsworth, CA). The supernatant was then purified following the Qiagen RNeasy mini kit protocol (Qiagen). The quality and quantity of total RNA extracted from samples was analyzed using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Cambridge, MA). Total RNA yield was approximately $230.30 \pm 141.41\text{ng}$ (260/280 ratio: 1.75) for BTM explants and $241.23 \pm 193.28\text{ng}$ (260/280: 1.83) for FDL explants. Total RNA was reverse transcribed using the AmpliTaq-Gold Reverse Transcription kit (Applied Biosystems, Foster City, CA) and real-time PCR was performed using the Applied Biosystems 7700HT instrument with SYBR Green Master Mix (Applied Biosystems). Primer pair sequences (Integrated DNA Technologies, Coralville, IA) are listed in Supplemental Data (Table S1). Briefly, we measured tendon markers (scleraxis, tenomodulin), matrix proteins (collagen types 1, 2, 3, decorin, biglycan, fibromodulin, aggrecan, sox9), matrix metalloproteinases (mmp-3 and mmp-13), one pro-inflammatory cytokine (IL-1 β), and apoptosis markers (caspase-3, caspase-9). Expression data for each gene were calculated from the threshold cycle (Ct) value, and normalized to the internal housekeeping gene GAPDH, which did not show any variation with days in culture (not shown). Each was then normalized to the average value for day 0 controls and the fold change was calculated. Calculations were performed in log space to maintain symmetry about zero and for better comparison between up- and down-regulated values.^{44,45}

Histological Evaluation

Analysis of tissue structure was performed via paraffin histology using standard techniques for samples collected from culture on days 0, 1, and 7. Briefly, samples were fixed in 4% paraformaldehyde, decalcified with a 12% formic acid solution (Immunocal, American MasterTech, Lodi, CA) for one week, processed, embedded in paraffin, and sectioned at $7\mu\text{m}$. Samples were then stained with hematoxylin & eosin (H&E) and toluidine blue (TB) to assess overall tissue structure, cell morphology and sGAG distribution. Specifically, H&E allows us to visualize collagenous tissue in pink and cell nuclei in purple, while TB stains cell nuclei in blue and polysaccharides in purple. Slides were then imaged using a standard light microscope. Additional sections were also stained using the TACS TdT (terminal deoxynucleotidyl transferase) using the In Situ Apoptosis fluorescence staining kit (R&D Systems, Minneapolis, MN), sealed with ProLong Gold Antifade reagent with DAPI as a counterstain, and imaged with a Nikon Elipse Ti-S inverted fluorescent microscope (Nikon Instruments, Melville, NY).

Cytokine Release into Culture Medium

For assessment of cytokine release, medium at days 1, 3, and 7 in culture was collected for the BTM and FDL and analyzed using the Meso Scale Discovery Mouse ProInflammatory 7-Plex multi-spot assay to quantify protein levels of IL-10, IL-6, IL-12p70, IFN- γ , IL-1 β , KC/GRO (mouse analog of IL-8), and TNF- α (Meso Scale Discovery, Rockville, MD). To investigate whether cytokines were released from the tendon, bone, or muscle, an additional three groups were added: (1) supraspinatus tendon only (TO), (2) supraspinatus muscle only (MO), and (3) humeral head bone only (BO). BTM explants were harvested from mice and the three tissues were separated just prior to immersion in culture medium. Samples were incubated in standard tendon culture medium as described above for up to 7 days, and medium from days 1 and 3 was analyzed using the pro-inflammatory multi-spot assay described above.

Cytokine Challenge of Flexor Tendons

To further confirm our hypothesis that cytokines released from muscle and bone could cause cell death and/or matrix degradation in our tendon explants, FDL explants were cultured in normal tendon medium for the first two days in culture and then challenged with cytokines for the duration of the culture period (7 days). Specifically, we investigated cytokine challenge using medium supplemented with (1) mouse recombinant IL-1 β at 10 ng/mL, (2) human recombinant IL-6 at 10 ng/mL, and (3) human recombinant TNF- α at 10 pg/mL (all from PeproTech, Rocky Hill, NJ). These cytokine concentrations were chosen based on the cytokine levels measured in the culture medium of the BTM group at day 1 of explant culture. We compared the results of these studies using exogenous cytokines with experiments using (4) conditioned medium taken from a 1:1 mixture of day 1 BTM culture medium and fresh tendon medium. Following the above studies (1)-(4), FDL explants were assessed for cell viability via live/dead staining as described above at days 3, 5 and 7 (n=2/group/day).

Statistical Comparisons

For cell viability, explant metabolism, gene expression, tendon biosynthesis and biochemical analyses, statistical comparisons were made within each explant group using a one-way ANOVA with Bonferroni-corrected post-hoc t-tests. To compare cytokine levels between groups, data were pooled over the complete culture period within a group and comparisons were made using a one-way ANOVA with Bonferroni-corrected post-hoc t-tests. For all comparisons, significance was denoted at $p < 0.05$.

Results:

Tenocyte viability from BTM explants was significantly decreased after 3 days in culture based on live/dead imaging (Fig. 2). This was confirmed quantitatively with significant decreases in the percentage of living cells from day 1 in culture onwards (Fig. 3A). Tenocytes appeared to be organized along the long axis of collagen fibers as expected up to day 5, after which there was no discernible cellular organization. In addition, tenocytes appeared to be elongated at initial harvest (day 0), but become rounder as the culture period continued. However, neither the overall wet weight of the tendon (not shown) nor the DNA

content (Fig. 3B) was altered during the culture period. BTM explants (including bone, tendon and muscle) exhibited increases in overall explant metabolism over the course of culture which reached significance by day 7 (Fig. 3C). BTM Explants exhibited overall cellular proliferation as measured by incorporation of radiolabeled thymidine which was significant following day 5 (Fig. 3D). Interestingly, the overall extracellular matrix content of the explants was not changed over the duration of the culture period. Specifically, there was no measured change in sGAG or total collagen content (Fig. 3E,F). However, synthesis of both sGAGs and total protein increased during the entire duration of culture (Fig. 3G,H), suggesting matrix turnover. Results for all biosynthesis and biochemical assays were similar for FDL explants (Supplemental Fig. S1) with the exception of cell viability, which remained constant over 7 days.

H&E stained sections did not show any discernible changes in collagen structure throughout the culture period. However, there was a noticeable rounding of cell nuclei over the culture period where cells appeared to have normal elongated tenocyte morphology at day 0, but appeared very rounded by day 7 (Fig. 4A-C, inset). TB stained sections showed some sulfated GAG loss with time occurring in the mineralized fibrocartilaginous region of the insertion site of the supraspinatus tendon in BTM cultures (Fig. 4D-F). Interestingly, there was also a marked loss in cell nuclei (blue) staining in both the marrow and trabecular bone of the humeral head (Fig. 4F). However, there did not appear to be a loss in tenocyte nuclei staining from day 0 to day 7 near the insertion site. Staining for apoptotic cells did not reveal any apoptosis in the tendon proper at days 0, 1 in culture (Fig. 4G,H), although positive control samples did show apoptosis in all cells (not shown). There may be a small amount of apoptotic cells present at day 7 in culture, but it was not substantial (Fig. 4I). There also was a loss in nuclear staining in the bone marrow cells and osteocytes similar to the TB staining (not shown).

Gene expression (mRNA levels) of type 1 collagen decreased throughout the culture period when compared to day 0 control samples (Fig. 5A). Type 2 collagen and aggrecan were upregulated, but it is important to note that type 2 collagen expression was very low in all samples. Biglycan, fibromodulin, and decorin expression were not significantly different from day 0 expression throughout culture, but collagen type 3 was downregulated significantly near the end of culture (Supplemental Fig. S2). Expression of tendon markers scleraxis and tenomodulin were downregulated throughout culture, with statistical significance at days 1, 3 and 7 (Fig. 5B). MMP-3 expression was no different from day 0 expression throughout culture, but MMP-13 was downregulated with significance at days 5 and 7 (Fig. 5C). Finally, there were no significant differences between control day 0 specimens at any point during culture in mRNA expression of either caspase-3 or caspase-9, although there may be downregulation for both markers with higher sample size (Fig. 5D). In addition, there were no statistically significant differences in the expression of either SOX-9 or IL-1 β (Supplemental Fig. S2). FDL explants exhibited downregulated collagen type 1, tenomodulin, fibromodulin, collagen type 2, and IL-1 β expression as well as upregulated expression of MMP-13 and MMP-3 throughout culture (Supplemental Fig. S3).

There was significant release of pro-inflammatory cytokines to the medium from the BTM, bone only (BO) and muscle only (MO) groups, while there was very little release from the

FDL and the tendon only (TO) groups (Fig. 6). Specifically, the concentrations released from the FDL and TO groups were either undetectable (below noise thresholds) or orders of magnitude lower than those found in the BO, BTM, and MO groups. The only cytokines with modest release in the FDL and TO groups were IL-6 and IL-1 β , which were similar between the two groups (Fig. 6E,F). In contrast, all groups with bone and muscle included had highly significant release of all 7 cytokines measured, with the largest release of IL-6, IL-1 β , IL-10, and TNF- α (Fig. 6E-G). Notably, concentrations of IL-6 and IL-1 β in the medium for these groups were on the order of nanograms/milliliter. For the most part, the levels of cytokines in the medium were maintained for the entire 1-week analysis period. However, while concentrations typically dropped after day 1 for the MO and BO groups, levels in the BTM group increased from day 1 to day 3 for several cytokines (Fig. 6).

In our cytokine challenge of FDL explants, control tendons maintained viability and tissue structure for the duration of the 7-day culture period (Fig. 7A). FDL explants subjected to BTM-conditioned medium, as well as medium with added IL-1 β , IL-6, and TNF- α all exhibited significant decreases in cell viability as soon as 1 day following the addition of cytokine, i.e., by day 3 in culture (Fig. 7B-E). This persisted for the duration of culture with the most cell death occurring in the groups exposed to BTM-conditioned medium and TNF- α .

Discussion:

To our knowledge, we present here the biological characterization of the first bone-tendon-muscle *in vitro* explant culture model. Unlike other previous explant models, ours includes crosstalk between the three adjacent rotator cuff-related tissues as well as the intricate hierarchical collagen structure, myotendinous junction, and enthesis. A major finding of this study was the marked cell death in BTM explants, compared to that of the tendon-only FDL explants. While live/dead images appear to show a dramatic difference between day 3 and day 5 in culture, quantification of many images suggest that there is a linear loss in cell viability beginning at the initial harvest. We did not find a significant decrease in the DNA content of tendon explants over the course of the first 7 days in culture, but we did find increased cellular proliferation, which could balance that from cell death, resulting in no net change in DNA content. Proliferation (by 3H-thymidine incorporation) and DNA content assays are performed on whole tendon; therefore, these trends represent averages over the entire tendon, including surrounding peritendon. In contrast, live/dead imaging is performed on selected (localized) tendon regions. Thus, increased proliferation could be associated with the tenocytes in tendon as well as the surrounding peritendon tissue which is generally more cellular. In addition to these viability analyses, histological examination also demonstrated loss of morphology where elongated and flat tenocytes were more rounded qualitatively by 7 days in culture. This, as well as a decrease in cell density, is to be expected since the tendon is not stimulated by mechanical loading in stress-deprived culture,⁴⁶ and is likely exacerbated by exposure to other factors in the medium. Based on all of these assays, it is clear that there is a distinct loss of cell viability and morphology in the tendon of our BTM explants, as is evident throughout culture, and this will be the immediate focus of future studies.

Interestingly, apoptosis staining did not demonstrate a significant amount of apoptotic cells in the tendon proper or insertion site at any of the time points investigated. Given that our live/dead imaging shows significant cell death between days 3 and 5 in culture, it is possible that the majority of the apoptotic response of cells occurs between days 3 and 5 and, thus, would not be present in any of the specimens gathered for histology. However, expression of caspase-3 and caspase-9, markers of cell apoptosis, appeared to be downregulated in culture, but unfortunately our sample size was not large enough to detect any statistical significance. Since inflammatory cytokines as found in our culture medium have been shown to induce cell death in chondrocytes and tenocytes,⁴⁷⁻⁴⁹ further studies are needed to identify and differentiate between mechanisms associated with cell death. It is possible, for example, that tenocytes in the BTM explants are experiencing necrosis due to cytokines and/or other mediators in the culture medium rather than specific programmed cellular apoptosis,⁵⁰ with the most likely target being rhabdomyolysis, or rapid breakdown, of the muscle. Future studies will investigate whether necrosis is occurring in any of the three tissues by measuring levels of high-mobility group B1 protein (HMGB1), which is released when cells undergo necrosis,⁵¹ or known products of muscle breakdown such as myoglobin, lactate dehydrogenase, or other markers of muscle damage, in the medium.

In the tendon, mRNA expression of matrix proteins was altered over the 1-week culture period. We did find a significant downregulation in type 1 mRNA expression and an upregulation in type 2 collagen and aggrecan expression, suggesting matrix turnover of both collagens and proteoglycans and potentially a transition to a more cartilage-like tissue which was not present in FDL explants. However, we also found downregulation of MMP-13 (a collagenase), contrary to previous studies in other models as well as our FDL data which demonstrates upregulation in both genes with mechanical injury, unloading and with the addition of pro-inflammatory cytokines.^{27,45,52} However, many of these studies investigated changes very early in culture, sometimes prior to our first time point in culture at day 1 and as early as 30 minutes after sacrifice.²⁷ Our data suggest that MMP-13 expression is reduced following the initial spike during the first two days of culture, while MMP-3 expression is no different from day 0 controls for the duration of the 1-week culture period. Further studies are necessary to determine how the presence of cytokines in the medium would affect tenocyte mRNA expression and how stress-deprivation would alter that response.

Regardless, mRNA expression changes did not result in net changes in matrix at the protein level during culture. While there did appear to be some loss of GAGs from the mineralized fibrocartilage revealed by histological evaluation, there were no substantial changes in collagen or sulfated GAG content in the tendon. We did not expect any changes in collagen protein content based on previous studies of both tendon and cartilage demonstrating that loss of collagen content or morphology does not occur during this early time period.^{26,47} Importantly, these findings were similar for the BTM explants and the FDL explants (Supplemental Figure S1) where there is no muscle or bone. Therefore, it's likely that the level of circulating inflammatory cytokines in the medium is not enough to cause rapid collagenolytic degradation by tenocyte-mediated processes, or in the absence of other metabolic processes.

However, there were statistically significant increases in both sGAG and total protein synthesis. These results agree with those of one other previous study of bovine tendon-alone explants in which biosynthesis of sGAG and total protein increased over 12 days in culture.²⁹ While the increased biosynthesis observed in the present study might be ascribed to the presence of serum in the culture medium,^{53,54} it is more likely associated with the trauma of dissection and/or rapid cellular proliferation. In bovine cartilage explant culture, proteoglycan synthesis rates increase during the first several days of culture, associated with proteoglycan loss immediately following explantation, and then stabilize for the duration of culture.⁵⁴ In fact, our FDL explants do reach a stable level of sGAG and protein synthesis following 5 days in culture (Supplemental Figure S1). We hypothesize that sGAG and protein synthesis remain elevated in our 1-week BTM cultures due to the continuous and rapid loss of sGAG, proteoglycan and cell loss from the fibrocartilage. It is also possible that this increase in synthesis is associated with increased synthesis rates of the newly proliferating cells, consistent with the cellular proliferation also found in this study. Regardless, given that there is no change in total sGAG or collagen content in the tissue based on our biochemical assessments, it is likely that matrix turnover and synthesis balance (i.e., proteolytic loss of initial matrix plus loss of newly synthesized molecules not incorporated into the tissue is balanced by neo-synthesis). Future studies will address this by measuring release to the medium of native proteins/proteoglycans and proteolytic fragments of these macromolecules, e.g. by Western analysis.

One of the most exciting findings is the large concentration of pro-inflammatory cytokines released to the culture medium of BTM explants. Based on previous studies, we initially hypothesized that there may be release of cytokines into the medium from the muscle and bone. Specifically, the unloading of muscle results in upregulation of TNF- α and IL-6, as well as atrophy and sometimes death of cells.⁵⁵⁻⁵⁹ In addition, muscle sepsis or trauma is also associated with release of IL-1.⁶⁰ Furthermore, the unloading and injury of bone is also associated with upregulation of TNF- α , IL-6, and IL-1.⁶¹⁻⁶⁵ In our explant culture system, we found release of all three of these cytokines into the media as well as several others associated with inflammation. Notably, these enhanced cytokine levels were found to be of the same order of magnitude as those detected in human synovial fluids following ACL tear.^{66,67} Furthermore, upregulation of TNF- α , IL-6, and IL-1 is also associated with tendon pathology.⁶⁸⁻⁷⁴ Looking closely at our data, it is also evident that the levels of these cytokines in the medium of the BTM explants may increase during culture due to a feedback loop, unlike that in the bone-only and muscle-only groups which typically decrease following day 1. This feedback loop, as well as the relationships between these and other inflammatory cytokines, has been explored in other studies^{75,76} and could provide evidence of the initiation of a diseased tendon state. Regardless, we also confirmed that cytokine exposure at these levels could induce cell death in otherwise viable FDL explants (Fig. 7). Many recent studies have explored the effect of these cytokines on tenocytes in monolayer culture of tenocytes and *in vivo*, leading to the conclusion that exposure to cytokines induces the expression of genes that lead to matrix degradation and cell death, such as matrix metalloproteinases (MMP-1,3).^{73,75,77} This could have significant clinical implications, particularly in the rotator cuff where multiple tendons, muscle, and bones interact inside the same joint space. Injuries to the muscle, bone, or cartilage surrounding the rotator cuff

tendons, such as tissue tears, dislocations or fractures, could incite a degenerative cascade thus leading to overall joint disease.

As with the development of any model system, there are a number of limitations that need to be addressed both in our study and in our system. The data in this study represent multiple experiments over an 8-month period and we noted some variability between experiments. In addition, due to the small size and delicate nature of the supraspinatus tendon, there were a number of samples lost to experimental error resulting in a variation of sample size per experiment. While some inherent variability is expected in biological models, future studies will focus on streamlining procedures for dissection and medium changes to minimize excessive handling of the tissue to limit the variation in sample number across experiments. Despite our thorough evaluation of many biological outcomes during this culture, there are other important parameters that were not measured in the present study. Specifically, evaluation of the mechanical function of our constructs is still ongoing and will be included in future studies. Additionally, we are also currently unable to determine if differences between FDL and the tendon of BTM explants are due to the circulating inflammatory cytokines in the medium or due to inherent differences in the response to stress deprivation. Additional experiments involving blockade of inflammatory cytokines should enable more direct comparisons between the two models. Finally, a significant concern for this model is the viability and function of the muscle and bone. This first study focused specifically on tendon homeostasis and the effect of the other tissues on the tendon, but ongoing studies are investigating homeostasis in bone and muscle. This model does induce injury to both tissues by virtue of dissection. If the injury can be mitigated initially or prevented with better dissection methods or culturing methods, we may be able to minimize cytokine release to the medium and its concomitant effects on tendon viability. At the same time, we recognize that the dissection and follow-on cytokine release may, indeed, represent an important injury model for use in discovery of therapeutics and other disease mitigating approaches. Thus, ongoing studies are currently evaluating various treatment methods that could reduce the effect of cytokines on tenocyte health, such as antibodies to block cytokine activity in the medium, therapeutic inhibitors, and antagonists of cytokine receptors.

Despite these limitations, we believe this model presents an innovative approach to understanding rotator cuff injury and tenocyte explant biology in a clinically-relevant tendon structure. We have shown that like the flexor tendon (FDL) model and rodent fascicle models, bone-tendon-muscle tendon explants are capable of maintaining their structure and organization in stress-deprived culture, regardless of cell viability, for the duration studies here. Interestingly, clinical cases of rotator cuff injury demonstrate the same pro-inflammatory cytokines in the synovial fluid surrounding the joint, suggesting similar phenomena to our model may be relevant to studies of rotator cuff injury. If we can understand and prevent this loss of viability, our model could ultimately be a powerful tool to investigate how mechanical and biological stimuli could alter normal tendon health and lead to rotator cuff tendon degeneration, as well as to explore additional relevant factors, such as age and gender, in this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of overall explant culture experiments. Rotator cuff bone-tendon-muscle (BTM) and flexor digitorum longus tendon (FDL) explants are harvested from the mouse at day zero and medium is changed every two days for the 7-day culture period. FDL explants and the tendon portion of BTM explants are harvested for characterization assays on days 1, 3, 5, and 7 post-harvest.

Figure 2.

Cell viability of supraspinatus tendons from BTM explants is imaged at days 1, 3, 5, and 7 in culture via fluorescent labeling with fluorescein diacetate (green, viable) and propidium iodide (magenta, non-viable). Images are representative of approximately 10-12 tendons and each image is a merged z-stack of multiple layers of cells visualizing approximately 110μm into the depth of the tissue.

Figure 3.

(A) Tendon cell viability is significantly decreased until day 5. (B) Tendon DNA content is unchanged throughout culture. (C) BTM explant metabolism is increased between day 3 and days 5 and 7 in culture. (D) Tendon cell proliferation was increased at days 5 and 7. There were no significant changes in (E) sulfated GAG or (F) total collagen content, but (G) GAG and (H) total protein synthesis were increased at days 5 and 7 in culture. All data are expressed as mean $\pm$ 95% confidence interval and bars represent significant comparisons where $p < 0.05$.

Figure 4.

Histological evaluation by (a-c) hematoxylin & eosin staining at 10x magnification and 4x zoom (inset) shows rounding of cells over the duration of culture. (d-f) Toluidine blue staining shows small loss of GAG content in mineralized fibrocartilage region and complete loss of nuclear staining in osteocytes and marrow cells at 10x magnification. [B = bone, FC = fibrocartilage, T = tendon] (g-h) TdT staining for apoptosis (green) with DAPI counterstain for cell nuclei (blue) at 10x magnification showed no apoptosis at days 0 or 1 and a small amount of apoptotic cells at day 7 in culture. Grey lines indicate tendon borders. All images are representative of 2-4 tendons.

Figure 5.

BTM explant supraspinatus tendon mRNA levels of (A) matrix proteins (type 1 collagen, type 2 collagen, aggrecan), (B) tendon markers (scleraxis, tenomodulin), (C) matrix metalloproteinases (MMP-3, MMP-13), and (D) apoptosis markers (caspase-9, caspase-3) during the culture period relative to day 0 control samples. All data are expressed as mean $\pm$ 95% confidence interval. Stars (*) represent significant comparisons with day 0 control levels where $p < 0.05$.

Figure 7.

Cell viability of FDL explants imaged during culture via fluorescent labeling with fluorescein diacetate (green, viable) and propidium iodide (magenta, non-viable). Experimental groups include (A) control FDL explants, (B) conditioned BTM medium (1:1 ratio of fresh medium to medium from BTM explant at D1), and medium supplemented with (C) 10 ng/mL IL-1 β , (D) 10 ng/mL IL-6, and (E) 10 pg/mL TNF- α . Images are representative of approximately 4-6 tendons and each image is a merged z-stack of multiple layers of cells visualizing approximately 110 μ m into the depth of the tissue.