Gap junctions in IL-1β-mediated cell survival response to strain

Jie Qi,1,2 Liqun Chi,3,4 Donald Bynum,3 and Albert J. Banes1,2,4

1Flexcell International, Hillsborough; 2Joint Department of Biomedical Engineering of North Carolina at Chapel Hill and North Carolina State University, Raleigh; 3Department of Orthopaedics, University of North Carolina, Chapel Hill; and 4Curriculum in Applied Sciences and Engineering, University of North Carolina, Chapel Hill, North Carolina

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Qi J, Chi L, Bynum D, Banes AJ. Gap junctions in IL-1β-mediated cell survival response to strain. J Appl Physiol 110: 1425–1431, 2011. First published January 6, 2011; doi:10.1152/japplphysiol.00477.2010.—Mechanical stimuli play important roles in proliferation and differentiation of connective tissue cells, and development and homeostatic maintenance of tissues. However, excessive mechanical loading to a tissue can injure cells and disrupt the matrix, as occurs in tendinopathy. Tendinopathy is a common clinical problem in athletes and in many occupational settings due to overuse of the tendon. Moreover, interleukin (IL)-1β is generally considered to be a “bad” cytokine, activating NF-κB and cell death and inducing matrix metalloproteinase (MMPs 1, 2, 3) expression and matrix destruction. However, activated NF-κB can also drive a cell survival pathway. We have reported that cyclic strain induced tenocyte death in three-dimensional (3D) cultures, and IL-1β could promote cell survival under strain. Therefore, it was hypothesized that (1) cyclic strain could induce cell death in tenocytes as observed in pathologic tendons in vivo; (2) a gene expression profile indicative of tendinopathy could be identified; and (3) low-dose IL-1β could protect cells from strain-induced, tendinopathy-like changes. Human tenocytes were cultured in 3D type I collagen hydrogels and subjected to 3.5% elongation at 1 Hz for 1 h/day for up to 5 days with or without IL-1β. Real-time RT-PCR data showed that cyclic strain regulated the expression of tendinopathy marker genes in a manner similar to that found in pathological tendons from patients and that addition of IL-1β reversed the gene expression changes to control levels. Results of further studies showed that IL-1β may modulate cell survival through upregulating the expression of connexin 43, which is involved in the modulation of cell death/survival in a variety of cells and tissues. The elucidation of the mechanisms underlying strain-induced cell death and recovery from strain injury will facilitate our understanding of the pathogenesis of tendinopathy and may lead to the discovery of new molecular targets for early diagnosis and treatment of tendinopathy.

interleukin-1β; strain; connexin; tendon; cell survival

MECHANICAL STIMULI ARE IMPORTANT for the proliferation and differentiation of cells, for the development of tissues, and for homeostatic maintenance of tissues (11, 13). However, excessive mechanical loading to tendons may cause damage to the tissues, as occurs in tendinopathy (5, 34). Tendinopathy is a chronic tendon pathology characterized by a combination of pain, redness, diffuse or localized swelling, and impaired performance (6, 34). It is a common clinical problem with athletes and injured workers and is difficult to diagnose and cure (1). Although the mechanisms involved in its pathogenesis are unclear, some major biomarker changes have been associated with tendinopathy. Signs include cell apoptosis, upregulation of matrix metalloproteinase (MMPs) expression, presence of cytokines and extracellular matrix proteins, matrix disorganization, thinning and microtearing of collagen fibres, extensive neovascularization, increased interfibrillar glycosaminoglycan expression, and increased glutamate, substance P, PGE2, and neuroactive peptide in the tissue (3, 6, 19, 23, 24, 26, 31, 34). Repeated, cumulative, minor damage to matrix and cells is thought to be the major factor inducing tendinopathy (34).

Tenocytes are responsible for the production and maintenance of the tendon matrix. Therefore, understanding the molecular effects of cyclic strain on tenocytes will help us understand the pathogenesis of tendinopathy (9, 15). We and other groups have reported that cyclic strain upregulates MMP expression and induces cell death of human tenocytes grown in three-dimensional (3D) collagen gels in a cell density-dependent manner (23, 30, 45). Expression of interleukin (IL)-1β was stimulated in stretched tenocytes and has been hypothesized to act as a negative modulator in cellular responses to cyclic strain (33, 40). We have reported that IL-1β makes tenocytes more tolerant to cyclic strain with the ability to survive excessive strain in a cell density-dependent manner (33). Although the mechanism(s) remains unknown, these results indicate that cell-cell connections and intercellular communication play a critical role in strain-induced cell death or survival. It is hypothesized that low concentrations of IL-1β may positively affect cell-cell communication to promote cell survival.

Gap junctions are specialized intercellular membrane channels connecting the cytoplasm of two or more neighboring cells to enable rapid, direct exchange of small molecules (<1,000 Da) (18). A gap junction is composed of two end-to-end connected hemichannels (also called connexons); each hemichannel contains six connexin (Cx) molecules (20). Gap junctions are ubiquitously expressed by cells of many types, including tenocytes (10, 20). Gap-junctional intercellular communication (GJIC) plays important roles in many physiological processes, including proliferation, differentiation, migration, maturation, morphogenesis, and pattern formation (39). Defects in connexins cause severe human diseases (39). Recently, a body of evidence suggests that gap junctions are also involved in the regulation of cell death and survival in a variety of cells and tissues (28, 30, 41). In both cases, the connexin most frequently involved is Cx43 (35). However, there are no reports addressing involvement of Cx43 in the modulation of strain-induced cell death/survival in connective tissues, such as tendons. Cx43 is the major connexin expressed in tendon tissues (10, 42). The half-life of Cx43 is 1.5–5 h (14). Therefore, it is believed that the expression of Cx43 is mainly transcriptionally regulated, although the degradation rate of Cx43 protein will also impact the abundance of Cx43 short-term (14, 28). We have developed an in vitro, human, bioartificial tendon (BAT) model and optimized the strain regimen to induce cell damage as gauged by live cell counting (21, 33).
It was hypothesized that Cx43 may be involved in IL-1β-mediated cell protection against strain. Seven genes, including collagen I, biglycan, fibronectin, transforming growth factor-β1 (TGF-β1), cyclooxygenase-2 (COX2), MMP27, and ADAMTS5 (a disintegrin and metalloproteinase domain with thrombospondin type 5 motif), were chosen as markers of cell strain damage to be investigated.

Real-time PCR experiments showed that 100 pM IL-1β dramatically upregulated the expression of Cx43 in human tenocytes. This is the first report that gap junctions are involved in the modulation of strain-induced cell death in tenocytes. Results in the present study indicate that the 3D BAT strain paradigm may act as an in vitro tendonopathy model and may facilitate the investigation of Cx43 as a therapeutic target in the pathogenesis of tendinopathy.

MATERIALS AND METHODS

Cell culture. Human tendon internal fibroblasts (HTIF) were isolated after surgery from discarded human flexor digitorum profundus tendon tissues, as described previously (8). HTIFs from passages 2 to 4 were used in this study. HTIFs were maintained in medium 199 (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 20 mM HEPES (pH 7.2, GIBCO), 0.1 mM ascorbate-2-phosphate, and 1% penicillin/streptomycin solution (GIBCO).

Fabrication and mechanical loading of 3D BAT cultures. The 3D BAT cultures were fabricated in Tissue Train culture plates (Flexcell International, Hillsborough, NC), as described before (33). This strategy culture plate allows for the molding of 3D, linear, cell-populated, matrix gels that are 30 × 4 × 4 mm. In brief, HTIFs were trypsinized, and cell number was determined using a Coulter particle counter (Beckman Coulter, Hialeah, FL). Cells were transfected with type I collagen (Vitrogen, 2.1 mg/ml; Cohesion, Palo Alto, CA) at 50,000 or 200,000 cells/100 μl-1·BAT-1. One hundred microliters (100 μl) of cell-gel suspension were transferred to a space created by vacuum deformation of the rubber membrane into an underlying trough loader jig (30 × 4 × 4 mm space). After gelation, vacuum was released, culture fluid was added, and the cells were cultured in the 3D BAT matrix for 48 h in medium 199 containing 10% FBS. Cells were then brought to quiescence by reducing the serum concentration to 2% for 24 h. The cultures were subjected to uniaxial strain for 1, 3, and 5 days at 3.5% elongation, 1 Hz, for 1 h/day with Flexcell’s Tension Plus cell strain system (model FX4000, Flexcell International, Hillsborough, NC) in the absence or presence of 100 pM IL-1β.

Staining of human tendon cells in BATs. At the end of experiments, human tenocytes in BATs were fixed with 3.7% formaldehyde in PBS, pH 7.2, at room temperature for 15 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 20 min, and rinsed with PBS twice. Actin filaments (microfilaments) were stained at room temperature for 1 h with rhodamine-phalloidin (Molecular Probes, Eugene, OR, at 1:400 dilution in PBS). The cells were then rinsed with PBS twice and mounted on glass slides. Cx43 proteins were stained with rabbit polyclonal anti-Cx43 antibody (1:100; Zymed, San Francisco, CA). The permeabilized BATs were blocked with PBS containing 5% BSA and 2% goat serum at room temperature for 2 h and washed with PBS twice. BATs were incubated with primary antibody at room temperature for 2 h and washed three times in PBS. Cells were incubated with a goat, anti-rabbit IgG, Alexa Fluor 488-labeled secondary antibody (1:250) in the dark for 1 h, followed by three washes in PBS. Cells were imaged with an Olympus BX-60 fluorescence microscope equipped with an Olympus FV II digital camera and image analysis software (Olympus Microsucite B3SV, OPELCO, Dullas, VA).

Apoptosis assay. At indicated time points, cells were released from the BATs with 0.1% type II collagenase ( Worthington, Lakewood, NJ) at 37°C for 15 min with rotation, washed with cold PBS buffer, and stained using an apoptosis assay kit from Invitrogen (Carlsbad, CA), according to the manufacturer’s protocol. Apoptotic cells were stained green with Alexa Fluor 488 annexin V, whereas dead cells were stained green and red with annexin and propidium iodide (PI), respectively. Live cells were unstained.

Quantitative real-time RT-PCR. Cells were collected on days 1, 3, and 5 after addition of IL-1β. Total RNA was isolated using an RNaseasy mini kit (QIAGEN, Valencia, CA), according to the manufacturer’s protocol. Complementary DNA was synthesized with SuperScriptIII (Invitrogen, Carlsbad, CA). The expression levels of target genes were determined by SYBR green real-time PCR using 18S rRNA as an internal control (Ambion, Austin, TX) with a Brilliant SYBR green QPCR master mix kit from Stratagene (La Jolla, CA). Primer sequences are listed in Table 1. The size of the PCR products was 300 bp. The PCR conditions were as follows: 30 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s.

Statistical analysis. All experiments were repeated at least three times. Statistical analyses were performed using Student’s t-test. A value of P < 0.05 was deemed as significant. The values are expressed as means ± SD.

RESULTS

Cyclic strain induced tendinopathy-like changes in human tenocytes grown in 3D type I collagen gels. It is believed that tendinopathy is caused by repetitive strain-induced microdamage to tissue and cells. Cell apoptosis is one of the major changes in the tissues (5, 34, 43). Therefore, we investigated whether cyclic strain could cause similar changes found in diseased tissues. Apoptosis assay results showed that strain-induced tenocyte apoptosis occurred from day 3 (~10% apoptotic cells), and over 95% of cells underwent apoptosis and/or cell death, staining positive at day 5 (Fig. 1). Seven marker genes from three groups were se-

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
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<tr>
<td>ADAMTS5</td>
<td>ATGAGGAGCCTCAGATGCAGCTA</td>
<td>CTGTTGAGTTGGCTGAGCAT</td>
</tr>
<tr>
<td>Biglycan</td>
<td>GTTCTGGGAGAACGAGAAGA</td>
<td>GCAGTTGAGTCTCAGAGCTT</td>
</tr>
<tr>
<td>Coll1</td>
<td>AGACAGTTTGGACTTTTGGGACCT</td>
<td>CTTTGGGAGTTGGCTCAGAGCT</td>
</tr>
<tr>
<td>COX2</td>
<td>CACCCATGTCAAAAAAGGGAGGTGT</td>
<td>AGGTTAGAAAGGCGAGCTCAGAC</td>
</tr>
<tr>
<td>Cx43</td>
<td>GGCTTTGTTTGTAAACATCTGAGA</td>
<td>ACGTTGAGTTGATCGAGCTGAA</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>AGCTGCGAGAGAAGCACAGAAGA</td>
<td>GCTGAGCTGAGGAGCTGGAAG</td>
</tr>
<tr>
<td>MMP27</td>
<td>AGCTTCTGACCTCTGTACTGAC</td>
<td>GTGCATGAGTGGAGTCAGAT</td>
</tr>
<tr>
<td>TGF-β</td>
<td>GTTCTGGGAGAACGAGAAGA</td>
<td>GCAGTTGAGTCTCAGAGCTT</td>
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</table>
lected: matrix proteins (coll1a1, fibronectin, biglycan), cytokines and signaling factors (TGF-β1, COX2), and enzymes (MMP27, ADAMTS5) (34). Real-time PCR performed on the samples from day 5 at 50,000 cells per BAT showed that cyclic strain dramatically upregulated the expression of these target genes (Fig. 2). All the genes in the matrix protein group showed over a 100% increase in expression (Fig. 2A). IL-1β treatment did not affect the expression of these genes in the non-stretched group (slightly increased the expression of fibronectin, but not statistically significant). However, IL-1β totally blocked cyclic strain-induced changes in the marker genes in the matrix protein group (Fig. 2A). Similar results were also found for TGF-β1 and MMP27 (Fig. 2B). Both strain and IL-1β increased the expression of COX2 to similar levels. IL-1β increased the expression of ADAMTS5 by 55% (P < 0.01). However, strain did not further increase its expression. There was no significant difference in results between the IL-1β-treated group and the group treated with both IL-1β and cyclic strain (Fig. 2).

Gap junctions are involved in strain-induced cell death. It was demonstrated that cells seeded at 100,000 cells per 100 μl per BAT, where cells form robust connections, survived when subjected to cyclic strain. However, cells seeded at low cell density and subjected to cyclic strain died (21). Results of cell density-dependency experiments indicated that cell-cell connections are involved in promoting cell survival. To determine whether a loss of function of gap junctions was involved in strain-induced cell death, a gap junction inhibitor (heptanol) was used. Heptanol alone, at concentrations of 100 and 500 μM, disrupted connexin-based cell-cell communication but did not induce cell death (Fig. 3). However, cells treated with 500 μM heptanol and cyclic strain had a dramatic change to a rounded cell shape even if cells were plated at higher density (200,000 cells per 100 μL per BAT) (Fig. 3). These rounded cells progressed to apoptosis. This change was similar to results found at low cell density, which resulted in the death of over 90% of the cell population in BATs (33).

IL-1β upregulated Cx43 expression in a time- and dose-dependent manner. Cx43, one of the two major connexins expressed in tendon tissues (10, 42), is reportedly involved in the modulation of cell death/survival (35). IL-1β dramatically (>300%) increased the expression level of Cx43 in human tenocytes as early as 8 h post-addition of 100 pM IL-1β (Fig. 4A). Even a minimal concentration of IL-1β (1 pM) could change Cx43 expression (Fig. 4B).

IL-1β altered cell morphology and cell-cell connections. As reported in our laboratory’s previous study, IL-1β altered cell morphology (Fig. 5) (33). Multiple, long, axon-like cell processes were observed on >70% of IL-1β-treated cells (Fig. 5, top right). Over 50% of IL-1β-treated cells were connected to each other through these long cell processes (arrows pointing to the cell-cell connections) compared with <10% of cells connected in the control group. Immunostaining showed that
the long cell processes on IL-1β-treated cells were positive for Cx43 proteins (Fig. 5, bottom right).

**DISCUSSION**

Data in the present study showed that cyclic strain can induce tenocyte apoptosis and tendinopathy-like changes in the gene expression profile of human tenocytes. Cx43 may be a new biotarget for understanding the pathogenesis and therapeutic treatment of tendinopathy.

Tendinopathy is a chronic, pathological feature of a non-rupture tendon injury caused by overuse or repetitive motion (34, 38). Tendinopathy accounts for 30–50% of all sports-related injuries and nearly half of all job-related injuries in the US (27). Changes in diseased tissues include altered cell morphology, increased apoptosis, increased matrix degradation and disorganization, changes in gene expression, and vascular ingrowth (6, 26, 27, 43). However, tendinopathy is difficult to treat due to uncertainty of its pathogenesis (1). Development of several animal models has revealed some mechanistic aspects of tendinopathy (3, 27, 31, 38). However, due to the cost, difficulty, invasiveness, lack of reproducibility, and varied time to induce injuries, it is difficult to perform these studies at molecular levels on animal models. An in vitro model is more controllable and preferred for some components of a mechanistic study. In the current presentation, a preliminary study on the development of an in vitro tendinopathy model was performed. In addition to cell death, a tendinopathy-like gene expression profile was also reproduced within the course of a 5-day, high-dose, cyclic strain regimen. Among the tested marker genes, MMP27 and ADAMTS5 did not show the same trend as that found in painful tendon tissues in which the expression of these two genes was reduced (24). These differences may represent the difference in the kinetics of tendinopathy development in vitro vs. in vivo. A longer term experiment is necessary to determine the gene expression profile associated with different stages of tendinopathy development. Histological studies will also be needed in the future to determine the effects of cyclic strain on extracellular matrix. These results should be helpful to refine a tendinopathy-inducing regimen. The purpose of this study is to develop an easy-to-use in vitro model for tendinopathy so that molec-
ular biology studies testing mechanisms can be easily performed. Due to presumed differences in the cell growth environment between in vitro and in vivo, the matrix structure and cell population between human tendons and the in vitro 3D cell culture model, the conditions driving a tendinopathy phenotype may be different but occur much more readily. Moreover, as cells compact the 3D matrix in a BAT, the cross-sectional area decreases so that the initial strain value of 3% actually creates more stress (increased force/unit area). IL-1β is a proinflammatory factor, which has been reported to be upregulated by cyclic strain (40). We and other groups have reported that cyclic strain can block IL-1β-induced expression of MMPs and that IL-1β can spare cells from strain-induced cell death (7, 33, 45). These data indicate that IL-1β and cyclic strain may interact. We further showed here that IL-1β reversed cyclic strain-induced changes in the expression of tendinopathy marker genes (except COX2). These results combined with results of previous studies strongly suggest that IL-1β may act as a protective factor at an early stage of cyclic strain-induced tissue/cell damage since prolonged treatment with IL-1β caused degeneration of tendon tissues (37).

**Fig. 5.** Staining of Cx43 and actin cytoskeleton on human tenocytes grown in type I collagen gels in the absence or presence of 100 pM IL-1β. Human tenocytes were fixed at 24 h post-addition of 100 pM IL-1β. Actin cytoskeleton was stained with rhodamine-phalloidin (red), and Cx43 was stained with anti-Cx43 polyclonal antibody (green). Scale bar is 50 μm. Cells at top were stained with Rhodamine-phalloidin. Arrows point to the cell-cell connections. Cells at bottom were stained with rhodamine-phalloidin and anti-Cx43 antibody.

**Fig. 6.** How IL-1β potentially promotes cell survival. IL-1β stimulates the release of ATP and PGE2 (28), which in turn may trigger a series of pathways to promote cell survival by activating P2 receptors and/or upregulating Cx43 expression (4, 29). It is still unknown whether P2 and adenosine receptors are involved in IL-1β-mediated cell survival under strain. The IL-1β signaling pathway was modified from Ref. 36 with permission from Elsevier.
Cx43 is one of the two major connexins expressed in tendon tissues and reportedly involved in the modulation of cell death/survival in neurons/astrocytes (Fig. 6) (10, 12, 29, 30, 42). Results in the present study suggest that Cx43 gap junctions may also play a critical role in modulating strain-induced cell death in tendon tissues. The detailed mechanisms are still unknown, but several pathways may be involved, including the P2 purinoceptor signaling pathway and Cx43 (Fig. 6). It is well known that mechanical stimulation increases ATP release (22, 32, 44). The P2 purinoceptor signaling pathway has been reported to be involved in regulating cell survival (4, 16, 17). A recent report showed that Cx43 hemichannels are able to secrete ATP on stimulation with electricity (25). Therefore, IL-1β may increase ATP release via upregulating Cx43 expression. However, other signaling pathways must also be involved since the integrity of gap junctions is needed to maintain cell survival.

In conclusion, the present study indicates that developing an in vitro tendinopathy model is possible by using a 3D BAT culture system. The development of an in vitro model should facilitate our understanding of the pathogenesis of tendinopathy, especially for the diagnosis of tendinopathy at early stages. Further studies on the mechanisms should lead to new targets for the diagnosis and treatment of tendinopathy.

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Current address of L. Chi: Dept. of Microbiology and Immunology, University of North Carolina at Chapel Hill, NC 27599.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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