Biochemical and Biophysical Research Communications 463 (2015) 667–672

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

IL-1β irreversibly inhibits tenogenic differentiation and alters metabolism in injured tendon-derived progenitor cells *in vitro*

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ARTICLE INFO

Article history:
Received 18 May 2015
Accepted 30 May 2015
Available online 4 June 2015

Keywords:
IL-1β
Tendon
Progenitor
Injury

ABSTRACT

Tendon injuries are common, and the damaged tendon often turns into scar tissue and never completely regains the original biomechanical properties. Previous studies have reported that the mRNA levels of inflammatory cytokines such as IL-1β are remarkably up-regulated in injured tendons. To examine how IL-1β impacts tendon repair process, we isolated the injured tendon-derived progenitor cells (inTPCs) from mouse injured Achilles tendons and studied the effects of IL-1β on the inTPCs *in vitro*. IL-1β treatment strongly reduced expression of tendon cell markers such as *scleraxis* and *tenomodulin*, and also down-regulated gene expression of *collagen 1*, *collagen 3*, *biglycan* and *fibromodulin* in inTPCs. Interestingly, IL-1β stimulated lactate production with increases in hexokinase II and lactate dehydrogenase expression and a decrease in pyruvate dehydrogenase. Inhibition of lactate production restored IL-1β-induced down-regulation of *collagen1* and *scleraxis* expression. Furthermore, IL-1β significantly inhibited adipogenic, chondrogenic and osteogenic differentiation of inTPCs. Interestingly, inhibition of tenogenic and adipogenic differentiation was not recovered after removal of IL-1β while chondrogenic and osteogenic differentiation abilities were not affected. These findings indicate that IL-1β strongly and irreversibly impairs tenogenic potential and alters glucose metabolism in tendon progenitors appearing in injured tendons. Inhibition of IL-1β may be beneficial for maintaining function of tendon progenitor cells during the tendon repair process.

1. Introduction

Tendon is a compositionally complex tissue and has an important mechanical function that translates muscular contractions into joint movement by transmitting forces from muscle to bone [1]. Tendon injuries are common, and the damaged tendon often turns into scar tissue and never completely regains its original biomechanical properties [2]. A better understanding of biological processes of tendon repair and degeneration is required for establishing strategies that stimulate tendon repair and induce its regeneration.

Previous studies have profiled expression of inflammatory cytokines in canine and rat tendon injury models [3,4], and found that gene expression of inflammatory cytokines including IL-1β is remarkably up-regulated. *In vitro* studies have shown that IL-1β makes tendon fibroblasts decrease the mRNA level of collagen 1 [5], suggesting that IL-1β disturbs function of tendon fibroblasts. However, the exact impact of increases in inflammatory cytokines in injured tendons has not been fully elucidated.

Recently, the presence of tendon stem/progenitor cells (TSPCs) has been demonstrated in various species including human, horse, rabbit, rat, and mouse [6–9]. These cells express stem cell-related markers, form adherent colonies and show multipotency *in vitro* and/or *in vivo*. Because they form tendon-like tissues in nude mouse or nude rat models [6,8], TSPCs are suggested to contribute to tendon repair. Using a mouse Achilles tendon injury model, we have recently reported that tendon progenitor-like cells (inTPCs) rapidly appear and are expanded in injured tendons [10]. These

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http://dx.doi.org/10.1016/j.bbrc.2015.05.122
0006-291X/

cells have characteristics as tendon progenitors and are integrated into regenerating tendon. On the other hand, they exhibit stronger spontaneous chondrogenic ability than the normal tendon progenitor cells and are integrated into heterotopic cartilaginous lesions induced in injured tendon [10]. Thus the inTPCs likely have the potential to contribute to tendon repair, but may be trans-differentiated into different cell lineages, negatively impacting tendon healing.

To understand how inflammatory cytokines affect the regenerative and degenerative potential of inTPCs, we focused on IL-1β, one of dominant cytokines that are up-regulated in injured tendons [3,4]and examined the effects of IL-1β on function of inTPCs. IL-1β strongly inhibited tenogenic, chondrogenic and osteogenic differentiation in inTPCs and altered glucose metabolism. Interestingly, the inhibitory effect on tenogenic differentiation was not recovered after removal of IL-1β while chondrogenic and osteogenic potential were maintained. The results indicate important implications in understanding the roles of IL-1β during the tendon repair process.

2. Materials and methods

2.1. Animals

All aspects of the research were conducted in accordance with the guidelines set by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. CD-1 female mice (6-8 weeks-old of age) were purchased from Charles River Laboratories (Charles River Laboratories International, Inc., Wilmington, MA).

2.2. Isolation of inTPCs

Injured tendon-derived progenitor cells (inTPCs) were isolated from the Achilles tendons of CD-1 mouse as we previously reported [10]. Briefly, the fibrous tissues that formed in the incised Achilles tendons were dissected 1 week after surgery. The dissociated cells were plated at a density of 140 cells/cm^2 in 100-mm dishes and cultured in DMEM containing 20% FBS (Gemini BioProducts, West Sacramento, CA). The inTPCs at passage 3 or 4 were used in the following experiments. We usually isolated inTPCs from 4 injured Achilles tendons and plated on 2 100-mm dishes.

2.3. Cultures of inTPCs

To perform cell proliferation assay, inTPCs were plated at 10^4 cells/well in a 96-well plate and allowed to adhere overnight. DMEM containing 10% FBS medium was supplemented with 1, 5 and 10 ng/ml mouse IL-1β (R&D Systems, Inc., Minneapolis, MN) for 3 days. Proliferation activity was then determined by measurement of cellular DNA contents using the CyQUANT NF Cell Proliferation Assay Kit (Life Technologies, Grand Island, NY) following the manufacturer's protocol. To evaluate tenogenic differentiation, inTPCs were plated on collagen 1 substrate (Cellmatrix, Nitta Gelatin Inc., Osaka, Japan) at a density of 1.5×10^5/well in a 12 well plate and were cultured in DMEM containing 10% FBS for 7 days in the presence or absence of IL-1β at concentrations of 1, 5 or 10 ng/ml with or without dichloroacetate (DCA, 1 mM, Santa Cruz Biotechnology, Inc. Santa Cruz, CA). For adipogenic or osteogenic induction, the inTPCs were plated on collagen 1 substrate at a density of 1.5×10^5/well in a 12 well plate and were cultured in DMEM containing 10% FBS and mouse StemXVivo Adipogenic or Osteogenic Supplement, respectively (R&D Systems, Inc., Minneapolis, MN) for 2 weeks in the presence or absence of IL-1 β at a concentration of 5 ng/ml. For chondrogenic induction, the cells were spotted on collagen 1 substrate at a density of 1.5×10^5/15 μl/well in a 12 well plate and cultured in DMEM containing 10% FBS and 50 μg/ml ascorbic acid for 7 days in the presence or absence of IL-1β at a concentration of 5 ng/ml. The inTPCs were also pre-treated with IL-1β in monolayer for 7 days in 100 mm dishes and then tested for tenogenic, chondrogenic, osteogenic and adipogenic differentiation under each condition described above. We used 2-3 different batches of inTPCs and obtained similar results. The concentrations of IL-1β used in this study were 1–10 ng/ml that have been usually used in other studies. Because 1–10 ng/ml of IL-1β significantly affected gene expression of tendon-associated markers in tenogenesis experiment, a single dose (5 ng/ml, the median dose) was used in some experiments.

2.4. RNA isolation and gene expression assay

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol and reverse-transcribed into cDNA. The resulting cDNA was subjected to a quantitative polymerase chain reaction (qPCR) assay. The qPCR was performed with an Applied Biosystems 7900HT Sequence Detection Systems running SDS 2.1 software using SYBR green reagents (Applied Biosystems, Foster City, CA). The average threshold cycle value (Ct value) was calculated from quadruplicate reactions. Standard curves were generated using 10-fold serial dilutions of cDNA of each gene with a correlation coefficient of >0.98. Relative expression levels were calculated based on a standard curve and normalized to *glyceraldehyde 3-phosphate dehydrogenase (Gapdh)*. The primer sequences used in this research are listed in Table 1.

2.5. Immunoblot analysis

The inTPCs plated on a density of 1.5×10^5/well in a 12 well plate and were cultured in DMEM containing 10% FBS in the presence or absence IL-1β at the concentration of 5 ng/ml for 6 or 24 h and then lysed in SDS sample buffer. Hexokinase II (HKII), lactate dehydrogenase (LDHA), pyruvate dehydrogenase (PDHA) and α-tubulin contents were examined by Immunoblot using the corresponding antibodies as described previously [11]. The anti-hexokinase II rabbit monoclonal antibody (#2867), anti-lactate dehydrogenase rabbit monoclonal antibody (#3582), anti-pyruvate dehydrogenase rabbit monoclonal antibody (#3205) were purchased from Cell Signaling Technology (Danvers, MA). The anti-α-tubulin mouse monoclonal antibody were purchased from Sigma–Aldrich (St. Louis, MO).

2.6. Lactate measurement

The inTPCs plated on a density of 1.5×10^5/well in a 12 well plate and were cultured in DMEM containing 10% FBS in the presence or absence IL-1β at the concentration of 5 ng/ml for 6, 24, 48 and 72 h with or without 1 mM DCA. The culture media were collected at each time point and lactate concentration in the conditioned medium was determined using the L-Lactate Assay Kit (Colorimetric) (Abcam Inc., Cambridge, MA) according to the manufacturer's protocol.

2.7. Statistical analysis

Results were analyzed using InStat 3 version 3.1a (GraphPad Software, Inc., La Jolla, CA). Student's T Test or one-way ANOVA followed by Dunnett tests were used to identify the differences. The threshold for significance for all tests was set as $p < 0.05$.

Table 1
Primers for qPCR.

Gene	Forward primers	Reverse primers	Accession no.
Scx	5′-TCAGCAACCAGAGAAAGTTGAGCAA-3′	5′-GGGTCAGTGTTCGGCTGCTTAGAGT-3′	NM_198885
Mkx	5′-AGTAAAGACAGTCAAGCTGCCACTG-3′	5′-TCCTGGCCACTCTAGAAGCG-3′	NM_177595
Egr1	5′-CAGCGCCTTCAATCCTCAAG-3′	5′-GCGATGTCAGAAAAGGACTCTGT-3′	NM_007913
Col3	5′-CAGAGCAACGGTCATACTCATTCACC-3′	5′-CAGCAACAGCAGAAGAGAAGCACC-3′	NM_009930
Col1	5′-GACATGTTCAGCTTTGTGGACCTC-3′	5′-GGGACCCTTAGGCCATTGTGTA-3′	NM_007729
Tnmd	5′-AACACTTCTGGCCCGAGGTAT-3′	5′-AAGTGTGCTCCATGTCATAGGTTTT-3′	NM_022322
Bgn	5′-TTTCTGAGCTTCGCAAGGATG-3′	5′-GGGCGTAGAGGTGCTGGAG-3′	NM_007542
Dcn	5′-CTATGTGCCCCTACCGATGC-3′	5′-CAGAACACTGCACCACTCGAAG-3′	NM_007833
Fmod	5′-CTCCAACCCAAGGAGACCAG-3′	5′-GGATCCACCAGTGAGAGTCTTC-3′	NM_021355
Lum	5′-TCGAGCTTGATCTCTCCTAT-3′	5′-TGGTCCCAGGTCTTACAGAA-3′	NM_008524
MMP13	5′-TCAGTCTCTTCACCTCTTTTGGGAATCC-3′	5′-TCAGTTTCTTTATGGTCCAGGCGATG-3′	NM_008607
Agg	5′-TCTGGAAATGACAACCCCAAGCAC-3′	5′-TGGCGGTAACAGTGACCCTGGAACT-3′	NM_007424
SOX9	5′-AGTTTGACCAATACTTGCCACCCAAC-3′	5′-TCCGTCTTGATGTGCGTTCGCT-3′	NM_011448
Osx	5′-CCAGCCTCTGGCTATGCAAA-3′	5′-AGGAAATGAGTGAGGGAAGGGT-3′	NM_130458
Runx2	5′-GGTCCCCGGGAACCAA-3′	5′-GGCGATCAGAGAACAAACTAGGTTT-3′	NM_001145920
Cebpa	5′-CAAGAACAGCAACGAGTACCG-3′	5′-GTCACTGGTCAACTCCAGCAC-3′	NM_007678
Cebpg	5′-CAGCACGGAAACTACAGCGA-3′	5′-ACTGCCCTGGGTTATCAGAAT-3′	NM_009884
Gapdh	5′-ATGAGCCCTTCCACAATGCCAAAG-3′	5′-AAGCCCATCACCATCTTCCAGGAG-3′	NM_008084

3. Results

3.1. Effects on tenogenic differentiation and glucose metabolism

We first examined the effect of IL-1β on tenogenic differentiation of inTPCs. The cells were treated with 1, 5 or 10 ng/ml IL-1β for 7 days and analyzed gene expression of tendon cell markers, *Scleraxis* (*Scx*) and *Tenomodulin* (*Tnmd*) [12,13]. Treatment with IL-1β significantly reduced the expression of *Scx* and *Tnmd* genes (Fig. 1A and B). IL-1β also down-regulated gene expression of *early growth response gene 1* (*Egr1*), *collagen 1* (*Col1*) and *collagen 3* (*Col3*) while it up-regulated *Mohawk* (*Mkx*) and *matrix metalloproteinase 13* (*Mmp13*) expression (Fig. 1C). It has been demonstrated that small leucine-rich proteoglycans play important roles in tendon repair and maintenance [14,15]. We therefore examined gene expression of these molecules and found decreases in *Biglycan* (*Bgn*) and *Fibromoduline* (*Fmod*) expression and a great increase in *Decorin* (*Dcn*) expression in the IL-1β-treated cultures (Fig. 1C). There are no significant differences for the gene expression of Lumican (*Lum*) between control and IL-1β-treated cultures (Fig. 1C).

IL-1β did not affect cell proliferation of inTPCs significantly (Fig. 2A), but we noticed that the medium of IL-1β-treated culture

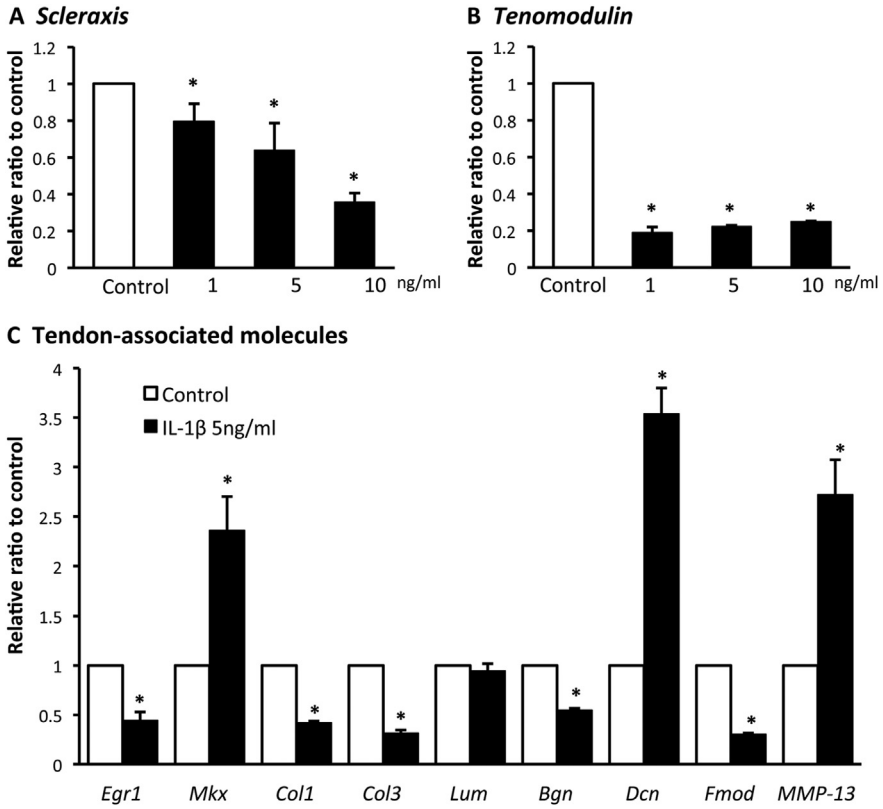

Fig. 1. Effects of IL-1β on tenogenic differentiation of inTPCs. The inTPCs were treated with the indicated concentrations of IL-1β for 7 days and subjected to qPCR for gene expression of *Scleraxis* (A), *Tenomodulin* (B) and tendon-associated molecules(C). Values are average and SD for 4 samples. *P < 0.05 to Control.

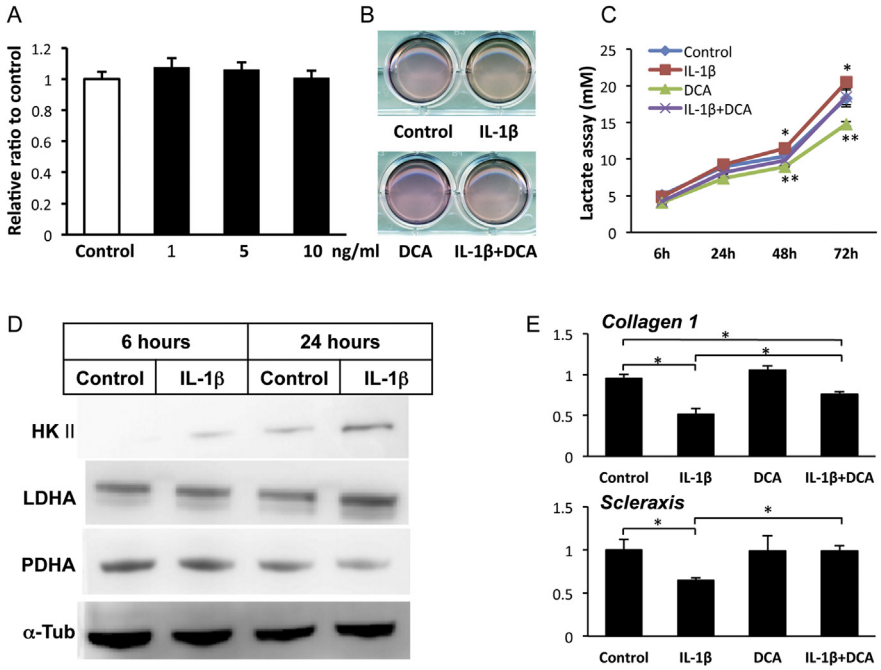

Fig. 2. IL-1β did not affect cell proliferation and stimulated lactate production with alterations of glucose metabolic enzymes in inTPCs. A, The inTPCs were treated with the indicated concentrations of IL-1β for 7 days and subjected to CYQUANT cell proliferation assay. B, Pictures of inTPC cultures treated in absence of presence of 5 ng/ml IL-1β and/or 1 mM DCA for 3 days. C, The media of inTPCs cultures were collected after treatment with vehicle (Control) or 5 ng/ml IL-1β for 6, 24, 48 or 72 h and subjected to lactate assay. *, $p < 0.05$ Control to IL-1β at 48 and 72 h; **, $p < 0.05$ DCA group to either Control or IL-β+DCA group at 48 and 72 h. D, The inTPC cultures were treated with vehicle (Control) or 5 ng/ml IL-1β and subjected to Immunoblot analysis for hekisokinase II (HKII), lactate dehydrogenase (LDHA), pyruvate dehydrogenase (PDHA) or α-tubulin (α-Tub). E, The inTPCs were treated with or without IL-1β (5 ng/ml) and DCA (1 mM) for 7 days and subjected to qPCR for gene expression of *Collagen 1* and *Scleraxis*. Values are average and SD for 4 samples. *, $p < 0.05$ to Control.

became acidic rapidly as determined by the medium color (Fig. 2B) and pH measurement (data not shown). To examine whether this change is due to changes in glycolysis activity and lactate synthesis, we measured the concentration of lactate in the culture medium 24, 48 and 72 h after treatment with IL-1β. IL-1β significantly increased lactate production over time (Fig. 2C). The increase in lactate production by IL-1β was completely abolished by co-treatment with dichloroacetate (DCA) that inhibits lactate synthesis [16] (Fig. 2C), suggesting that IL-1β shifted glucose metabolism toward an anaerobic respiration mode. Consistently, the contents of hexokinaseII (HK II) that catalyzes the first rate-limiting step of glucose catabolism and lactate dehydrogenase (LDHA) that catalyzes the conversion of pyruvate to lactate were increased by IL-1β. In contrast, pyruvate dehydrogenase (PDHA) that catalyzes the conversion of pyruvate and coenzyme A into acetyl coenzyme A was inhibited by IL-1β after 24 h (Fig. 2D). Furthermore, DCA treatment recovered IL-β inhibitory effects on *collagen 1* and *scleraxis* gene expression (Fig. 2E).

3.2. Effects on multipotency

Next, we studied whether IL-1β modulates multipotency of inTPCs. The cells were cultured under chondrogenic, osteogenic or adipogenic conditions in the presence or absence of IL-1β and subjected to gene expression analysis for corresponding differentiation markers: *Aggrecan* (Agg) and *Sex-determining region Y-box 9 (SOX9)* for chondrogenesis; *Osterix (Osx)* and *Runt-related transcription factor 2* (*Runx2*) for osteogenesis; and *CCAAT/enhancer-binding protein alpha* (*Cebpa*) and *CCAAT/enhancer binding protein gamma* (*Cebpg*) for adipogenesis. The results showed that IL-1β decreased expression of chondrogenic, osteogenic and adipogenic differentiation markers (Fig. 3A, B and C, respectively).

Gene expression of inflammatory cytokines is initially up-regulated in the injured site of tendon, but would eventually decrease. We asked whether the phenotype of inTPCs is consequently altered once they are exposed to IL-1β or not. To ask this question, we pretreated inTPCs with IL-1β for 7 days and then re-plated them and cultured under various differentiation inductive conditions in the absence of IL-1β. The IL-1β pre-treated inTPCs showed decreases in gene expression levels of *Scx*, *Tnmd*, *Col1* and *Col3* compared to those in the control culture that had not been pre-treated with IL-1β (Fig. 4A). The expression levels of chondrogenic and osteogenic differentiation markers in the pre-treated inTPCs were comparable with those in the control culture without IL-1β pre-treatment (Fig. 4B and C). In contrast, expression levels of adipogenic differentiation markers were lower in the IL-1β pretreated cells than those in the control (Fig. 4D).

4. Discussion

It has been acknowledged that the use of anti-inflammatory drugs should be carefully considered for therapies of tendon disorders [17]. Treatment with anti-inflammatory drugs may result in negative effects on the biomechanical properties of affected tendons [18,19]. In turn, the *in vitro* results indicate that inhibition of inflammatory cytokine actions would be beneficial to tendon healing. The inflammatory cytokines such as IL-1β cause matrix destruction and loss of tendon biomechanical properties by inducing inflammatory mediators such as cytosolic phospholipase A2 (cPLA2), cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2), and increase expression or activities of matrix metalloproteinases (MMPs) such as MMP1, MMP3 and MMP13 in tenocytes [5,20–22]. However, current information on the effects of inflammatory cytokines on tendon cells is largely limited to the cells isolated from uninjured tendons.

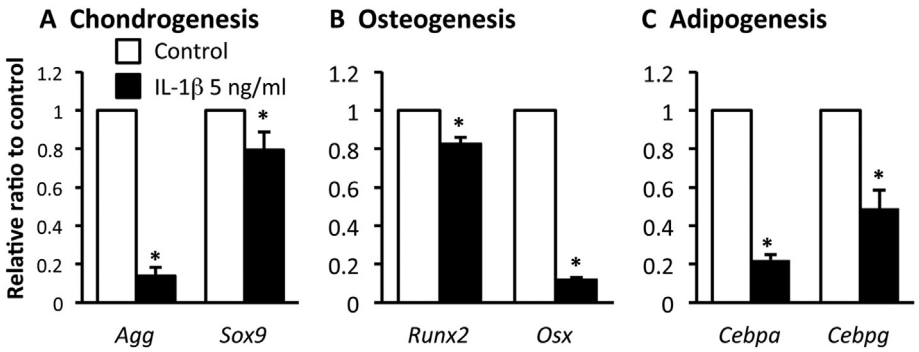

Fig. 3. Effects of IL-1β on gene expression of chondrogenic, ostegenic and adipogenic differentiation in inTPCs. The inTPCs were cultured in micromass (A) or monolayer (B and C) under chondrogenic (A), osteogenic (B) or adipogenic (C) inductive condition in presence or absence of IL-1β (5 ng/ml). Total RNAs were prepared from the cultures after 7 days (A) or 14 days (B and C) and subjected to gene expression analysis for indicated genes. Values are average and SD for 4 samples. *, $p < 0.05$ to Control.

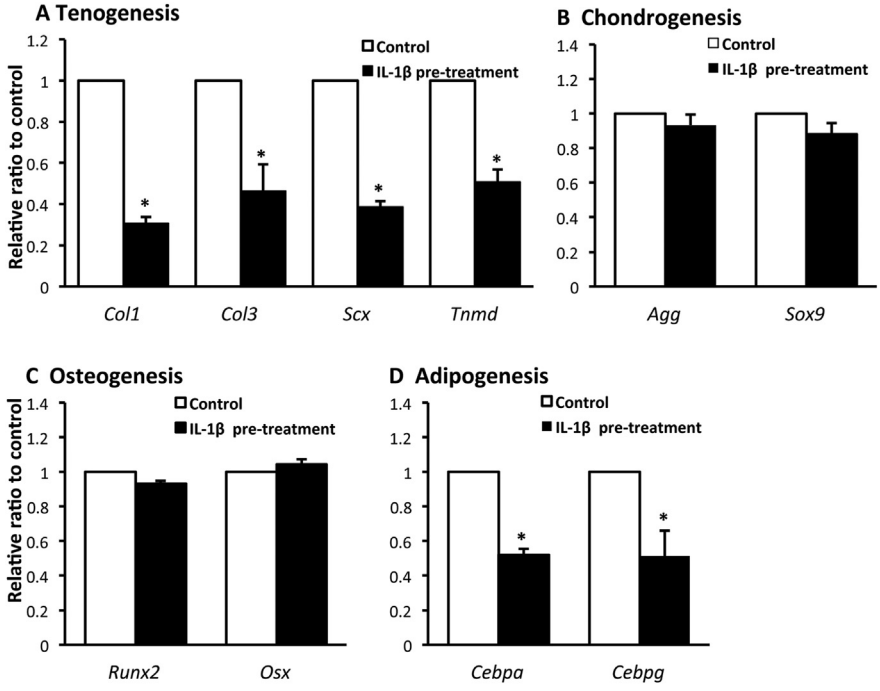

Fig. 4. Effects of IL-1β pre-treatment on function of inTPCs. The inTPCS were cultured with (IL-1β pre-treatment) or without (Control) 5 ng/ml IL-1β for 7 days in monolayer and re-plated in monolayer culture (A, C and D) or micromass culture (B) without IL-1β under tenogenic (A), chondrogenic (B), osteogenic (C) or adipogenic (D) induction. Total RNAs were prepared from the cultures after 7 days (A and B) or 14 days (C and D) and subjected to gene expression analysis for indicated genes. Values are average and SD for 4 samples. *$P < 0.05$ to Control.

Our results showed that IL-1β up-regulated gene expression of MMP13 in tendon progenitors derived from injured tendons. Furthermore, IL-1β reduced expression of tenogenic differentiation markers (*Scx* and T*nmd*), the main tendon associated collagens (*Col1* and *Col3*) and *Egr-1*, a transcriptional factor that plays an important role in tendon [23]. More interestingly, the inhibitory effect of IL-1β on tenogenic differentiation is likely irreversible, and IL-1β-pretreated cells continued showing inferior tenogenic ability even after IL-1β was removed. This irreversible response was detected for adipogenic differentiation, but not for chondrogenic and osteogenic differentiation. These findings indicate that exposure of tendon progenitors to IL-1β at an early stage of tendon injury permanently impairs tenogenic differentiation of tendon progenitor cells. In contrast, the exposed progenitor cells likely maintain the ability to differentiate into chondrogenic and osteogenic cells. Interestingly, we found that IL-1β stimulated lactate-producing glycolysis, presumably by up-regulation of key enzymes such as HKII and LDHA and down-regulation of PDHA. These findings indicate that IL-1β affects glucose metabolic mode of inTPCs, possibly resulting in inhibition of collagen 1 synthesis.

Our results indicate that expression of *Bgn* and *Fmod* was inhibited in inTPCs by IL-1β. These actions of IL-1β would alter the microenvironment of the niche of tendon progenitors and impair the recovery of biomechanical properties of regenerating tendons. In contrast, we detected up-regulation of expression of *Dcn* and *Mkx* which is a homeobox gene involved in tendon development. Importance of *Dcn* in formation of tendon collagen fibers has been demonstrated by different lines of evidence [24–26]. Ito et al. have reported that *Mkx* mutant mice have hypoplastic tendons throughout the body and smaller collagen fibril diameters; they have also indicated that Mkx plays a critical role in tendon development by regulating type I collagen production [27]. Significance of Dcn and Mkx by IL-1β needs to be further investigated.

Tendon mineralization can be found as a result of tendon injuries or a feature of tendinopathy [28,29]. Previous studies reported that mineralization was found in 14–62% of cases following percutaneous or open repair of the Achilles tendon [30] and is thought to be a cause of pain and tendon weakness [31,32]. Our data showed that IL-1β suppressed the chondrogenic and osteogenic differentiation ability in inTPCs, indicating that IL-1β may not directly participate in tendon mineralization in injured tendons, but rather may play an inhibitory action on tendon mineralization during the inflammatory stage. Chondrogenic and osteogenic differentiation of inTPCs was not affected after the removal of IL-1β while their tenogenic differentiation was suppressed, suggesting that tendon progenitor cells irreversibly decrease the tenogenic ability, but preserve chondrogenic and osteogenic potential after exposure to IL-1β. Thus once IL-1β concentration would decrease, the progenitors would contribute to these inductive events.

In summary, our study showed that IL-1β irreversibly inhibits tenogenic differentiation of inTPCs, indicating that inflammatory cytokines, at least IL-1β proteins, strongly affect function of tendon progenitor cells appearing in injured tendons. It is therefore suggested that proper timing of the control of inflammatory cytokine actions is critical for stimulation of tendon regeneration.

Authors contribution

Research design (K. Z and M. E.-I.); the acquisition and analysis of data (K. Z.); the interpretation of data (K.Z., M. E.-I., S. A. and B.Y.); drafting the paper or revising it critically (K.Z., M. E.-I., S. A. and B. Y.).

Conflict of interest

None.

Acknowledgment

We thank Miss L. Cantley, A. T. Gunawardena and R. Berger for technical assistance. This study was supported by the Penn Center for Musculoskeletal Disorders Pilot and Feasibility Grant (NIH/NIAMS P30AR050950), the NIH R21AR062193 Grant and the interdepartmental fund of the Children's Hospital of Philadelphia.

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