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TGF- β Rescues Extracellular Matrix Turnover in Rotator Cuff Pathology

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Abstract

Introduction: Tendinopathies are the most frequent causes of chronic shoulder pain. Long head of the biceps (LHB) tendon lesions are often associated with massive rotator cuff (RC) tears. Palliative LHB tenotomy decreases RC disease patient's pain and disability. The aim of this work was to identify the biological changes of LHB in RC disease and assess its association with clinical manifestations.

Methods: RC disease patients submitted to LHB tenotomy was evaluated using a clinical protocol in order to retrieve information regarding shoulder pain duration and intensity (visual analogue scale) and shoulder function (Constant score). LHB tendon samples from these patients were compared with cadaver controls. Tendon tissue was qualitatively studied by conventional histology and immunohistochemistry was used to access semi quantitatively the presence of substance P and calcitonin gene-related peptide (CGRP). Tendon cell cultures were used to determine the gene expression of several extracellular matrix genes with and without stimulation with transforming growth factor (TGF)-β, TNF, IL-10 or dexamethasone.

Results: Histologically, LHB tendon from RC patients and cadaver controls had similar characteristics. RC patients had a significantly higher CGRP immunohistochemistry score as compared to controls ($p=0.010$) but there was no correlation with patient clinical features. On the contrary, regarding substance P no differences were found between RC patients and controls immunohistochemistry score but a correlation with shoulder pain ($r=0.828$, $p=0.021$) was identified. Through gene expression analysis we found a downregulation of the extracellular matrix genes type I collagen and thrombospondin 4, as well as vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) in patients with RC disease. However, in vitro stimulation of RC tenocytes with TGF-β rescued their ability to produce type I collagen and VEGF.

Conclusion: LHB tendon from RC disease patients had neurotransmitter disturbances that could be related to shoulder pain. Moreover, we demonstrated that LHB from RC disease patients had a downregulation of extracellular matrix genes, as well as of VEGF and NGF genes. We showed that TGF-β can partially normalize the expression of these genes, suggesting that modulating TGF-β could be a therapeutic opportunity for improving tendon quality in the context of chronic tendinopathies.

Keywords: Long head of biceps; Rotator cuff tear; Gene expression; CGRP; Substance P; TGF-β

Introduction

Rotator cuff (RC) disease is the most frequent cause of chronic shoulder pain [1-3], inducing disability and impairment of quality of life [4-6]. Its prevalence increases with age. In fact, nearly 50% of patients over 60 years suffer from rotator cuff disease [7-9]. Long head of the biceps (LHB) tendon lesions are often involved in this rotator cuff disease. The macroscopic LHB lesions associated to RC tears vary in degree from tendinitis, delamination and subluxation on the medial rim of the bicipital groove to frank dislocation or even joint entrapment due to hypertrophy and spontaneous rupture. In 1990, after observing the analgesic effect of spontaneous rupture of the LHB tendon in patients with massive irreparable rotator cuff tear, Walch et al. [10] proposed arthroscopic LHB tenotomy as a palliative treatment for these patients. Moreover, Boileau et al. [11] have shown that arthroscopic LHB tenotomy can effectively treat severe pain or dysfunction caused by an irreparable rotator cuff tear associated with a biceps lesion. However, the physiopathological basis that justifies the success of this surgical procedure is still unclear.

Classically, it was thought that tendons could not undergo matrix turnover and tissue healing. However, recent evidence has shown that tenocytes actively synthesize matrix components and are capable of tissue regeneration [12]. During healing, type III collagen synthesis increases in relation to type I and type I collagen fiber bundle structure is disturbed, leading to impairment of the biomechanical properties [13].

After tendon injury, a healing non-specific response occurs. An initial inflammatory phase begins when inflammatory cells enter into the site of injury. Monocytes and macrophages predominate and pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF) seem to be pivotal for leading the ongoing tendon inflammatory process [14]. Transforming growth factor (TGF)-β is strongly associated with tissue repair, promoting new matrix synthesis [15]. During this phase, vasoactive and chemotactic factors are released, particularly vascular endothelial growth factor (VEGF) and nerve growth factor (NGF), leading to angiogenesis [16] and proliferation of nerve endings [17]. Furthermore, the neuropeptides substance P and calcitonin gene-related peptide (CGRP) are released in the microenvironment where they are responsible for vasodilatation and endothelial permeability, contributing to both inflammation and shoulder pain. Recently, Backman et al. demonstrated that substance P produced by tenocytes in response to mechanical loading regulate cell proliferation through an autocrine loop [18]. During the following

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proliferative phase, activation of tendon cells and recruitment of more inflammatory cells take place [19].

The aim of this work was to identify the biological changes of LHB tendons from RC disease patients that could also lead to pain and disability. We also aimed at testing the effect of TGF-β on these biological disturbances.

Patients and Methods

Patients

Patients with chronic RC disease who underwent shoulder surgery were consecutive enrolled in this study during a period of one year. Although they underwent several surgical procedures, LHB tenotomy was always performed. RC disease was defined by identification of tendon tear through ultrasonography or MRI.

In the pre surgery visit, a week before the surgery, a questionnaire was applied in order to retrieve information regarding shoulder pain duration and intensity (visual analogue scale (VAS)) and shoulder function (Constant score) [20].

Written informed consent was obtained from all patients and this study was performed according to the declaration of Helsinki as amended in Seoul 2008, and was approved by Institutional Ethics Committee.

Tendons from cadavers with no more than 48 h post-mortem, age and gender matched, were used as controls and the tissue was harvested by experienced orthopedic surgeons by the same method. The specimens were macroscopically inspected in order to exclude the presence of rotator cuff tears. This procedure was conducted with the approval of the National Institute of Forensics Medicine.

Tendon evaluation

Each tendon harvested from patient or cadaver was marked indicating proximal and distal ends. The tissue was divided into three equal parts; the proximal and distal ends were used for histological staining and immunohistochemistry while the central fragment was used for *in vitro* cell culture.

Tissue histology: Tendon tissue was fixed in a solution of paraformaldehyde 10% and embedded in paraffin. Transversal sections were cut (5 μm) and stained with hematoxylin-eosin to observe tenoblasts and tenocytes, blood vessels and nerve endings. Wright giemsa stain was performed to analyze the presence of inflammatory cells infiltrating the tendon tissue. Tenocytes and tenoblasts were identified by their different morphology in ten fields (78732 μm^2 of field area) with 400x magnification while blood vessels and nerve endings were counted in the whole specimen at 400x magnification.

The presence of substance P and CGRP was studied by immunohistochemistry. Paraffin sections were cut (4 μm) and antigen recovery was performed using 20 μg/ml of proteinase K (Sigma, Germany). The sections were stained either with 1 μg/ml of substance P monoclonal antibody (Santa Cruz Biotechnology, USA) or 1:50 CGRP monoclonal antibody (Abnova, USA) and the peroxidase-based EnVision (Dako, Denmark) was used as secondary antibody. Color was developed with diaminobenzadine-tetrahydrochloride (Sigma, USA) and a 0.5% H_2O_2 solution. Slides were counterstained with hematoxylin and mounted. As a positive control, a sample of skin was used; while for negative control the primary antibody was replaced by 1% bovine serum albumin (Sigma, Germany). Slides were analyzed by two independent observers using a semiquantitative score (0 - no immunohistochemical stain, 1 - moderate stain, 2 - marked stain).

Primary cell culture: Explant cultures of tendon cells were performed as described elsewhere [21]. Briefly, small fragments of tissue (2 mm^3) were cultured in Dulbecco's Modified Eagle Medium (D-MEM) (Gibco, Spain) supplemented with 50000 units of penicillin/streptomycin (Gibco, Spain), 2 mM of L-glutamine (Gibco, Spain) and 10% fetal bovine serum (FBS) (Gibco, Spain). Cells were cultured at 37ºC, 5% CO_2. Medium was changed after 10 days of culture and then every 2-3 days. When cells reached 90% confluence, they were seeded in a 24-well plate at a density of 10^6 cells/well. Cells were stimulated either with 10 ng/ml TGF-β (Sigma, Germany) or 10 ng/ml TNF (Sigma, Germany) or 10 ng/ml IL-10 (Immunotools, Germany). RNA was collected before and 48h after stimulation. Dexamethasone at 0.1 μM (Sigma, Germany) was also used to mimic treatment. Cells were stimulated for 24 h and RNA was collected at time zero and after the simulated treatment.

RNA extraction: Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was cleaned from DNA contaminants using DNaseI (Qiagen, Germany). RNA concentration and purity were determined spectrophotometrically in Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) and RNA samples were stored at -80ºC until further use.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR): cDNA synthesis was performed from 40 ng of each RNA sample using the DyNAmo cDNA synthesis kit (Finnzymes, Finland) and 300 ng of random hexamer primers according to the manufacturer's instructions. cDNA template (2 ng/ml) was amplified with DyNAmo Flash SYBR green qPCR kit (Finnzymes, Finland) on a Rotor-Gene thermocycler (Qiagen, Germany), according to the manufacturer's instructions. The efficiency of quantitative real time PCR was controlled by the standard curve method for each target gene and each reaction was validated by the presence of a single peak in the melting curve analysis. Primers for the housekeeping and target genes (Table 1) were

Gene	GenBank accession number	Primer sequence	Transcript size
B2M	NM_004048.2	F: ctatccagcgtactccaaagattc	176 bp
		R: cttgctgaaagacaagtctgaatg	
COL1A1	NM_000088.3	F: gggattccctggacctaaag	63 bp
		R: ggaacacctcgctctcca	
COL3A1	NM_000090.3	F: ctggaccccagggtcttc	75 bp
		R: catctgatccagggtttcca	
IL1b	NM_000576.2	F: tacctgtcctgcgtgttgaa	76bp
		R: tctttgggtaatttttgggatct	
THBS4	NM_003248.4	F:ctaccgctggttcctacagc	66 bp
		R: gagccttcataaaatcgtaccc	
TNC	NM_002160.2	F: cggggctatagaacaccagt	75 bp
		R: aacatttaagtttccaatttcaggtt	
TNF	NM_000594.2	F: cagcctcttctcctgat	123 bp
		R: gccagagggctgattagaga	
NGF	NM_002506.2	F: tccggacccaataacagttt	75 bp
		R: ggacattacgctatgcacctc	
VEGF	NM_001025366.2	F: agtgtgtgcccactgagga	60 bp
		R: ggtgaggtttgatccgcata	

B2M: b-2-Microglobulin; COL1A1: Collagen type Ial; COL3A1: Collagen type IIIal; F: Primer forward; IL1b: Interleukin 1b; NGF: Nerve Growth Factor; bp: base pairs; R: Primer reverse; THBS4: Trombospondin-4; TNC: Tenascin C; TNF: Tumor Necrosis Factor; VEGF: Vascular Endothelial Growth Factor

Table 1: Real time PCR primer sequences.

designed using the software Probefinder (Roche, Switzerland) in order to anneal in separate exons preventing amplification of genomic DNA. β_2-microglobulin (B2M) was used as housekeeping gene.

Statistical Analysis

Values are presented as median [interquartile range] or frequencies for continuous or categorical variables, respectively. For continuous variables, non-normality was determined using Shapiro-Wilk test and groups were compared using the Mann-Whitney test and Wilcoxon-T test. For categorical data, chi-squared or Fisher exact test were used. Spearman test was used to correlate biologic with clinical parameters. Statistical significance was set for p-value<0.05. Analysis was performed using the Statistical Package for the Social Sciences software SPSS version 17.0 (Chicago, USA).

Results

Characteristics of the study population

Eight patients with chronic RC disease that underwent LHB tenotomy during surgical treatment were enrolled in this study. The median age was of 61 [8] years and 38% of them were men. Shoulder pain started 18 [6] months before surgery and the VAS for pain was of 45mm. Shoulder function assessed by the Constant score before surgery was of 51 [21].

As a control group, tendon biopsies were collected from 8 cadavers without macroscopic signs of shoulder pathology, age (p=0.202) and gender (p=0.315) matched.

There were no differences in LHB tendon histological characteristic between RC disease patients and controls

The number of tenoblasts, tenocytes (Figure 1A), blood vessels and nerve endings (Figure 1B) were counted in both the proximal and distal ends and we did not found any differences between the two extremities. Moreover, we did not observe any significant differences in the number of tenocytes, tenoblasts, blood vessels and nerve endings (Figures 1C-1F) when comparing RC disease patients and controls. Regarding inflammatory signs we did not observe any cell infiltrate in patients or controls biopsies by giemsa staining.

CGRP immunohistochemistry score was significantly higher in RC disease patients as compared to controls

Both substance P and CGRP bind to receptors expressed by tendon cells, therefore they were visible around these cells (Figures 2A and 2B). For substance P we did not find any statistical difference, neither between distal and proximal ends nor between patients and controls. Regarding CGRP total score, we found a statistical significant difference between RC disease patients and controls (p=0.010). Specifically, in the proximal end, there was also a difference between the two groups, with a higher score in the RC disease group (p=0.041). Although no differences were found regarding substance P score between groups, RC disease patients had a correlation between substance P score and VAS for pain (r=0.828, p=0.021) but not with Constant score. No correlation was found between CGRP score and patients clinical characteristics.

Extracellular matrix turnover related genes were downregulated in RC disease patients

A group of genes related with the extracellular matrix turnover, cell metabolism and inflammation was analyzed in cultured cells from patients and controls (Figure 3).

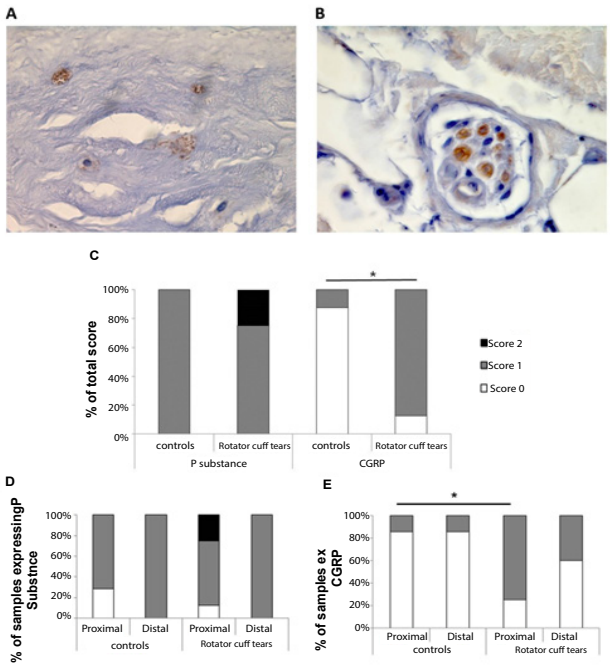

Figure 1: Histological evaluation of tendon tissue from RC disease patients and controls. Hematoxylin-eosin staining allowed the identification of tenocytes (square), tenoblasts (arrow) (A), blood vessels (arrow) and nerve endings (square) (B). The number of tenocytes (C), tenoblasts (D), blood vessels (E) and nerve endings (F) was counted both in controls and patients (magnification 400x).

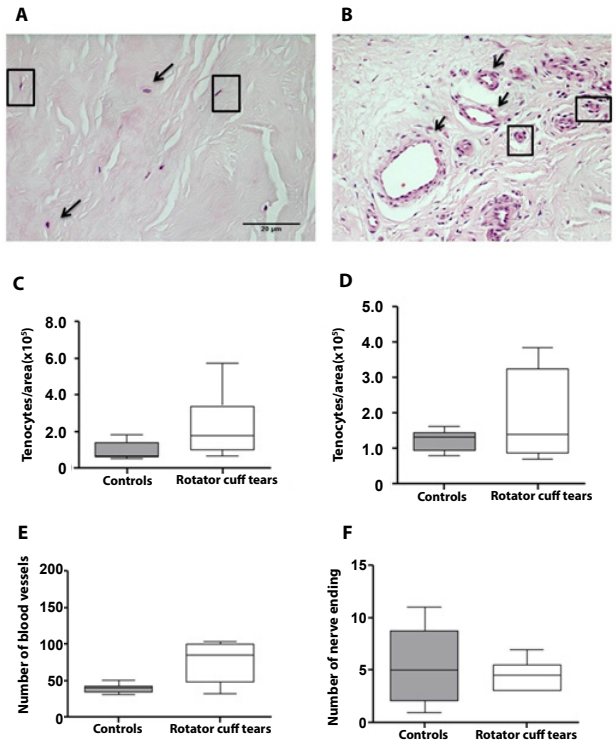

Figure 2: Neuropeptide identification in the tissue. Immunohistochemistry was performed for evaluation of substance P (A) and CGRP (B). (A-magnification 400x and B- magnification 1000x). * p-value<0.05.

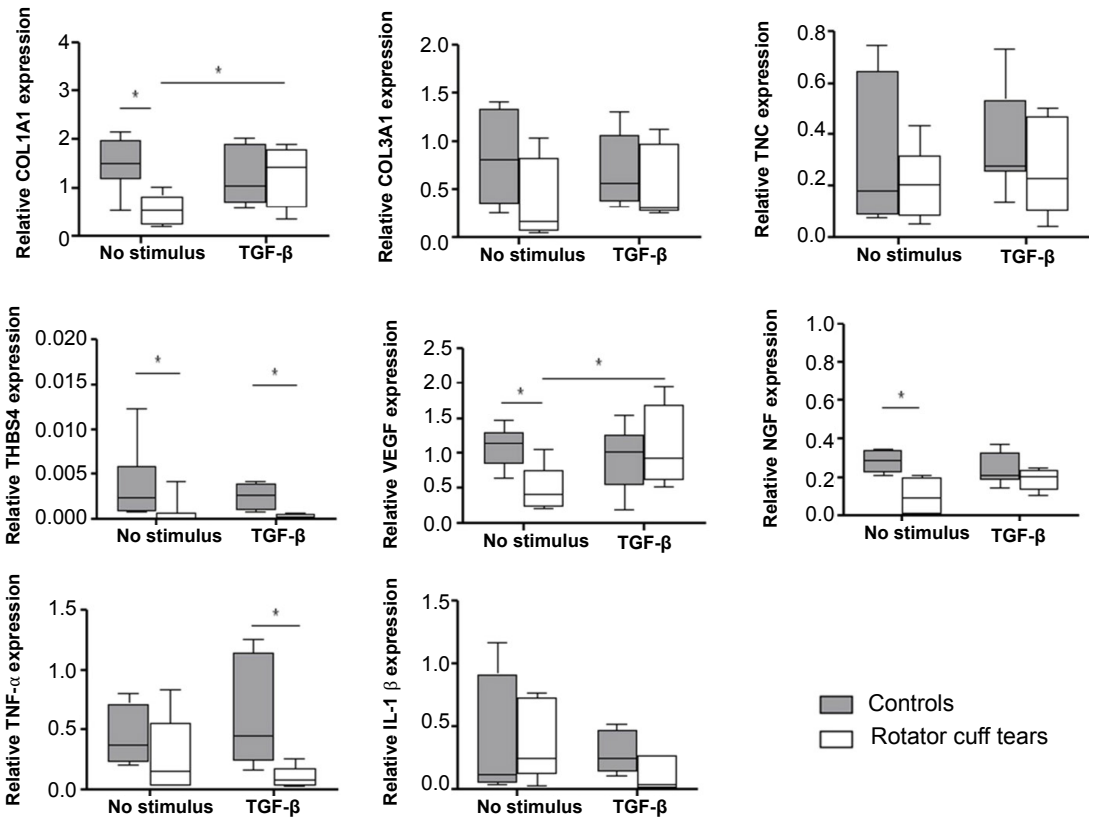

Figure 3: Relative expression levels of extracellular matrix turnover, cell metabolism and inflammation genes with and without stimulation with TGF-β. The expression of each gene was normalized to the housekeeping gene, B2M. *p-value<0.05
(B2M: β-2-Microglobulin; COL1A1: Collagen type Iαl; COL3A1: Collagen type IIIαl; IL1-β: Interleukin 1β; NGF: Nerve Growth Factor; TGF-β: Transforming Growth Factor β; THBS4: Trombospondin 4; TNC: Tenascin C; TNF: Tumor Necrosis Factor; VEGF: Vascular Endothelial Growth Factor)

Collagen type I (COL1A1) and type III (COL3A1) were both downregulated in patients (p=0.004 and p=0.077, respectively). Tenascin C (TNC) and thrombospondin-4 (THBS4), two tendon-selective genes coding for glycoproteins present in the extracellular matrix were also evaluated [22]. While TNC was found to be similar between patients and controls (p=0.456), THBS4 was significantly reduced in the RC disease group (p=0.022). The growth factors VEGF and NGF were both significantly downregulated in the patients group (p=0.007 and p=0.006, respectively). On the other hand, no significant differences were found regarding tissue expression of inflammatory cytokines, such as TNF (p=0.429) and IL-1β (p=0.943).

In addition, the expression of the same genes was assessed after stimulation with TGF-β, TNF, IL-10 and dexamethasone. Although no changes were observed in gene expression in the control group after TGF-β stimulation, in the RC disease group an upregulation of type I collagen (p=0.048) and VEGF (p=0.034) was noticed. No other differences were detected with TNF, IL-10 and dexamethasone stimulation.

Discussion

The main goal of this work was to identify the biological changes in the LHB tendon in the context of RC disease. We found higher amounts of the neuropeptide CGRP in LHB tendons from RC disease patients as compared to controls. Moreover, we found that the genes that code for matrix proteins and growth factors are downregulated in LHB tendons from RC disease patients, suggesting that the cellular machinery responsible for the tissue healing is disturbed. Interestingly, *in vitro* TGF-β stimulation of tenocytes partially corrects these effects.

RC disease has been classically described as a progressive disorder of the rotator cuff tendon often with LHB involvement, which begins with an acute tendinitis that progress to tendinosis with degeneration and, finally, complete rupture occurs [23]. However, histological studies of RC disease have found minimal to none inflammatory cells in the rotator cuff and LHB tendons [24-26]. In accordance, we also did not find any inflammatory cells in LHB tendons of RC disease patients, neither an upregulation of IL-1β or TNF expression levels in tenocytes. The absence of inflammation in the LHB tendon of RC patients does not exclude the possible contribution of inflammatory signals coming from the surrounding structures, namely the shoulder capsule and rotator cuff tendons.

Substance P induces vascular permeability and cytokine and growth factors release by immune cells [27] enhancing cell proliferation and angiogenesis. CGRP has a similar effect, since it plays an important role in vasodilation and plasma extravasation [28]. In a cadaveric study with four male individuals, Alpantaki et al. demonstrated that the LHB tendon is innervated by a network of sensory and sympathetic fibers which are clustered in its proximal part [28]. Other studies detected CGRP and substance P in human biceps brachii [25] and Andersson et al. [29] found the presence of substance P and its receptor in tenocytes of human Achilles tendon. In this work we have detected the

presence of both substance P and CGRP in the LHB of patients with RC disease although only CGRP score was significantly higher in RC disease patients as compared to controls. Moreover, substance P was found to be positively correlated to pain (measured by VAS for pain) strengthening the possible link between this findings and the cause of pain in these patients.

Additionally, we have also observed a downregulation of matrix turnover genes, particularly of type I collagen, in RC disease patients. Genes that code for VEGF and NGF were also downregulated. TGF-β, a cytokine involved in tissue repair and wound healing [30], was able to partially correct this disturbance. Previous studies have shown the presence of one of the three isoforms of TGF-β and an increase of their receptors in chronic tendon pathology [31]. These results suggest that TGF-β play a relevant role in restoring cellular activity in the context of chronic tendon pathology. Thus, modulating TGF-β could be a therapeutic opportunity for improving tendon quality in the context of chronic tendinopathies.

Conclusion

In summary, we demonstrated that the LHB tendon of RC disease patients has neurotransmitter disturbances that could be related to shoulder pain. Moreover, we demonstrated that LHB tendons of RC disease patients present a downregulation of extracellular matrix genes, as well as of the VEGF and NGF genes, that can be partially corrected by TGF-β stimulus.

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