Ultrasound Stimulation of Types I and III Collagen Expression of Tendon Cell and Upregulation of Transforming Growth Factor β

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ABSTRACT: Traumatic tendon injuries are commonly treated with ultrasound. However, previous research has not examined the molecular mechanism of this therapeutic effect on collagen synthesis of tendon cells. This study was designed to determine the effect of ultrasound on the expression of type I and type III collagen of tendon cells intrinsic to rat Achilles tendon. Whether a correlation exists between this effect and the expression of transforming growth factor β (TGF-β), which enhances collagen synthesis, was also investigated. Tendon cells after ultrasound treatment and protein expression of types I and III collagen were determined by immunocytochemistry. The mRNA expressions of a1(I) procollagen, a1(III) procollagen, and TGF-β were determined by reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, the concentration of TGF-β in conditioned medium was evaluated by enzyme-linked immunosorbent assay (ELISA). Immunocytochemical staining revealed that ultrasound-treated tendon cells were stained more strongly for types I and III collagen than were control cells. Upregulation of procollagen a1(I) gene, procollagen a1(III) gene, and TGF-β at the mRNA level was confirmed by RT-PCR. A dose-dependent increase in the concentration of TGF-β in conditioned medium obtained from cells treated with ultrasound was demonstrated by ELISA assay \( p = 0.043 \). In conclusion, ultrasound stimulates the expression of type I and type III collagen in a process that is likely mediated by the upregulation of TGF-β.

INTRODUCTION

Therapeutic ultrasound is commonly used in physical medicine to treat sports-related tendon injury. Although the widespread popularity and therapeutic usage of this physical modality suggest its efficacy, scientific evidence of the molecular mechanism on collagen synthesis of tendon cells remained limited.

Tendons act as transducers of force from muscle to bone. Their basic constituent is collagen, which accounts for 70% of the dry weight of a tendon.1 Approximately 90% of collagen in normal tendons is type I and less than 10% is type III collagen.2 Type I collagen is organized into fibrils grouped in parallel to form organized bundles while type III collagen is almost completely confined to the endotendineum which surrounds the bundles.3 Tendon cells (fibroblasts), which are its basic cellular component of tendon, are the source of collagen production, protein mediators of repair, and matrix proteoglycans.1 It appears that the physiologic response of tendon cells to trauma induces production of both types I and III collagen.4 In the process of tendon healing, type III collagen is the major constituent,5 later being replaced by type I collagen.6

The effect of ultrasound on enhancing tendon healing is well recognized in animal studies. Moreover, the tensile strength of surgically repaired Achilles tendon has been shown to increase after ultrasound exposure.7 The breaking strength of rat Achilles tendons following partial rupture also increased after ultrasound treatment.8,9 One of the proposed mechanisms for ultrasound accelerating the rate of tissue healing was by increasing collagen and general protein synthesis in fibroblasts.10 However, the effect and molecular mechanism of ultrasound on collagen synthesis of tendon cell have yet to be investigated.
Transforming growth factor β (TGF-β), a member belonging to a family of cytokines with pleiotrophic effects on fibroblasts, has emerged as a pivotal mediator in tissue repair. It stimulates production of matrix molecules such as collagen and fibronectin from several fibroblast cell lines of human, mouse, rat, and chicken origin. TGF-β is known to play a prominent role in the healing process of injured tendon or ligament by directing the fibroblast migration and secretion of extracellular matrix proteins. It was demonstrated that pulsed ultrasound exposure could enhance the TGF-β secretion of osteoblasts. It is possible that ultrasound could also stimulate TGF-β secretion, and thus enhance the collagen synthesis of tendon cells.

This study attempted to assess the effects of ultrasound on collagen synthesis (expression) of tendon cells, and to explore the relationship between these effects and the secretion of TGF-β.

METHODS

The following procedures were approved by the Institutional Review Board prior to commencement of the study.

Primary Culture of Rat Achilles Tendon Cells

The Achilles tendons from Sprague-Dawley rats (weighing 200–250 g) were excised. The excised tendon was soaked in povidone–iodine for 3 min and washed twice in phosphate-buffered saline (PBS). Each tendon was then cut into small pieces of approximately 1.5 to 2.0 mm³ (six pieces in six-well culture plates. After 5 min of air drying for better adherence, 0.5 mL of Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Logan, UT), with 10% fetal bovine serum (FBS; Cansera, Rexdale, Ontario, Canada), 100 U/mL penicillin, and 100 μg/mL streptomycin was added to each well. The explants were then incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air. After migrating out from the explants, the cells started to grow rapidly and the confluent culture was subcultured by trypsin digestion at a 1:3 dilution ratio. Tendon cells between passages 2 and 4, with proper growth rate and normal fibroblast shape, were used in the following experiments.

Ultrasound Treatment

Custom-designed ultrasound apparatus was used for signal delivery (Therasound 3.5, Rich-Mar Corporation). The ultrasound transducer was sterilized by 75% alcohol and ultraviolet light before the experiments. The ultrasound transducer at a frequency of 1.0 MHz was secured over a 35 mm collagen-coated dish, which was filled with a total volume of 5 mL of DMEM with 2.5% FBS. The transducer head was immersed vertically into the culture well, just touching the surface of the medium and then fixed. In this way the distance between the transducer head and the bottom of the dish was approximately 4 to 5 mm. A pulsed mode of 2 ms ON and 8 ms OFF was used. To assess the dosage effect of pulsed mode ultrasound on collagen synthesis of tendon cell, four separate treatment groups at exposure dosage of 0 (control), 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm² were investigated. Tendon cells at 50% to 60% confluence were treated with ultrasound once for 5 min and experiments were performed 24 h later. The control group underwent the same experimental treatments with the ultrasound power off. Temperature was measured using thermocouple probe inserted into the culture medium after receiving ultrasound treatment.

Immunocytochemistry

For the purpose of direct microscopic examination on the results of immunocytochemical analysis, tendon cells were subcultured and then seeded directly on the collagen-coated glass coverslips, where cells behaved similarly as on the collagen-coated plastic dishes. Subconfluent tendon cells grown on glass coverslips placed on the bottom of plastic dishes containing growth medium were then treated with 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm² or without ultrasound for 5 min. Twenty-four hours later, the cells were fixed in 4% paraformaldehyde in PBS (pH 7.5) for 15 min at room temperature. All immunostaining procedures were performed directly on the coverslips at room temperature. The coverslips were first immersed for 30 min in blocking solution that contained 1% bovine serum albumin (BSA) and 1% goat serum in PBS. After three washings in PBS, the cells were incubated for 1 h with rabbit anti-rat monoclonal antibodies against type I or III collagen (Novotec, Saint Martin La Garenne, France) diluted in blocking solution. Negative control was performed following the same procedures except excluding the primary antibody form the incubation. The signal was detected with DAKO labeled streptavidin–biotin system and color development was performed by incubation with diaminobenzidine substrate–chromogen (DAKO, Via Real, Carpinteria, CA) for 5 to 10 min. After counterstaining the cell nuclei with hematoxylin, the coverslips were mounted on a glass slide with the cell layer down. The experiments were performed in triplicate.

RNA Isolation

Total cellular RNA from control or ultrasound treated cells was isolated by lysis in a guanidine–isothiocyanate buffer followed by one-step phenol–chloroform–isoamyl alcohol extraction. Briefly, 5 × 10⁶ cells were lysed in 0.5 mL solution D containing 4M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5 % sodium sarcosine, and 0.1M β-mercaptoethanol with vigorous vortexing. Sequentially, 50 μL of 2M sodium acetate...
(pH 4.0), 0.5 mL of phenol, and 100 µL of chloroform–isoamyl alcohol (49:1 v:v) were added to the homogenate. After vortexing for 30 s, the solution was centrifuged at 10,000 g for 15 min at 4°C. The RNA was precipitated by the addition of 0.5 mL isopropanol to the aqueous phase and kept at –20°C for 1 h. RNA was pelleted by centrifuging the solution at 10,000 g for 15 min at 4°C. After rinsing the RNA pellet in ice-cold 75% ethanol, the dry RNA was dissolved in DEPC-treated ddH2O.

Reverse Transcription-Polymerase Chain Reaction

One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) by incubation with 200 U of reverse transcriptase in 20 µL of reaction buffer containing 0.25 µg of random primers and 0.8 mM dNTPs at 42°C for 1 h. Two microliters of the cDNA was used as a template for the PCR reaction. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control to monitor the difference in cDNA loading, and control for PCR variation. The PCR was performed in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 µM of each primer, and 5 U Taq DNA polymerase for 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The amplification results were assessed by using 1.5% agarose gel electrophoresis. Oligonucleotide sequences for the specific primers used in this study were summarized in Table 1. The experiments were performed in triplicate.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to measure the concentration of TGF-β1 in conditioned medium (culture supernatant) of tendon cells. After 24 h of ultrasound treatment, the medium was aspirated and transferred to the wells of a 96-well ELISA plate that were precoated with mouse anti-TGF-β1 antibody (360 µg/mL, 100 µL/well; R&D Systems, Minneapolis, MN) overnight at room temperature. After three washes with PBS-Tween (PBST), each well was blocked with 150 µL of block buffer (5% Tween 20, 5% sucrose in PBS with 0.05% NaN3) at room temperature for 1 h. After three washes with PBST, 100 µL each of the samples and standards were added and incubated in wells for 2 h at room temperature. The wells were then washed three times with PBST, and then biotinylated anti-TGF-β1 antibody (54 µg/mL, 100 µL/well, R&D Systems) was added to each well and incubated for 2 h at room temperature. After three washes with PBST, streptavidin horseradish peroxidase (100 µL/well) was added to each well and incubated for 20 min at room temperature in the dark. After three washes with PBST, 100 µL of substrate solution (1:1 mixture of H2O2 and tetramethylbenzidine) was added for color development and then the reaction was terminated with 2 N H2SO4 as a stop solution. The plate was then read with a microplate reader at 450 nm absorbance (Dynex Technologies). Recombinant TGF-β1 was serially diluted ranging from 0 to 2000 pg/mL and the readings were used to make a standard curve. The experiments were performed in triplicate.

Table 1. Oligonucleotides Used in RT-PCR

<table>
<thead>
<tr>
<th>Type</th>
<th>Oligonucleotides</th>
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<tbody>
<tr>
<td>Type I Collagen</td>
<td>sense: 5’-TGG-AGA-CAG-GTC-AGA-CCT-G-3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’-TAT-TCG-ATG-AGC-GTC-TTG-CC-3’</td>
</tr>
<tr>
<td>Type III Collagen</td>
<td>sense: 5’-TAA-AGG-GTG-AAC-GGG-GCA-GT-3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’-ACG-TTC-CCC-ATT-ATG-GCC-AC-3’</td>
</tr>
<tr>
<td>TGF-β</td>
<td>sense: 5’-TGG-GAT-TGT-AAC-TGT-GAA-CTG-3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’-TGG-TGG-CTA-GGA-TGT-CCT-T-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>sense: 5’-TTC-ATT-GAC-CTC-AAC-TAC-AT-3’</td>
</tr>
<tr>
<td></td>
<td>antisense: 5’-GAG-GGG-CCA-TCC-ACA-GTC-TT-3’</td>
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Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Comparisons between the ELISA results and of the ultrasound treated and control cells were performed using the Kruskal–Wallis test. The level of statistical significance was set at a p value of 0.05.

RESULTS

Cells started rapid growth after migrating out from the explants, and confluence was reached in 2 weeks. Cells at 50% to 60% confluence were treated with various dosages of ultrasound to examine the effect on collagen synthesis and TGF-β expression of tendon cells. Expression of types I and III collagen was analyzed by immunocytochemical staining. In the control group, type I and type III collagen were expressed in the majority of tendon cells and exclusively localized in the cytoplasm (brown color). However, the amount of type I and type III collagen expression increased significantly in ultrasound-treated tendon cells, reflected by much stronger staining (deep brown) for type I (Fig. 1) and type III collagen (Fig. 2). This finding indicated that ultrasound increased protein expression of types I and III collagen in tendon cells. In addition, the intracellular and intercellular fibril formation of collagen I was clearly demonstrated in ultrasound-treated tendon cells, but not in control cells.

To further investigate the expression of types I and III collagen at transcriptional level, the mRNA expression of pro α1(I) chain of type I collagen and the pro α1(III) chain of type III collagen in ultrasound-treated tendon cells were analyzed by RT-PCR. Ultrasound stimulated the gene expression of
procollagen $\alpha_1$ (I) gene, and procollagen $\alpha_1$ (III) gene of tendon cells in a dose-dependent manner (Fig. 3).

The mRNA expression of TGF-\(\beta\) in ultrasound-treated tendon cells was analyzed to understand the potential molecular mechanism underlying the ultrasound stimulation of collagen synthesis of tendon cells. The mRNA expression of TGF-\(\beta\) was upregulated dose dependently after pulsed ultrasound treatment (Fig. 3). The gradual increase in TGF-\(\beta\) expression at the mRNA level was a function of ultrasound intensity and correlated well with the stimulatory effect on collagen synthesis of tendon cells. Furthermore, the TGF-\(\beta\) concentration of conditioned medium of tendon cells treated with
ultrasound increased dose dependently (255.0 ± 11.5 pg/mL for the control group and 309.0 ± 25.5 pg/mL, 310.0 ± 29.0 pg/mL, and 363.3 ± 34.6 pg/mL for the cultures treated with 0.1 W/cm², 0.5 W/cm², and 1.0 W/cm² of pulsed ultrasound, respectively; \( p = 0.043; \) Fig. 4).

The temperature rise of the culture medium after ultrasound treatment was 3.0 ± 0.06°C at 0.1 W/cm², 3.0 ± 0.11°C at 0.5 W/cm², and 2.9 ± 0.03°C at 1.0 W/cm², respectively (\( n = 3, p > 0.05 \)). No further temperature increment was noted in culture medium treated with higher intensity of ultrasound treatment.

**DISCUSSION**

Healing of the injured tendon proceeds through three overlapping phases: inflammation, regeneration, and remodeling/maturation. The initial phase involves an inflammatory response with an influx of cellular elements. During the regenerative phase, tendon cells migrate to the repair site and proliferate. Finally, the extracellular matrix begins collagen production once a sufficient number of tendon cells have colonized the repair site. Initially, type III collagen in a woven pattern is rapidly deposited. Type III collagen is characterized by a small fibril that is deficient in cross-linking. The remainder of the repair process is characterized by shift to the deposition of type I collagen, which continues for an indeterminate period in the final maturation phase. Thus, type I and type III collagen synthesis is fundamental to the healing process of the injured tendon. Collagen is essential to maintain the mechanical properties of tendon. This study documented the ultrasound stimulation of type I and type III collagen expression of tendon cells. The enhanced collagen synthesis and the promoted fibril formation of type I collagen by ultrasound thus could exert a positive effect on the repair process of tendon injury.

Ultrasound is known to exert both thermal and nonthermal effects on the cells and tissues. Thermal effects are used in physical therapy for the treatment of chronic sprains, strains, and pain relief. Nonthermal effects are used in the stimulation of tissue regeneration, protein synthesis in fibroblasts, and tendon repair. Physiologically, pulsed ultrasound tends to produce nonthermal effects such as cavitation and microstreaming but less temperature rise in tissue than dose continuous ultrasound. Regarding the effect on collagen synthesis, the present experiments demonstrated the dose-dependent stimulatory effect of pulsed ultrasound on collagen expression. Nonthermal effects were primarily considered to be responsible for these dose-dependent stimulatory effects because the temperature rise was always around 3°C with different intensities of ultrasound treatment. Transient temperature increase by 3°C could not cause any change on the gene expression of type I collagen, type III collagen, and TGF-\( \beta \) in tendon cells (unpublished data).

In our previous study, ultrasound was demonstrated to stimulate tendon cell proliferation up to 25% with ultrasound at intensity of 1.0 W/cm².20

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**Figure 3.** Ultrasound stimulates the expression of type I collagen, type III collagen, and TGF-\( \beta \) at transcriptional level. Gel electrophoresis of RT-PCR products. GAPDH was used as a reference gene. The resultant DNA bands of GAPDH, type I collagen, type III collagen, and TGF-\( \beta \) were identified at the 467 bp, 409 bp, 401 bp, and 161 bp position, respectively. Band intensity indirectly reflected the mRNA expression level.

**Figure 4.** The TGF-\( \beta \) concentration of conditioned medium of tendon cells treated with ultrasound increased dose dependently (\( n = 3; p = 0.043 \)).
However, in this study ultrasound could enhance the TGF-β secretion of tendon cells as high as 42%. After excluding the confounding factor of cell numbers on the measured concentration of TGF-β, the stimulation effect of ultrasound on the production of TGF-β is still significant.

The most commonly used frequencies of therapeutic ultrasound in physical therapy are in the range of 0.8 to 1.1 MHz and the most clinically used intensities are in the 0.5 to 2.0 W/cm² ranges. The delivery can be continuous or pulsed with treatment duration of 5 to 10 min per site. To contrast the ultrasound used to treat bone fracture and nonunion, pulsed mode with remarkably lower intensity was used. This study showed that ultrasound with intensity of 1.0 W/cm² has significant stimulatory effect on collagen expression and TGF-β secretion of tendon cells. The parameters of ultrasound used in this study are consistent with those of clinically used therapeutic ultrasound.

The cellular mechanisms underlying the therapeutic ultrasound have been postulated. At the cellular level, an increase in temperature increases the rate of metabolism. Besides, the mechanical force of ultrasound could serve as extracellular information, which is transmitted to cells and modulates the expression of genes that regulate cell growth and differentiation. The effect of mechanical pressure at the cell membrane could activate the stretch receptor type of cation channel. Such change in ion, protein transport, or cation concentration could consequently modify intracellular signals for gene expression.

Furthermore, the mechanical force transmitted by ultrasound to cytoskeleton could affect cell metabolism and gene expression. Increased activation of TGF-β has also been demonstrated for fibroblasts subjected to strain. It was proposed that upregulation of type I collagen expression in response to cellular stresses is mediated by TGF-β stimulation. This study is the first to document that ultrasound treatment stimulates secretion of TGF-β and collagen expression of tendon cells at the transcriptional level in a dose-dependent manner.

Results obtained in the present study are based on an in vitro model, though valuable, can not be extrapolated into an in vivo condition directly. Direct application of ultrasound on the tendon cells may be different from an in vivo application through the skin in terms of the ultrasound dosage and the tissue absorption. Theoretically, if similar in vitro responses to the ultrasound treatment occur in vivo, a higher dosage or longer time to achieve the change may be needed. Ultrasound has been demonstrated to promote tendon repairing process in human and different animal models including rabbit and rat. Some of these studies were associated with the findings of increased collagen synthesis. All these studies were usually done by multiple ultrasound treatments and analyzed several days later. However, because our findings on the ultrasound-induced expression of three specific genes including type I collagen, type III collagen, and TGF-β were novel, it should be confirmed in in vivo animal models to reach a more clinically meaningful conclusion.

In conclusion, this study provided novel information on the molecular events associated with the stimulation effect of ultrasound on collagen expression of tendon cells. The molecular mechanism that accounts for the stimulatory effect on collagen synthesis may be modulated through a TGF-β-related pathway.

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