The Pentapeptide KTTKS Promoting the Expressions of Type I Collagen and Transforming Growth Factor-β of Tendon Cells

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ABSTRACT: Pentapeptide KTTKS, a subfragment of type I collagen propeptide, has been demonstrated to promote the extracellular release of collagen in fibroblasts. The present study was designed to investigate the effect and molecular mechanism of KTTKS on type I collagen expression of rat Achilles tendon cells. Genes related to the upregulation of collagen expression such as transforming growth factor-β (TGF-β), and mRNA-binding proteins (mRBP) E1 and K were also examined. MTT assay revealed cell viability was not affected by KTTKS treatment. Protein expression of type I collagen was determined by immunocytochemistry and Western blot analysis. Results showed that KTTKS induced type I collagen expression of tendon cells. The mRNA expressions of α1(I) procollagen, TGF-β, mRBP K, as determined by reverse transcription-polymerase chain reaction (RT-PCR), were also enhanced after KTTKS treatment. The stability of preexisting α1(I) procollagen mRNA after the addition of α-amanitin was analyzed by RT-PCR at 24 and 48 h. Results showed that KTTKS slowed down the degradation of α1(I) procollagen mRNA ($p = 0.021$). Furthermore, the concentration of TGF-β in conditioned medium, as determined by enzyme-linked immunosorbent assay, increased dose dependently in cells treated with KTTKS ($p = 0.001$). In conclusion, KTTKS promotes the expression of type I collagen and maintains its mRNA stability in a process associated with the upregulation of TGF-β. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:1629–1634, 2007

Keywords: pentapeptide KTTKS; type I collagen; transforming growth factor-β; tendon cells

INTRODUCTION

Tendons act as transducers of force from muscle to bone. Their basic constituent is collagen, which accounts for 70% dry weight of the tendon.[1] Tendon cells (fibroblasts), the basic cellular component of tendon, are the source of collagen production, protein mediators of repair, and matrix proteoglycans.[1] Type I collagen is a major component of the extracellular matrix (ECM) from the tendon, and is the predominant collagen species produced during the postinflammatory regeneration. Several investigations have focused on the use of chemical and physical agents to stimulate collagen synthesis of tendon cells and thus accelerate tendon healing.[2–4]

An in vivo study has demonstrated that a pentadecapeptide could promote healing of transected rat Achilles tendon.[5] Meanwhile, it was postulated that collagen peptide could promote both procollagen type I biosynthesis and the repair process in a dermal wound.[6] Furthermore, in vitro studies have suggested that the NH2- or COOH-terminal propeptides of collagens, once removed from their parent procollagen molecules, may exhibit specific regulatory activities; including among these is the influence of propeptides on the synthesis of collagen itself.[7–9] A subfragment of type I collagen with a minimum necessary sequence of KTTKS (Lys-Thr-Thr-Lys-Ser) has been reported to dramatically augment ECM production in fibroblasts.[9] It seems possible that this pentapeptide KTTKS could also stimulate type I collagen synthesis of tendon cells. However, this issue has yet been investigated.

Transforming growth factor-β (TGF-β), a member belonging to a family of cytokines with pleiotrophic effects on fibroblasts, has emerged as a

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pivotal mediator in tissue repair.[10] It stimulates production of matrix molecules such as collagen and fibronectin from several fibroblast cell lines of human, mouse, rat, and chicken origin.[11,12] TGF-β is known to play a prominent role in the healing process of injured tendon,[13] by directing the fibroblast migration and secretion of ECM proteins.[14] It was postulated that production of types I collagen by tendon cells is associated with TGF-β expression.[4] Meanwhile, the induction of type I collagen by TGF-β is accompying by an upregulation of collagen mRNA-binding proteins.[15] These heterogenous nuclear ribonucleoprotein (hnRNP), especially hnRNP E1 and K, act as an mRNA stabilizer and contribute significantly to the post-transcriptional upregulation of collagen synthesis. Possibly this pentapeptide could stimulate the TGF-β secretion and thus also the upregulation of RNA binding proteins, to enhance the collagen synthesis and stability in tendon cells.

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

MATERIALS AND METHODS

The following procedures were approved by the Institutional Review Board.

Primary Culture of Rat Achilles Tendon Cells

The method of cultivating tendon cells form Sprauge–Dawley rats (weighing 200 to 250 g) was described as previous study.[4] Tendon cells between passages 2 and 4, with proper growth rate and normal fibroblast shape, were used. All the following experiments were performed in at least triplicate.

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) Assay

MTT assay was used to detect cell survival after tendon cells were treated with pentapeptide. Twenty-four hours after pentapeptide (0, 0.02, 0.2, 2.0 μg/mL) treatment, MTT assay was performed. MTT 50 μg/mL was added and incubated at 37°C for 1 h. Then, the MTT solution was discarded and 1 mL dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals. Aliquots were then read at 570 nm.

Immunocytochemistry

For the purpose of direct microscopic examination on the results of immunocytochemical analysis, tendon cells were seeded directly on the glass coverslips and placed on the bottom of plastic dishes containing growth medium and then treated with or without pentapeptide (2.0 μg/mL) for 24 h. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.5) for 15 min at room temperature. After 30 min in blocking PBS containing 1% bovine serum albumin and 1% goat serum and washed in PBS, cells were incubated for 1 h with rabbit antirat monoclonal antibodies against type I collagen (Novotec, Saint Martin La Garenne, France) diluted in blocking solution. Negative control was performed following the same procedures excluding the primary antibody from the incubation. The signal was detected with DAKO labeled streptavidian–biotin system, and color development was performed by incubation with diaminobenzidine substrate-chromogen (DAKO, Via Real, Carpiateria, CA) for 5 min. After counterstaining the cell nuclei with hematoxylin, the coverslips were mounted on a glass slide.

Western Blot Analysis

Cell extracts were prepared in lysis buffer containing Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM PMSF, and 1% Triton X-100 followed by the sonication method. Protein concentration of the cell extracts was determined by Bradford assay (Bio-Rad Laboratories, Richmond, CA). Samples with identical protein quantities were then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane. The membrane was incubated at room temperature in blocking solution (1% BSA, 1% goat serum in PBS) for 1 h, followed by a 2-h incubation in blocking solution containing an appropriate dilution of primary antibody, for example, tubulin (NeoMarks, Fremont, CA) or rabbit antirat monoclonal antibodies against type I collagen (Novotec). After washing, the membrane was incubated in PBS containing appropriate secondary antibody conjugated with horseradish peroxidase (Sigma, St. Louis, MO) for 1 h. The membranes were washed and the positive signals developed with enhanced chemiluminescence reagent (Amershan Pharmacia Biotech, Little Chalfont Buckinghamshire, England).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was isolated by lysis in a guanidine-isothiocyanate buffer followed by one-step phenol-chloroform-isoamyl alcohol extraction as described in previous study.[4] 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. Oligonucleotide sequences for the specific primers used are listed in Table 1.

Messenger RNA Stability

To clarify whether pentapeptide could regulate Type I collagen expression by modifying transcripts stability,

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Table 1. Oligonucleotides Used in RT-PCR

GAPDH
Sense: 5′-TTC-ATT-GAC-CTC-AAC-TAC-AT-3′
Antisense: 5′-GAG-GGG-CCA-TCC-ACA-GTC-TT-3′
mRNA-binding protein E1
Sense: 5′-CAA-CCC-AGT-GGA-AGG-CTC-TTC-TCT-TC-3′
Antisense: 5′-CAG-AGG-AAA-GCC-TGG-CAT-TGC-ATT-G-3′
mRNA-binding protein K
Sense: 5′-ACC-GAA-ACC-AAT-GGT-GAA-TTT-GAA-TTT-G-3′
Antisense: 5′-CGA-AAT-TCA-ACC-ATC-TCA-TCA-G-3′
TGF-β
Sense: 5′-TGG-GAT-TGT-AAC-TGT-GAA-CTG-3′
Antisense: 5′-TGG-TGG-CTA-GGA-TGT-CTT-T-3′
Type I collagen
Sense: 5′-TGG-AGA-CAG-GTC-AGA-CCT-G-3′
Antisense: 5′-TAT-TCG-ATG-ACT-GTC-TTG-CC-3′

tendon cells with or without pentapeptide treatment (2.0 μg/mL) for 24 h were then treated with 5 μg/mL α-amanitin to stop the newly synthesized mRNA transcripts. After adding α-amanitin, total RNA was extracted at 0, 24, and 48 h to analyze the relative mRNA expression of procollagen α1(I) by RT-PCR. The semiquantitative measurement of the band density was calculated by using 1D Digital Analysis Software (Kodak Digital Science™, Eastman Kodak Company, Rochester, NY). The band intensity of procollagen α1(I) was normalized to the band intensity of 18S ribosomal RNA in the same sample. Normalized data, representing the relative mRNA abundance of type I collagen, at 0 h was expressed as 100% and data at 24 and 48 h were expressed as the percentage of that at 0 h.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to measure the concentration of TGF-β1 in culture supernatant of tendon cells. After 24 h of pentapeptide treatment, the medium was aspired and transferred to the wells of a 96-well ELISA plate following the manufacturer's procedures. The plate was then read with a microplate reader at 450 nm absorbance (Dynex technologies, Chantilly, VA). Recombinant TGF-β1 was serially diluted ranging from 0 to 2000 pg/mL, and the readings were used to make a standard curve.

Statistical Analysis

All nonparametric data were expressed as mean ± SEM. Comparisons between the MTT assay and ELISA results of the pentapeptide-treated and control cells were performed using Kruskal–Wallis test. Mann–Whitney test was used to compare the ratio of procollagen α1(I) mRNA at different time points and post hoc analysis of MTT assay and ELISA results. The level of significance was set at a p-value of 0.05.

RESULTS

Tendon cells at 50–60% confluence were treated with various dosages of pentapeptide to examine the effect on collagen synthesis and TGF-β expression.

MTT assay for tendon cell viability revealed that the OD_{570} (optic density) value did not show significant change after pentapeptide treatment (0.69 ± 0.04 for the control group, 0.66 ± 0.39, 0.67 ± 0.73, and 0.69 ± 0.97 for the cultures treated with 0.02, 0.2, and 2.0 μg/mL of pentapeptide, respectively; $p = 0.933$) (Fig. 1). The results indicated that pentapeptide in this concentration range did not exert toxic effect on tendon cells.

For immunocytochemistry, the brown stain of type I collagen, in the control group, was detected in the majority of tendon cells and exclusively localized in the cytoplasm (Fig. 2A). However, the amount of type I collagen expression increased significantly in pentapeptide-treated tendon cells, reflected by much stronger staining (deep brown) for type I collagen (Fig. 2B). Western blot analysis confirmed the protein expression of type I collagen was upregulated as a function of pentapeptide concentration (Fig. 2C). At the transcriptional level, the results of RT-PCR revealed that pentapeptide stimulated the gene expression of procollagen α1(I) gene of tendon cells dose dependently (Fig. 3).

The control of mRNA stability is known to play a role in regulating the gene expression of α1(I) procollagen; we therefore examined whether the decay of α1(I) procollagen mRNA could be modulated by the pentapeptide. The level of α1(I) procollagen mRNA declined progressively in tendon cells after

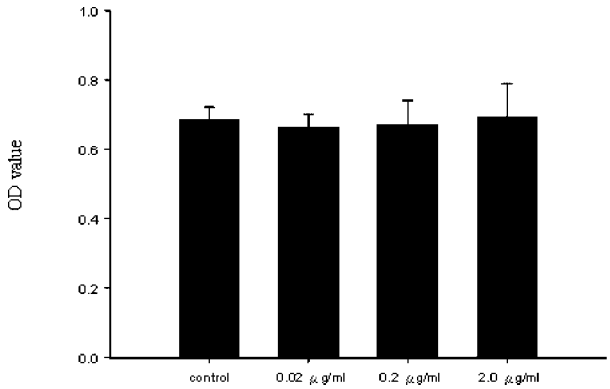

Figure 1. MTT assay revealed that the OD value did not change significantly after peptapeptide treatment ($p = 0.933$; $n = 3$).

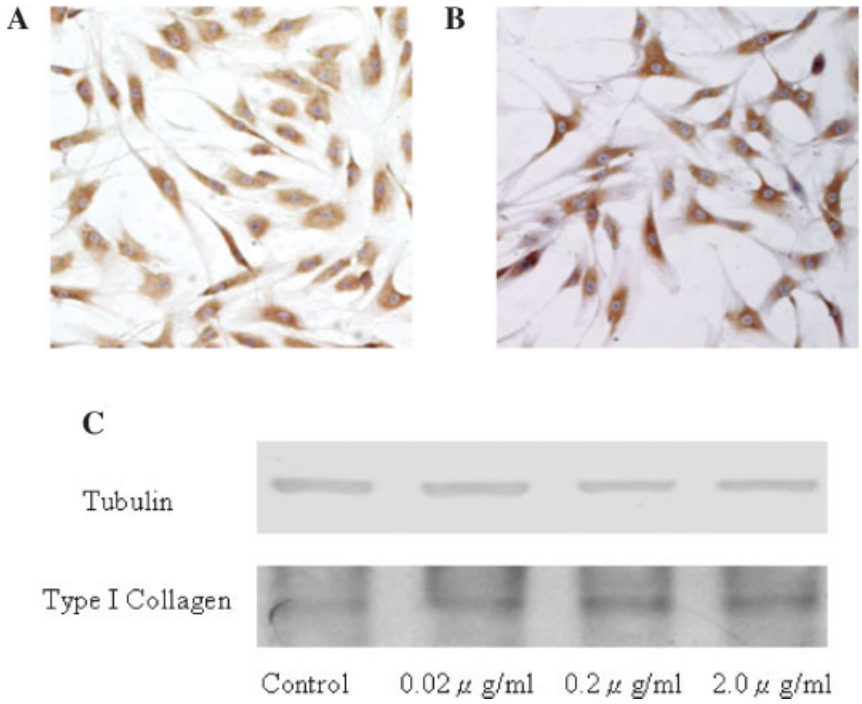

Figure 2. Type I collagen staining in tendon cells without pentapeptide treatment (A) and with pentapeptide treatment (2.0 μg/mL) ($n = 3$) (B). Cytoplasm stained with brown color indicated positive type I collagen expression. The pentapeptide treated cells displayed stronger staining (brown) for type I collagen (original magnifcation 400×). Western blot analysis also revealed expression of type I collagen was upregulated by pentapeptide ($n = 3$) (C).

α-amanitin treatment (Fig. 4A). The calculated ratio of α1(I) procollagen mRNA in control cells and pentapeptide-treated cells at 24 h after α-amanitin treatment were $90.8 \pm 1\%$ and $91.9 \pm 2\%$ ($p = 0.564$), respectively. However, The ratio of α1(I) procollagen mRNA in control cells and pentapeptide-treated cells at 48 h after α-amanitin treatment were $68.8 \pm 1\%$ and $80.9 \pm 2\%$ ($p = 0.021$), respectively. The above results implied that pentapeptide could stabilize α1(I) procollagen mRNA. Furthermore, the mRNA expression of hnRNP E1 and hnRNP K was analyzed to understand the potential molecular mechanism underlying the pentapeptide stimulation of collagen synthesis of tendon cells. The mRNA expression of hnRNP K was upregulated dose dependently after pentapeptide treatment (Fig. 4B).

The TGF-β concentration of conditioned medium of tendon cells treated with pentapeptide increased dose dependently (157.2 ± 4.5 pg/mL for the control group, and 164.7 ± 6.0 pg/mL, 212.7 ± 2.8 pg/mL, and 240.4 ± 17.4 pg/mL for the cells treated with 0.02, 0.2, and 2.0 μg/mL of pentapeptide, respectively; $p = 0.001$) (Fig. 5A). RT-PCR of TGF-β also revealed gradual increase in TGF-β expression at the mRNA level as a function of pentapeptide concentration (Fig. 5B).

DISCUSSION

Healing of the injured tendon proceeds through three overlapping phases: inflammation, regeneration, and remodeling/maturation.[1] 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, especially collagen production by tendon cells begins. This study documented the pentapeptide stimulation of type I collagen expression of tendon cells. The implication of this study is that the pentapeptide which promotes the collagen synthesis

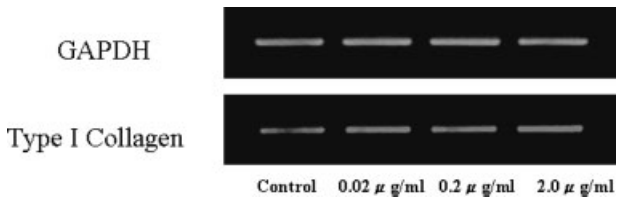

Figure 3. Gel electrophoresis of RT-PCR products revealed pentapeptide stimulates the expression of α1(I) procollagen gene at transcriptional level. The DNA bands of GAPDH and α1(I) procollagen were identified at the 467- and 409-bp position, respectively ($n = 3$).

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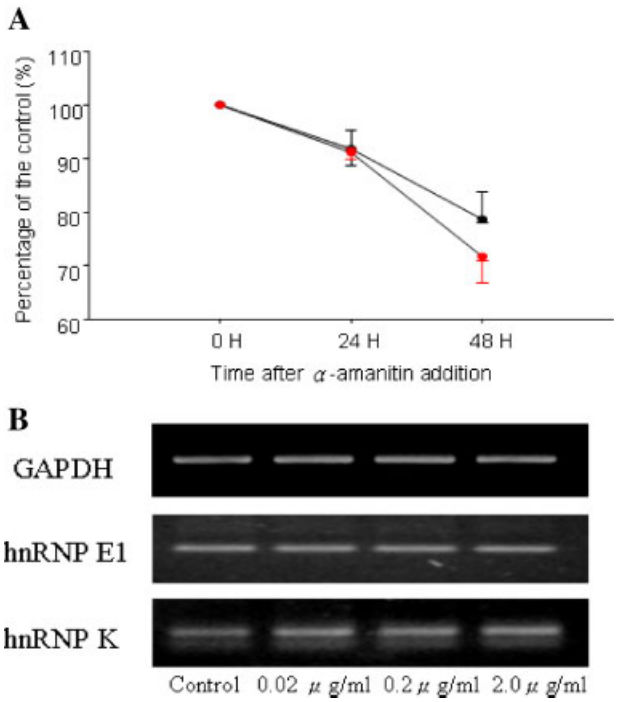

Figure 4. (A) The ratio of mRNA of α1(I) procollagen is significantly reduced in control cells (lower line) than that of cells treated with pentapeptide (upper line) at 48 h after α-amanitin treatment. The levels of α1(I) procollagen were determined at time 0, at 24 h and at 48 h after the addition of α-amanitin ($n = 3$). (B) Results of RT-PCR revealed pentapeptide stimulates the expression of hnRNP K at transcriptional level. The resultant DNA bands of hnRNP E1 and hnRNP K were both at the 100-bp position ($n = 3$).

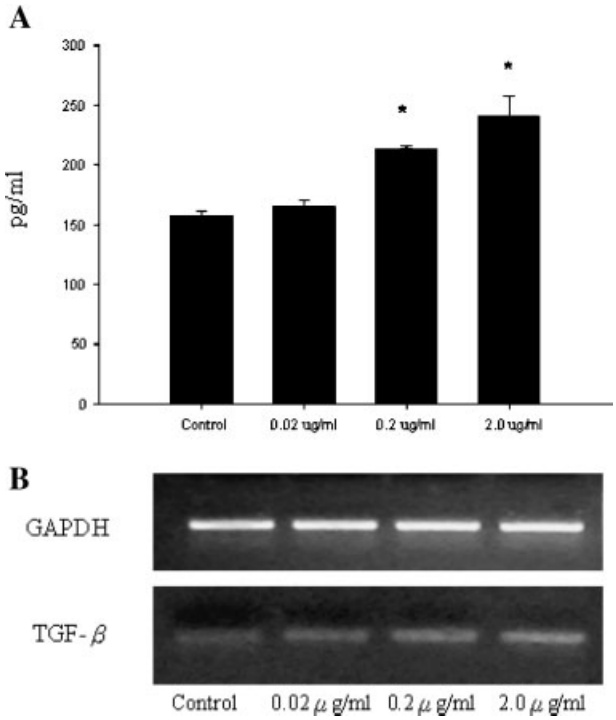

Figure 5. (A) The TGF-β concentration of conditioned medium of tendon cells treated with pentapeptide increased dose dependently ($^*p < 0.05$; $n = 5$). (B) Pentapeptide stimulates the expression of TGF-β at the transcriptional level. The resultant DNA band of TGF-β was identified at the 161-bp position ($n = 3$).

of tendon cells could lead to promising treatment for a damaged tendon.

The results of this study, revealing increased gene expression of type I collagen of tendon cells and mRNA stability after pentapeptide treatment, suggested that the regulatory mechanisms could be transcriptional and posttranscriptional. Studies on cellular binding and uptake of the collagen fragments are currently very limited. However, it has been shown that the N-terminal propeptides of either the type I or type III collagen can bind to the membranes of fibroblasts or liver endothelial cells, respectively, with subsequent internalization by receptor-mediated endocytosis.[16,17]

TGF-β is a cytokine that predominately affects epithelial cells and has a role in extracellular matrix production, cell migration, and cell proliferation during tendon healing.[18] It has also been reported to increase α1(I) procollagen mRNA by enhancing mRNA stability.[19] It was reported that two mRNA-binding proteins, hnRNP E1 and K, as positive effectors of type I collagen synthesis, act at the posttranscriptional level by interaction with the 3′-untranslated regions of procollagen α1(I) and procollagen α2(I) mRNAs.[15] Results of this study, revealing the upregulation of hnRNP K expression after pentapeptide treatment, implied that post-transcriptional control of collagen synthesis at the mRNA level may substantially be caused by alteration of the expression of RNA-binding proteins. The RNA-binding protein may effectively promote collagen synthesis in this way. These findings were further supported by increasing α1(I) procollagen mRNA stability after pentapeptide treatment. The result of this study is in accordance with a previous study that TGF-β dose not activate solely collagen synthesis but synergistically the synthesis of hnRNP K as well.[15]

This study document for the first time that pentapeptide promotion of type I collagen expression and that is associated with TGF-β expression. The increased secretion of TGF-β of tendon cells could exert both autocrine and paracrine effects on themselves or neighboring cells to stimulate more collagen synthesis.

Collagen peptide could affect the size of collagen fibrils in the Achilles tendon and improve the mechanical properties of the Achilles tendon.[20]

Meanwhile, a pentadecapeptide was reported to accelerate healing of transected rat Achilles tendon without effect on tendon cell growth.[5] The above conclusion for the effect of peptide on tendon cells was in accordance with the observation in our study that pentapepetide did not affect tendon cell proliferation but promote collagen synthesis of tendon cells. These studies also demonstrated the successful use of short peptides in vivo, either by ingestion or intraperitoneal injection, to improve the tendon functions in animal models. Therefore, the present findings on the pentapeptide-induced expression of three specific genes including type I collagen, hnRNP K and TGF-β of tendon cells, although simply an in vitro study, strongly implicated the potential clinical use of pentapeptide KTTKS to improve the process of tendon healing in the future.

In conclusion, this study provided novel information on the molecular events associated with pentapeptide KTTKS acting to stimulate collagen expression and enhancing mRNA stability of tendon cells. The molecular mechanism that accounts for the stimulatory effect on collagen synthesis may be modulated through a TGF-β-related pathway.

ACKNOWLEDGMENTS

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