Thyroid hormones increase collagen I and cartilage oligomeric matrix protein (COMP) expression *in vitro* human tenocytes

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Summary

Background: **we previously demonstrated the presence of high levels of thyroid hormones (THs) receptors isoforms in healthy tendons, their protective action during tenocyte apoptosis, and the capability to enhance tenocyte proliferation *in vitro*. In the present study we tested the ability of THs to influence ECM protein tenocyte secretion in an *in vitro* system.**
Methods**: primary tenocyte-like cells were cultivated for 1, 7 and 14 days in the presence of T_3 or T_4 individually or in combination with ascorbic acid (AA).**
Results**: THs (T_3 or T_4) in synergism with AA increase significantly the total collagen production after 14 days. THs in synergism with AA increase significantly the expression of collagen I,biglycan and COMP, after some days.**
Conclusion**: THs play a role on the extra cellular matrix of tendons, enhancing *in vitro* the production of several proteins such as collagen I, biglycan and COMP. THs receptors are active on human tenocytes, and can play a role in tendon ailments.**

KEY WORDS: ascorbic acid, collagen I, COMP, tenocytes, thyroid hormones, tendons.

Introduction

Tendinopathies and tendon ruptures are the most common musculoskeletal disorders with high social costs. Metabolic disorders (e.g. diabetes, hypercholesterolemia, hyperuricaemia and obesity) and congenital metabolic disorders are an emerging co-factors involved in the pathogenesis of tendon diseases[1,2]. The potential role of thyroid hormones during tendons diseases has been investigated only for calcific tendinopathy of rotator cuff[3,4].
Tendons are fibrous connective tissues that are composed of cells within a complex extra cellular matrix (ECM) rich of collagens, proteoglycans, glycoprotein and water[5]. The fibril-forming type I collagen is the major component responsible for the tensile strength of the ECM. Between and within the type I collagen fibers are other important matrix molecules such as type III and V collagens, that contains proteoglycans (PGs) and glycoproteins. The PGs (decorin, biglycan, fibromodulin, etc.) are anionic molecules that have collagen-binding proprieties and interact with the collagen fibers and other matrix molecules, regulating the ECM assembly, including fibrillogenesis[6,7].
COMP (Cartilage Oligomeric Matrix Protein) or Trombospondin 5, first identified in cartilage, is an abundant glycoprotein particularly present in tendon exposed to compressive load. COMP belongs to the thrombospodin gene family with the ability to bind to type I, II, and IX collagen molecules as well as fibronectin. COMP acts as a catalyst in collagen fibrillogenesis *in vitro*, and there are also indications that COMP acts as a regulator *in vivo* in the extracellular fibril assembly[8-15].
The essential role of THs, T_3 (triiodothyronine) and T_4 (thyroxine) in the development and metabolism of many tissues and organs, both in early and adult life, is mediated mainly through T_3, which regulates gene

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expression by binding to the TH receptors (TR)-α and -β. Thyroid hormones have been reported to modulate cells morphology, differentiation, and proliferation[16-18], and to regulate ECM organization and synthesis[19-24]. We recently showed that thyroid receptors (TRα/β isoforms) are present on rotator cuff tendons, that THs impacts favorably on the growth of human healthy tenocytes *in vitro* culture in a dose dependent manner, and that THs protect from apoptosis human healthy tenocytes isolated from human tendon, again in a dose dependent manner[25].
Theoretically hyperthyroidism may be accompanied by increased catabolism of both soluble and insoluble collagen. Hypothyroidism seems to be accompanied by decreased rates of catabolism of collagen[26-30].
However, despite these observations, the effects of thyroid hormones on the biosynthesis of ECM proteins by the tenocytes have not yet systematically studied. In the present study we tested the ability of THs to influence ECM protein tenocyte secretion in an *in vitro* system.

Materials and methods

Patients and methods

All the procedures described in this investigation were approved by our local ethics committee. All the patients gave written informed consent to be included in this study. Tendon samples were harvested from healthy supraspinatus tendons biopsy specimen in 5 patients who were operated arthroscopically for shoulder instability (3 men, 2 women, age: 28 ± 5 year). Systemic conditions such as thyroid disorders, diabetes, gynecological condition, neoplasia, rheumatic diseases, epilepsy and any previous or concomitant rotator cuff disease were considered exclusion criteria.

Tendon cell cultures

Primary human tendon derived cell cultures were established as previously described[25]. In brief, cells were isolated from tissue samples by washing several times with phosphate buffered saline Dulbecco's W/O Ca and Mg (PBS)+ 1% penicillin/streptomycin (Invitrogen, Life Technologies, Carlsbad, CA, USA). Small pieces of fresh tendon were carefully dissected and mechanically disaggregated with the aid of fine watchmaker forceps to maximize the interface between tissue and medium. The tendons were immediately placed on Petri dishes of 60 mm in diameter (Greiner CELLSTAR dish, Sigma-Aldrich, Saint Louis, MO, USA), containing 5 mL of α-MEM supplemented with 20% heat-inactivated foetal calf serum (FCS) and 1% L-glutamine and 1% penicillin/streptomycin (Gibco, Invitrogen, Life Technologies) at 37° C in 5% CO_2 and air with a change of medium every 2-3 days. Tenocytes were then harvested by Stem Pro Accutase (Life Technologies Carlsbad, CA, USA) and centrifugation at 1,500 rpm for 5 min when the cells migrated out of tendon pieces, and reached 60-80% of confluence (19 days). Collected tenocytes were immediately used for culture to avoid phenotype drift with further *in vitro* passages[31]. The tenocyte phenotype was confirmed by assessing the expression of a tenocyte-specific gene (scleraxis) and genes for collagens α1(I), α2(I) and α1(III) in real-time PCR assays with specific primers (data not shown).
The tenocyte-like cells were seeded with 5x10³ vital cells per well in a 24-well plate (Greiner CELLSTAR dish, Sigma-Aldrich), and in triplicates. Cells were cultured in alpha-MEM supplemented with 1% L-glutamine and 1% penicillin/streptomycin and 20% FBS. Every 3 days, 50% of the medium was changed. After 24 h, the cultured cells were exposed to two different doses of T_3 or T_4 (10^{-7} and 10^{-6} M) with or without L-ascorbic acid (10^{-7} M) (AA) (Sigma Aldrich). Fresh aliquots of T_3 or T_4 were added each 24 h, while AA was added every 3 days. At day 1, 7 and 14 after seeding, a Sirius red assay was performed to analyze total collagen production.

Picrosirius red staining

The quantification of total collagen tenocyte-seeded 24-well culture was assessed after 1, 7 and 14 days of incubation as previously described using a modification of the method described by Lopez-de Leon and Rojkind[32]. At the end of TH exposure, cells were fixed with Bouin's solution [acetic acid 5% (Sigma Aldrich), formaldehyde 9% (ICN Biomedicals Inc., Ohio USA), picric acid 0.9% (Carlo Erba Reagents, Chaussée du Vexin, France)], for 1 hour at room temperature. After fixation, the cell layers were stained with 0.1% Sirius red-Direct Red 80 in saturated picric acid for 1 h, after which excess Sirius red was removed by washing under running tap water. The dye was then eluted with 0.1 N NaOH/methanol (1:1) (Sigma-Aldrich), and the collagen quantified by spectrophotometry at 540 nm. The values were then normalized against total protein concentration. All assays were performed in triplicate. Tenocyte-like cells culture without any treatment were used as control.

Immunofluorescence staining

The primary tenocyte-like cells were seeded with 5x10³ vital cells per well in a 2-well chamber slides (Thermo Fisher Scientific, Inc., Rochester, NY, USA), in triplicates and cultured as previous described. After 1, 7 and 14 days of culture the tenocytes were fixed with pure acetone for 10 min at -20° C. Then, washed a few minutes with PBS. Cells were incubated for 30 min at room temperature with PBS containing 5% of Bovine Serum Albumin (BSA) (Kedrion Group S.P.A., Lucca, Italy) for protein blockage. Primary antibodies for Anti-type I (1:2000), Anti-type III (1:500) (Sigma-Aldrich), anti-type V collagen molecules (1:500) (Chemicon International, Inc., Temecula, California, USA), anti-Biglycan (1:100), anti-COMP

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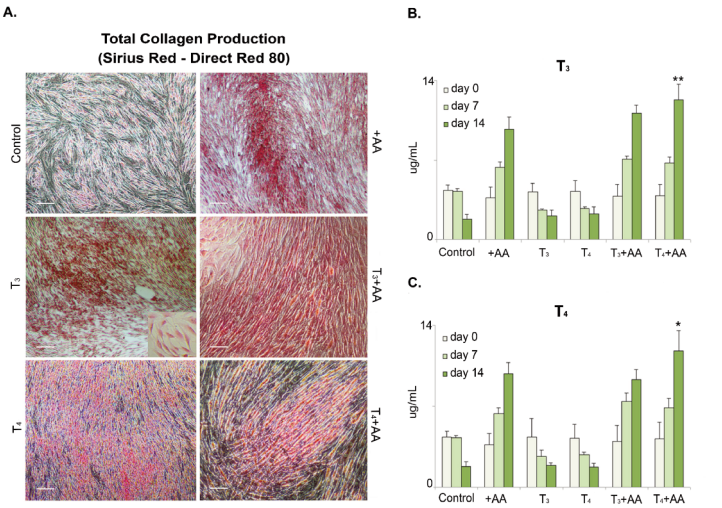

Figure 1. Total collagens production of primary tenoyte-like cells in vitro culture. A) primary tenocyte-like cells isolated from 5 healthy patients, were cultivated for 1, 7 and 14 days in the presence of T3 or T4 or T3+ AA and/or T4+AA as control, we used cells without any treatment and cells in the presence of AA were used as positive control for the secretion and accumulation of collagen from primary tenocyte-like cells. Red color show the accumulation of total collagen in the 24 well plates after 14 days of culture followed by Sirius red staining; B), C) quantification of total collagen after 1,7 and 14 days of in vitro culture in the presence of T3 or T4. The collagen was quantified by spectrophotometry at 540 nm. Representative images from 8 independent experiments. The data are expressed as mean ± SD for 8 independent experiments for samples run in triplicate.(n = 8, $^{*}P$ <0.05, $^{**}P$ <0.01).Scale bar(A): 50 µm.

(1:1000), anti-Fibromodulin (1:200) (Abcam, Cambridge UK) and secondary antibodies were diluted in 5% BSA. Cells were incubated overnight at 4° C with primary antibodies, 1 h with the appropriate fluorochrome antibody at room temperature and then washed a few times with PBS containing 5% BSA. Molecule's staining Alexa Fluor 488 (Life Tecnologies) was used for type Icollagen and Alexa Fluor 568 (Life Tecnologies) for type IIIcollagen, PE was used for type V collagen (Serotec, Oxford UK) and FITC (Sigma Aldrich) for anti-Byclican and anti-COMP staining. After washing with PBS plus 5% BSA, slides were mounted with 25 µL VECTASHIELD® Hard Set Mounting Medium and then were examined with ECLIPSE Ti-U inverted, fluorescent microscope (Nikon Instruments INC., Melville, NY, USA). Digital images were captured with NIS-Elements Imaging Software (Nikon Instruments INC.).

Statistical Analysis

Data are typical results from a minimum of three replicated experiments and are expressed as mean ±SD. Comparison of individual treatment was conducted using Student's *t* test. Statistical significance in comparison with the corresponding control values was indicated by * P < 0.05. *versus* control; ** P < 0.01 *versus* control.

Results

Total Collagen production

In the first series of experiments, we examined whether THs may play a role in the induction of the total collagen production from the primary tenocyte-like cells. Furthermore, as AA regulates collagen synthesis,[33,34] we used AA individually as positive control, and in combination with T_3 or T_4. When T_3 or T_4 were used in combination with AA, collagen production of the primary tenocyte-like cells seeded in the 24 wells demonstrated a statistically significant progressively increase after 1, 7 and 14 days of culture (n =8,$^{*}P$ <0.05,$^{**}P$<0.01), while, in the presence of T_3 or T_4 individually, there was no collagen production, compared with control cells and +AA positive control (Fig. 1 A, B, C). In addition, T_3+AA or T_4+AA produced an increase in total collagen production in a dose-dependent manner. For example, at the dose of 10^{-7}M of T_3 plus AA in the culture cells, the production of total collagen was 11,14 ± 1.5 µg/ml compared with 9,75 ± 1.6 µg/ml of AA culture cells. Greater differences were observed at the dose of 10^{-6}M (12,5 ± 1.2 µg/ml of T_3+AA culture *vs* 9,75 ± 1,6 µg/ml of AA culture cells). The higher increase was obtained by adding T_3+AA *vs* T_4+AA (10^{-6}M: 12,5 ± 1.2 µg/ml *vs* 11,8 ± 1.8 µg/ml) (n = 4, $^{*}P$ <0.05, $^{**}P$ <0.01) (Fig. 1 B, C).

T3 and T4 in synergism with AA modulate the expression of type I Collagen

Next, we determined and quantified the type of collagen deposed from primary tenocyte-like cells after stimulation with T_3 or T_4 alone and or in combination of AA. Collagen accumulation was evaluated by immunofluorescence staining of cells cultured on chamber slides. Primary tenocyte-like cells revealed a high amount of collagen I expression (Fig. 2A, B with T_3+AA and T_4+AA). Furthermore, the expression of collagen I was higher in T_3+AA than T_4+AA, and was significantly higher compared to AA used as a control (Fig. 2 B). No presence of collagen III was demonstrated in any culture conditions (Fig. 2 C). Primary

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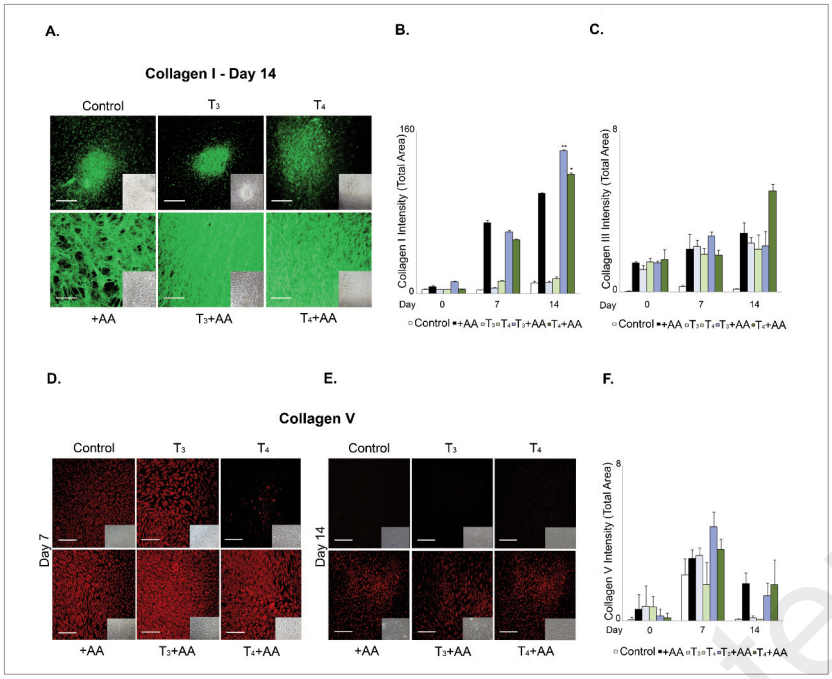

Figure 2. Collagen I, III and V expression of primary tenocyte-like cells in vitro culture isolated from 5 healthy patients, and stained as described in material and methods, section *Immunofluorescence Staining*. Representative images from 4 independent experiments. A) Expression of Collagen I after 14 days of culture in green fluorescence and in natural light, showing the primary tenocyte-like cells. B) Collagen I Intensity (Total Area was quantified by anti-collagen I) it was measured by Nikon software. C) Collagen III Intensity (Total Area). D) Expression of Collagen V after 7 and 14 days of culture in red fluorescence and in regular light image showing the primary tenocyte-like cells. E) Collagen V Intensity (Total Area). F) Data are expressed as mean ± SD for 4 independent experiments for samples run in triplicate ($n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$).Scale bar(A, D, E): 50 µm.

tenocyte-like cells showed similar collagen V expression in all culture conditions, except control cells. Immunofluorescence staining at day 7 reveal any deposition of collagen V from the primary tenocyte-like cells but only an intracytoplasmic staining. (Fig. 2 D, F). Moreover, at day 14 the expression of collagen V decreased drastically in T_3+AA, T_4+AA and control AA, and no staining was detected in all the other experimental conditions (Fig. 2 E, F).

Fibromodulin, Biglycan and COMP expression

Non-collagenous proteins such as fibromodulin, byglican and COMP are present within tendon and play an important role[35]. Immunofluorescence staining of primary tenocyte-like cells cultured on chamber slides revealed no expression of fibromodulin in any of the experimental conditions (data not shown). Moreover, after 14 day of culture in the presence of both T_3+AA or T_4+AA, the cells showed low expression of biglycan; this was higher when T_3+AA was added to the cells culture (Fig. 3 A). COMP expression was presents after 14 day of culture in all culture condition for exception of control cells (Fig. 3 B). In addition, evaluating the amount of immunofluorescence staining through the NIS-Elements Imaging Software, there was a significantly higher intensity in the presence of both T_3+AA and T_4+AA. This was more significant with T_3+AA($^{*}P < 0.05$,$^{**}P < 0.01$)[36] (Fig. 3 C).

Discussion

In the present work, we provide evidence that THs-treated tenocytes modulate ECM proteins secretion *in vitro*. THs treatment, in synergism with AA, increases the production of collagen I, Byglican and COMP. The present findings, together with those we previously reported[25], suggest a binary role for THs on tendons, directly, on tenocyte proliferation, and in synergism with other factors such as AA on tenocyte ECM protein recreations.

Collagen metabolism have been reported to be affected by THs[37,38]. However, the effect of THs on expression of collagen in human tenocytes had not been previously reported. THs stimulate the synthesis of total collagen in a dose dependent manner during 14 days of culture. The expression of collagen I, the major fibrillar collagen of the tendon, is significantly increased in the primary tenocyte-like cells *in vitro* after 14 days of culture. This increase of expression leads to collagen synthesis and accumulation. In contrast THs do not affect the expression of collagen III that normally is less abundant in tendon, and increase only during early phase of remodeling[39] and in tendinopathic tendons[40]. The lack of collagen III productions under TH stress should be considered a protective factors for tendons.

THs in synergism with AA increases the intracytoplasmic expression of collagen V after 7 days of culture to decrease drastically after 14 days. Collagen V it is known to regulate the characteristic of fibrillar structure in tendon[39,41].

While the most abundant protein in tendons is collagen I, PGs, comprising 1-5% of tendon dry weight, are extracellular matrix proteins that play an important role in collagen fibril formation and in resisting compressive loads[42-45]. Biglycan and fibromodulin are PGs expressed in tendon. Biglycan is a small leucine-rich proteoglycan; to date, its role is largely unknown; it is present in the short-term repair response to injury

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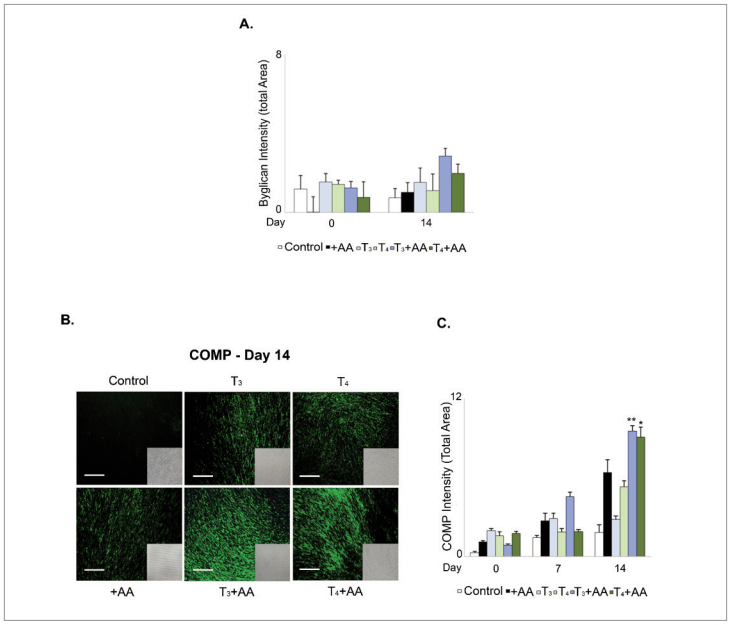

Figure 3. Biglycan and COMP expression of primary tenoyte-like cells *in vitro* culture. A) Byglican Intensity (Total Area) after 1 and 14 days of culture. B) Expression of COMP after 14 days of culture in green fluorescence and in regular light image showing the primary tenocyte-like cells. C) COMP Intensity (Total Area). Representative results from 5 independent experiments. Data are expressed as mean ± SD for 5 independent experiments for samples run in triplicate. (*P <0.05, **P <0.01).Scale bar(B): 50 µm.

and active remodeling[46]. Our data show that, after exposure to THs combined with AA primary tenocyte-like cells, increase the expression of biglycan. In according with previous report where, it was shown that THs up-regulated biglycan gene in tail fibroblasts[47]. THs do not affect, in our hands, the change in expression of fibromodulin as compared to AA or to control cells. COMP is another protein located in the extracellular matrix surrounding the cells. COMP has been identified in many musculoskeletal structures, including ligaments, meniscus, tendons, and synovium. The normal function of COMP is not fully known[48-51]. It is believed to play a role in cell proliferation and apoptosis, as well as in the regulation of cell migration and attachment. Research has also shown that COMP binds strongly to calcium. Interestingly, our results show that THs in combination with AA significantly enhance the expression of COMP. In our knowledge, this is the first time that it is demonstrated a close association between COMP expression and THs. Recently, cleaved fragments of COMP protein were associated with early and late stage tendon injury[52]. In addition, COMP levels are associated with age: postmenopausal women have increased levels of COMP, that decrease after received hormone replacement therapy[51].

In conclusion, this is the first study that provides evidence of a significant association between thyroid hormones and tenocyte ECM proteins secretion. In particularly, there are significant associations with collagen I, biglycan and COMP expression. There are many biological questions that remain to be explore, and translational areas that need to be expanded. Nevertheless, this study provides a first framework that may help clinician in the diagnosis and management of tendinophaties.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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