Interleukin-6: A growth factor stimulating collagen synthesis in human tendon

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Abstract

Human connective tissue e.g. tendons respond dynamically to physical activity, with collagen synthesis being increased after both acute and prolonged exercise or training. Markers of collagen synthesis and degradation as well as concentration of several potential growth factors have been shown to increase markedly in the peritendinous tissue around the human Achilles tendon following exercise. Of these potential growth factors Interleukin-6 (IL-6) showed the largest fold increase suggesting that IL-6 may be involved in transforming mechanical loading into collagen synthesis in human tendon tissue. In the present study the tissue levels of type I collagen turnover markers (procollagen type I N-terminal propeptide (PINP) and C-terminal telopeptide of type I collagen (ICTP)) were measured by the use of microdialysis in peritendinous tissue of the Achilles tendon in 14 male volunteers, who had recombinant human IL-6 infused into the peritendinous tissue of the Achilles' tendon in one leg, with the other leg serving as control. Subjects were randomly assigned to either a resting group or an exercise group performing a 1-hour treadmill run (12km/h, 2% uphill) prior to infusion. In addition to IL-6, serum concentrations of collagen turnover markers PINP, ICTP and ICTX (C-terminal telopeptide of type I collagen) were measured. The peritendinous concentration of PINP rose markedly in response to rhIL-6 infusion in both the exercise and the rest group demonstrating that infusion of IL-6 significantly stimulates collagen synthesis in the peritendinous tissue in humans. Exercise alone did not result in an increased collagen synthesis. This indicates that IL-6 is involved in the collagen synthesis and supports the hypothesis that IL-6 is an important growth factor of the connective tissue in healthy human tendons.
Introduction

The human tendons are composed of densely arranged collagen fibres, elastin and proteoglycans with type I collagen as the dominant connective tissue protein. During the last decade, new methods like the use of microdialysis catheters has enabled experiments demonstrating that the collagen tissue responds dynamically to altered levels of physical activity and that collagen synthesis rates can be increased by both an acute bout of exercise e.g. a prolonged run (36 k) (14; 20) and by repeated sessions or weeks of training (8; 17). These data indicate that the human tendon has the potential to adapt to training by increased tissue synthesis influencing morphology and mechanical properties leading to a strengthening of the tissue (16; 17; 28). Recent data however demonstrate that 1 hour of uphill running (3%) stimulate the collagen synthesis to a much lesser extent than after 36 kilometers of running (8). This indicates that a relationship exists between the amount of loading and the collagen synthesis. In support of this a recent study on healthy and chronically overloaded human tendons respectively demonstrated that only the injured Achilles tendons responded to a 12 weeks rehabilitation program containing eccentric exercises with increased collagen synthesis in contrast to the healthy tendons (12). Thus collagen synthesis can be stimulated if the relative load is high enough. However it is not known whether it is the loading alone or the involvement of released growth factors that leads to the adaptation of the tissue.

It is well accepted that mechanical loading of the human tendon results in a marked interstitial increase in concentration of various growth factors, and that growth factors such as IGF-1 (2), TGF-beta (8; 19; 24), PDGF-bb (3), IL-1-beta (6) and IL-6 (19) can stimulate the synthesis of collagen at least in-vitro. We have performed several studies on humans...
showing an increase in the above-mentioned growth factors, but the exercise-induced release of Interleukin-6 (IL-6) was the most profound of all growth factors so far measured (13). In response to a prolonged run (36 km) the interstitial concentrations of IL-6 increased markedly in peritendinous region around the Achilles tendon (13). This increase was 40 fold higher than the increase ever found in serum IL-6 and 10 fold higher than the IL-6 concentration found in exercise stimulated muscle suggesting a local role of IL-6 (13).

These data suggest that IL-6 could be released from the extracellular matrix or connective tissue potentially having a paracrine role in this tissue (8; 17). Tendon tissue is dominated by fibroblasts which produces extracellular matrix components and in addition to this is known to release IL-6 (10; 29). Thus, the process of mechanotransduction could involve IL-6 as an important growth factor released in a paracrine fashion. In studies on humans, it has been shown, that the release of IL-6 is directly correlated to the length of the exercise (9; 13; 21; 23), and it is generally believed that IL-6 serves as a regulator of metabolism during exercise (4; 7; 13; 23; 27).

We hypothesize, that IL-6 acts as a growth factor for the connective tissue stimulating collagen synthesis of the healthy human Achilles tendon following loading. Thus the purpose of the present study was to investigate the role of IL-6 in the stimulation of collagen synthesis in the human Achilles tendon in combination with or in the absence of mechanical loading. By the use of the microdialysis technique, recombinant human IL-6 (rhIL-6) was infused into the peritendinous tissue ventral to the Achilles tendon in concentrations reflecting the released IL-6 concentration earlier found in the same tissue in response to a 3 h run (13). To investigate the effect of exercise compared to a high concentration of IL-6 alone, the experiment was done both in resting subjects and in subjects completing a 1-hour run just prior to the infusion. The mechanical loading used in
the present study in itself is expected only to generate minor if any stimulation of the collagen synthesis. The microdialysis was performed in both legs of each subject, but IL-6 was only infused in one leg with the other leg serving as a control. We hypothesize that an infusion of Interleukin-6 in the peritendinous tissue around the human Achilles tendon will result in an increased collagen synthesis. Furthermore it is expected that the degree of collagen synthesis will be further increased when preceded by mechanical loading.
Methods and materials

Subjects

Fourteen young men were included in this study (mean age 23 years (range 18-29 years); weight 74 kg (60-85); height 180cm (165-190); mean body mass index (BMI) 23 (19-26)) approved by the regional Ethical Committee of Region Hovedstaden (KF: H-A-2007-055). All subjects were in good health, non-smokers and used no medication. Elite athletes were excluded, but the subjects did regular training (mean MET score representing physical activity level 44 (range: 32-58))(1), and were able to complete a 1-hour run. Prior to inclusion informed written and oral consent was obtained from each subject in adherence to the Declaration of Helsinki.

Experimental protocols

Two protocols were used in this experiment. Subjects were via the envelope method randomly allocated to either an exercising group (n=7) or a resting group (n=7). To measure both the acute and prolonged effect of IL-6 and exercise all subjects were seen twice, on the day of exercise (1 h running/no exercise; time 0 h) and three days later (time 72 h; Figure 1). On both experimental days the subjects arrived after an overnight fast and were told not to perform any kind of exercise for 48 hours prior to the experiments and on the two days between the experimental days.

Exercising protocol

Seven subjects performed a 1-hour run on a treadmill (12 km/h). After completion of the exercise bout, the microdialysis fibers were positioned in the peritendinous tissue ventral
to the Achilles tendons in both legs under local anesthesia (16). The microdialysis fibers were perfused for a total of 4½ hours, and samples from the interstitial fluid were collected each hour for later analysis. The sample collected during the first 30 minutes was not used for analysis due to the risk of the insertion of the catheter could influence the results (16). On the dominant leg the microdialysis fiber was perfused with a 20 % human albumin suspension containing an IL-6 concentration of 8000pg/ml. Based on a 50% recovery in microdialysis catheters this concentration resample the concentration found in the tendon tissue after a 3 hour run (rhIL-6 (B.K. Pedersen, Rigshospitalet, Copenhagen; Denmark))(13). On the non-dominant leg, the microdialysis fibers were perfused with the albumin suspension only. On the second experimental day (72 h) the microdialysis procedure was repeated, though this time both of the fibers were perfused with the albumin suspension only.

**Resting protocol**

In resting protocol the sampling of tissue fluid was performed using microdialysis exactly as in the exercising protocol with the only difference being that the subjects did not perform any exercise prior to the microdialysis procedure. As in the exercising protocol the dominant leg was perfused with a perfusate added rhIL-6 on the first experimental day (8000pg ml⁻¹)(13). All samples were immediately stored at - 80°C until analyses were done. During analysis the evaluator was blinded to which group the subjects were allocated.

**Microdialysis**

One microdialysis catheter was inserted in each leg, via a canulla, from the medial side through the peritendinous space to the lateral side just ventral to the Achilles tendon (16).
The active part of the membrane covered the area from 30 to 60 mm proximal to the Achilles' tendon insertion to the calcaneus bone, measured by palpating the insertion of the tendon and using a tape measure. The microdialysis fibers were all high molecular mass cut-off fibers (3000kDa, membrane length 30mm, catheter outer diameter 0.05mm). The fibers were custom made and sterilized in the laboratory. The catheters were perfused via a high precision syringe pump (CMA 100) at a rate of 2 µl min\(^{-1}\).

To prevent the Interleukin-6 from sticking to the inside of the microdialysis catheter the perfusate used contained Human Albumin “ZLB” 20% solution (ZLB Behring)(C Fischer, personal communication). To determine the in vivo recovery labeled glucose (Glucose D-[3-\(^3\)H], 250µCi in 2.5 ml ethanol/water (9:1), Perkin Elmer) was added to the perfusate (2 µl 10ml\(^{-1}\)) using the internal reference method (25). Labeled glucose was used, as no radioactively labeled IL-6 was available.

**Blood samples**

Blood samples were taken before running as well as at times 0 h, 5 h and 72 h after completing the 1-hour run.

The antecubital vein was used for blood sampling. Blood samples for determination of collagen turnover and plasma concentrations of IL-6 and IGF-1 were centrifuged at 3000 G for 10 min at 4° C, and the supernatant was stored at -80°C for subsequent analysis.

Blood samples for determination of plasma concentrations of Creatine Kinase (CK) were done to ensure that the subjects had not been exercising before the experiment. The samples were stored on ice and immediately analyzed.
Analyses

Collagen synthesis and degradation

For determination of collagen synthesis procollagen type I N-terminal propeptide (PINP) was analyzed in both plasma and dialysate using PINP ELISA kit (custom made in the laboratory). The microdialysis samples used for determination of PINP were collected from 90 to 150 min after insertion of the microdialysis catheters. Collagen degradation was determined by analyzing plasma and dialysate concentrations of C-terminal telopeptide of type I collagen (ICTP) and plasma concentrations of C-terminal telopeptide of type I collagen (ICTX). ICTP was analyzed using UniQ ICTP EIA enzyme immuno-assay (Orion Diagnostica) and ICTX using serum. The microdialysis samples used for determination of ICTP were collected from 30 to 90 min after insertion of the microdialysis catheters.

Inflammatory mediators and growth factors

The plasma interleukin-6 concentration was measured using an ELISA high sensitive immunoassay kit (Quantikine HS, R&D systems, Minneapolis MN, USA).

Insulin-like Growth Factor 1 (IGF-1) was analyzed in plasma and in dialysate. The microdialysis samples used for this were collected from 210 to 270 min after insertion of the catheters.

Statistics analysis

The level of statistical significance was set to p<0.05 (two-tailed). All results are represented as means ± SEM. When a significant difference was found over time (determined by one-way repeated measures ANOVA), the Bonferroni’s multiple comparison tests were used as post-hoc test. The microdialysis concentrations (calculated...
as differences between 0 h post and 72 h post) were analysed using a 2-way ANOVA with IL-6 as the dependent variable. GrafPad prism and PASW Statistics 18 were used for statistical analysis.
Results

All 14 subjects completed the two protocols.

Collagen synthesis – tissue levels of PINP

Peritendinous concentrations of PINP were measured immediately after (0 h post) and 72 hours after IL-6 infusion. Infusion of rhIL-6 resulted in a significant increase in PINP 72 hours following the infusion in both the resting and exercising leg (Figure 2). This was not found in the leg were no IL-6 were infused neither during rest nor following exercise (Figure 2).

Collagen synthesis - serum levels of PINP

No overall significant effect of the interventions could be detected in any of the two groups on PINP concentrations in the blood (Table 1).

Collagen degradation – tissue levels of ICTP

No significant changes were found in peritendinous concentrations of ICTP in response to rhIL-6 infusion neither during rest or following exercise (Figure 3).

Collagen degradation – serum levels of ICTP and ICTX

Serum levels of ICTP increased significantly as an immediate response to exercise (overall: p=0.0014; pre exercise 4.1 ± 0.4 µg l⁻¹; 0 h post exercise 7.0 ± 1.4 µg l⁻¹; p<0.05).

Thereafter the level of ICTP decreased to baseline. No significant changes were seen in the resting group (and no significant difference in the ICTP serum concentration was found at any time point when the two protocols were compared.
Serum concentration of ICTX decreased significantly 5 hours after IL-6 infusion in the
resting group (pre infusion: 1,0 ± 0,1 ng ml⁻¹; 5 h post infusion: 0,5 ± 0,1 ng ml⁻¹; p<0,05)
returning to basal level at 72 hours after IL-6 infusion. A similar pattern was found in the
exercising group with a significant decrease 5 h post infusion.

**Inflammatory mediators – IL-6 in serum**

Interleukin-6 concentrations in serum (sIL-6) increased almost 5 fold in an immediate
response to exercise and returned to basal level when measured 5 hours later (Figure 4).
At 72 hours after exercise the level of sIL-6 had decreased to a significant lower level than
both pre exercise and 5 hours after exercise (0,6 ± 0,1 pg ml⁻¹; p<0,05; Figure 4). The
resting group did not show any changes in serum IL-6 concentrations (Figure 4).

**Growth factors - IGF-1 in serum**

The Insulin-like Growth Factor 1 (IGF-1) serum concentrations in the two groups did not
change significantly in response to exercise or in the rest group.

**Growth factors – IGF-1 in dialysate**

Interstitial concentrations of IGF-1 in the resting group were significantly higher in the IL-6
infused leg than in the control leg measured 0 h post intervention (p=0,006; Table 2). No
difference was seen in the exercise group (p > 0.05; Table 2).
Discussion

The present study demonstrates that infusion of recombinant human Interleukin-6 (rhIL-6) by the use of the microdialysis technique results in a significant increase in collagen synthesis in the peritendinous tissue around the Achilles tendon in humans, and that the effect of the infused IL-6 is not dependent of the tissue being stimulated by exercise prior to the infusion. In fact the 1-hour run alone is not a sufficient load to stimulate the collagen synthesis alone (Figure 2).

To test the hypothesis of IL-6 being involved in local collagen synthesis rhIL-6 was infused in the peritendinous tissue resulting in a marked increase in the peritendinous concentrations of PINP(Figure 2). This increase was both found in the subjects that had been exercising and in the resting control subjects. The rhIL-6 concentration infused aimed at reflecting the concentration of IL-6 previously found in peritendinous tissue in response to a 36 km run (13). The fact that the local concentration within the peritendinous tissue of IL-6 following a 36 km run has been found to be much higher than the concentration in both the skeletal muscles and serum concentrations supports the notion of IL-6 being released from extracellular matrix or connective tissue. In support IL-6 being released from the peritendinous tissue in response to exercise an in-vitro study has found an increased IL-6 secretion from human tendon fibroblasts in response to mechanical stretching (26). As a result of the 36 km run not only were IL-6 found to be increased but also collagen synthesis was found to increase as much as 3-fold in the peritendinous area around the Achilles tendon in response to the long run (20) indicating a potential role of IL-6 as a growth factor in this adaptive response. However 36 km running may represent a superphysiological exercise for most humans. In a previous study on the effect of a shorter
exercise bout (12 km running, 3% incline) we demonstrated that the response in collagen synthesis was less pronounced (8) or even absent (unpublished data). The present study aimed at combining a relative shorter exercise (1-hour running) and the presence of a high tissue concentration of IL-6 equal to the concentration released from the tissue during the prolonged run (13).

The present data shows that the combination of the 1-hour exercise and infusion of high concentrations of rhIL-6 leads to a local increase in the marker of collagen synthesis (PINP) in the peritendinous tissue (Figure 2). The increase in local collagen synthesis is however also present in the resting subjects not subjected to exercise when rhIL-6 is infused (Figure 2). This indicates a strong link between high tissue IL-6 concentrations and local collagen formation (PINP) in the peritendinous tissue around the human Achilles tendon.

When 1-hour of exercise was performed without additional infusion of rhIL-6 we observed a significant four-fold increase in IL-6 in serum underlining that IL-6 is produced in response to the 1-hour exercise (Figure 4). The increase in serum IL-6 concentration immediately after exercise is in line with previous studies on IL-6 release during exercise (5; 7; 13; 22; 27). Although serum concentration of IL-6 increased significantly to approximately 5 pg/ml in response to the one-hour bout of exercise, this was 800 fold lower than the concentration earlier measured locally in the tissue in response to the prolonged run (13) and not enough to lead to a detectable significant increase in local collagen synthesis (Figure 2). However when additional rhIL-6 was infused mimicking the local concentration of IL-6 measured in the tissue during a prolonged run (3 h, 36k)(13) a doubling of the collagen synthesis was demonstrated. This increase in collagen synthesis was comparable to the increase found during rhIL-6 infusion alone. This suggests that IL-6
has a role in stimulating the local fibroblasts to increased collagen synthesis. This is also supported by data from animal studies involving IL-6 knockout mice where the mechanical and organizational properties of tendons three weeks after an injury were found to be reduced in the IL-6 knockout mice compared to control mice (18).

The infusion of the very high but physiological concentration of rhIL-6 (13) lead to a significant increase in local collagen synthesis, but although the infused rhIL-6 concentration reflected the concentration measured in response to a 3-hour run the collagen synthesis was only doubled and thus lower than the 3 fold increase previously found following the prolonged exercise (17; 20). These data altogether supports a role for IL-6 in the exercise-induced type I collagen production in human tendon although the data indicate that more factors and processes seems to be involved in the transfer of mechanical forces to cellular response during exercise.

In the present study serum concentrations of PINP did not change in response to exercise alone or as a result to the microdialysis procedures (control group)(Figure 2). In previous studies markers of collagen type I synthesis in serum (PINP) increased 3 days after prolonged exercise returning to basal level 5 days after (15; 17). The same tendency has been found in peritendinous tissue with concentrations of collagen type I synthesis markers peaking 3 days after prolonged exercise (17; 20). In addition it was found that the tissue elevation in collagen synthesis rate remained elevated after weeks of physical training (14). In the present study the one-hour exercise alone did not lead to an increase in local collagen synthesis. The fact that we did not find a change in collagen synthesis after 1 hour of running is in agreement with the dose response relationship previously suggested for the exercise-induced local tendinous collagen production (8). Furthermore it has been reported that studies on the effect of exercise bouts shorter than 36 km have
failed to show any changes in the synthesis rate for collagen type I (15). It has however to be taken into account that the subjects participating in the present study were very fit with a mean MET score of 44 (equal to PAL (daily physical activity level) of 1,83). This is significant higher than for age matched average adults in the Nordic countries (average MET 38,4)(Nordic Council of Ministers, 2004) indicating that the exercise load in the present study is insufficient to stimulate an adaptive response in collagen metabolism in very fit subjects.

As a marker for collagen degradation we measured ICTP in both serum and in the peritendinous space around the Achilles tendon (Figure 3). We found that the serum concentration of ICTP increased immediately after exercise followed by a decrease to baseline level after five hours. This is in agreement with previous data (15; 17). When measured in the tissue no effect on the collagen degradation of the one-hour run could be detected (Figure 3), indicating that the determined increase in collagen synthesis might result in an increased collagen deposition with an adaptive increase in mechanical properties (11).

In conclusion, the present study is the first attempt to examine collagen synthesis in peritendinous area in the direct response to locally infused IL-6. We demonstrate that infusion of recombinant human Interleukin-6 (rhIL-6) by the microdialysis technique results in a significant increase in collagen synthesis of the peritendinous tissue in humans and that the effect of the infused rhIL-6 is not dependent of the tissue being stimulated by exercise prior to the infusion. The response to the infusion of rhIL-6 is similar to what has been found in response to exercise. Although not conclusive the present study indicates that locally infused rhIL-6 stimulate local production of type I collagen in human peritendinous tissue.
Acknowledgement

We would like to thank Bente Klarlund Pedersen for making rHIL-6 available and Allan Flyvbjerg for skilled technical assistance with the IGF-1 analysis. This study was supported by grants from the Danish Rheumatism Association, The Danish Ministry of Culture Committee for Sports Research, Danish Medical Research Counsel (22-04-0191), and NovoNordic Foundation.
328  **Legends to figures**

330  **Figure 1.** Experimental design protocol 1 and protocol 2. In the samples obtained from the tissue via the microdialysis catheters concentrations of procollagen type I N-terminal propeptide (PINP), C-terminal telopeptide of type I collagen (ICTP), Insulin-like Growth Factor 1 (IGF-1) and Interleukin-6 (IL-6) were measured at all timepoints. In the blood samples concentrations of procollagen type I N-terminal propeptide (PINP), C-terminal telopeptide of type I collagen (ICTP), Insulin-like Growth Factor 1 (IGF-1), C-terminal telopeptide of type I collagen (ICTX), Creatine Kinase (CK) and Interleukin-6 (IL-6).

338

339  **Figure 2.** Interstitial concentrations of procollagen type I N-terminal propeptide (PINP) in the peritendinous tissue around the Achilles tendon measured immediately after and 72 hours after 1 hour of running (treadmill; exercise group) or rest (rest group). The concentrations of PINP are measured in both legs with rhIL-6 infusion or resting legs (control leg). Values are corrected for relative recovery and given as differences between concentrations 0 h post and 72 h post. Error bars represent SEM. *P < 0.05.

349

350  **Figure 3.** Interstitial concentrations of C-terminal telopeptide of type I collagen (ICTP) in the peritendinous tissue around the Achilles tendon measured immediately after and 72 hours after 1 hour of running (treadmill; exercise group) or rest (rest group). The concentrations of ICTP are measured in IL-6 infused leg and control leg. Values are corrected for relative recovery and given as differences between concentrations 0 h post and 72 h post. Error bars represent SEM. *P < 0.05.

360  **Figure 4.** Serum concentrations of Interleukin-6 (IL-6) measured before, immediately after, 5 and 72 hours after 1 hour of running on a treadmill (exercise group). Error bars represent SEM. *P < 0.05.


12. **Langberg H, Ellingsgaard H, Madsen T, Jansson J, Magnusson SP, Aagaard P and Kjaer M.** Eccentric rehabilitation exercise increases peritendinous type I collagen


Figure 1

Protocol 1

Microdialysis
Blood samples

Run 1 h

(4½ h)

0 h post-exe.

(4½ h)

72 h post-exe.

↑

↑

↑

↑

Protocol 2

Microdialysis
Blood samples

(4½ h)

(4½ h)

↑

↑

↑

↑
Figure 2
Figure 3

Collagen degradation
\( \Delta (72h-0h) \)

1CTP [ng/ml]

-2.5  0.0  2.5  5.0  7.5

R  R +IL6  Ex  Ex +IL6
Figure 4
Collagen synthesis – serum levels of PINP

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<th>72 h post intervention</th>
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<td>115 ± 13,9 ng ml(^{-1})</td>
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<tr>
<td>Resting group</td>
<td>118,9 ± 9,9 ng ml(^{-1})</td>
<td>127,1 ± 12,1 ng ml(^{-1})</td>
<td>110,2 ± 12,6 ng ml(^{-1})</td>
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Table 1

Growth factor – IGF-1 in dialysate

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<th>Intervention leg (IL-6 infusion)</th>
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<td>4,0 ± 0,3 ng ml(^{-1})</td>
<td>7,2 ± 1,2 ng ml(^{-1})</td>
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<tr>
<td>Resting group</td>
<td>5,5 ± 1,0 ng ml(^{-1})</td>
<td>4,5 ± 0,9 ng ml(^{-1})</td>
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Table 2