CLINICAL STUDY

GH and IGF1 levels are positively associated with musculotendinous collagen expression: experiments in acromegalic and GH deficiency patients

Simon Doessing, Lars Holm, Katja M Heinemeier, Ulla Feldt-Rasmussen¹, Peter Schjerling, Klaus Qvortrup², Jytte O Larsen³, Rie H Nielsen, Allan Flyvbjerg⁴ and Michael Kjaer

Department of Orthopaedic Surgery M, Faculty of Health Sciences, Institute of Sports Medicine, Bispebjerg Hospital and Centre for Healthy Aging, University of Copenhagen, Bispebjerg Bakke 23, Copenhagen DK-2400, Denmark, ¹Department of Endocrinology, Copenhagen University Hospital Rigshospitalet, Copenhagen DK-2100, Denmark, ²Department of Biomedical Sciences, Faculty of Health Sciences and ³Department of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen DK-2200, Denmark and ⁴Department of Endocrinology and Internal Medicine and the Medical Research Laboratories, Clinical Institute, Aarhus University Hospital, Aarhus DK-8000, Denmark

(Correspondence should be addressed to S Doessing; Email: simondoessing@gmail.com)

Abstract

Objective: Disproportionate growth of musculoskeletal tissue is a major cause of morbidity in both acromegalic (ACRO) and GH-deficient (GHD) patients. GH/IGF1 is likely to play an important role in the regulation of tendon and muscle collagen. We hypothesized that the local production of collagen is associated with the level of GH/IGF1.

Design and methods: As primary outcomes, collagen mRNA expression and collagen protein fractional synthesis rate (FSR) were determined locally in skeletal muscle and tendon in nine ACRO and nine GHD patients. Moreover, muscle myofibrillar protein synthesis and tendon collagen morphology were determined.

Results and conclusions: Muscle collagen I and III mRNA expression was higher in ACRO patients versus GHD patients ($P < 0.05$), whereas collagen protein FSR did not differ significantly between ACRO and GHD patients in muscle ($P = 0.21$) and tendon ($P = 0.15$). *IGF1Ea* and *IGF1Ec* mRNA expression in muscle was higher in ACRO patients versus GHD patients ($P < 0.01$). Muscle *IGF1Ea* mRNA expression correlated positively with collagen I mRNA expression ($P < 0.01$). Tendon collagen fibrillar area tended to be higher in GHD patients relative to ACRO patients ($P = 0.07$). Thus, we observed a higher expression for collagen and *IGF1* mRNA in local musculotendinous tissue in ACRO patients relative to GHD patients. Moreover, there was a tendency towards a higher collagen protein FSR and a smaller collagen fibril diameter in ACRO patients relative to GHD patients. The results indicate a collagen-stimulating role of local IGF1 in human connective tissue and add to the understanding of musculoskeletal pathology in patients with either high or low GH/IGF1 axis activity.

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Introduction

Disproportionate growth of musculoskeletal tissue is a major cause of morbidity in patients with acromegaly and GH deficiency (GHD) (1, 2), and symptoms from bone, joints, tendon and muscle have a major impact on the quality of life in both patient groups (3–6). GH and insulin-like growth factor 1 (IGF1) play an important role in the regulation of protein synthesis, including collagen and myofibrillar protein, the two most abundant proteins in musculoskeletal tissues. However, the possible effect of altered GH/IGF1 levels on the synthesis of collagen and myofibrillar protein has not been determined locally in musculoskeletal tissues in patients with acromegaly and GHD.

Acral overgrowth of hands and feet and coarsened facial features in acromegalic patients (7) suggest an

association between GH/IGF1 and the production of collagen. Moreover, indirect measures of whole-body collagen synthesis appear to correlate with circulating GH/IGF1 and respond to hormone-regulating treatment (8). It is, however, presently unknown whether collagen protein synthesis in specific musculoskeletal tissues correlates with the level of GH/IGF1 years after remission. This is clinically relevant, because the detrimental effect of prior GH excess on musculoskeletal tissue is not fully reversible by hormone-regulating treatment (1), a finding that questions the association between GH/IGF1 and collagen. Moreover, it remains to be established to what extent the level of GH/IGF1 affects the synthesis of collagen protein locally in musculoskeletal tissue of GHD individuals, as suggested by the observed increase in collagen gene

expression of hypopituitary men after short-term GH supplementation (9).

Locally produced IGF1 is gaining attention as a possible key regulator of musculoskeletal protein synthesis (10, 11), and short-term GH supplementation was associated with increased *IGF1* mRNA expression in skeletal muscle in both normoendocrine participants and GHD patients (9, 12). It is currently not known how chronically altered GH levels may affect the local expression of musculoskeletal IGF1 isoforms in acromegalic patients, and importantly, how well local IGF1 expression and local collagen expression are associated in these patients.

Collagen morphology is a major determinant of the mechanical properties in tissues such as bone and tendon. One study in GHD rats suggested that bone collagen fibril diameter and GH levels were inversely correlated (13), which could in part explain the high incidence of bone fractures seen in GHD patients (14). However, a possible association between GH/IGF1 titre and collagen fibril diameter is unexplored in man.

GH/IGF1 excess clearly yields a muscle anabolic effect in growing animals (15–18), whereas myofibrillar protein synthesis in healthy human adults appear to be unaffected by supplementation of GH (12, 19, 20). In acromegalic patients, excess GH does not result in increased muscle mass or strength (21–23), while the observed increase in muscle strength and endurance experienced by GHD patients following GH treatment appear to be a result of altered metabolic functions as well as a stimulation of protein synthesis, suppression of protein oxidation and increased muscle mass (24–27). Taken together, it is currently not clear whether GH/IGF1 and skeletal muscle myofibrillar protein synthesis are positively correlated in man, but investigating both acromegalic and GHD patients offers a unique possibility to address this question.

In this study, we hypothesized that the level of GH/IGF1 in acromegalic and GHD patients is positively associated with collagen synthesis in tendon and muscle, whereas the effect on myofibrillar protein is limited. Outcomes were collagen and *IGF1* mRNA expression, as well as rate of collagen and myofibrillar protein synthesis in skeletal muscle and collagen synthesis in tendon. Moreover, quantitative collagen morphology in tendon was determined.

Subjects and methods

Patients

Eighteen male patients, nine with acromegaly (38 ± 9 years) and nine with GHD (35 ± 9 years), were recruited from the Department of Endocrinology, Copenhagen University Hospital, Rigshospitalet (Table 1). The patients gave informed consent to a protocol adhering to the Helsinki Declaration, which was approved by the

Table 1 Patient clinical characteristics.

Patients	Disease/ duration (years)	Treatment	Age (years)
1	ACRO/2	Somatostatin analogue (20 mg/4 weeks)	31
2	ACRO/0	Somatostatin analogue (20 mg/2 weeks)	31
3	ACRO/7	Surgery + somatostatin analogue (30 mg/4 weeks)	32
4	ACRO/6	Surgery + somatostatin analogue (30 mg/3 weeks)	32
5	ACRO/1	Somatostatin analogue (20 mg/4 weeks)	33
6	ACRO/2	Surgery + somatostatin analogue (10 mg/4 weeks)	37
7	ACRO/6	Surgery	47
8	ACRO/6	Somatostatin analogue (20 mg/4 weeks)	49
9	ACRO/2	Surgery + somatostatin analogue (10 mg/4 weeks)	52
10	GHD/1	GH (0.3 mg/day)	21
11	GHD/4	GH (0.2 mg/day)	28
12	GHD/3	GH (0.8 mg/day)	30
13	GHD/5	GH (0.4 mg/day)	32
14	GHD/3	GH (0.6 mg/day)	32
15	GHD/3	GH (0.2 mg/day)	38
16	GHD/8	GH (0.6 mg/day)	39
17	GHD/8	GH (0.5 mg/day)	43
18	GHD/7	GH (0.6 mg/day)	50

Ethics Committee of Copenhagen and Frederiksberg (KF-01-241/03). Upon recruitment, the patients had been involved in hormone-regulating treatment for 4 years (± 2 years). All nine GHD patients received GH substitution when included, but were withdrawn from the treatment 2 weeks prior to the study in order to bring the patients closer to a state of GHD. The number of patients who accepted the invasive protocol was limited, and consequently the patient groups displayed heterogeneity with regards to duration of disease, prior treatment strategies and present hormone-regulating treatment. The present study therefore focused strictly on the association between GH/IGF1 titre and collagen/myofibrillar protein synthesis measured at a single time point.

Nine healthy age-matched male participants were included as controls with regards to circulating GH, IGF1, and IGF-binding protein (IGFBP) levels.

Tissue biopsy procedure

Biopsies were taken in a post-absorptive state (10 h fasting). The sample sites were prepared with local anaesthetics (lidocaine, 1%), and tendon and muscle were sampled from the non-dominant leg. Tendon samples were taken from the patella tendon (Bard Magnum Biopsy Instrument, C R Bard, Inc., Covington, GA, USA) with a 14 g needle (28). The total wet weight of each tendon sample was from 5 to 10 mg. Half of the tendon biopsy was frozen in liquid nitrogen and stored

at -80°C and subsequently used for protein fractional synthesis rate (FSR) analysis, while the other half was fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.4, for transmission electron microscopy (TEM) analysis. Muscle was sampled from the vastus lateralis (m. quadriceps) muscle using a 5 mm Bergström biopsy needle (Stille, Stockholm, Sweden) with suction (29). The total wet weights of the muscle samples were from 50 to 80 mg. Muscle samples were divided and frozen in liquid nitrogen and stored at -80°C for subsequent mRNA expression and FSR analysis. In order to establish tissue natural abundance of $[1-^{13}\text{C}]$ proline for determination of FSR, a skin biopsy was obtained with a 4 mm Dermal Biopsy Punch (Miltex, Inc., York, PA, USA) from the back, frozen in liquid nitrogen and stored at -80°C .

Muscle mRNA measurements

The amount of mRNA for collagen isoforms $\alpha 1(\text{I})$ (*COL1A1*) and $\alpha 1(\text{III})$ (*COL3A1*), as well as *IGF1* isoforms *IGF1Ea* and *IGF1Ec*, was measured with real-time RT-PCR (Table 2). Muscle was homogenized in TriReagent (Molecular Research Centre, Cincinnati, OH, USA) using a bead mixer with steel beads (Biospec Products, Bartlesville, OK, USA). Following homogenization, bromochloropropane (Molecular Research Centre) was added in order to separate the samples into an aqueous and an organic phase. Following isolation of the aqueous phase, RNA was precipitated using isopropanol, washed in ethanol and dissolved in RNase-free water. All muscle samples were weighed prior to RNA extraction. RNA concentrations were determined by spectroscopy, and good RNA quality was ensured by gel electrophoresis.

Synthesis of cDNA was performed using the Omniscript reverse transcriptase (Qiagen) on 500 ng of RNA in 20 μl . For each target mRNA, 0.25 μl cDNA was amplified in 25 μl Quantitect SYBR Green Master Mix (Qiagen) with specific primers (100 nM each, Table 1) on a real-time PCR machine (MX3000P, Stratagene, La Jolla, CA, USA). The thermal profile was 95°C , 10 min $\rightarrow$ (95°C , 15 s $\rightarrow$ 58°C , 30 s $\rightarrow$ 63°C , 90 s) $\times$ 50 $\rightarrow$ 95°C , 60 s $\rightarrow$ 55°C , 30 s $\rightarrow$ 95°C , 60 s. Signal intensity was acquired at the 63°C step, and the threshold cycle (C_t) values were related to a standard

curve made with the cloned PCR product. Specificity was confirmed by melting curve analysis after amplification (the 55 to 95°C step). We analysed the tissue samples for both *IGF1Ea* and *IGF1Ec*. A 49 bp insertion in *IGF1Ec* makes this isoform distinguishable from *IGF1Ea*. In the *IGF1Ea* assay, the sense primer was placed over the insertion point ensuring that the last two bases fitted downstream of the insertion point. This creates a single base mismatch between *IGF1Ea* and *IGF1Ec* in the 3' end (...GGA-3' in *IGF1Ea* versus...GTA-3' in *IGF1Ec*), which should inhibit amplification from *IGF1Ec* cDNA (30). Indeed, there was no amplification of the 49 bp larger *IGF1Ec* isoform in the *IGF1Ea* assay, based on agarose gel and melting curve analysis. For the *IGF1Ec* assay, the sense primer was placed in the *IGF1Ec*-specific insertion. The large ribosomal protein P0 (*RPLP0*) mRNA, which was stably expressed relative to both *GAPDH* mRNA and total RNA (data not shown), was chosen as an internal control.

Collagen and myofibrillar protein FSR

Measurement of collagen protein FSR was carried out according to previous techniques (31). Skin biopsy was used to establish the natural abundance of $[1-^{13}\text{C}]$ proline in collagen protein, thereby eliminating the need for a pre-infusion basal tissue biopsy. To label collagen protein in muscle and tendon, a flooding dose of $[1-^{13}\text{C}]$ proline (1 g labelled proline (>99 atoms%, Cambridge Isotope Laboratory, Andover, MA, USA) plus 3 g unlabelled proline (AppliChem, Darmstadt, Germany), 4 g total) was given. Blood samples were drawn every 10–30 min during the 2 h experiment to establish area under the curve (AUC) for the precursor.

For extraction of proteins, all tissues were homogenized (FastPrep 120A-230, Thermo Savant, Holbrook, NY, USA) in 1 ml ice-cold extraction buffer (0.02 M Tris-HCl, 0.15 M NaCl, 0.1 M EDTA and 0.1% Triton X-100, pH 7.4) and subsequently centrifuged (1600 *g* at 4°C for 20 min). In skin and tendon samples, this procedure pelleted the total collagen protein fraction. The pellets were then washed twice with 70% ethanol. In muscle samples, the homogenization procedure pelleted the myofibrillar and collagen proteins. After discarding the supernatant, the pellet was resuspended in 0.7 M KCl, in which the myofibrillar proteins

Table 2 Primers for real-time RT-PCR.

mRNA	Sense	Antisense	GenBank accession number
<i>IGF1Ea</i> ^a	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC	NM_00618.3
<i>IGF1Ec</i> ^a	GCCCCATCTACCAACAAGAACAC	CGGTGGCATGTCACTCTTCACTC	NM_001111283.1
<i>COL1A1</i>	GGCAACAGCCGCTTCACCTAC	GCGGGAGGACTTGGTGGTTTT	Z74615.1
<i>COL3A1</i>	CACGGAAACACTGGTGGACAGATT	ATGCCAGCTGCACATCAAGGAC	NM_000090.3
<i>GAPDH</i>	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT	NM_002046.3
<i>RPLP0</i>	GGAAACTCTGCATTCTCGTTCCT	CCAGGACTCGTTGTACCCGTTG	NM_053275.3

^aThe *IGF1Ec*-specific 49 bp insertion (58) is discriminated by an insertion-specific sense primer (*IGF1Ec*) versus an insertion point overlapping (last two 3' bases) sense primer (*IGF1Ea*).

solubilize and the collagen proteins remain precipitated. After centrifugation (1600 *g* at 4 °C for 20 min), the myofibrillar proteins were precipitated from the supernatant using 2.3 × vol. ice-cold ethanol and left at 4 °C for 1 h. After pelleting the myofibrillar proteins (1600 *g* at 4 °C for 20 min), the myofibrillar protein fraction was washed twice with 70% ethanol. All isolated protein fractions were hydrolysed in 6 M HCl at 110 °C overnight, and the amino- and imino acids were purified using cation exchanger (Dowex 50WX8, Bio-Rad) and eluted by 2 M NH₄OH. The amino- and imino acids were derivatized as their *N*-acetyl-*n*-propyl esters, which were analysed by capillary gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS; Delta Plus XL, Thermo Finnigan, Bremen, Germany) using a capillary column DB 1701, 30 m × 0.25 mm, 1.5 µm film (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA) to separate proline. Plasma-free proline was derivatized as their *tert*-butyldimethylsilyl derivative according to standard procedures and analysed by GC–MS (Trace GC 2000 and Automass Multi, Thermo Quest Finnigan, Paris, France) using a capillary column CP-Sil 8 low bleed, 30 m × 0.32 mm, 0.25 µm column (Chrompack, Varian, Palo Alto, CA, USA). The rate of tissue protein synthesis was calculated according to the precursor-product principle as follows: fractional protein synthesis (FSR%/h) = $\Delta E_p \times 100 / \text{AUC}_p$; where ΔE_p is the change in proline labelling over time in tissue protein, and AUC_p is the AUC of venous proline labelling with time in hours.

Transmission electron microscopy and stereological analysis of tendon

Fixed tendon samples were rinsed in 0.15 M sodium cacodylate buffer, pH 7.4, and post-fixed for 2 h in 1% OsO₄ in 0.12 M sodium cacodylate buffer, pH 7.4. The samples were dehydrated in graded series of ethanol, transferred to propylene oxide and embedded in Epon (Hexion, Houston, TX, USA) according to standard procedures. Ultra thin sections were cut with a Reichert-Jung Ultracut E microtome and collected on Formvar membranes. Sections were stained with uranyl acetate and lead citrate and examined with a Philips CM 100 TEM (80 kV) equipped with a SIS MegaView2 camera. Digital images were recorded with the analySIS software package.

From each biopsy cross section, ten randomly sampled electron micrographs were recorded and subsequently analysed stereologically (C.A.S.T.-grid software, The International Stereology Center at Olympus, Denmark) to quantify the individual fibril diameter and area, as well as the cross-sectional number of fibrils per area as described previously (32, 33).

Hormone assays

Blood samples drawn from the antecubital vein were separated (3200 *g* at 4 °C), and serum was stored at

–80 °C. Serum GH (sGH) and serum IGF1 (sIGF1) concentrations were determined by a time-resolved immunofluorometric assay (Perkin Elmer, Turku, Finland). Serum IGFBP-1 (sIGFBP-1) was determined by an in-house RIA as described previously (34). sIGFBP-3 was measured by a commercially available IRMA (BioSource Europe, Nivelles, Belgium). All assays had intra- and inter-assay coefficients of variation below 5 and 10% respectively.

Statistical analysis

Using SigmaStat 3.5 (Systat Software, Inc., San Jose, CA, USA), differences between acromegalic and GHD patients were tested with unpaired, two-sided *t*-tests. Outcome was the difference between patient groups with 95% confidence interval (CI) and significance level (*P* value). Pearson's correlation analysis was used to determine baseline correlation between systemic IGF1/local IGF1 mRNA and collagen mRNA. Patient characteristics are presented as mean ± s.d. All other results are presented as mean ± s.e.m. mRNA data were log-transformed before statistical analysis and are presented as geometric mean ± back-transformed s.e.m. Differences were considered significant when *P* < 0.05.

Results

Systemic GH, IGF1 and IGFBP

Relative to age-matched controls, the acromegalic patient group had higher levels of sGH, sIGF1 and sIGFBP-3, while the GHD patient group had lower levels of sGH, sIGF1 and sIGFBP3 compared to healthy participants (all *P* < 0.05; Table 3). sGH was 30-fold (95% CI: 13; 46; *P* < 0.01) higher in acromegalic patients compared with GHD patients, and sIGF1 was 2.9-fold higher (95% CI: 1.5; 4.3; *P* < 0.01) in acromegalic patients relative to GHD patients. sIGFBP3 was 1.4-fold (95% CI: 1.2; 1.5; *P* < 0.01) higher in acromegalic patients compared to GHD patients, whereas sIGFBP1 did not differ between patient groups (*P* = 0.50) nor between acromegalic/GHD patients and controls (*P* = 0.13 and 0.40 respectively; Table 3).

Table 3 Levels of serum GH, insulin-like growth factor 1 (IGF1), IGF binding protein 1 (IGFBP1) and IGFBP3. Data are means ± s.e.m.

	GH-deficient patients	Age-matched controls	Acromegalic patients
sGH (µg/l)	0.16 ± 0.05	0.72 ± 0.26 ^{*,†}	2.40 ± 0.47 [‡]
sIGF1 (µg/l)	128 ± 15	189 ± 11 ^{*,†}	316 ± 53 [‡]
sIGFBP1 (µg/l)	37.1 ± 11.1	33.5 ± 2.5	46.7 ± 7.8
sIGFBP3 (µg/l)	3406 ± 157	3993 ± 151 ^{*,†}	4567 ± 134 [‡]

n = 9 for each group. Results are compared using unpaired, two-sided *t*-test. ^{*}Difference between controls and GH deficiency (*P* < 0.05). [†]Difference between controls and acromegaly (*P* < 0.05). [‡]Difference between GH deficiency and acromegaly (*P* < 0.01).

Two weeks of discontinued GH treatment in GHD patients did not result in a change (difference between pre GHD and post GHD) in the levels of sGH, sIGF1, sIGFBP1 or sIGFBP3 (all $P > 0.22$; data not shown).

Local collagen expression and collagen/myofibrillar protein synthesis

Muscle collagen I mRNA expression was 1.7-fold (95% CI: 1.1; 2.7; $P = 0.03$) higher and muscle collagen III mRNA expression was 1.6-fold (CI: 1.2; 2.1, $P = 0.02$) higher in acromegalic patients relative to GHD patients (Fig. 1C and D). Collagen protein FSR did not differ significantly between acromegalic and GHD patients in neither muscle ($P = 0.21$) nor tendon ($P = 0.15$; Fig. 2A and B). Pooled muscle and tendon collagen FSR was 1.2-fold (CI: 1.1; 1.4, $P = 0.04$) higher in acromegalic patients relative to GHD patients. Muscle myofibrillar protein FSR was similar in acromegalic and GHD patients ($P = 0.42$; Fig. 2C).

Local IGF1 expression

Local *IGF1Ea* mRNA expression in muscle was 2.5-fold (95% CI: 1.8; 3.4; $P < 0.01$) higher in acromegalic patients compared with GHD patients, and muscle *IGF1Ec* mRNA expression was 5.4-fold (95% CI: 2.2; 13.2; $P < 0.01$) higher in acromegalic patients relative to GHD patients (Fig. 1A and B).

Collagen morphology

There was a tendency towards a higher overall tendon collagen fibrillar area in GHD patients relative to acromegalic patients of 1.3-fold (95% CI: 0.9; 1.7; $P = 0.07$; Fig. 3B and Fig. 4). Tendon collagen fibrillar distribution tended towards a relatively larger concentration of small collagen fibrils in acromegalic patients relative to GHD patients (Fig. 3A and Fig. 4).

IGF1/collagen correlation

Local muscle *IGF1Ea* mRNA expression correlated significantly with both muscle collagen I mRNA expression ($r^2 = 0.55$, $P < 0.01$) and muscle collagen III mRNA expression ($r^2 = 0.59$, $P < 0.01$; not shown). sIGF1 did not correlate significantly with either collagen I mRNA expression ($r^2 = 0.12$, $P = 0.20$) or collagen III mRNA expression ($r^2 = 0.005$, $P = 0.79$; data not shown).

Average tracer to tracee ratios in plasma and tissue

$^{13}\text{C}/^{12}\text{C}$ proline was 0.18854 in plasma. In tissue, $^{13}\text{C}/^{12}\text{C}$ proline was 0.01091 in tendon collagen protein, 0.01090 in muscle collagen protein and 0.01090 in muscle myofibrillar protein.

Figure 1 Skeletal muscle collagen mRNA expression. Acromegalic and GH-deficient (GHD) patients ($n = 9$ for each patient group) were studied. Data are geometric means $\pm$ back-transformed S.E.M. Results were compared using unpaired, two-sided *t*-test. (A) *IGF1Ea* mRNA; (B) *IGF1Ec* mRNA; (C) collagen I mRNA; (D) collagen III mRNA. *Difference between acromegalic and GHD patients $P < 0.02$. **Difference between acromegalic and GHD patients $P < 0.01$.

Figure 2 Skeletal muscle and tendon protein fractional synthesis rate (FSR). Acromegalic and GH-deficient (GHD) patients ($n=9$ for each patient group) were studied. Data are means $\pm$ s.e.m. Results were compared using unpaired, two-sided t -test. (A) Tendon collagen protein fractional synthesis rate; (B) muscle collagen protein fractional synthesis rate; (C) muscle myofibrillar protein fractional synthesis rate. No significant differences were seen.

Discussion

The primary finding in the present study is that higher availability of GH and IGF1 in patients with acromegaly compared with GHD patients is associated with a higher expression of collagen in skeletal muscle, and a tendency towards a higher synthesis of collagen protein in muscle and tendon. Tendon collagen fibril

diameter tended towards an inverse relationship with GH/IGF1 levels, whereas the level of contractile myofibrillar protein synthesis was not associated with GH/IGF1 levels.

Collagen and myofibrillar protein

The significantly higher collagen expression observed in acromegalic patients versus GHD patients strongly suggests the involvement of GH/IGF1 in the regulation of musculoskeletal collagen production in man, and shows an increased potential for collagen synthesis in musculoskeletal tissue after years of disease despite hormone-regulating treatment. The clear differences in mRNA expression for collagen in muscle between acromegalic and GHD patients were accompanied by non-significant differences in tissue-specific collagen protein synthesis. This lack of a clear association between tissue-specific collagen mRNA and collagen protein synthesis differences is in contrast to previous observations made in healthy participants following 2 weeks of GH supplementation (12), and is likely a consequence of inherent differences in mRNA expression and protein FSR measurements. The measure of collagen synthesis, expressed as a fraction of the total collagen pool size, is only completely comparable between groups with similar collagen pool sizes (35). However, as acromegalic patients presumably have substantially larger collagen tissues compared to both healthy controls and GHD patients (36, 37), our data imply that despite a significantly larger collagen pool, the FSR tended to be higher in patients with acromegaly. This means a higher musculoskeletal collagen turnover rate in acromegalic patients when expressed in absolute terms. Although measuring protein synthesis as a fraction would thus seem less suitable for the type of experiment presented here,

Figure 3 Quantitative tendon collagen morphology. Acromegalic and GH-deficient (GHD) patients ($n=9$ for each patient group) were studied. Data are means $\pm$ s.e.m. Results were compared using unpaired, two-sided t -test. (A) Tendon collagen fibril size distribution; (B) mean tendon collagen fibril area. *Difference between GHD and acromegaly $P=0.07$.

Figure 4 Transmission electron microscopy analysis of tendon. Acromegalic and GH-deficient (GHD) patients ($n=9$ for each patient group) were studied. Images illustrate differences in collagen fibril size distribution and collagen fibril area. (A) Acromegalic patients; (B) GHD patients.

the lack of a well-established method for determining total tissue collagen pool size makes absolute collagen synthesis measurements impossible.

It cannot be excluded that compartmental non-steady-state proline tracer dynamics can be influenced by GH/IGF1 via altered water and compartmental tissue distribution. If present, we find that this could result in an increased concentration of non-labelled proline and thus a relatively lower proline enrichment in acromegalic patients versus GHD patients, resulting in an underestimation of protein FSR in acromegalic patients relative to GHD patients.

Finally, the possibility of a type 2 error cannot be ruled out, given the relatively small number of patients in this study as well as the observation that collagen FSR was significantly higher in acromegalic patients relative to GHD patients when muscle and tendon data were pooled.

The observation that local IGF1 expression is positively correlated with local collagen expression, and is also probably associated with the absolute synthesis of collagen, is supported by *in vitro* studies showing a dose-dependent effect of IGF1 on collagen synthesis in animal tendon explants (38–40). However, it has to be acknowledged that the limited number of *in vivo* studies in animals measuring the effect of GH supplementation on tendon/muscle collagen synthesis have yielded somewhat conflicting results (41, 42). Correlations between circulating collagen propeptides (e.g. collagen I carboxyterminal propeptide (PICP)) and GH/IGF1 are observed in acromegalic and GHD patients (43, 44) and are compatible with our observations. However, despite commonly being described as a marker of bone-specific collagen synthesis (43, 45), circulating PICP measurements are clearly inconclusive regarding tissue-specific changes, such as tendon and skeletal muscle, because collagen I from other tissues likely contributes substantially to circulating PICP (46).

Myofibrillar protein synthesis was not statistically different between acromegalic and GHD patients. This was an expected result based on previous findings in healthy adult individuals (12, 19), and suggests that

myofibrillar protein, unlike collagen, is unaffected by GH or IGF1 in fully-grown individuals (18).

There was no correlation between collagen mRNA or collagen protein synthesis and duration of disease or type of treatment in neither acromegalic nor GHD patients. Nor was collagen mRNA/protein synthesis correlated with s-testosterone (data not shown) or glucocorticoid treatment.

Systemic and local levels of IGF1

As expected, acromegalic patients had significantly higher levels of circulating GH/IGF1, and conversely, GHD patients had significantly lower levels of circulating GH/IGF1 relative to healthy controls (Table 3). This observation is of fundamental importance for the study, and confirmed that the patients differed from the normal population with respect to systemic GH/IGF1, despite being involved in hormone-regulating treatment.

We measured IGFBP1, which is believed to act as a blocker of IGF1 actions (47), as well as IGFBP3, which is thought to be a buffer for IGF1, thereby facilitating a pool of IGF1 in local tissues and thus IGF1 receptor ligand binding (48). The observation that sIGFBP1 levels were similar in both patient groups, whereas sIGFBP3 was relatively higher in acromegalic patients (Table 3), confirms previous observations (49, 50), and show that the final outcomes in the present study were not influenced by unexpected fluctuations in IGFBP1 and IGFBP3.

The expression of *IGF1Ea* and *IGF1Ec* mRNA in skeletal muscle was elevated in acromegalic patients compared with GHD patients (Fig. 1), suggesting that long-term elevated GH levels mediate a continuous activation of the local autocrine/paracrine IGF1 signalling system. A significant correlation between local *IGF1* mRNA and collagen mRNA in muscle was demonstrated, whereas no significant correlations between sGH or sIGF1 and collagen expression were observed (data not shown). This suggests a role for local IGF1 signalling, a notion that is supported by animal studies, showing that circulating IGF1 has little effect on overall growth in mice (10), while locally produced IGF1 is a prerequisite for GH-stimulatory effects in muscle and cartilage (51, 52). IGF1 is known to induce collagen synthesis in animal tendon explants (39, 40, 53), and in mice that overexpress human IGF1 locally, it was demonstrated that the collagen content was elevated in heart muscle (54). These findings are in accordance with our results and support the view that IGF1, rather than GH, is directly responsible for the elevated collagen expression and protein synthesis seen in the present study. Measuring local IGF1 could prove a relevant diagnostic tool in monitoring the treatment of acromegalic and GHD patients with musculoskeletal disorders, supplementing current monitoring strategies using circulating IGF1 (55).

Quantitative collagen morphology

There was a tendency towards smaller tendon collagen fibril area and a higher concentration of small collagen fibrils in acromegalic patients compared with GHD patients (Figs 3B and 4). This suggests that a chronically high level of GH/IGF1, with a concomitant increase in collagen expression and in absolute collagen synthesis, correlates to smaller collagen fibril area in tendon. In the only other *in vivo* study correlating the level of GH to collagen fibril dimensions, bone collagen fibril diameter was found to be larger in GHD rats compared to wild-type controls (13), which is in support of the observations in the present study. GH/IGF1 could potentially affect known regulators of collagen fibril diameter/area such as proteoglycans including decorin (56, 57). However, the effect of GH on fibrillar diameter/area could also be due to a consequence of changes in collagen synthesis *per se*, which would suggest a general negative correlation between collagen synthesis and collagen fibril diameter. It is possible that the observed association between GH/IGF1 and collagen morphology in human tendon also exists in other musculoskeletal tissues. This is interesting, given that GHD rats have significantly decreased bone strength (13), and that GHD patients suffer from a high incidence of bone fractures (14).

Study design

An important limitation of the present study design is the lack of a healthy control group to establish tissue collagen expression and synthesis in persons with normal GH/IGF1 levels. The presence of a healthy control group would clearly have allowed for a more complete understanding of the role of GH/IGF1 under physiological and pathological conditions.

Conclusion and perspectives

Our study shows that the level of circulating GH/IGF1 is positively associated with local *IGF1* mRNA expression in musculotendinous tissue when comparing acromegalic and GHD patients. This indicates that a long-term elevation in GH and IGF1 levels was causing a continuous elevation in human collagen production. The lack of a significant difference in myofibrillar protein synthesis rate between patient groups indicates that myofibrillar protein synthesis, and thus muscle size/strength, is not affected by elevated levels of GH/IGF1. Low collagen fibril area was associated with high levels of GH/IGF1 and elevated collagen production in acromegalic patients, which suggests an involvement of GH/IGF1 in the regulation of collagen morphology and thus tissue quality. Despite the limited number of patients and the potential influence of other hormonal factors, the present observation contributes to the

understanding of the connective tissue pathways in patients with either high or low GH/IGF1 axis activity. Moreover, these findings strongly suggest that the level of GH/IGF1 is correlated with both the quality and the regenerative capacity of collagen tissue. This is relevant from a clinical perspective given the large number of acromegalic and GHD patients suffering from musculoskeletal disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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