CLINICAL STUDY

Increased serum and bone matrix levels of transforming growth factor β1 in patients with GH deficiency in response to GH treatment

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Abstract

Objective: Patients with adult onset GH deficiency (aoGHD) have secondary osteoporosis, which is reversed by long-term GH substitution. Transforming growth factor β1 (TGFβ1 or TGFB1) is abundant in bone tissue and could mediate some effects of GH/IGFs on bone. We investigated its regulation by GH/IGF1 in vivo and in vitro.

Design and methods: The effects of GH substitution (9–12 months, placebo controlled) on circulating and cortical bone matrix contents of TGFβ1 were investigated in patients with aoGHD. The effects of GH/IGF1 on TGFβ1 secretion in osteoblasts (hFOB), adipocytes, and THP-1 macrophages as well as the effects on release from platelets were investigated in vitro.

Results: In vivo GH substitution increased TGFβ1 protein levels in cortical bone and serum. In vitro, GH/IGF1 stimulation induced a significant increase in TGFβ1 secretion in hFOB. In contrast, no major effect of GH/IGF1 on TGFβ1 was found in adipocytes and THP-1 macrophages. Finally, a minor modifying effect on SFLLRN-stimulated platelet release of TGFβ1 was observed in the presence of IGF1.

Conclusion: GH substitution increases TGFβ1 in vivo and in vitro, and this effect could contribute to improved bone metabolism during such therapy, potentially reflecting direct effect of GH/IGF1 on bone cells.

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Introduction

Patients with adult onset GH deficiency (aoGHD) have secondary osteoporosis characterized by reduced bone mass, decreased bone turnover, and increased fracture risk (1). These conditions are reversed by long-term GH substitution and are associated with increases in cortical bone matrix contents of insulin-like growth factor 1 (IGF1), substantiating that the beneficial effects of GH are partly mediated by locally produced IGFs (2). However, other growth factors and cytokines could contribute to the effects of GH on bone remodeling (3, 4).

Transforming growth factor β1 (TGFβ1 or TGFB1) is an important regulator of bone metabolism and bone extracellular matrix (ECM) is a major storage site for TGFβ1, stored in a latent form bound to TGFβ-binding protein 1 (LTBP1) (5–7). Once released from ECM and activated by resorbing osteoclasts, TGFβ may act as a coupling factor coordinating bone resorption and subsequent bone formation by influencing the remodeling pathway, including effects on osteoclast formation and activity, recruitment and proliferation of osteoblast precursors, and stimulation of mature osteoblasts to produce bone matrix proteins as well as by exerting anti-apoptotic effects (6). Importantly, LTBP1 is a substrate for matrix metalloproteinases (MMPs), and LTBP1 proteolysis may be a physiological mechanism for the release of TGFβ from ECM-bound stores (7).

In vitro, TGFβ1 expression is regulated by systemic hormones, growth factors, and cytokines present in the bone microenvironment (6, 8). Both GH and IGF1 may increase the activity of relevant MMPs in human osteoblasts potentially promoting TGFβ release (9, 10), and there are several reports on the effect of IGF1 and TGFβ on bone metabolism showing enhancing but also attenuating effects when combined (11–13). However, the relationship between GH/IGF1 and TGFβ1 in bone metabolism in aoGHD is unknown, and there is no in vivo data on the interaction between these mediators in these patients.
The aim of this study was to examine the relationship between GH/IGF1 and TGFβ1 in aoGHD by comparing bone matrix and circulating TGFβ1 levels in patients with aoGHD receiving GH substitution. In addition, we examined the effects of GH/IGF1 on TGFβ1 in cultures of various cells in relevance to GHD (e.g. human osteoblasts and adipocytes).

Materials and methods

Study populations

The study comprised of two study populations (Table 1) as described previously (2, 14). In study 1, we examined bone matrix levels of TGFβ1 in cortical bone powder after extraction (see details below), available from 20 patients with aoGHD of at least 12 months duration (2). Additional hormone replacement was continued unchanged throughout the study, and no patients had previously been treated with GH. The study was double-blind, randomized, and placebo-controlled, with duration of 12 months. Patients were stratified according to sex, age above or below 50 years, and pituitary disease (Cushing/non-Cushing). After randomization, patients were assigned GH (Norditropin; Novo Nordisk A/S, Gentofte, Denmark), administered s.c. as self-injections daily at bedtime in a dosage of 2.0 IU/m² per day or placebo preparations administered in a similar fashion. From each patient, fasting blood samples and an iliac crest bone biopsy were obtained under local anesthesia at baseline and after 12 months, using a modified Bordier trephine (inner diameter 9 mm) from the standard site 2 cm below the iliac crest and 2 cm behind the anterior superior iliac spine. The samples were frozen at −40 °C immediately after removal. Whilst frozen, the biopsies were later sawed carefully to divide cortical and trabecular bone (2).

In study 2, serum TGFβ1 levels were measured in fasting blood samples from 55 subjects who had severe aoGHD of at least 2 years duration, randomized to GH (Genotropin; Pfizer, Inc., New York, NY, USA) or placebo (Table 1). Each treatment period lasted 9 months, with a 4-month washout interval between periods. GH replacement therapy was dosed individually to maintain serum IGF1 concentrations between the mean±1 s.d. of the sex- and age-related reference range. Additional hormone replacement was continued unchanged throughout both studies.

An independent ethics committee approved both study protocols, informed consent was obtained from all subjects, and the studies were conducted according to the Declaration of Helsinki II and the guidelines of Good Clinical Practice.

Table 1 Characteristics of the study populations

<table>
<thead>
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<th>Study</th>
<th>Placebo (n=10)</th>
<th>GH (n=10)</th>
<th>n=55</th>
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<td><strong>Age (mean±s.d.)</strong></td>
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<td>51±6</td>
<td>49±10</td>
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<td><strong>Sex (male/female)</strong></td>
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<td>6/4</td>
<td>31/24</td>
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<tr>
<td><strong>BMI (mean±s.d.)</strong></td>
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<td>18</td>
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</table>

Bone samples were treated as described previously (2). Briefly, cortical bone specimens were washed with water to remove soft tissue and blood, defatted in trichloroethylene for 6 days (changed every second day) at 4 °C and dried by immersion in ethanol/ether (1:1). The samples were pulverized in a liquid nitrogen-cooled mortar and pestle, passed through an 84 μm sieve, and stored at −40 °C until use. For determining TGFβ1 and total protein contents, 15 mg of bone powder were extracted once with 1.5 ml of 0.5 mol/l ammonium EDTA (pH 6.2) and once with 1.5 ml of 4 mol/l guanidinium–HCl (pH 7.4), both containing protease inhibitors as described (2). Extractions were carried out for 18 h at 4 °C by rotation, the solution was centrifuged (12 000 g for 30 min), and the supernatant was separated from the remaining bone residues. Supernatants from both extractions were combined and desalted in Sephadex PD-10 columns (Amersham Pharmacia Biotech), lyophilized in a Speed Vac Concentrator (Savant Instruments, Hicksvill, NY, USA), and stored at −80 °C until assayed. We have previously determined albumin in our bone samples as a marker of blood contamination and found no detectable levels (2).

Body composition

In study 2, total body composition including total lean mass, fat mass, and bone mineral density was measured by DEXA (DPX-L, software version 1.31; Lunar Corp., Madison, WI, USA).

Cell culture

The hFOB cell line 1.19 was obtained from American Type Culture Collection (Rockville, MD, USA) and cultured as described previously (15). hFOB cells were differentiated for 2 days, followed by culturing with and without recombinant human GH and IGF1 (R&D Systems, Minneapolis, MN, USA) for 3, 6, and 24 h. In some experiments, a blocking antibody against the IGF1 receptor (IGF1R) was used (concentration 20 μg/ml, R&D Systems). The primary human subcutaneous and visceral preadipocytes (Lonza Walkersville, Inc., Walkersville, MD, USA) were cultured in preadipocyte
basal medium-2 (PBM-2; Lonza) supplemented with FCS (10%), glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 µg/ml). Cells were maintained at 37 °C in a humidified 5% CO2 and seeded at a density of 40,000 cells/well in 12-well plates (Costar, Cambridge, MA, USA). Confluent preadipocyte cultures were induced to differentiate using differentiation media (PBM-2 with FCS (10%), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), insulin (0.5 µM), dexamethasone (0.1 µM), indomethacin (50 µM), and isobutyl-1-methylxanthine (0.5 mM)). Adipocyte differentiation was documented by staining with AdipoRed reagent (Lonza). The ratio of differentiated cells/undifferentiated cells after 14 days of differentiation exceeded 10 as assessed by relative fluorescence units. In differentiation experiments, confluent preadipocytes were washed twice in PBS and incubated with differentiation media without FCS and cultured overnight in differentiation media without FCS, with different concentrations of GH and IGF1 in quadruplicate. In separate experiments, cells were cultured for 14 days in differentiation media, washed twice with PBS, and cultured overnight in differentiation media without FCS, with different concentrations of GH and IGF1 in quadruplicate. The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MA, USA) was differentiated into macrophages by incubation with phorbol myristate acetate (100 nM; Sigma) (16) for 72 h before stimulation with GH or IGF1 (200 ng/ml each) for 6 and 24 h. Preparation and stimulation of citrated platelet-rich plasma were performed as described previously (17), incubated at 22 °C with Tris-buffered saline or 10 µM of the thrombin receptor agonist SFLLRN (The Biotechnology Centre of Oslo, Oslo, Norway), alone or in combination with GH or IGF1 (200 ng/ml each). After 90 min, aliquots were centrifuged at 13,000 g for 5 min to obtain platelet-free plasma. In all experiments, cell-free supernatants were stored at indicated time points at −80 °C until analysis.

Blood sampling and immunoassays

Serum for both populations was isolated from blood samples drawn after an overnight fast and frozen at −80 °C until analysis. Serum IGF1 was measured as described previously in studies 1 and 2. IGF1 in bone extracts was measured as described (2, 18).

Assay for TGFβ1

The lyophilized ammonium and guanidine extracts were redissolved in sample diluent consisting of 1.4% delipidized BSA in PBS and 0.05% Tween. Bone matrix, serum, and conditioned media levels of TGFβ1 were quantified by enzyme immunoassay (ELISA) using matched commercially available antibodies (R&D Systems) after activation according to the manufacturer’s instructions. Intra-assay (n = 8) and inter-assay (n = 4) variation for bone homogenates was 4.8 and 5.9% respectively. Sensitivity defined as 3σs.d. of the blanks was 21 pg/ml. The mean recovery of two samples spiked with different concentrations of recombinant TGFβ1 was 115%. Parallel dilution of two samples gave a recovery of 101, 103, and 96% of expected values at 1:2, 1:4, and 1:8 times dilution respectively. To evaluate the extraction efficiency for bone matrix TGFβ1, ten cortical bone specimens were extracted with two additional guanidinium–HCl extractions, and all supernatants were analyzed separately. The extraction efficiency after the two extractions used in the study was 98.7% (17.6 ± 1.5% (mean ± s.d.) for the ammonium EDTA extraction and 81.1 ± 1.6% after the first guanidinium–HCl extraction). TGFβ1 concentrations in bone matrix were adjusted for total protein (Bio-Rad Laboratories, Inc. GmbH). For serum samples, we observed an intra-assay coefficient of variation (CV) of 3.4% (n = 8) and an inter-assay CV of 3.7% (n = 4). The mean recovery of two serum samples spiked with different concentrations of recombinant TGFβ1 was 105%. Parallel dilution of two samples gave a recovery of 93, 92, and 97% at 1:2, 1:4, and 1:8 times dilution respectively. The detection limit was similar as for bone homogenates. For serum, we also investigated the effect of repeated freeze thaw cycles and long-term storage on TGFβ1 levels. The mean CV between fresh samples and aliquots that were thawed 1, 2, and 3 times was 3, 5, and 11% respectively (decrease observed with increase in cycles). The mean CV between the immediately frozen sample and the samples exposed to room temperature for 1, 4, and 24 h was 13, 15, and 21% respectively (decrease observed over time).

RNA extraction and real-time quantitative PCR

Culture media were collected; cells were harvested; total RNA was isolated, and RNA integrity and quantity were assessed as described (15). RT was performed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) with total RNA between 0.5 and 1 µg. For real-time RT-PCR, sequence-specific oligonucleotide primers for TGFβ1 (forward AATTGAGGCCTTCCGCCTTAG and reverse CCGGTAGTAAACCCTTGAT) were designed using Primer Express software version 2.0 (Applied Biosystems). SYBR Green assay was performed with the qPCR Master Mix for SYBR Green I (Eurogentec, Seraing, Belgium). Quantification of mRNA was performed using the standard curve method of the ABI Prism 7500 (Applied Biosystems). Data were normalized to β-actin (forward AGGCACCAGGCGTGAT and reverse TCGTCCAGTTGGTGAGCT). Statistical analysis

In study 1, the parameters were not normally distributed, and the effects of GH treatment were tested by comparing changes in the measured parameters...
from baseline to 12 months in the two groups with the Mann–Whitney U test. Within-group changes were tested with the Wilcoxon signed-rank test. In study 2, data were normally distributed and parametric statistics were used throughout. The effect of GH therapy compared with placebo was estimated by the crossover effect, calculated by the difference between GH and placebo (baseline to 9-month changes). Using mean values, a point estimate and a two-sided 95% confidence interval of the crossover differences were calculated. Only subjects participating in the study for at least 3 months of the second period were included in the analyses.

In vitro experiments were evaluated with unpaired t-tests. Relationships between variables were tested by the Spearman (study 1) and Pearson (study 2) correlation analyses, and the level of significance was set at P<0.05.

Results

The association between TGFβ1 and GH/IGF1 in aoGHD in vivo: study 1

While there were no changes in cortical bone matrix protein levels of TGFβ1 during placebo in study 1, GH substitution increased TGFβ1 protein levels in this compartment (Fig. 1B), resulting in a significant treatment effect compared with placebo (median and interquartile range: −8 (−21, 12) vs 46 (32, 67) ng/mg total protein, placebo versus GH, respectively, P<0.001). In cortical bone, baseline TGFβ1 was correlated with IGF1 (Fig. 1B), but not with circulating IGF1 (r = −0.06, P = 0.818). In contrast, the change in cortical TGFβ1 was associated with the change in serum IGF1 (Fig. 1C), but not with changes in cortical IGF1 (r = 0.32, P = 0.176).

The association between TGFβ1 and GH/IGF1 in aoGHD in vivo: study 2

In study 2, we found that while GH substitution induced an increase in serum levels of TGFβ1, there were no changes in TGFβ1 levels during placebo substitution, resulting in a significant treatment effect of GH (Fig. 2A). The change in serum TGFβ1 tended to correlate with the change in IGF1 (Fig. 2B) and was associated with a significant decrease in total body fat mass, but not in total bone density, and baseline levels of TGFβ1 and total body fat mass also tended to be inversely correlated (Fig. 2C).

Effects of GH/IGF1 on TGFβ1 in hFOB

Our findings may suggest a significant relationship between GH/IGF1 and TGFβ1 in aoGHD in vivo, in particular within bone. To further elucidate this interaction, we investigated the effects of GH/IGF1 on TGFβ1 secretion in different relevant cell types. In osteoblasts (hFOB), 6 h of GH stimulation induced a significant decrease in TGFβ1 mRNA with no effects of IGF1 (Fig. 3A). In contrast, GH significantly increased TGFβ1 protein secretion after 6 h compared with
unstimulated levels (Fig. 3B). A similar but more modest effect was observed for IGF1 at 200 ng/ml (Fig. 3B). When looking at the time course during GH and IGF1 stimulation (both 200 ng/ml), no significant effects on TGF\(\beta1\) mRNA were observed (Fig. 3C). For secreted protein, a similar increase in TGF\(\beta1\) was observed at 3 and 6 h after GH and IGF1 stimulation, with a slightly more potent effect of GH (Fig. 3D). To investigate whether the effects of GH are mediated by IGF1, we investigated the combined effect of GH/IGF1 on TGF\(\beta1\) synthesis and release as well as using an anti IGF1R antibody when stimulating with GH. As shown in Fig. 3E, no effects were observed from these experiments on TGF\(\beta1\) mRNA levels at 6 h. For secreted TGF, under these conditions were not detectable in conditioned media. When mature subcutaneous and visceral adipocytes were stimulated with 100 and RT-PCR, indicating a large amount of TGF\(\beta1\) copy numbers. Compared with the relatively modest amounts secreted by osteoblasts (100–300 pg/ml), this could indicate a large intracellular pool of TGF\(\beta1\) in these cells.

**Effects of GH/IGF1 on TGF\(\beta1\) in adipocytes**

TGF\(\beta1\) is produced by adipocytes and may regulate adipogenesis and adiposity (19). We therefore investigated the effects of GH and IGF1 in regulating TGF\(\beta1\) synthesis and secretion in differentiating as well as in mature adipocytes. Figure 4A shows the presence of intracellular lipid droplets in mature but not in undifferentiated subcutaneous adipocytes. When differentiating subcutaneous were stimulated with 100 and 500 ng/ml GH and IGF1 after 7 and 14 days, no effects on TGF\(\beta1\) mRNA expression were observed, except for an increase by 100 ng/ml GH at 14 days (Fig. 4B). Secreted TGF under these conditions were not detectable in conditioned media. When mature subcutaneous and visceral adipocytes were stimulated with 100 and
500 ng/ml GH and IGF1 for 6 h, no effects were observed on TGFβ1 mRNA expression (Fig. 4C and D). Similarly, no effects were observed on TGFβ1 mRNA expression when subcutaneous and visceral adipocytes were stimulated with 100 ng/ml (or 500 ng/ml, data not shown) GH and IGF1 for 3, 6, and 12 h (Fig. 4E and F). Again, secreted TGF under these conditions was not detectable in conditioned media.

**Effects of GH/IGF1 on TGFβ1 release from inactivated platelets and macrophages**

Macrophages and, in particular, blood platelets may be a source of circulating TGFβ1, and we next examined whether GH and IGF1 could regulate the release of TGFβ1 in these cells. When THP-1 macrophages were stimulated 200 ng/ml GH and IGF1 for 6 and 24 h, a small decrease in TGFβ1 was observed after 24 h (Fig. 5A). Finally, the thrombin receptor agonist SFLLRN (10 μM) markedly enhanced the release of TGFβ1 from platelets, and as shown in Fig. 5B, GH but not IGF1 (both 200 ng/ml) significantly enhanced the effect of SFLLRN, although the effect was rather modest.

**Discussion**

GHD serves as a model in which the effect of chronic GH on skeletal metabolism can be studied. In this study, we show that GH replacement therapy in aoGHD induced a significant increase in TGFβ1 levels as shown both in bone matrix and in serum. In vitro, no effect of GH or IGF1 was observed on TGFβ1 mRNA levels, but a significant effect of IGF1 and, in particular, GH on TGFβ1 secretion was observed in human osteoblasts. Together, these data indicate that both GH and IGF1 may stimulate the release of TGFβ1 from osteoblasts, which could contribute to the beneficial effects of GH substitution on bone metabolism.

The effects of GH on bone tissue are mediated through a complex interaction of circulating GH, IGFs, IGF-binding proteins, and locally produced IGFs and binding proteins, acting in an autocrine and a paracrine way (1). Thus, we have previously demonstrated that long-term GH treatment increases the accumulation of IGF1 in cortical bone in patients with GHD (2), and that acromegalic patients, characterized by excess GH, circulating IGF1, and bone mass, have increased cortical bone content of IGF1 (18). This study extends these findings and shows that GH substitution increases the accumulation of TGFβ1 in cortical bone. Similar to IGFs, TGFβ1 can be released from the ECM during bone resorption to act as a local determinant of site-specific coupled bone formation (6, 7, 20). Consequently, an increase in bone matrix TGFβ1 during GH substitution may reflect increased bone turnover with effects of GH/IGF1 on bone resorption and release/activation of latent TGFβ1, which may contribute to a synchronized increase in bone turnover and in due course a higher bone mass and improved skeletal biomechanical competence during GH substitution in GHD.

The tight correlation between bone matrix IGF1 and TGFβ1 as well as between the change in serum IGF1 and bone matrix TGFβ1 supports an association between GH/IGF1-driven bone turnover and release of TGFβ1 from ECM but may also suggest direct effects of GH/IGF1 on TGFβ1 synthesis in osteoblasts. Indeed, an effect of IGF1 and, in particular, GH on secretion of TGFβ1 in hFOB was observed. In contrast, we observed no effect of GH or IGF1 on TGFβ1 mRNA levels, indicating that the increased TGFβ1 protein levels are largely due to increased secretion or potentially post-transcriptional modification and not de novo synthesis. Both GH and TGFβ1 play critical roles in bone biology, and although in this study we cannot elucidate a direct interplay between these at the molecular level, the decrease in TGFβ1 mRNA after 6 h could reflect some interactions between these pathways, unrelated to the GH-stimulating effect on TGFβ1 protein release. Moreover, we observed no combined effect of GH/IGF1 on TGFβ1 secretion or inhibiting effect of an antibody against the IGF1R when stimulating with GH. Thus, although we cannot exclude some interaction, these data suggest that the effects of GH are not primarily dependent on de novo IGF1 synthesis.

Taken together, these data may indicate that the increase in bone matrix TGFβ1 during GH therapy could partly reflect an enhancing effect of GH and IGF1 on TGFβ1 secretion from osteoblasts. In addition to the latent stored pool of TGFβ1 in ECM, TGFβ1 is also abundantly localized in the cytoplasm, especially in the Golgi apparatus and endoplasmic reticulum prior to secretion (21, 22). However, the mechanism by which GH and IGF1 may increase the secretion of latent TGFβ1 from intracellular storage in osteoblasts is unknown and will have to be investigated in future studies. Our data may also suggest that osteoblasts contain a large pool of TGFβ1 that is not released upon GH/IGF1 activation.

Although the skeleton is a major storage site, it is unknown whether the increase in serum TGFβ1 during GH substitution reflects an increase and spillover from
bone matrix due to increased bone turnover. TGFβ1 is expressed in most cells, and the negative correlation between changes in total body fat mass and TGFβ1 during therapy may suggest other sources as well. Adults with GHD are characterized by central adiposity and low-grade systemic inflammation (23), which are reversed by GH substitution. TGFβ1 may attenuate inflammation (24), and an increase in TGFβ1 during GH treatment could therefore represent immunomodulating effects of GH on adipose tissue including adipocytes and macrophages. However, in contrast to the increase in hFOB, no significant effects of GH/IGF1 on TGFβ1 mRNA or protein secretion were observed in subcutaneous or visceral adipocytes. Finally, platelets are a major source for circulating TGFβ1 (25), and the IGF1R are present on platelets and may enhance platelet aggregation when activated (26, 27). Indeed, stimulation with GH combined with the thrombin receptor agonist SFLRN, a potent activator of platelet activation in relevance to the in vivo situation, revealed a modest but significant enhancing effect on TGFβ1 release from platelets, which could contribute to the circulating pool of TGFβ1 during GH substitution.

In this study, we observed an increase in circulating and cortical bone matrix protein levels of TGFβ1 in response to 9–12 months of treatment with GH. Stimulation of human osteoblasts with IGF1 and, in particular, GH increased the secretion of TGFβ1. This could contribute to the improved bone metabolism during such therapy in GHD, potentially reflecting direct effect of GH/IGF1 on bone cells.

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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