

Growth hormone stimulates osteoprotegerin expression and secretion in human osteoblast-like cells

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Abstract

It is presently thought that osteoprotegerin (OPG) is a cytokine involved in the regulation of osteoblast/osteoclast crosstalk and maintenance of bone mass. Recent studies showed that GH replacement therapy in GH-deficient patients was able to induce a significant increase of OPG in the plasma, as well as in the cortical and the trabecular bone. In order to determine whether GH could directly modulate OPG secretion, the effect of GH on human osteoblast-like cells (hOB) in primary culture was studied. After detecting the presence of the mRNA for the GH receptor (GHR) by RT-PCR, hOB were exposed to increasing concentrations of GH, from 0.1 to 25 ng/ml, for 24 h. The results showed that GH exposure was able to stimulate OPG secretion in a concentration-dependent manner. In addition, the OPG mRNA levels were increased, indicating that the

hormone has a stimulatory effect on gene expression. The stimulatory effect on OPG expression and production was prevented by exposing the cells to tyrphostin AG490 (10 µM), an inhibitor of Janus kinase 2, which is one of the kinases involved in the intracellular pathway activated by the binding of GH to its receptor. Similar results were obtained when the cells were exposed to a receptor antagonist of GH, pegvisomant at 50 nM. GH exposure neither induced an increase in IGF-I expression nor secretion in hOB. These results suggest that the stimulation of OPG production induced by GH in hOB is specific and receptor mediated and further support the view that GH is able to modulate bone remodeling by directly influencing osteoblast-osteoclast crosstalk.

Journal of Endocrinology (2007) **192**, 639–645

Introduction

Besides growth hormone (GH) effect on skeletal growth, the hormone plays a regulatory role on bone remodeling through the concerted action of GH itself, insulin-like growth factors (IGFs), and IGF-binding protein (IGFBP) produced either by the liver or locally (Ohlsson *et al.* 1998, Ueland 2004). The GH regulatory role in bone remodeling and maintenance of bone mass has been clearly established in clinical studies, where patients with acquired GH deficiency in adulthood (GHD) were found to have secondary osteoporosis, characterized by reduced bone mass, increased fracture risk, and decreased bone remodeling (Holmes *et al.* 1994, Wuster *et al.* 2001). Replacement therapy in GHD patients induces a dose-dependent increase of bone remodeling, as evaluated by relevant biochemical markers that peak at 3–6 months after the start of treatment and remain elevated throughout the 2 years of observation time (Nielsen *et al.* 1991, Joannsson *et al.* 1996). Acromegalic patients, who have chronic systemic GH and IGF-I excess, are characterized by increased bone turnover and biochemical markers of bone formation and bone resorption that correlate with circulating GH and IGF-I levels, suggesting the activation of both osteoblasts and osteoclasts in modulating bone turnover (Ezzat *et al.* 1993).

Maor *et al.* (1989) showed that GH is able to directly induce bone formation *in vitro*. The effects of GH on bone cells are mediated through the functional GH receptors (GHRs). Expression of the GHR has been reported in cultured human osteoblast-like cells (hOB; Nilsson *et al.* 1995) and in rat osteoblast-like cell lines (Barnard *et al.* 1991). The receptor is a member of the cytokine/hematopoietic receptor superfamily that has no intrinsic tyrosine kinase activity (Kelly *et al.* 1993). Binding of GH to GHR promotes receptor dimerization and initiates a cascade of events leading to protein phosphorylation and activation of nuclear proteins and transcription factors. In osteoblasts, binding of GH to its receptor leads to the activation of a cytoplasmic tyrosine kinase, Janus kinase 2 (Jak2), that phosphorylates its own and GHR tyrosine residues (Argetsinger *et al.* 1993), which in turn activates several members of the STAT family of transcriptional factors (Gerland *et al.* 2000). As summarized by Ueland (2004), GH and IGF-I/-II may regulate osteoblast proliferation (Kassem *et al.* 1993, Nilsson *et al.* 1995), while GH has IGF-independent effects on differentiation. In fact, the hormone induces an increase in the markers of osteoblast differentiation, such as osteocalcin and alkaline phosphatase (Kassem *et al.* 1993). GH is able to stimulate osteoclastic bone resorption through direct and indirect actions on osteoclast

differentiation and through indirect activation of mature osteoclasts, possibly through local IGF-I/-II (Nishiyama *et al.* 1996, Kanatani *et al.* 2000).

Critical for the bone resorptive process is the balance between the recently discovered member of the tumor necrosis factor (TNF) ligands and family of receptors, osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL) produced by osteoblasts, which are the dominant mediators of osteoclastogenesis under osteoblast control. OPG acts as an endogenous inhibitor of osteoclast differentiation and activation by binding to RANKL, and thus neutralizing and preventing its binding to the specific receptor RANK expressed on osteoclasts and their precursors (Hofbauer *et al.* 2000), resulting in decreased osteoclastogenesis and osteoclast function. Several hormones, growth factors, cytokines, and prostaglandins regulate the OPG/RANKL/RANK system through their direct effects on bone cells (Cheung *et al.* 2003, Suda *et al.* 2004). Even GH appears to modulate this system, since GH replacement therapy enhances OPG levels both in blood (Lanzi *et al.* 2003) and in trabecular and cortical bone explants from GH-deficient patients (Ueland *et al.* 2002). Since the production of OPG is required for the maintenance of bone mass and GH has anabolic effects on the bone, the aim of the present study was to investigate whether GH could modulate OPG expression and production in hOB. On the basis of the results obtained, it was evaluated whether GH effects on hOB were direct or mediated by the locally produced IGF-I.

Materials and Methods

Cell culture

Bone cells were established in culture by a modification of the Gehron Robey & Termine (1985) procedure from trabecular bone samples obtained from waste material from orthopedic surgery for degenerative diseases or traumatic fractures of the femoral neck requiring osteotomy procedures. Donors gave their informed consent for the use of the waste material. None of the patients (51–73 years old) submitted to surgery had any malignant or metabolic bone diseases other than senile osteoporosis and osteoarthritis. Briefly, the trabecular bone was cut into small pieces (2×2×2 mm) and washed thoroughly with commercial standardized Joklik's modified MEM serum-free medium (Sigma) to remove nonadherent marrow cells. The bone pieces were incubated with the same medium containing 0.5 mg/ml collagenase (type IV; Sigma) at 37 °C for 30 min, with rotation. The collagenase digestion was stopped by adding Iscove's modified Dulbecco's medium (IMDM; Eurobio, Les Ulis, France) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). The bone pieces (8–10 from each patient) were then placed in 25 cm² flasks and cultured in IMDM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 U/ml mycostatin, and 0.25 µg/ml amphotericin B. Cells began to migrate within

1–2 weeks and reached confluence after 1 month. Culture medium was changed every 2–3 days. The cell population was tested for alkaline phosphatase and osteocalcin production after the addition of 10⁻⁸ M 1,25(OH)₂D₃ to ensure that the cells were endowed with osteoblast characteristics. Alkaline phosphatase was determined in the cell layer solubilized with 0.5 ml of 0.1% SDS by measuring the *p*-nitrophenol phosphate reduction (Roche Diagnostics). Osteocalcin was measured by IRMA (Nichols, San Juan Capistrano, CA, USA). All cells were used at the first passage to reduce the possibility of phenotype changes.

Reverse transcriptase PCR

Total RNA was extracted from hOB using TRIzol according to the manufacturer's instructions (Invitrogen, Inc.). RNA pellets were dissolved in sterile distilled water and their concentrations were determined spectrophotometrically (optical density (OD)_{260/280}). One microgram total RNA was retrotranscribed in a total volume of 25 µl using an oligodT primer (0.5 µM), 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase, deoxynucleotides (0.5 mM), M-MLV reaction buffer 1×, and rRNasin ribonuclease inhibitor 1 U/µl (Promega). According to the sequences published in GenBank, we constructed specific primers for the human GHR (GenBank NM000163: Forward (Fw)5'-CCCTATATTGACAACAT CAGTTCC-3' and Reverse (Rev)5'-TTTCCTTCCTT GAGGAGATCTGG-3') that amplified a 330 bp sequence and for IGF-I (GenBank NM000618: Fw5'-AGCAGTC TTCCAACCCAATTA-3' and Rev5'-CACGGACAGAGC GAGCTG-3') that amplified 355 bp (Luo *et al.* 2005). PCR was performed in a final volume of 20 µl containing cDNA (4 µl RT-PCR ± solution), 1 µM primers, 10 µM of each deoxyNTP, Taq polymerase (0.5 U), and PCR buffer 1× supplied with MgCl₂ (2 mM; Promega). The PCR was performed with a thermal cycler (Tpersonal, Whatman Biometra, Goettingen, Germany) using the following conditions: (a) for GHR: an initial denaturation at 94 °C for 5 min, followed by 30 PCR cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 90 s, and polymerization at 72 °C for 90 s; (b) for IGF-I: an initial denaturation at 94 °C for 5 min followed by 25, 28, and 34 PCR cycles of denaturation at 94 °C for 45 s, annealing at 48 °C for 45 s, polymerization at 72 °C for 60 s, and then a final extension of 10 min at 72 °C. The products of PCR were finally analyzed on a 2% tris acetate EDTA (TAE) agarose gel. The DNA ladder used was PCR Low Ladder Set (100 bp ladder; Sigma). IGF-I expression was compared with the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) used as an internal control.

Real-time PCR

OPG and IGF-I mRNA relative expressions were evaluated by real-time PCR in hOB obtained from four and three different donors respectively. For OPG mRNA determination, after 48-h serum-free medium incubation, hOB at

confluence were treated with 5 ng/ml human GH for 4, 6, and 24 h, or with both pegvisomant (50 nM) or tyrphostin AG490 (10 μ M) alone, or in association with GH for 24 h. For IGF-I mRNA determination, after 48-h serum-free medium incubation, hOB at confluence were treated with 5 ng/ml GH for 6 and 24 h. As previously described, total RNA was isolated with TRIzol and reverse transcribed (1 μ g in 25 μ l reaction buffer) using M-MLV reverse transcriptase and oligo-(dT)₁₆ priming according to the manufacturer's protocol (Promega). Relative quantification of OPG and *IGF-I* gene expression were performed on an ABI PRISM 7700 sequence detector (Perkin-Elmer, Norwalk, CT, USA) using 10 ng cDNA of the RT-PCR solution in a final volume of 25 μ l. Primers for OPG and IGF-I amplification and minor group binding (MGB) probes (FAM dye-labeled) were provided by Assay-on-demand Gene expression Assay Mix (Applied Biosystems, Foster City, CA, USA). Primers for the housekeeping gene *GAPDH* were used as an endogenous control (Applied Biosystems, Foster City, CA, USA). All primers were chosen to either span exon junction or lie in different exons to prevent amplification of genomic DNA. Real-time PCR was run according to the following protocol: an initial step of 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. mRNA levels were quantified using the comparative threshold-cycle (C_T) method. The amount of target mRNA in each sample was normalized to the amount of the *GAPDH* mRNA, designated as a calibrator, to give ΔC_T ($C_{T\text{target}} - C_{T\text{GAPDH}}$). The amounts of target mRNA in the samples were expressed using the formula: amount of target mRNA = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T(\text{sample } 1)} - \Delta C_{T(\text{control sample})}$, assuming that the efficiencies of the PCR were close to 1. Cells from different donors were analyzed in triplicate for each experimental point.

Assays

Primary cultures of human hOB were seeded into six-well multiwell plates and allowed to grow. At confluence, after 48-h serum-free medium incubation, the cells were treated for 24 h with increasing concentrations of GH (0.1–10 ng/ml; 4.5×10^{-12} – 4.5×10^{-10} M) and/or in the presence of pegvisomant (5×10^{-8} M) or tyrphostin AG490 (10 μ M). OPG production was measured in the conditioned media with a commercial kit (Immundiagnostik, Bensheim, Germany) and is expressed as femtomole/ 10^5 cells. IGF-I was measured using an ELISA kit (Biosource Europe SA, Nivelles, Belgium) and is expressed as nanogram/well.

Statistical analysis

The data were statistically analyzed using the statistical package GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). The significance of differences between groups was

assessed by means of a one-way ANOVA for nonparametric values (Kruskal–Wallis test) and a multiple comparison test (Dunn's test).

Results

The expression of the receptor for GH in hOBs was controlled by RT-PCR. As expected (Nilsson *et al.* 1995), these cells constitutively express GHRs (data not shown). The incubation of hOBs for 24 h with increasing concentrations of GH (0.1–25 ng/ml) induced a rise in OPG production, which reached a plateau at 1–10 ng/ml GH (Fig. 1); the higher concentrations of 25 ng/ml were less effective, showing a bell-shape trend. The maximal effect was a twofold increase in the protein production ($P < 0.001$). Similar effects were obtained on OPG mRNA production; GH at 5 ng/ml was able to induce an increase in the expression of mRNA for OPG within 4-h GH exposure, which was statistically significant ($P < 0.05$) after 6 h and returned to basal levels after 24 h (Fig. 2). To evaluate whether or not the rise in OPG production was a specific effect of GH, hOB were treated with an antagonist of GH, pegvisomant (50 nM) for 15 min before GH. Pegvisomant *per se* did not induce any effect on OPG production when compared with basal secretion (untreated cells), whereas it prevented the increase in OPG induced by GH (Fig. 3A). A specific involvement of GHRs in OPG secretion by hOB was confirmed by pretreating the cells with an inhibitor of one of the kinases (Jak2) involved in the intracellular pathway activated by GH, tyrphostin AG490 (Tyr). Tyr treatment (10 μ M, 15 min before GH) inhibited GH-induced OPG secretion, whereas the inhibitor *per se* did not change OPG secretion when compared with untreated cells (Fig. 3B). The real-time PCR results showed that

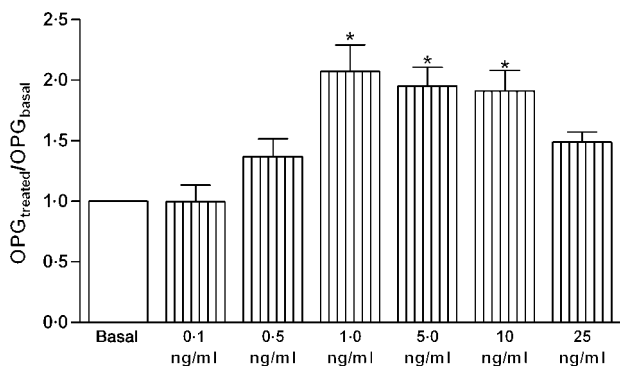


Figure 1 Concentration-dependent increase in osteoprotegerin (OPG) secretion in human osteoblast-like cells after 24-h exposure to GH concentrations ranging from 0.1 to 25 ng/ml (4.5×10^{-12} – 4.5×10^{-10} M). Data are means \pm S.E.M. of six experiments, using cells from six different donors. Basal represents OPG secretion from untreated cells. The results are expressed as the ratio of the OPG (femtomole/ 10^5 cells) produced by treated and untreated cells. * $P < 0.001$ versus basal (Kruskal–Wallis test for nonparametric data and Dunn's multiple comparison test).

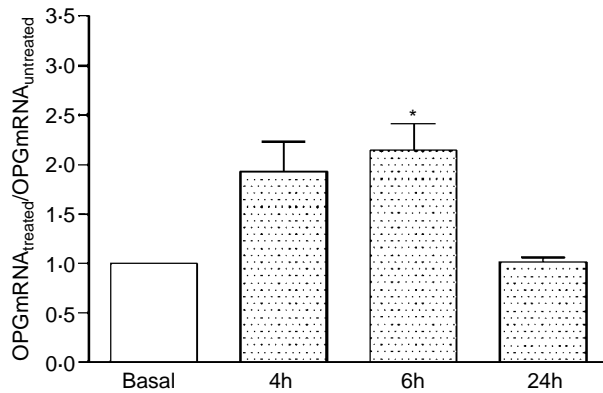


Figure 2 Real-time PCR showing the effect of GH (5 ng/ml) on OPG mRNA expression in hOB after 4-, 6-, and 24-h incubation. Basal represents OPG mRNA expression in untreated cells. The results were normalized to GAPDH and the mRNA in the samples from treated cells are expressed as a ratio of the amount measured in the samples from untreated cells (see Materials and Methods for details). The results are the mean \pm S.E.M. of four experiments performed in triplicate using cells from four different donors. * $P < 0.05$ versus basal (Kruskal–Wallis test for nonparametric data and Dunn’s multiple comparison test).

pretreatment of hOB with Tyr also prevented the GH-induced rise of OPG mRNA (Fig. 4). Twenty-four hours of GH exposure (5 ng/ml) did not induce any significant change in IGF-I secretion (untreated cells: 1.07 ± 0.54 ng/well; treated cells: 0.63 ± 0.08 ng/well). In addition, the expression of mRNA for IGF-I after 6- and 24-h GH (5 ng/ml) exposure did not significantly change as shown by semi-quantitative-PCR analysis (Fig. 5A) and real-time PCR (Fig. 5B). IGF-I mRNA was poorly detectable even after 34 amplification cycles (Fig. 5A).

Discussion

This study shows that GH is able to upregulate OPG secretion and expression by acting directly on osteoblast-like cells. These data are in line with previous *in vivo* studies demonstrating that serum OPG levels, as well as cortical and trabecular bone OPG contents, increase following GH replacement therapy in patients with GHD (Ueland *et al.* 2002, Lanzi *et al.* 2003). The fact that hOB express GHRs and that a specific GHR antagonist is able to prevent the increase in OPG secretion indicate that the effect of GH is specific and receptor mediated. This is also supported by the fact that inhibition of the activity of Jak-2, which is one of the first events that follow binding of GH to its receptor (Gerland *et al.* 2000), inhibits OPG upregulation by GH.

The response of hOB to GH exposure appears to be direct, not mediated by IGF-I, as GH did not increase IGF-I expression or secretion in these cells. Unlike hOB, in osteogenic sarcoma cells (SaOS-2) and murine osteoblasts,

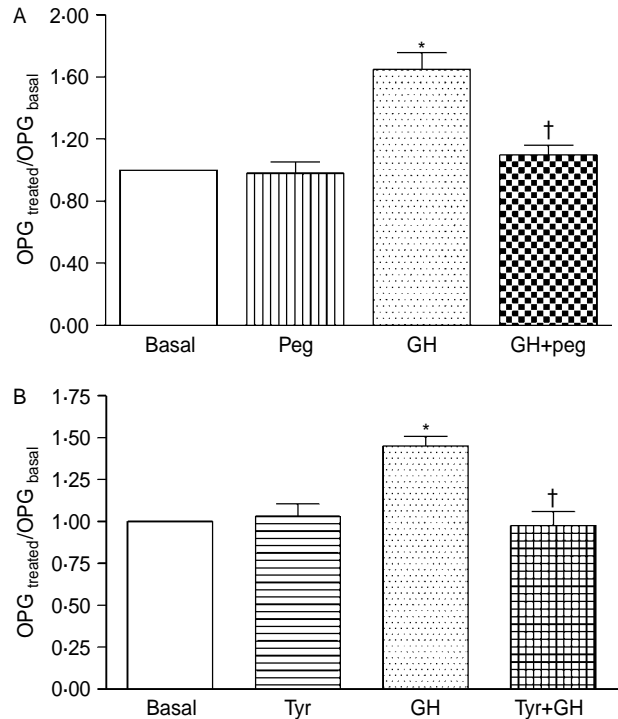


Figure 3 (A) Effect of the GH antagonist pegvisomant (Reg; 5×10^{-8} M, 15 min before GH) on GH (5 ng/ml) stimulation of osteoprotegerin (OPG) secretion measured 24 h after GH exposure in human osteoblast-like cells (hOB). The results are expressed as the ratio of the OPG (femtomole/ 10^5 cells) produced by treated and untreated cells; basal represents the OPG production (26.83 ± 4.52 fmol/ 10^5 cells) of untreated cells. Data are the mean \pm S.E.M. of four experiments using cells from different donors. (B) Effect of pretreatment with tyrphostin AG490 (Tyr; 10 μ M), an inhibitor of JAK2, on OPG secretion measured 24 h after GH exposure (5 ng/ml) in hOB. The results are expressed as the ratio of the OPG (femtomole/ 10^5 cells) produced by treated and untreated cells; basal represents the OPG production (26.83 ± 4.52 fmol/ 10^5 cells) of untreated cells. Data are the mean \pm S.E.M. of four experiments using cells from different donors. * $P < 0.01$ versus basal; † $P < 0.01$ versus GH (Kruskal–Wallis test for nonparametric data and Dunn’s multiple comparison test).

GH treatment increases the secretion of IGF-I (Wong *et al.* 1990, Scheven *et al.* 1991). However, the induction of IGF-I by GH in hOBs has not been detected by others either (Kassem *et al.* 1993, Kanzaki *et al.* 1995), thus supporting the idea that GH could exert direct anabolic effects on osteoblasts. IGF-I independent effects of GH on OPG secretion by hOB are further supported by the fact that IGF-I, in the same cellular system, downregulates OPG (Rubin *et al.* 2002). The reason for the discrepancy between GH and IGF-I effects on OPG secretion is unclear, particularly by considering that GH raises IGF-I serum levels by inducing hepatic IGF-I synthesis. However, it should be considered that the final effect on bone cells is the balance between circulating levels of GH, IGFs, IGFBP, and locally produced IGFs and IGFBPs, acting in an autocrine and paracrine way (Ueland 2004).

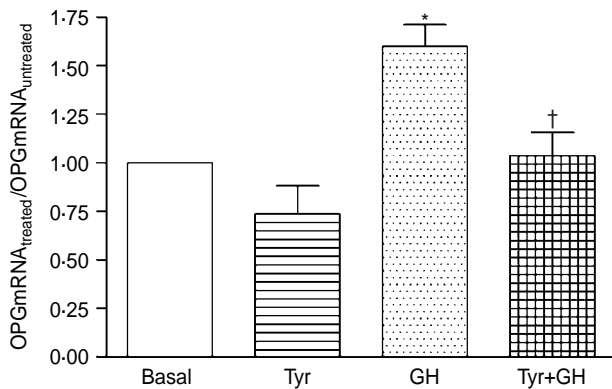


Figure 4 Real-time PCR showing the effect of tyrphostin AG490 (Tyr; 10 μ M), an inhibitor of JAK2, on OPG mRNA expression in hOB after 6-h incubation with 5 ng/ml GH. The results were normalized to GAPDH and the mRNA in the samples from untreated cells are expressed as a ratio of the amount measured in the samples from untreated cells (see Materials and Methods for details). Basal represents OPG mRNA expression in untreated cells. The results are the mean \pm s.e.m. of four experiments performed in triplicate using cells from four different donors. * $P < 0.01$ versus basal; [†] $P < 0.05$ versus GH (Kruskal–Wallis test for nonparametric data and Dunn's multiple comparison test).

OPG is important in maintaining bone mass, as transgenic mice without OPG develop osteoporosis (Mizuno *et al.* 1998), whereas mice overexpressing OPG develop osteopetrosis (Yamashita *et al.* 2002). The increase of OPG in hOB suggests that GH may set bone remodeling outcome (i.e. increased or decreased bone mass) by balancing the ratio OPG:RANKL in the microenvironment. Following GH treatment, despite the overall activation of bone turnover (Joannsson *et al.* 1996), the osteoclast activity may be relatively reduced by the increase of OPG with a subsequent positive shift toward bone neodeposition at each remodeling unit (Lanzi *et al.* 2003).

It has recently been observed that OPG, besides being a decoy receptor for RANKL, can also have a direct effect on purified osteoclasts as it modulates the expression of key proteases involved in bone resorption activity (Wittrant *et al.* 2002), suggesting a more complex regulation of bone resorption by this cytokine than just preventing binding of RANKL to RANK. OPG, in fact, directly inhibits the expression of cathepsin K and TRAP mRNA and stimulates metalloproteinases-9 (MMP-9; Wittrant *et al.* 2002). Tartrate resistant-acid phosphatase and cathepsin K are predominantly involved in the bone matrix solubilization (Garnero *et al.* 1998) and MMP-9 is essential for the initiation of bone resorption (Wittrant *et al.* 2002). The stimulatory effect on MMP-9 synthesis and expression, and the inhibition of tartrate resistant-acid phosphatase and cathepsin K by OPG are likely to stimulate further research to explain the effect of GH through OPG upregulation in hOB.

Understanding the mechanism involved in the regulation of osteoclast and osteoblast activities by a hormone that stimulates both terms of the remodeling equation in a mature

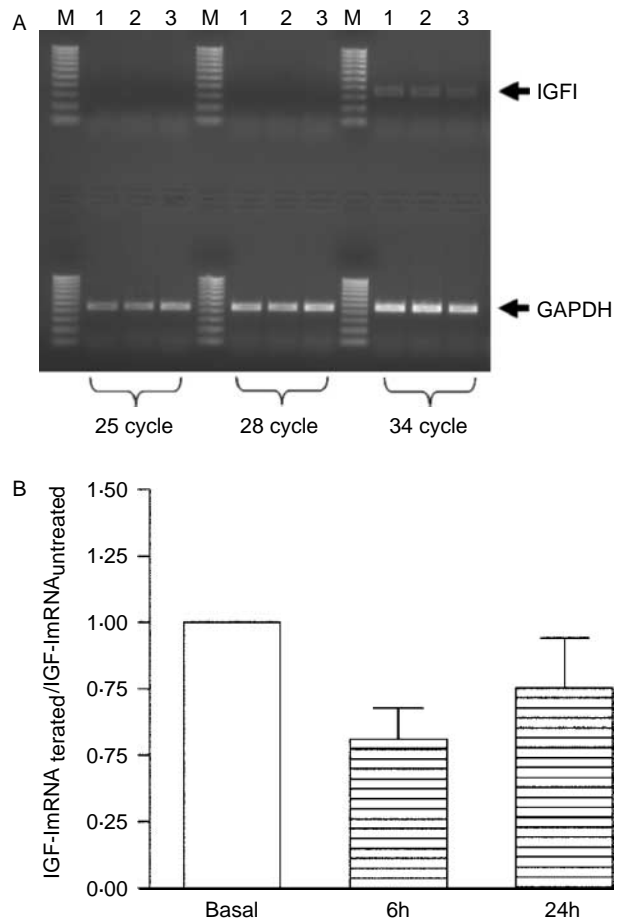


Figure 5 (A) IGF-I mRNA expression in primary cultures of human osteoblast-like cells after 6- and 24-h exposure to GH (5 ng/ml). Lane M, DNA ladder; lane 1, PCR product of untreated cells; lane 2, PCR product of cells treated with GH (5 ng/ml) for 6 h; and lane 3, PCR product of cells treated with GH (5 ng/ml) for 24 h. GAPDH expression was used as an internal reference. The reaction was stopped at different cycles of the PCR to demonstrate that the effect is not due to the plateau phase. The experiment was carried out thrice with cells from three different donors. (B) Real-time PCR showing the effect of GH (5 ng/ml) on IGF-I mRNA expression in hOB after 6- and 24-h incubation. The results were normalized to GAPDH, and the mRNA in the samples from treated cells are expressed as a ratio of the amount measured in the samples from untreated cells (see Materials and Methods for details). Basal represents IGF-I mRNA expression in untreated cells. The results are the mean \pm s.e.m. of three experiments performed in triplicate using cells from three different donors.

skeleton is essential to elucidating the physiological role on bone metabolism. In fact, in adults who lose bone because resorption cavities are incompletely repaired, an increase in bone turnover would most likely amplify the rate of bone loss (Parfitt 1991). However, this is not the case with GH, as several clinical studies have shown that GH administration increases bone turnover, as well as bone mass in elderly men (Rudman *et al.* 1990), in postmenopausal women (Landin-Wilhelmsen *et al.* 2003), and in GHD patients (Schlemmer *et al.* 1991). Such an effect requires, as

hypothesized by Parfitt (1991), a favorable shift in the focal balance between resorption and formation during each remodeling cycle as can be produced by other hormones, such as PTH (Tashjian & Gagel 2006).

In conclusion, this study has demonstrated that GH directly affects OPG synthesis and expression in hOB. The effect is specific and does not require the involvement of IGF-I. The OPG:RANKL equilibrium achieved under GH stimulus might determine a positive bone mass outcome as it relatively reduces the resorption phase of the activated bone remodeling sequence to a pro-formative activity. Based upon these results, the concern about recombinant human GH treatment of osteoporosis, raised by the editorial of Rosen & Wüster (2003), should be reconsidered.

Acknowledgements

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 23 November 2006

Accepted 12 December 2006

Made available online as an Accepted Preprint

27 December 2006