Osteo-Anabolic Effects of Human Growth Hormone with 22K- and 20K Daltons on Human Osteoblast-Like Cells

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Abstract. Human growth hormone with 22,000 Dal (22K-hGH) stimulates proliferation and differentiation of osteoblasts as well as production of interleukin-6 in vitro and bone formation and remodeling in vivo. To investigate whether hGH isoform with 20Kd (20K-hGH), which accounts for 10% of circulating hGH, elicits similar metabolic effects on skeletal tissues, we studied the biological effects of 20K-hGH in cultured human osteoblast-like cells (HOB). HOB were obtained from trabecular bone explants and cultured in α-MEM supplemented with 10% FCS. In subconfluent cultures, 22K- and 20K-hGH stimulated [3H]thymidine incorporation by 62 ± 27% and 63 ± 23%, respectively (mean ± SD, n=8, P>0.1). In confluent cultures, 22K- and 20K-hGH increased alkaline phosphatase activity by 38 ± 23% and 41 ± 23% (P>0.1), respectively, and increased the osteocalcin concentration in the presence of 10⁻⁹ M 1,25-(OH)₂D₃ by 50% and 47% (P>0.1), respectively. Furthermore, both hGHs doubled the interleukin-6 (IL-6) concentration in the conditioned medium. RT-PCR analysis revealed that 22K- and 20K- hGH increased IL-6 gene expression 2.2 ± 0.6 and 2.4 ± 0.7-fold, respectively. In summary, we have demonstrated that 20K-hGH elicits equipotent anabolic effects on HOB and stimulates to the same extent the production of IL-6, a cytokine which initiates osteoclastogenesis. These in vitro findings suggest that 22K- and 20K-hGH may equipotently stimulate bone remodeling and elicit anabolic effects on skeletal tissue when administered in vivo.

Key words: Human GH (hGH), 20K-hGH, Osteocalcin, Interleukin-6, Osteoblasts

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likely that hGH stimulates not only bone formation but also bone resorption, leading to the acceleration of bone remodeling [3].

Previous studies have shown that 20K-hGH elicits the same bioactivity as 22K-hGH did in various growth promoting assays [1, 2], but different effects on carbohydrate and lipid metabolism have been reported [1, 2]. These differences have been attributed to diminished insulin-like activity of 20K-hGH [9-11] and diminished affinity for GH receptors of rat lymphoma cells (Nb-2 cells), human liver and lymphoblastoid cells (IM-9 cells) [12-15]. Recently, recombinant 20K-hGH with a natural sequence was synthesized and it was found that the recombinant 20K- and 22K hGH increases body weight in rats equipotently [16] and that both hGHs have the same affinity constant for hGH receptors transiently expressed on CHO cells [17].

Since human osteoblast-like cells express receptors for hGH [18], we studied the osteotrophic effects of 20K-hGH, and compared them with those elicited by 22K-hGH in human osteoblast-like cells.

Materials and Methods

Materials

The recombinant 22K-hGH (Genotropin, Sumitomo Pharmaceuticals Co., Osaka, Japan) and 20K-hGH (supplied by Mitsui Pharmaceutical Inc., Tokyo, Japan) [16] were dissolved in 0.9% saline containing 0.2% BSA and stored in aliquots at -80 °C as stock solutions. Cell culture media (a-MEM) and reagents were supplied by GIBCO (Grand Island, NY, USA). Fetal calf serum (FCS) was purchased from Filtron Pty Ltd. (Brooklyn, Australia). IGF-I was from Becton Dickinson Labware, (Bedford, MA, USA). Human endothelin-1 (ET-1) was obtained from Cosmo Bio Co. Ltd. (Tokyo). 1,25-(OH)2D3 was from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). [Methyl-3H]thymidine was obtained from Amersham Corp (Arlington Heights, IL, USA). All reagents used were of analytical grade and the biochemicals were obtained from Sigma.

Cell culture

Cultured HOB were established from trabecular bone explants as described previously [19]. Trabecular bone was obtained from vertebra or iliac crest during orthopedic surgery. The bone samples were obtained from 4 females, aged 26-48 yr (median 37.3 yr) and 4 males aged 17-45 yr (median 33.3 yr). None of the patients had any known systemic bone disease or neoplastic disorder. The study was approved by the ethical committee of Tokyo Women's Medical University in Japan. The bone fragments were washed extensively and repeatedly with culture medium to remove adherent marrow cells and to expose the trabecular surface of the bone. Small bone chips (1 × 1 × 1 mm) were then placed in culture flasks (75 cm²) each containing 15 ml a-MEM supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (50 µg/ml) (α-MEM/10%FCS), and cultured at 37 °C in a humidified atmosphere with 5% CO2. Cell outgrowth from the trabecular bone surfaces was apparent after 5 days, and the osteoblast-like cells became confluent after 10-14 days of culture. Cell passages were performed by incubating confluent cells in 0.25% trypsin diluted in calcium- and magnesium-free phosphate-buffered saline and replating the cells at a density of 1 : 3. Experiments were usually performed with HOB subcultured at first and second passage. Under the culture conditions employed, HOB produced osteocalcin for more than 12 passages (data not shown).

[3H]thymidine incorporation into DNA of HOB

HOB were plated in 24-multiwell dishes in the α-MEM/10%FCS (Nunclon). Twenty-four h after plating, the medium was changed to α-MEM/0.1%FCS to induce growth arrest. After an additional 48 h, the medium was changed and the cells were incubated for another 24 h with serum-free medium supplemented with 1% bovine serum albumin (BSA) in the presence of hGH or vehicle. [3H]thymidine (2 µCi/ml, specific activity 40-60 Ci/mol) was added in the last 5 h of the incubation period. After the cell monolayer was washed with Hanks' solution (pH 7.4) and extracted with cold 5% trichloroacetic acid, the resulting precipitates
were then washed with ethanol-ether (volume ratio at 4:1) and solubilized with 1 N sodium hydroxide. The radioactivity was determined with a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). All determinations were performed in quadruplicate.

**Alkaline phosphatase activity of HOB**

Effects of 22K- and 20K-hGH on osteoblast differentiation were assessed in confluent cultures. Twenty-four h after plating, the medium was changed and cells were allowed to grow for 96 h in α-MEM/10% FCS. This medium was replaced with serum-free medium with 1% BSA or 2%FCS containing various concentrations of hGH. After the cells were cultured for an additional 96 h, Al-P activity was determined as described previously [20].

In several experiments, confluent HOB were cultured in α-MEM/2%FCS containing various concentrations of hGH for 96 h in the presence or absence of 1,25-(OH)2D3. Osteocalcin released into the culture medium over a 96 h incubation period was measured by an immunoradiometric assay (ELSA-Osteo, CIS Diagnostic K.K., Sakura-shi, Chiba). This assay measures the 1–49 human osteocalcin and human osteocalcin peptide 1–43 [21]. The detection limit was less than 0.4 ng/ml. The intra- and interassay coefficients of variation were less than 5% and 10%, respectively. Osteocalcin production was corrected for variations in the number of cells and expressed as ng osteocalcin per 10^5 cells.

**Measurements of IL-6 in the conditioned medium**

IL-6 levels in the supernatant of HOB cultures were measured by ELISA [22] after the confluent cells were cultured in α-MEM/0.5%FCS containing various concentrations of hGH for 24 h.

**Reverse transcription-PCR**

HOB were cultured in α-MEM/10%FCS in 10 cm dishes. When the cells reached confluence, the medium was changed to α-MEM/0.5%FCS supplemented with various concentrations of 22K- and 20K-hGH. After 24 h of culture, total RNA was extracted, and RT-PCR was performed as described previously [23]. The primer pair for IL-6 were 5'-ATGAACTCTTCTCCACAAGCC-3' (sense ) and 5'-GAAGAGCCCTCAAGGCTGGACTG-3' (antisense), and those for β-actin were 5'-GTGGGGGCGCCAGAAGGACCA-3' (sense) and 5'-CTCCCTTATGTCAAGCAGATTTGC-3' (antisense) [24]. Each PCR cycle included 1 min of denaturation at 94 °C, 1 min of primer annealing at 60 °C, and 1.5 min of extension/synthesis at 72 °C. After the last cycle, all samples were incubated for an additional 5 min at 72 °C. The values for IL-6 gene expression divided by β-actin expression were used for comparison of gene expressions, with the control value for HOB defined as 1.00.

**Statistical analysis**

All values are expressed as the mean ± SD. Analysis of variance was studied by using Kruskal-Wallis's test followed by Dunnet's test to determine the significance of differences in multiple comparisons. Differences at P<0.05 were considered statistically significant.

**Results**

**Effects of 22K- and 20K-hGH on [3H]thymidine incorporation**

Consistent with previous reports [4–6], 22K-hGH increased [3H]thymidine incorporation into HOB in a dose-dependent manner (P<0.01, n=8)(Fig. 1). 20K-hGH also increased [3H]thymidine incorporation to the same extent as 22K-hGH does. At 0.5 nM, which is well within the physiological range of the hormone, 22K- and 20K-hGH induced 25.7 ± 19.8 and 23.2 ± 12.3% increases in [3H]thymidine incorporation, and stimulated it maximally at 25 nM, by 62 ± 27% and 63 ± 23%, respectively. There was no significant difference between them (P>0.05).

In a few experiments, anabolic effects of hGH were compared with those of IGF-1 and endothelin-1. As shown in Table 1, IGF-1 most potently stimulated [3H]thymidine incorporation into HOB. As reported previously [20], endothelin-1 also stimulated [3H]thymidine incorporation into HOB, which was less than that induced by GH.
Basal Al-P activity of HOB was 139.4 ± 53.5 μU/mg protein in confluent cultures (Mean ± SD of 8 experiments). After 4 days of treatment with either 22K- or 20K-hGH, Al-P activity increased in a dose-dependent manner. 22K-hGH and 20K-hGH increased Al-P activity by 38 ± 23% and 41 ± 23% of controls at 25 nM, respectively (Fig. 2). There was no significant difference between them.

To further compare anabolic effects of hGH on human osteoblasts, effects of 22K-hGH and 20K-hGH on osteocalcin production were compared. Neither hGH significantly stimulated osteocalcin production in the absence of 1,25-(OH)2D3, but they significantly stimulated osteocalcin production at 1.6 nM in the presence of 1,25-(OH)2D3 (Fig. 3). The dose-response curve was biphasic: both 22K- and 20K-hGH maximally stimulated osteocalcin production at 40 nM. There was no significant difference between them in osteocalcin production.

HOB grown in α-MEM/0.5%FCS spontaneously secreted IL-6 (1.2 ± 0.1 ng/104 cells). After 24 h culture with hGH, both hGHs increased IL-6 production in a concentration-dependent manner.

### Table 1. Effects of 22K-hGH, 20K-hGH, IGF-I, and ET-1 on [3H]thymidine incorporation in HOB

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]thymidine incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>214.0 ± 21.8</td>
</tr>
<tr>
<td>22K-hGH</td>
<td>0.91 × 10^-9 M (20 ng/ml)</td>
</tr>
<tr>
<td>20K-hGH</td>
<td>1.0 × 10^-9 M (20 ng/ml)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>1.0 × 10^-8 M (200 ng/ml)</td>
</tr>
<tr>
<td>ET-1</td>
<td>2.6 × 10^-10 M (20 ng/ml)</td>
</tr>
<tr>
<td>22K-hGH</td>
<td>1.0 × 10^-9 M (20 ng/ml)</td>
</tr>
<tr>
<td>20K-hGH</td>
<td>1.0 × 10^-9 M (20 ng/ml)</td>
</tr>
</tbody>
</table>

Subconfluent HOB were cultured in α-MEM/0.1%FCS for 48 h. The hormones were added and the cells were cultured for an additional 20 h, followed by the addition of [3H]thymidine. After another 5 h of culture, [3H]thymidine incorporated into HOB was counted as described in Materials and Methods. Values are means ± SD of quadruplicate samples. * P<0.05.

### Effects of 22K- and 20K-hGH on Al-P activity

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### Effects of 22K- and 20K-hGH on osteocalcin production

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### Effects of 22K- and 20K-hGH on IL-6 release

HOB grown in α-MEM/0.5%FCS spontaneously secreted IL-6 (1.2 ± 0.1 ng/104 cells). After 24 h culture with hGH, both hGHs increased IL-6 production in a concentration-dependent manner.
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There was no significant difference between them. When HOB was cultured with 5 nM 22K-hGH and 20K-hGH for 72 h, the IL-6 concentration in the conditioned medium increased to 11.8 ± 3.9 and 10.8 ± 1.5 ng/ml at 24 h (P<0.05) and 41.2 ± 8.4 and 38.1 ± 5.4 ng/ml at 72 h (P<0.01), respectively.

Effects of 22K- and 20K-hGH on IL-6 gene expression

Consistent with the above findings, significant expression of the IL-6 gene was observed in cultured HOB in all the experiments (Fig. 5). Exponential amplification of IL-6 and β-actin cDNA was observed at 21–30 cycles. When samples were subjected to 24 cycles of PCR for IL-6, 22K-hGH and 20K-hGH at 5 nM caused 2.2 ± 0.6 and 2.4 ± 0.7-fold increases in the IL-6 gene expression, respectively.

Discussion

With osteoblast-like cells derived from normal adult human trabecular bone, we have clearly demonstrated that 22K- and 20K-hGH exerted equipotent osteotrophic effects, in terms of
stimulating $[^3]H$thymidine incorporation, Al-P activity and osteocalcin production at 0.5-5.0 nM, namely at the physiological concentration of the hormones in the human blood [2]. These in vitro findings are consistent with the recent observation that 22K- and 20K-hGH have identical binding affinities (0.41 nM) and exert full agonistic effects on CHO cells transfected with hGH receptor cDNA [16, 17]. Furthermore, the present in vitro findings are in keeping with the in vivo data showing that 20K hGH has the same weight gain activity as 22K hGH in hypophysectomized rats [16]. Since Al-P activity of bone origin and osteocalcin are markers for bone formation, and since the metabolic clearance rate of 20K-hGH is slower than that of 22K-hGH in rats [25], the present in vitro findings suggest that 20K-hGH will be capable of eliciting equipotent anabolic effects on skeletal tissue when administered in human subjects in vivo.

In conclusion, our data revealed that 20K-hGH and 22K-hGH equally stimulated $[^3]H$thymidine incorporation, Al-P activity, and the production of osteocalcin in human osteoblast-like cells. Furthermore, 20K-hGH stimulated IL-6 production to as much as 22K-hGH. These findings indicate that 20K-hGH will stimulate bone remodeling, and exhibit equipotent anabolic effects on the skeletal
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