

## 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  $\alpha$ -MEM supplemented with 10% FCS. In subconfluent cultures, 22K- and 20K-hGH stimulated [<sup>3</sup>H]thymidine incorporation by  $62 \pm 27\%$  and  $63 \pm 23\%$ , respectively (mean  $\pm$  SD,  $n=8$ ,  $P>0.1$ ). In confluent cultures, 22K- and 20K-hGH increased alkaline phosphatase activity by  $38 \pm 23\%$  and  $41 \pm 23\%$  ( $P>0.1$ ), respectively, and increased the osteocalcin concentration in the presence of  $10^{-9}$  M  $1,25-(OH)_2D_3$  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  $2.2 \pm 0.6$  and  $2.4 \pm 0.7$  -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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HUMAN GH (hGH) is a mixture of peptides, mainly composed of a single chain polypeptide of 191 residues with a molecular weight of 22,000 (22K-hGH). In addition to this authentic hGH, there is a variant of hGH with 20,000 Dal, which is synthesized by deletion of residues 32–46 of 22K hGH [1, 2]. This hGH isoform (20K-hGH) consists

of 5–10% of the hGH in the pituitary gland and 15% of the hGH in serum [1, 2].

When 22K-hGH is administered *in vivo*, it not only stimulates growth of skeletal tissues but also influences protein, carbohydrate and fat metabolism [1–3]. Several *in vitro* studies have shown that 22K-hGH directly stimulates the proliferation and differentiation of osteoblast-like cells and increases the IGF-I concentration in the medium in osteoblasts cultured in serum-free medium [4–6]. Furthermore, 22K-hGH stimulates interleukin-6 (IL-6) production in human osteoblast-like cells [7]. Since IL-6 is involved in osteoclastogenesis in the bone marrow, leading to bone resorption [8], it is

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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  $-80^{\circ}\text{C}$  as stock solutions. Cell culture media ( $\alpha$ -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).  $1,25\text{-(OH)}_2\text{D}_3$  was from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). [Methyl- $^3\text{H}$ ]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 ( $1 \times 1 \times 1$  mm) were then placed in culture flasks ( $75 \text{ cm}^2$ ) each containing 15 ml  $\alpha$ -MEM supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (50  $\mu\text{g}/\text{ml}$ ) ( $\alpha$ -MEM/10%FCS), and cultured at  $37^{\circ}\text{C}$  in a humidified atmosphere with 5%  $\text{CO}_2$ . 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).

### *[ $^3\text{H}$ ]thymidine incorporation into DNA of HOB*

HOB were plated in 24-multiwell dishes in the  $\alpha$ -MEM/10%FCS (Nunc). Twenty-four h after plating, the medium was changed to  $\alpha$ -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. [Methyl- $^3\text{H}$ ]thymidine (2  $\mu\text{Ci}/\text{ml}$ , 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  $\alpha$ -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  $\alpha$ -MEM/2% FCS containing various concentrations of hGH for 96 h in the presence or absence of 1,25-(OH)<sub>2</sub>D<sub>3</sub>. 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<sup>5</sup> 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  $\alpha$ -MEM/0.5% FCS containing various concentrations of hGH for 24 h.

#### *Reverse transcription-PCR*

HOB were cultured in  $\alpha$ -MEM/ 10% FCS in 10 cm dishes. When the cells reached confluence, the medium was changed to  $\alpha$ -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'-ATGAACCTCTTCTCCACAAGCGC-3' (sense) and 5'-GAAGAGCCCTCAGGCTGGACTG-3' (antisense), and those for  $\beta$ -actin were 5'-GTGGGGCGCCCCAGGCACCA-3' (sense) and 5'-CTCCTTAATGTCACGCACGATTTC-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  $\beta$ -actin expression were used for comparison of gene expressions, with the control value for HOB defined as 1.00.

#### *Statistical analysis*

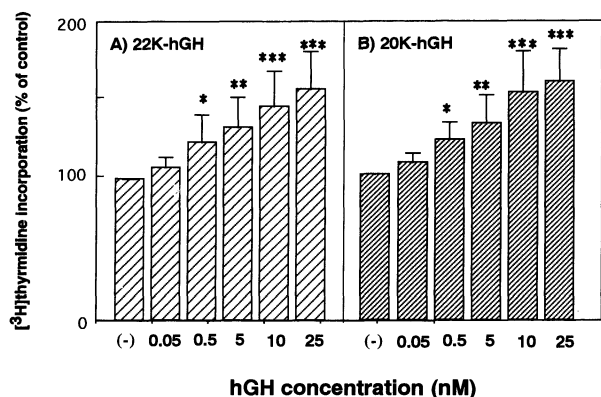
All values are expressed as the mean  $\pm$  SD. Analysis of variance was studied by using Kruskal-Wallis's test followed by Dunnett' test to determine the significance of differences in multiple comparisons. Differences at  $P < 0.05$  were considered statistically significant.

## **Results**

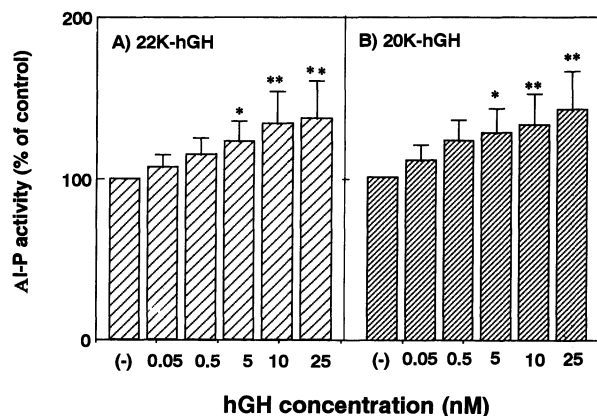
#### *Effects of 22K- and 20K-hGH on [<sup>3</sup>H]thymidine incorporation*

Consistent with previous reports [4–6], 22K-hGH increased [<sup>3</sup>H]thymidine incorporation into HOB in a dose-dependent manner ( $P < 0.01$ ,  $n = 8$ ) (Fig. 1). 20K-hGH also increased [<sup>3</sup>H]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  $25.7 \pm 19.8$  and  $23.2 \pm 12.3\%$  increases in [<sup>3</sup>H]thymidine incorporation, and stimulated it maximally at 25 nM, by  $62 \pm 27\%$  and  $63 \pm 23\%$ , 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-I and endothelin-1. As shown in Table 1, IGF-1 most potently stimulated [<sup>3</sup>H]thymidine incorporation into HOB. As reported previously [20], endothelin-1 also stimulated [<sup>3</sup>H]thymidine incorporation into HOB, which was less than that induced by GH.



**Fig. 1.** Effects of different doses of recombinant 22K- and 20K-hGH on [ $^3$ H]thymidine incorporation in HOB. Subconfluent HOB were cultured with  $\alpha$ -MEM/0.1%FCS for 48 h. Thereafter, the hormones were added and the cells were cultured for an additional 20 h. [ $^3$ H]thymidine was present during the last 5 h of culture. Results are the means  $\pm$  SD for 8 different experiments. Data are shown as % increase compared with control cultures. Absolute values for control cultures were  $2312 \pm 1482$  cpm/well (mean  $\pm$  SD,  $n=8$ ). A; 22K-hGH, B; 20K-hGH. \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ , with vs. without hGH.



**Fig. 2.** Effects of different doses of recombinant 22K- and 20K-hGH on Al-P activity in HOB. Confluent HOB were cultured in  $\alpha$ -MEM containing 0.2% BSA and various concentrations of hGH (0.05–25 nM). After 4 days of culture, Al-P activity in the cell lysate was measured as described in Materials and Methods. Results are the mean  $\pm$  SD % for 8 different experiments. Data are shown as % increase compared with control cultures. Absolute values for control cultures were  $156.4 \pm 202.6$  mU/mg protein. A) 22K-hGH, B) 20K-hGH. \*  $P<0.01$ , \*\*  $P<0.01$ : with vs. without hGH.

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

[ $^3$ H]thymidine incorporation (cpm)		
Control		$214.0 \pm 21.8$
22K-hGH	$0.91 \times 10^{-9}$ M (20 ng/ml)	$301.0 \pm 33.0^*$
	$0.91 \times 10^{-8}$ M (200 ng/ml)	$379.0 \pm 50.6^*$
20K-hGH	$1 \times 10^{-9}$ M (20 ng/ml)	$286.0 \pm 39.8$
	$1 \times 10^{-8}$ M (200 ng/ml)	$374.4 \pm 49.4^*$
IGF-I	$2.6 \times 10^{-10}$ M (2 ng/ml)	$392.2 \pm 47.8^*$
	$2.6 \times 10^{-9}$ M (20 ng/ml)	$707.0 \pm 82.6^*$
Endothelin-1	$1 \times 10^{-7}$ M	$307.0 \pm 28.2^*$

Subconfluent HOB were cultured in  $\alpha$ -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 [ $^3$ H]thymidine. After another 5 h of culture, [ $^3$ H]thymidine incorporated into HOB was counted as described in Materials and Methods. Values are means  $\pm$  SD of quadruplicate samples. \*  $P<0.05$ .

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

Basal Al-P activity of HOB was  $139.4 \pm 53.5$   $\mu$ U/mg protein in confluent cultures (Mean  $\pm$  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

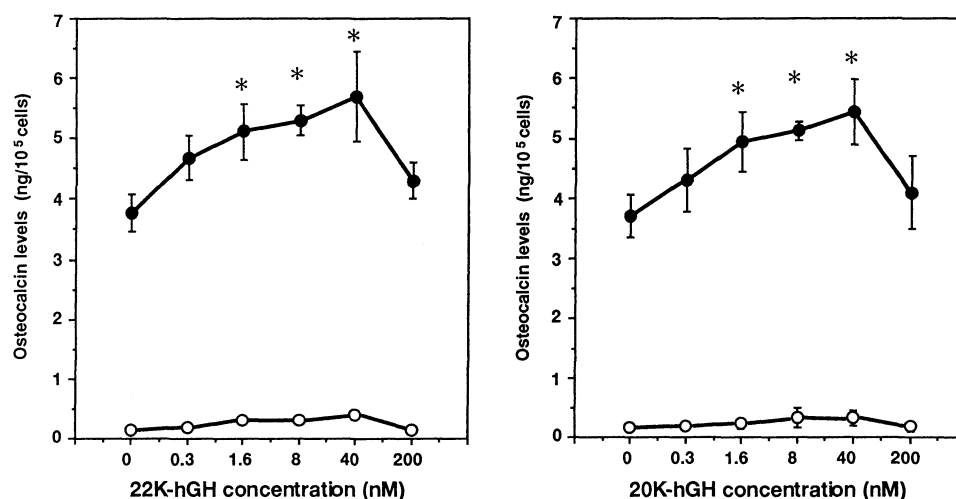
increased Al-P activity by  $38 \pm 23\%$  and  $41 \pm 23\%$  of controls at 25 nM, respectively (Fig. 2). There was no significant difference between them.

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

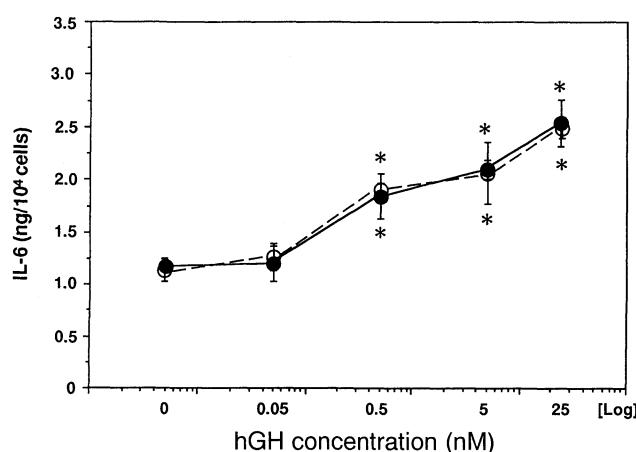
To further compare anabolic effects of hGH on human osteoblasts, effects of 22K-hGH and 20K-hGH on osteocalcin production were compared. Neither hGH significantly stimulated osteocalcin production in the absence of  $1,25-(\text{OH})_2\text{D}_3$ , but they significantly stimulated osteocalcin production at 1.6 nM in the presence of  $1,25-(\text{OH})_2\text{D}_3$  (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.

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

HOB grown in  $\alpha$ -MEM/0.5%FCS secreted IL-6 spontaneously ( $1.2 \pm 0.1$  ng/ $10^4$  cells). After 24 h culture with hGH, both hGHs increased IL-6 production in a concentration-dependent manner



**Fig. 3.** Effects of 22K- and 20K-hGH on osteocalcin produced by HOB. Confluent HOB were cultured in  $\alpha$ -MEM/10% FCS containing various concentrations of hGH (0.3–200 nM) in the presence or absence of  $10^{-9}$  M  $1,25-(\text{OH})_2\text{D}_3$ . After culturing for 96 h, the conditioned medium was harvested and the osteocalcin concentration was determined as described in Materials and Methods. Levels of osteocalcin were expressed as ng per  $10^5$  cells. Data are shown as the mean  $\pm$  SD for quadruplicate cultures. Representative data from 3 experiments are shown.  $\bullet$ — $\bullet$ ;  $1,25-(\text{OH})_2\text{D}_3$  (+),  $\circ$ — $\circ$ ;  $1,25-(\text{OH})_2\text{D}_3$  (-). \*  $P < 0.05$ ; with vs. without  $1,25-(\text{OH})_2\text{D}_3$ .



**Fig. 4.** Dose-response effects of 22K- and 20K-hGH on IL-6 released into the culture medium. HOB were cultured in  $\alpha$ -MEM/0.5%FCS for 40 h. Then, the medium was changed to fresh medium supplemented with various concentrations of hGH (0.05–25 nM). After culturing for 24 h, the conditioned medium was harvested and the IL-6 concentration was determined as described in Materials and Methods. Data are shown as mean  $\pm$  SD for quadruplicate cultures.  $\bullet$ — $\bullet$ ; 22K-hGH,  $\circ$ — $\circ$ ; 20K-hGH, \*  $P < 0.05$ ; with vs. without hGH.

(Fig. 4). There was no significant difference between them. When HOB was cultured with 5

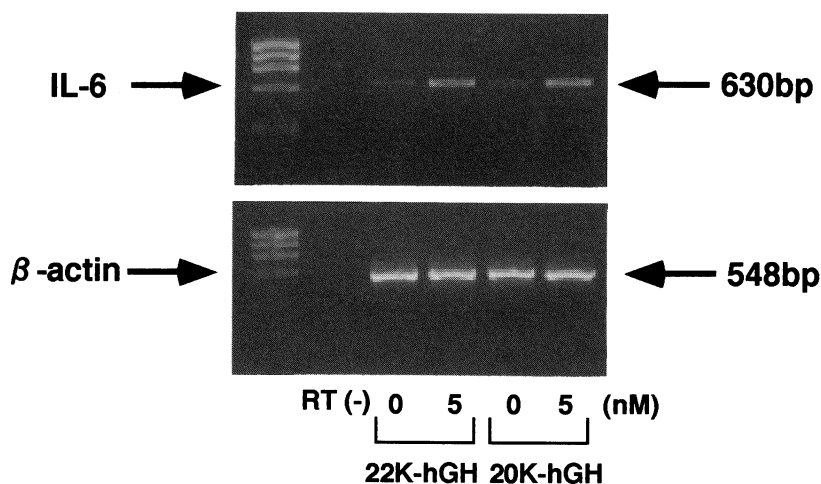
nM 22K-hGH and 20K-hGH for 72 h, the IL-6 concentration in the conditioned medium increased to  $11.8 \pm 3.9$  and  $10.8 \pm 1.5$  ng/ml at 24 h ( $P < 0.1$ ) and  $41.2 \pm 8.4$  and  $38.1 \pm 5.4$  ng/ml at 72 h ( $P < 0.01$ ), respectively.

#### 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  $\beta$ -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  $2.2 \pm 0.6$  and  $2.4 \pm 0.7$ -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 osteotropic effects, in terms of



**Fig. 5.** Effects of 22K- and 20K-hGH on IL-6 gene expression. HOB were cultured in  $\alpha$ -MEM/0.5%FCS for 40 h. Then, the medium was changed to fresh medium supplemented with 5 nM 22K- or 20K-hGH. After culturing for 24 h, the cells were harvested and total RNA was prepared. RT-PCR were performed as described in Materials and Methods. Lane 1: RT (-), Lanes 2-5: RT (+), Lanes 2,4: hGH (-), Lane 3: 22K-hGH (5 nM), Lane 5: 20K-hGH (5 nM).

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 contrast to HOB, 20K-hGH has less potent growth-promoting activity in Nb-2 cells than 22K-hGH. This different activity is accounted for by the fact that prolactin receptors, which are abundantly expressed in Nb-2 cells [26], bind to 22K-hGH better than 20K-hGH [27]. Although prolactin receptor is also expressed in murine bone cells [26], our present findings of equipotent

growth-promoting effects on HOB suggest that the expression levels of prolactin receptors in human adult osteoblasts would be negligible compared to that of GH receptors.

We have also demonstrated that 20K-hGH stimulated IL-6 release from HOB, as 22K-hGH did [7]. Both hGHs more than doubled the IL-6 concentration in the conditioned medium, and this was accompanied by an increase of IL-6 gene expression. Since IL-6 is known to initiate osteoclastogenesis in the bone marrow [8] and has been implicated in the regulation of GH effects on bone turnover and remodeling, as postulated in patients with primary hyperparathyroidism and hyperthyroidism [28], the present *in vitro* findings are consistent with the clinical observation that both bone formation and resorption were activated during a GH treatment study of GH deficient patients [29, 30].

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

tissues, when administrated *in vivo* to human subjects.

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