Regulation of *growth-hormone-receptor* gene expression by growth hormone and pegvisomant in human mesangial cells

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Background. Mice transgenic for growth hormone develop mesangial proliferation, glomerular hypertrophy, and progressive glomerular sclerosis suggesting that the growth hormone–insulin-like growth factor I (IGF-I) pathway plays an important role. Therefore, we studied the impact of variable concentrations of 22 kD, 20 kD growth hormone, as well as of the growth hormone receptor antagonist pegvisomant (B2036-PEG), on both the *growth hormone receptor (GHR/GHBP)* gene expression and growth hormone binding protein (GHBP) formation in a human glomerular mesangial cell line. Further, the impact on collagen, IGF-I and IGF binding protein-1 (IGFBP-1) formation was studied.

Methods. In order to assess transcription, quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used.

Results. Physiologic doses of 22 kD or 20 kD growth hormone caused a dose-dependent and significant ($P < 0.01$) up-regulation of *GHR/GHBP* gene transcription, whereas supraphysiologic doses (50 and 500 ng/mL) resulted in down-regulation ($P < 0.001$). Whenever pegvisomant was used, there was no increase in GHR/GHBP expression. These data were confirmed using run-on experiments. Further, the assessment of GHBP presented a constant, dose-dependent increase, which was completely abolished in the experiments where pegvisomant was used.

Conclusion. We present data showing that growth hormone has a direct impact on *GHR/GHPB* gene transcription and that pegvisomant is a potent growth hormone receptor antagonist in human mesangial cells. In addition, although the *GHR/GHBP* gene transcription is down-regulated by supraphysiologic growth hormone concentrations, this effect was not found when GHBP levels were measured. This finding may reflect a self-inhibitory effect of growth hormone on the level of *GHR/GHBP* gene transcription, which does not involve the regulation of the shedding of GHBP and may, therefore, be of physiologic interest.

Glomerular hypertrophy and/or hyperplasia in experimental renal diseases in rodents have been implicated as a cause of progressive glomerular sclerosis and chronic renal failure [1]. However, although the precise mechanisms involved in the development of glomerulosclerosis have not been fully elucidated, the growth hormone–insulin-like growth factor-I (IGF-I) axis, including its autocrine activation of signaling pathways, as well as IGF-binding protein-1 (IGFBP-1), are suspected to play an important role for several reasons [1–12]. First, mice transgenic for either bovine or human growth hormone expressing elevated growth hormone levels present a giant phenotype, develop mesangial proliferation, glomerular hypertrophy, and progressive glomerular sclerosis, leading to renal failure and death by 8 to 9 months of age [1, 13, 14]. Second, in the aging rats, there is a strong correlation between the severity of glomerular sclerosis and plasma growth hormone levels [15]. Third, there are reports indicating that glomerulosclerosis may be associated with acromegaly, suggesting that the overproduction of growth hormone may be partly responsible for the development of human glomerulosclerosis [16, 17]. In addition, as compensatory renal growth in uninephrectomized adult mice seems to be growth hormone–dependent, growth hormone–deficient rats are somewhat protected from the development of glomerular sclerosis after partial nephrectomy [18, 19]. Moreover, in streptozotocin-induced diabetes in the rat, renal growth hormone receptor (GHR) mRNA levels were found to be increased, leading to speculation that the increased concentration of growth hormone in circulation and, therefore, its action may contribute to diabetic glomerulosclerosis [20]. Further, in dwarf mice transgenic for a mutated growth hormone form (G119K), which acts as an antagonist of endogenous growth hormone, glomeruli were reported to be normal and these mice were protected from streptozotocin-diabetes–induced progressive glomerular sclerosis [21, 22]. Increased growth hormone, as well as IGF-I activity, is associated with increased glomerular produc-

tion of extracellular matrix proteins, and the glomerular lesions might resemble diabetic nephropathy [1, 23, 24].

As an extensive body of evidence supports the permissive role of growth hormone in the development of glomerulosclerosis and experimental evidence suggests that different domains of the growth hormone molecule (mice transgenic for 20 kD and 22 kD growth hormone) may cause variable effects on linear growth and/or organ hypertrophy but an identical effect on the development of glomeruscloeris [25], the aim of our study was to analyze the impact of variable concentrations of 22 kD, 20 kD growth hormone, and of the GHR antagonist pegvisomant (GHR-A; B2036-PEG) (Sensus Corp., Austin, TX, USA) on the *GHR/growth hormone binding protein* (*GHR/GHBP*) gene expression as well as on the GHBP formation in a human glomerular mesangial cell line in order to define the direct effect of growth hormone on the regulation of the expression of its own receptor. Further, the impact of growth hormone, growth hormone variant, and GHR-A on types I and IV collagen, IGF-I and IGFBP-1 secretion into the cell culture medium was studied.

METHODS

Cell culture, mutant growth hormones, and pegvisomant (B2036-PEG)

Following double transfection of human mesangial cells (HMC) with T-SV40 and H-ras oncogene, stable cell lines of T-SV40 immortalized human glomerular mesangial (T-HMC) cells were obtained [26]. As these cells do express the GHR, functional studies on the *GHR/GHBP* gene expression can be performed. T-HMC were maintained in monolayer culture as previously described [26]. When they had reached approximately 70% confluency, the medium was aspirated, the cells were washed twice with phosphate-buffered saline (PBS), pH 7.4 (Sigma Chemical Co., St. Louis, MO, USA), and 1 mL of a serum-free hormonally defined medium was added. Serum-free hormonally defined medium contained 0.4 mmol/L ornithine; 2.25 μg/mL L-lactic acid; 2 ng/mL glucagon; 2.5×10^{-8} mol/L selenium; 5×10^{-8} mol/L hydrocortisone; 1×10^{-6} mol/L ethanolamine; 2 ng/mL choleratoxin; 1 μg/mL insulin, 1 μg/mL transferrin, 25 ng/mL endothelial growth factor (EGF) but, as previously reported, no growth hormone, IGF-I, IGF-II, or IGFBP-1 [27]. After overnight incubation, the medium was aspirated and the cells were washed twice with PBS (pH 7.4). Thereafter, different samples containing variable forms of growth hormone (22 kD, 20 kD growth hormone) and pegvisomant were added to the culture medium at various concentrations (12.5, 25, 50, and 500 ng/mL of growth hormone variants and, in pegvisomant studies, an additional experiment using 20 μg/mL pegvisomant was performed) and incubation was continued

for 0, 1, 3, or 6 hours. The additional dose of 20 μg/mL of pegvisomant was chosen with reference to a clinical trial [28] in which, due to the reduced affinity of pegvisomant for the GHR compared to 22 kD growth hormone, patients were treated for acromegaly with a standard dose of 20 mg pegvisomant/ day and were found to have a mean serum concentration of 20 μg/mL. Further, the incubation times were chosen based on previous experiments, where marked differences in transcription rates of the *GHR/GHBP* gene were seen at these times [29]. In addition, maximum internalization of growth hormone is reported after 2 hours, whereas hormone accumulation in the nucleus was saturated after 1 hour [30]. Each experiment was repeated eight times. Moreover, in order to determine the effect on types I and IV collagen, IGF-I, and IGFBP-1, the cells were incubated over a period of 72 hours.

RNA extraction

RNA was extracted as previously described [29], using the acid guanidium thiocyanate-phenol-chloroform method [31]. The RNA concentration was measured by absorbance at 260 nm using a double-beam spectrophotometer (UV 150-02, Shimadzu Corporation, Tokyo, Japan).

Construction of the internal control and synthesis of internal control RNA

The plasmid for the preparation of synthetic internal control RNA (cRNA) was constructed by inserting a 50 base pair (bp) fragment of the rat prolactin (PRL) receptor DNA into a portion of the GHR cDNA, as previously described [32]. Briefly, the subsequent chimeric plasmid of GHR was cleaved with *Eco*RV and *Eco*RI, and a 545 bp fragment was obtained. This fragment was subcloned into the *Sma*I-*Eco*RI sites of bluescript SK + (Stratagene Cloning System, San Diego, CA, USA). Following digestion with *Sal*I, the chimeric GHR construct served as a template for in vivo transcription by T7 RNA polymerase to generate internal cRNA. The cRNA was purified with phenol-chloroform extraction and subsequently by oligo (deoxythymidine) chromatography. The absolute number of cRNA molecules was calculated using spectrophotometric absorbance at 260 nm, the molecular weight of cRNA molecule (216,600 g/mol) and Avogadro's number.

Oligonucleotide primers used for amplification

Oligonucleotide primers were purchased from Mycrosynth, Balgach, Switzerland. The sense primer was 5′-CC CTATATTGACAACATCAGTTCC-3′; nucleotide 624 to 647 (exon 7) [33] and the antisense primer was 5′-TT CCCTTCCTTGAGGAGATCTGG-3′ nucleotide 931 to 954 (exon 9) [33].

cDNA synthesis and polymerase chain reaction (PCR) amplification

Four micrograms of total RNA and 2.0×10^6 molecules of internal control were reverse transcribed (RT) with 200 U Moloney murine leukemia virus reverse transcriptase (RT-M-MLV, Gibco BRL, Life Technologies, Basel, Switzerland) primed with 1 µg oligo (deoxythymidine)$_{12-18}$ primer (Roche, Rotkreuz, Switzerland). The RT reaction was carried out in 50 µL (total volume) RT-buffer (50 mmol/L KCl, 5 mmol/L Mg$_2$Cl, and 20 mmol/L Tris-HCl, pH 8.3), 200 µmol/L deoxy-nucleotide-triphosphate (NTPs), 25 pmol sense and antisense primers, 5 µL of 50% formamide, 1×10^6 cpm ^{32}P-end-labeled sense primer of the GHR, and 1.5 U Ampli-Taq-DNA (Perkin-Elmer, Rotkreuz, Switzerland). Sense primer was 5-end-labeled with [gamma-^{32}P] adenosine triphosphate (ATP) (>5000 Ci/mmol) (Amersham, Zürich, Switzerland) using T4 polynucleotide kinase (Amersham). After the initial denaturation at 95°C for 1½ minutes, the PCR amplification was carried out in 24 cycles as follows: denaturation 45 seconds at 94°C, 1½ minutes annealing at 55°C, and 1½ minutes of extension at 72°C. Amplification was completed with an additional extension step at 72°C for 10 minutes. PCR was performed in an AMS (Protocol Thermal Cycler, AMS, Lugano, Switzerland).

Analysis of the PCR-amplified cDNA product

PCR products (21 µL) were separated on 2% Metaphor gel (FMC, Bioproduct, BioConcept, Allschwill, Switzerland) and stained with ethidium bromide. The bands corresponding to each specific PCR product were excised from the gels, and the amount of incorporated radioactivity was determined in a β-scintillation counter (MR-300, Automatic Liquid Scintillation System, Kontron, Zeiss AG, Zürich, Switzerland). Radioactivity (counts per minute) was plotted against the amount of template (cRNA or target molecule). Identically sized gel pieces of the negative control were excised at the position of each positive band. The radioactivity of the negative control values served as background.

Quantification of GHR mRNA

The radioactivity recovered from each gel slice was plotted against either the amount of total RNA (nanograms) or cRNA (molecules). Previous studies have shown that the two curves are parallel and exponential for both cRNA and GHR/GHBP mRNA, suggesting that the quantitative PCR is equally efficient for both controls and target RNA without any competition that could interfere with reliable quantification [32].

Nuclear run-on transcription

Nuclear run-on assays were performed as previously described [29]. Briefly, nuclei were isolated from freshly washed pellets of growth hormone–treated or untreated human mesangial cells (T-HMC) and initiated transcripts were allowed to extend in the presence of [α-^{32}P] carbamylated protein (CTP) (3000 Ci/mmol, Perkin Elmer Life Sciences, Hünenberg, Switzerland). Each run-on probe was hybridized with one of four identical filters dot-blotted with 10 µg of each GHR clone, β-tubulin clone pDβ-1SK1 DNA, and plasmid Bluescript SK+ DNA as a negative control. After cross-linking of the DNA to the filters (Hybond-C, Amersham), the filters were prehybridized overnight at 42°C. Run-on probe (6×10^7 cpm) synthesized from treated or untreated T-HMC nuclei was added, and the filters hybridized at 42°C for 72 hours. The filters were washed, dried, and autoradiographed. The strength of each autoradiographic signal was determined by liquid scintillation spectroscopy of excised filter pieces [29]. The ratio of the counts corresponding to the GHR mRNA/β-tubulin mRNA at time 3 hours without any growth hormone added was arbitrarily set as 1.0 U, and other data were expressed as GHR/GHBP run-on transcription units using this base-line. β-tubulin mRNA levels in T-HMCs did not change as a result of growth hormone treatment and could, therefore, be used to control for small variations in the quantity of RNA present in each sample analyzed. The values obtained were checked against values obtained by scanning densitometry (Bio-Rad Model 620 Video Densitometer, Bio-Rad Laboratories, Hemel, Hempstead, UK) of the autoradiograph, and were found to correlate significantly (r, 0.93; $P < 0.001$). In order to analyze whether the changes in the rate of *GHR/GHBP* gene transcription were dependent upon protein synthesis, a second set of experiments was performed where cycloheximide was added to the culture medium, at a concentration of 10 µg/mL, 30 minutes before either growth hormone (25 and 500 ng/mL) and/or GHR-A (12.5 and 500 ng/mL pegvisomant). In an additional set of experiments, 22 kD growth hormone (25 ng/mL) was combined with 12.5 ng/mL pegvisomant.

Determination of GHBP

GHBP was measured as described previously [34, 35], using a minor modification of the method originally published by Baumann et al [36]. Briefly, 100 µL of medium derived from the cell culture experiments was incubated with 30,000 cpm ^{125}I growth hormone for 45 minutes at 37°C, followed immediately by gel chromatography (Sephacryl S200, column 1.8 ×100 cm) at 4°C using PBS. Radioactivity in the eluate (1 mL fractions) was quantified and the portion of ^{125}I growth hormone bound to GHBP, with an apparent molecular weight of 85 K, was calculated. The area of each peak was quantified using an objective computer program (PeakFit, Jandel Scientific, Erkrath, Germany). Chromatography on a long column separates an additional binding protein of low affinity/

high capacity, and thereby reduces nonspecific binding [37]. A separate chromatography step to determine nonspecific binding is therefore superfluous. Growth hormone was determined in each medium sample by immunoradiometric assay (IRMA) (Medgenix, Brussels, Belgium). The apparent binding of ^{125}I growth hormone was corrected for the growth hormone concentration in the sample, based on a displacement curve using increasing amounts of unlabeled growth hormone [34].

Determination of collagen, IGF-I, and IGFBP-1 synthesis

In order to test the impact of various concentrations of growth hormone and growth hormone variants, as well as pegviosmant (12.5, 25, 50, and 500 ng/mL) on collagen types I and IV, IGF-I, and IGFBP-1 production, the immortalized human glomerular mesangial cells were incubated for 72 hours.

Each experiment was repeated five times. According to the protocol provided by the company (Cedarlane, Ltd., Hornby, Ontario Canada), 96-well flexible assay microplates (Microtest III, Falcon, 3912, Fisher Scientific, Wohlen, Switzerland) were coated with 200 μL of conditioned media and supernatant overnight at 4°C. Thereafter, the wells were washed three times for 5 minutes each in PBS at 20°C. For determination of types I and IV collagen, wells were incubated with 100 μL, 1:200 dilution of a purified rabbit anti-human collagen types I and IV (Cedarlane Ltd.) in PBS/3% bovine serum albumin (BSA) for 90 minutes at 20°C under moderate stirring. The wells were then washed three times for 5 minutes each in PBS (at 20°C) and incubated with 100 μL, 1:100 dilution (PBS/3% BSA) of the secondary antibody-peroxidase conjugate (peroxidase-labeled rabbit anti-IgG; Bio-Rad 75011, Bio-Rad Laboratoires AG, Rheinach, Switzerland) for 90 minutes at 20°C under moderate stirring. Final detection was performed by incubating the wells with 100 μL of the chromogen solution [50 mL PBS, 118 mg phenol, 24 mg 4 aminoantipyrine (AMP), 30% H_2O_2 10 μL added extemporaneously] for 90 minutes at 20°C and by assessing optical density of the stained reaction with the spectrophotometer (Microplate reader, Thermo$_{max}$, Molecular Devices/Cytion SA, Epalinges, Switzerland) at 450 nm. Regression analysis showed a linear detection of types I and IV collagen between 20 and 45 ng/well ($r^2 = 0.97$) and 10 and 40 ng/well ($r^2 = 0.96$), respectively. All concentrations were normalized to 10^5 cells.

IGF-I in the supernatant was measured using the IGF-I IRMA kit (Nichols Institute, San Juan Capistrano, CA, USA). In order to ensure removal of IGFBPs and/or IGFBP fragments, the samples were acidified to separate the IGF-I and IGFBPs. Then, excess IGF-II was added in the assay to block the IGFBP binding sites from recombining with the released IGF-I [38, 39]. Intra-assay and interassay coefficient of variance was at 5 ng/mL

5.8% and 9.7%, respectively. IGBP-1-enzyme-linked immunosorbent assay (ELISA) was performed according to the kit instructions of the company (IBL, Hamburg, Germany; http://www.IBL-Hamburg.com). Intra-assay and interassay coefficient of variance was at 3 ng/mL 3.6% and 8.1%, respectively.

Statistical analysis

Statistical differences among experimental groups were determined by Student unpaired two-tailed t test. The interassay coefficient of variation (CV) for the quantification experiments and the run-on transcription was 6% and 8%, respectively. Values of $P < 0.05$ were considered significant. Values are expressed as means ($\pm$SEM) unless otherwise stated.

RESULTS

Quantitative RT-PCR

T-HMC were cultured in the presence of different forms of growth hormone (22 kD, 20 kD) and of pegvisomant at variable concentrations. By using quantitative RT-PCR, the abundance of GHR/GHBP transcripts was measured at the beginning of the experiments as well as after 0, 1, 3, and 6 hours. The overall data of the growth hormone experiments performed are summarized in Table 1 and Figure 1.

Effect of addition of 22 kD recombinant human growth hormone. A dose-dependent increase in *GHR/GHBP* gene expression was seen when physiologic doses of 12.5 and 25 ng/mL growth hormone were added to the culture medium (Table 1 and Fig. 1A). In contrast, already the treatment with a concentration of 50 ng/mL growth hormone resulted in a significant ($P < 0.001$) decrease of GHR/GHBP mRNA expression, which was observed during the first 3 hours of the assay. After 6 hours, the level of expression increased, closely approximating the 1-hour level (Fig. 1A and Table 1). However, the 6-hour level of GHR/GHBP expression was still significantly decreased ($P < 0.01$) when compared to the 0-hour level. When a supraphysiologic concentration of 500 ng/mL growth hormone was given to the T-HMCs, a constant and significant ($P < 0.001$) decrease of GHR (GHR/GHBP) mRNA molecules given as number of molecules $\times$ $10^6/\mu g$ total RNA was observed in the first 3 hours of the assay and expression remained constantly decreased for the duration of the experiments (3 hours to 6 hours) (Fig. 1A and Table 1).

Effect of addition of 20 kD recombinant human growth hormone. The data obtained did not show any significant difference to the results obtained in the experiments where 22 kD growth hormone was used (Table 1, Fig. 1A).

Effect of addition of the GHR antagonist "pegvisomant." The addition of any dose of pegvisomant (12.5 ng/mL, 25 ng/mL, 50 ng/mL, and 500 ng/mL, as well as 20 μg/mL)

Table 1. Number of growth hormone receptor (GHR) mRNA molecules in TSV40 cells using quantitative polymerase chain reaction (PCR)

|		0 hour	P value	1 hour	P value	3 hours	P value	6 hours
		GHR/GHBP mRNA ($\times 10^6$ molecules/µg total RNA) Incubation times						
22 kD growth hormone								
0 ng/mL		1.07 ± 0.02	<0.001	0.92 ± 0.01	<0.001	0.80 ± 0.02	<0.01	0.72 ± 0.01
12.5 ng/mL		1.03 ± 0.02	<0.01	1.09 ± 0.02	NS	1.10 ± 0.02	NS	1.09 ± 0.01
25 ng/mL		1.09 ± 0.02	<0.01	1.16 ± 0.02	<0.01	1.27 ± 0.03	<0.01	1.37 ± 0.02
50 ng/mL		1.11 ± 0.02	<0.01	0.99 ± 0.03	<0.01	0.83 ± 0.04	<0.001	0.98 ± 0.03
500 ng/mL		1.14 ± 0.02	<0.001	0.86 ± 0.02	<0.001	0.54 ± 0.01	NS	0.53 ± 0.02
20 kD growth hormone								
12.5 ng/mL		1.02 ± 0.02	NS	1.09 ± 0.02	NS	1.11 ± 0.02	NS	1.10 ± 0.02
25 ng/mL		1.04 ± 0.01	<0.001	1.18 ± 0.02	<0.01	1.27 ± 0.03	NS	1.30 ± 0.03
50 ng/mL		1.08 ± 0.02	<0.01	1.01 ± 0.02	<0.001	0.82 ± 0.03	NS	0.91 ± 0.03
500 ng/mL		1.12 ± 0.02	<0.001	0.73 ± 0.02	<0.001	0.56 ± 0.03	<0.01	0.46 ± 0.02
GHR-A (pegvisomant)								
12.5 ng/mL		1.03 ± 0.01	<0.001	0.70 ± 0.03	NS	0.67 ± 0.02	NS	0.64 ± 0.03
25 ng/mL		1.01 ± 0.02	<0.001	0.74 ± 0.02	<0.01	0.65 ± 0.03	<0.01	0.58 ± 0.02
50 ng/mL		1.06 ± 0.02	<0.001	0.72 ± 0.01	<0.001	0.60 ± 0.02	<0.01	0.52 ± 0.02
500 ng/mL		1.07 ± 0.02	<0.001	0.71 ± 0.02	<0.01	0.64 ± 0.01	NS	0.61 ± 0.02
20 µg/mL		1.11 ± 0.01	<0.001	0.76 ± 0.02	<0.01	0.67 ± 0.01	NS	0.63 ± 0.01
GHR-A (pegvisomant)	22 kD growth hormone							
12.5 ng/mL	12.5 ng/mL	1.01 ± 0.01	<0.001	0.73 ± 0.03	NS	0.71 ± 0.02	NS	0.68 ± 0.03
12.5 ng/mL	25 ng/mL	1.04 ± 0.01	<0.001	0.78 ± 0.02	NS	0.72 ± 0.03	<0.01	0.64 ± 0.02
12.5 ng/mL	50 ng/mL	1.05 ± 0.01	<0.001	0.74 ± 0.02	<0.01	0.67 ± 0.02	<0.01	0.56 ± 0.02
12.5 ng/mL	500 ng/mL	1.08 ± 0.02	<0.001	0.79 ± 0.02	<0.001	0.53 ± 0.01	<0.01	0.47 ± 0.02

GHBP is growth hormone binding protein.
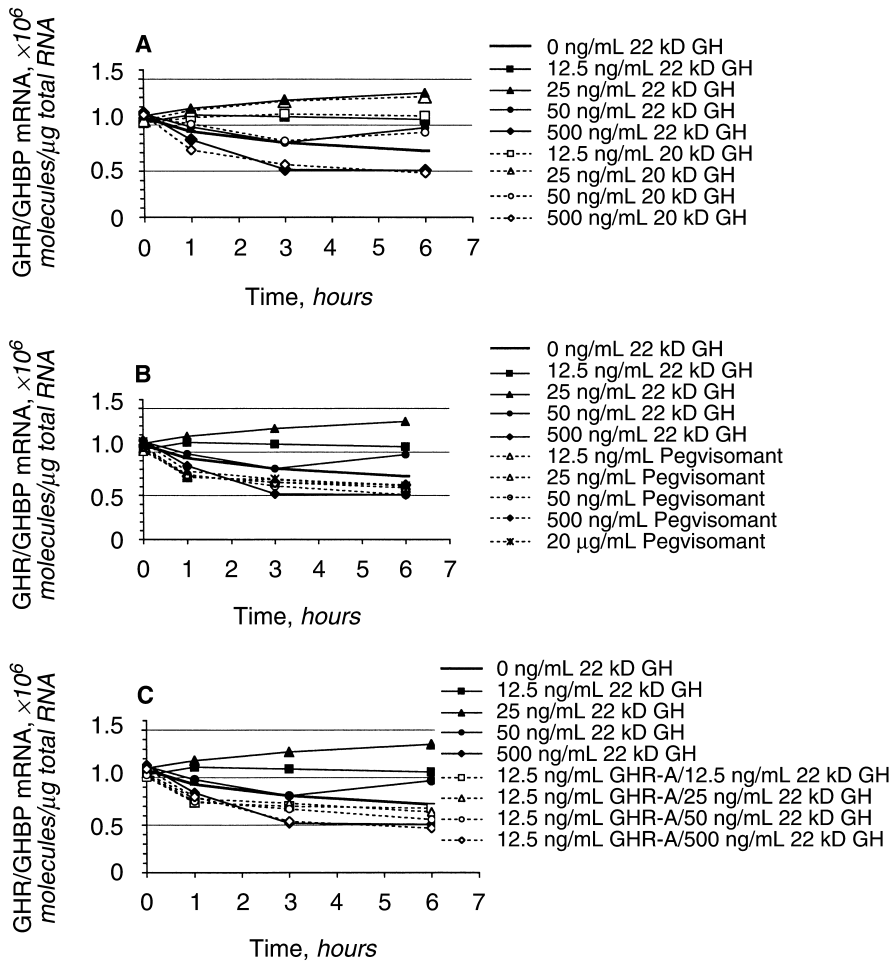
Fig. 1. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) amplification. Time course of effects of different concentrations of 22 kD and 20 kD growth hormone (GH) (*A*), pegvisomant (B2036-PEG) (*B*) as well as the inhibitory effect of B2036-PEG (GHR-A) (*C*) on *GHR/GHBP* gene expression [growth hormone receptor/growth hormone binding protein (GHR/GHBP) mRNA] in a human glomerular mesangial cell line (T-HMC) is depicted. The cells were cultured in serum-free hormonally defined medium and were harvested for RNA extraction at 0, 1, 3, and 6 hours after the addition of either 22 kD, 20 kD growth hormone, pegvisomant, or the variable concentrations of growth hormones and pegvisomant. Values plotted are means of values obtained from eight individual cultures (Table 1).
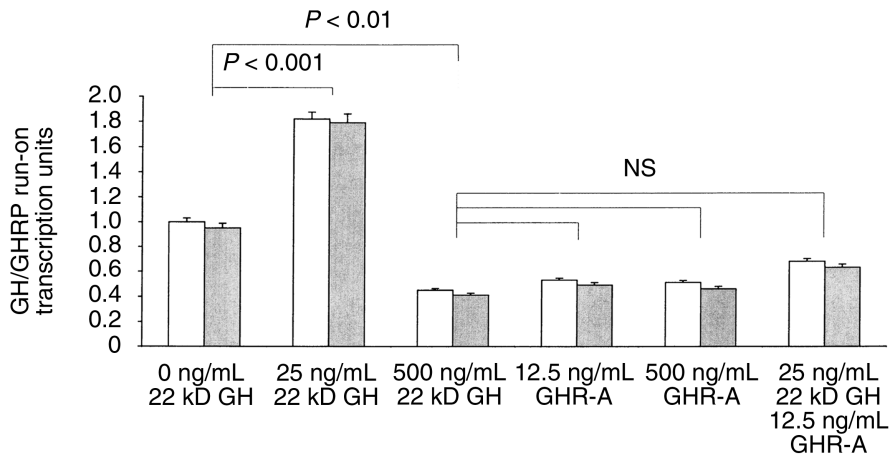
Fig. 2. Run-on experiments. Effects of different concentrations of 22 kD growth hormone (GH) (0, 25, and 500 ng/mL) or pegvisomant (B2036-PEG) (12.5 and 500 ng/mL) following 3 hours incubation time on transcription of *GHR/GHBP* gene in human glomerular mesangial cells (T-HMC) are shown. The autoradiographic signals were quantified by liquid scintillation counting of filter pieces as described in the **Methods** section. The ratio obtained with growth hormone receptor/growth hormone binding protein (GHR/GHBP) target DNA to that obtained with the β-tubulin target DNA was calculated for each set of culture conditions. This ratio was arbitrarily set at 1 U for untreated T-HMC cells, and other values were adjusted accordingly and plotted. Values shown are means of values obtained in eight parallel experiments from eight individual cultures. Symbols are: (□) without cycloheximide; (■) with cycloheximide. The lines above the bar indicate SEM.

did not augment GHR/GHBP expression over the time period studied (Table 1 and Fig. 1B). Most interestingly, the different levels reached were even significantly ($P < 0.01$) decreased in comparison to the data obtained in the experiments where no growth hormone at all was added (Table 1). Obviously the GHR/GHBP expression rates were significantly different ($P < 0.001$) at all times and concentrations when compared with the expression levels obtained in the experiments where either 22 kD or 20 kD growth hormone was used (Table 1).

To study the antagonistic impact of pegvisomant on the GHR expression, variable concentrations of the 22 kD growth hormone form were combined with 12.5 ng/mL pegvisomant and added to the cell culture. Even the lowest concentration of pegvisomant (12.5 ng/mL) used was able to completely and significantly suppress the changes in *GHR/GHBP* gene expression (Table 1 and Fig. 1C).

Run-on assay

By performing nuclear run-on experiments, we also examined the question of whether the changes in *GHR/GHBP* gene transcription levels were real, and therefore the results of a changed rate of transcription. T-HMCs were cultured under the same conditions as the cells used for RNA quantification for 3 hours in the presence of different concentrations of growth hormone (25 ng/mL and 500 ng/mL) and pegvisomant (12.5 ng/mL and 500 ng/mL). The doses of 25 ng/mL and 500 ng/mL of growth hormone were chosen because the most significant differences in transcription rate could be expected here. In addition, nuclei were prepared from freshly isolated cells and radiolabeled run-on probes were synthesized and hybridized to filters carrying GHR/GHBP and β-tubulin cDNA fragments (Fig. 2). The data were consistent with the results obtained in the quantitative RT-PCR experi-

ments. In order to analyze whether the changes in the rate of *GHR/GHBP* gene transcription depend on new protein synthesis, cycloheximide (10 μg/mL) was added to the culture medium 30 minutes before the growth hormone. The levels of the run-on transcripts remained unchanged in all the experiments (Fig. 2). This indicates that the regulating effects of growth hormone on the *GHR/GHBP* gene transcription were dependent, at least partly, on preexisting factors and did not require protein synthesis.

Determination of GHBP levels in the culture medium

In order to define whether changes in *GHR* gene expression might be reflected in GHBP levels following the incubation with 22 kD growth hormone and pegvisomant, GHBP levels were determined in the cell culture medium using the same experimental procedure as for the RNA quantification. The binding of ^{125}I growth hormone, after correction for the amount of growth hormone present in the culture medium, is presented as corrected percentage binding and values shown are indicated as mean ± SD. As shown in Figure 3, binding of ^{125}I growth hormone significantly ($P < 0.001$) increased following the addition of more than 12.5 ng/mL growth hormone. The addition of pegvisomant in variable concentrations (12.5, 25, 50, and 500 ng/mL), however, did not present any significant changes of GHBP measured when compared with the experiments where neither growth hormone nor pegvisomant was added.

Determination of types I and IV collagen, IGF-I and IGFBP-1 levels in the culture medium

In order to further analyze the impact of the various growth hormone and GHR-A concentrations on mesangial cells, the secreted quantity of types I and type IV collagen as well as IGF-I and IGFBP-1 was measured
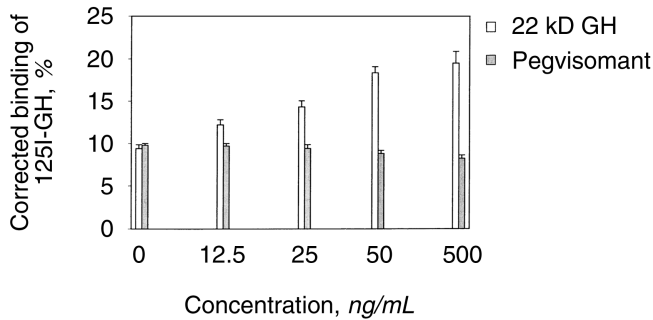
Fig. 3. Different concentrations (0, 12.5, 25, 25, 50, and 500 ng/mL) of 22 kD as well as pegvisomant (B2036-PEG) were separately added to the culture medium as described in the Methods section. Thereafter, growth hormone binding protein (GHBP) was measured following 2 hours of incubation. Values plotted are means of values obtained in eight parallel experiments from eight individual cultures.

in the supernatant of the cell culture following 72 hours of incubation (Table 2). Most important, there was a significant, growth hormone dose-dependent increase of the type IV collagen concentration in the medium, whereas type I collagen only increased following incubation at high growth hormone concentrations (500 ng/mL). This stimulatory effect was not seen after the incubation with pegvisomant, types I and IV collagen did not change at all. Further, even the addition of the lowest dose of pegvisomant (12.5 ng/mL) to the cell culture medium blocked the stimulatory effect of 500 ng/mL 22 kD growth hormone on collagen secretion (Table 2). Moreover, IGF-I concentration in the supernatant was not measurable when the cells were exposed to no or low growth hormone concentration (12.5 ng/mL) or following the pegvisomant stimulation. The addition of increasing growth hormone concentrations to the cell culture medium, however, resulted in an increase in the secreted IGF-I level. Further, IGFBP-1 concentration in the supernatant after 72-hour cell culture incubation increased parallel to the IGF-I levels.

DISCUSSION

Although recent evidence suggests that constitutive autocrine secretion of IGF-I as well as activation of its signaling pathways in the kidney participate in the development and progression of glomerulosclerosis [1, 6, 40], previous data derived from mice transgenic for IGF-I demonstrated renal and glomerular hypertrophy but no glomerulosclerosis. Mice transgenic for growth hormone, however, developed severe glomerulosclerosis, leading to uraemia and eventually death [13]. Therefore, based on many reports it is postulated that growth hormone may play an important role in the development of glomerular sclerosis in any clinical situation where increased levels of growth hormone are found [1–3, 8–10, 13, 14, 16–19, 21, 22]. This notion is further strengthened by

data obtained from studies in which the development of glomerulosclerosis was prevented in mice transgenic for a growth hormone antagonist [22, 41]. Furthermore, it was shown that the overexpression of a growth hormone antagonist, which suppressed endogenous growth hormone, also prevented the development of glomerulosclerosis in mice presenting with uncontrolled hyperglycaemia [41]. However, the mechanisms that cause or prevent the histologic lesions in glomeruli are still not fully understood. It has been suggested that the absence of glomerulosclerosis where there is a lack of functional growth hormone may be due to the interruption of a growth hormone–mediated increase in glomerular type IV collagen and laminin synthesis as well as the absence of its accumulation [8].

As mesangial cells are most important and central to the physiology and pathophysiology of the glomerulus, the aim of our studies was to analyze the impact of different growth hormone variants and pegvisomant at variable concentrations, on the *GHR (GHR/GHBP)* gene transcription in stable cell lines of T-SV40 immortalized glomerular mesangial cells [26, 42]. Human growth hormone consists of several structural modified isoforms that have been detected in the pituitary gland and the placenta as well as in the circulation [43–45]. The major physiologic component of pituitary gland–secreted growth hormone (about 85%) is a single-chain polypeptide of 191 amino acids with a molecular mass of 22 kD. Using the 22 kD growth hormone exclusion assay, an immunomagnetic extraction method for measurement of non-22 kD growth hormone isoforms in human blood [46], an average proportion of 8.1% (range, 3.2% to 13.9%) of non-22 kD growth hormone isoforms in normal prepubertal children were reported, of which the 20 kD growth hormone variant represents the major proportion [46]. Therefore, in our studies the 20 kD variant of growth hormone, which represents a growth hormone form with deletion of amino acids 32 to 46 of the regular amino acid sequence, was examined as well. As previously reported in a hepatoma cell line, the 22 kD and the 20 kD growth hormone forms showed similar effects on *GHR/GHBP* gene regulation. In these experiments using human glomerular mesangial cells, there was a constant and dose-dependent increase of growth hormone/GHBP mRNA concentration whenever physiologic doses of growth hormone (12.5 ng/mL and 25 ng/mL) were used. At supraphysiologic doses of growth hormone (50 ng/mL and 500 ng/mL) a dose-dependent and statistically significant down-regulation of the GHR/GHBP transcription was found (Table 1 and Fig. 1A). This finding might, partly, explain the reduced IGF-I mRNA production in the kidney as reported in diabetes mellitus, possibly caused by a specific growth hormone resistance [9, 11]. Importantly, all these data were confirmed using run-on experiments. This fact confirms that the data ob-

Table 2. Secreted amount of type I and IV collagen, IGF-I and IGBP-1 into the culture medium

		Collagen type IV ng/well per 10^5 cells	Collagen type I ng/well per 10^5 cells	IGF-I ng/mL per 10^5 cells	IGFBP-1 ng/mL per 10^5 cells
22 kD growth hormome					
0 ng/mL		14.3 ± 2	26.7 ± 6	Not measurable	Not measurable
12.5 ng/mL		23.8 ± 4[a]	29.3 ± 5	1.3 ± 1	Not measurable
25 ng/mL		33.7 ± 3[b]	26.2 ± 6	2.5 ± 1	3.1 ± 1
50 ng/mL		36.6 ± 5[b]	31.1 ± 7	12.3 ± 4[c]	7.8 ± 2[b]
500 ng/mL		38.5 ± 7[b]	49.2 ± 5[a]	15.8 ± 5[c]	8.4 ± 2[b]
20 kD growth hormone					
12.5 ng/mL		22.9 ± 6[a]	23.9 ± 6	Not measurable	Not measurable
25 ng/mL		34.4 ± 5[b]	28.4 ± 3	2.1 ± 1	3.9 ± 1
50 ng/mL		38.7 ± 5[b]	25.2 ± 7	9.8 ± 2[b]	5.2 ± 1[a]
500 ng/mL		40.9 ± 6[b]	36.7 ± 8[a]	13.4 ± 1[c]	9.5 ± 1[b]
GHR-A					
12.5 ng/mL		11.8 ± 4	24.8 ± 5	Not measurable	Not measurable
25 ng/mL		13.1 ± 5	29.3 ± 5	Not measurable	Not measurable
50 ng/mL		14.5 ± 4	32.0 ± 9	Not measurable	Not measurable
500 ng/mL		13.7 ± 2	29.3 ± 7	Not measurable	Not measurable
20 μg/mL		12.3 ± 5	23.7 ± 7	Not measurable	Not measurable
GHR-A	22 kD growth hormone				
12.5 ng/mL	500 ng/mL	16.1 ± 5	27.3 ± 6	Not measurable	Not measurable

Abbreviations are: IGF-I, insulin-like growth factor-I; IGFBP1, insulin-like growth factor binding protein 1; GHR, growth hormone receptor.
[a] $P < 0.05$
[b] $P < 0.01$
[c] $P < 0.001$

tained in the quantitative PCR experiments are real. As the addition of cycloheximide to the culture medium did not significantly change the transcription rate during the run-on experiment, it is clear that the synthesis of new enzymes is not necessary. Furthermore, like other polypeptide hormones, growth hormone exerts its effect by binding to a specific, high-affinity cell surface protein, the GHR. In addition, a specific serum binding protein for growth hormone (GHBP), with high affinity and low capacity for growth hormone, has been characterized in humans [47–49] and several other mammalian species, including rabbit [50], mouse [51], rat [52], pig, and dog [53]. The amino acid sequence of this GHBP has been shown to be identical to the extracellular domain of the liver membrane GHR [47]. In rats and mice, GHR and GHBP are encoded by two distinct mRNAs generated by alternative splicing of a single primary transcript [54, 55]. In mammals, however, there is evidence that GHBP is produced by specific proteolysis of the GHR [56–58]. Therefore, as in our experiments human glomerular mesangial cell were studied, we were able to obtain information on both GHR and GHBP transcription. In order to study the GHBP formation, the GHBP was measured in the cell culture medium. These analyses were performed to study the possible impact of the variable growth hormone forms on the regulated GHBP formation/release under these different culture circumstances. Interestingly, there was a constant increase of GHBP found in the cell culture medium. This fact may be because the cells are preventing themselves from being overfloated by masses of growth hormone. Similar data have already been found and reported in liver cells [29].

In order to obtaine more detailed information about the effects of growth hormone on the regulation of GHR/GHBP transcription, a GHR antagonist with enhanced affinity for the human GHR (pegvisomant) was studied at different concentrations. Pegvisomant is a member of a GHR antagonist family recently developed for the human GHR that has been successfully used in acromegalic patients [59]. As the mean serum concentration of pegvisomant in the patients treated with a daily dose of 20 mg was about 20 μg/mL, this additional concentration was used in our experiments [59]. The findings were most impressive. Pegvisomant was able to inhibit *GHR/GHBP* gene transcription at any concentration used. These data are consistent with previously reported data in diabetic mice [9, 12, 60], but the major new finding of the present study is that a GHR-A directly inhibits *GHR/GHBP* gene transcription at the cellular level in human mesangial cells.

As short-term experiments (incubation up to 6 hours) to test the impact of these various growth hormone and pegvisomant concentrations on the amount of types I and IV collagen, IGF-I, and IGFBP-1 secreted showed no effect, cell culture studies were expanded for 72 hours and the supernatant was analyzed thereafter. Importantly, after this prolonged incubation, there was an increase in collagen secretion into the cell culture medium, which paralleled the increase in growth hormone concentration added to the cell culture medium. Pegvisomant, however, did not demonstrate any secretory effect and inhibited the effect of growth hormone (500 ng/mL) on collagen secretion into the cell culture medium at even the lowest dose (12.5 ng/mL). Further, on increasing

growth hormone concentration a significant increase in IGF-I and IGFBP-1 concentration in the supernatant following incubation for 72 hours could be found. These data are in agreement with the hypothesis that IGFBP may prevent the degradation of collagen [61].

CONCLUSION

We present data showing that 22 kD and 20 kD growth hormone have the same bioactivity and a specific and direct impact on *GHR/GHPB* gene transcription, and that pegvisomant is a potent GHR antagonist not only in liver cells but also in human glomerular mesangial cells. In addition, although the *GHR/GHBP* gene transcription is down-regulated by supraphysiologic concentrations of growth hormone, this effect was not found when GHBP levels were measured in the cell culture medium. This finding may reflect a self-inhibitory effect of growth hormone on the level of *GHR/GHBP* gene transcription but not on the regulation of the shedding of GHBP and may, therefore, be of physiologic interest.

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