Alteration in bovine growth hormone histidine 22 results in transgenic mice with an enhanced diabetogenic profile

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1. Introduction

Growth hormone (GH), a 191 amino acid polypeptide hormone secreted by the anterior pituitary gland in mammals, is not only necessary for normal linear growth, but is also involved in other aspects of metabolism (15). GH displays an array of biological effects on protein, carbohydrate, and lipid metabolism. The structure of GH is 54% helical, containing four α-helices arranged in a tightly packed helix bundle (1,13). GH from numerous species share a high degree of amino acid identity (27,38). Helix I of bovine (b) GH encompasses residues 7-34. This α-helix, encoded by exon II of bGH, is distinctly amphiphilic (or amphipathic) with a strongly hydrophobic region (Fig. 1). These hydrophobic amino acids are highly conserved across numerous species and members of the GH-prolactin (PRL) family (27). Previous studies have shown that portions of the amino-terminus of GH may be involved in the insulin-like and/or diabetogenic activities, as well as receptor binding of the hormone. For example, amino terminal fragments (6-13 and 4-15) of hGH were found to be hypoglycemic both *in vivo* in rats and by isolated adipocyte glucose uptake (26,23). Similarly, hGH 1-43 was found to enhance the sensitivity of adipose tissue to insulin action and also to be an insulin potentiator *in vivo* by various studies (17,32,33). An amino terminal

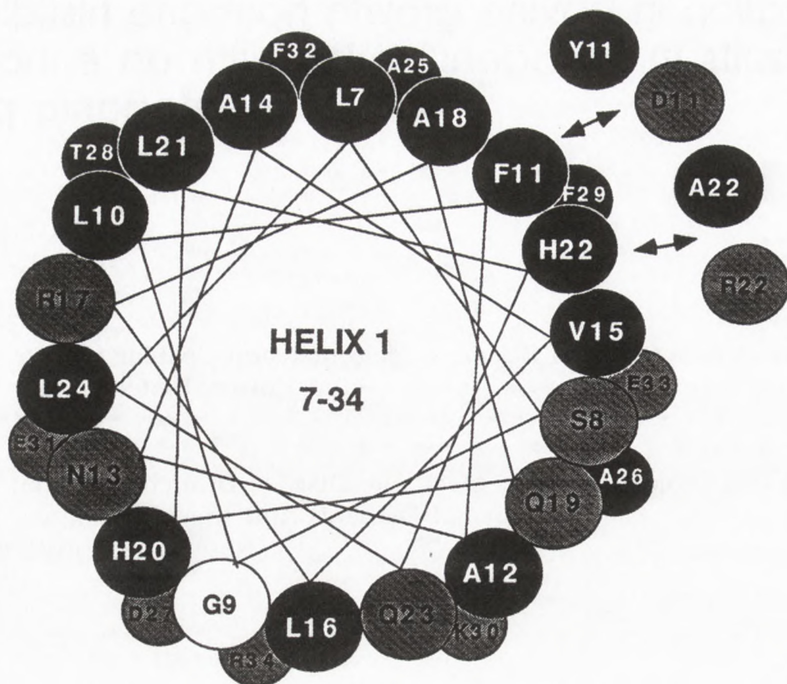

Fig. 1. GH helix 1 diagram with substitutions. Shown is bGH α-helix 1 with amino acid substitutions at positions 11 and 22 identified to the right of the wild-type helical wheel. The hydrophobic amino acids are black, white are neutral, and gray are hydrophilic according to the hydrophilicity scale of Hopp and Woods (19).

deletion mutant was found to have substantially decreased diabetogenic activities by glucose tolerance testing in obese mice (36). Additionally, Cunningham and Wells (9) proposed that hGH Phe 1O, which corresponds to bGH Phe 11, is involved in receptor binding.

Supraphysiological concentrations of GH in the peripheral tissues of transgenic mice, such as the kidney, may lead to certain pathological conditions such as glomerulosclerosis. Thus, mice expressing a GH transgene may be used not only to study growth, but also as a model of glomerular disfunction or to model the clinical condition of acromegaly. In this study, site-specific mutagenesis of GH and transgenic mouse production were used in concert to help define structure/function relationships of the first α-helix of bGH. Specific amino acids were targeted in bGH that might be involved in the diabetogenic, insulin-like, and/or receptor binding of the hormone. Sites targeted for mutagenesis were chosen mainly by comparison of published GH sequences from a number of species. Phe 11 and His 22 are highly conserved throughout GH, PRL, and PL families and across species (Fig. 1). Specifically, following mutagenesis and expression in cultured mu-

rine cells, bGH-F11D, F11Y, H22A, and H22R genes were used to generate transgenic mice which express the bGH analogs. Various parameters of these animals were examined including body growth, *in vivo* diabetogenic activity, kidney histology, and relevant blood chemistry parameters.

2. Materials and methods

2.1. Plasmid Mutagenesis

For oligonucleotide-directed site-specific mutagenesis, plasmid pbGH10Δ16 was used as the parental plasmid (6). This plasmid is pBR322 based and contains the mouse metallothionein I transcriptional regulatory element (MT-I)-along with bGH exons I-V and two introns (*a* and *d*). Mutations were made between sites Nar I and Bst B1 restriction sites in pbGH10Δ16 using double-stranded oligonucleotides encoding the desired amino acid substitution. The mutations were confirmed by dideoxy sequence analysis (Sequenase; United States Biochemical) (data not shown). These amino acid substituted bGHs encoded by the mutated genes are referred to as bGH analogs throughout this manuscript.

2.2. Transgenic Mice

Transgenic mice were produced by the direct microinjection of the DNA of interest into the male pronucleus of fertilized mouse eggs obtained from B6SJLF1/J (C57BL/6J x SJL/J) as described by Wagner et al., (37). After microinjection, the eggs were transplanted into the uteri of foster mothers and carried to term. DNA extracted from mouse tails was analyzed for the transgene by slot blot hybridization analyses as previously described (6). Mice were fed *ad libitum* and body weight was monitored on a weekly basis.

Serum IGF-1 levels of the bGH mice were determined using a heterologous RIA kit for IGF-1 (Nichols Inst., San Juan Capestrano, CA). Prior to the RIA, the transgenic mice sera were acid-ethanol extracted to dissociate the IGF-1 binding proteins/IGF-1 complexes and the assay was set-up according to the manufacturer's instructions.

Regression analysis was used to prepare a standard curve and determine IGF-1 concentrations. The IGF-1 values were recorded in ng/ml ± STD.

Blood urea nitrogen (BUN) and serum creatinine (SCR) concentrations were determined using a Kodak Ektachem DT6O analyzer, an accompanying DTSC module, and the appropriate Ektachem dry chemistry slides (Eastman Kodak, Rochester, NY). Serum samples (10 μl) from bGH-analog transgenic mice and NTG were tested in duplicate or triplicate. Results from each age group for a given bGH analog were recorded as the mean (mg/dl) ± STD.

Radio-immuno assays (RIA) for bGH and insulin were performed by standard methods. Briefly for the bGH RIA, assays were performed in duplicate

with 10 μl of samples or standards, ^{125}I-pGH, and anti-bGH 1° antibody (anti-bGH AFP#55, from Dr. Parlow, Harbor-UCLA) and RIA buffer. Samples were vortexed thoroughly and incubated overnight (for a minimum of 16 hours) at room temperature. Following overnight incubation, 2° antibody (goat anti-monkey IgG, Sigma, St. Louis, MO) and Pansorbin Cells (Protein A, Calbiochem, La Jolla, CA) were added to the mixture and vortexed completely. Following incubation and centrifugation, the samples were evaluated by gamma counting.

Similarly, for the insulin RIA 10 μl of the serum sample was added to RIA buffer, radiolabelled human insulin (diluted in RIA buffer to 5000 cpm/200 μl), and guinea pig anti-insulin (Amersham, Arlington Heights, IL). The secondary antibody used for the insulin RIA is goat anti-guinea pig IgG (Sigma). Regression analysis was used to generate a standard curve from which the bGH or insulin concentration was determined.

2.3. *In Vivo* Diabetogenic Activity

Glucose clearance tests were performed on bGH and bGH analog transgenic mice and their nontransgenic (NTG) littermates that had been fasted for 16 hours. A fasting blood sample was obtained from the tail at time zero. Mice were injected (ip) with 100-250 μl of glucose solution (2 mg glucose/g body weight). Blood samples were collected starting at 15 min and at intervals of 30 and 60 min over a period up to 4 hours post glucose injection. The collected samples were immediately analyzed for glucose using a One Touch II blood glucose meter (Lifescan Inc., Ca). The values were recorded in mg/dL ± STD.

2.4. Kidney histology

Upon sacrifice, the kidneys of the mice were removed and immediately placed in Carnoy's fixative. Coronal kidney sections from bGH-analog transgenic mice were embedded in methacrylate and stained with hematoxylin and eosin and PAS (Periodic acid-Schiff). Sections of 4 μm were examined using a light microscope, graded on a scale of 0-4^{+} according to the severity of the glomerulosclerosis with four being the most severe, and examined for tubulointerstitial lesions (39).

3. Results

3.1. Transgenic Mice

In order to test the ability of these bGH analogs to promote growth, transgenic mouse lines were established which express bGH-F11Y, bGH-F11D, bGH-H22A, and bGH-H22R. Mouse lines were found to contain approximately 2-5 copies of bGH DNA. Shown in Figure 2 is a comparison of body weight

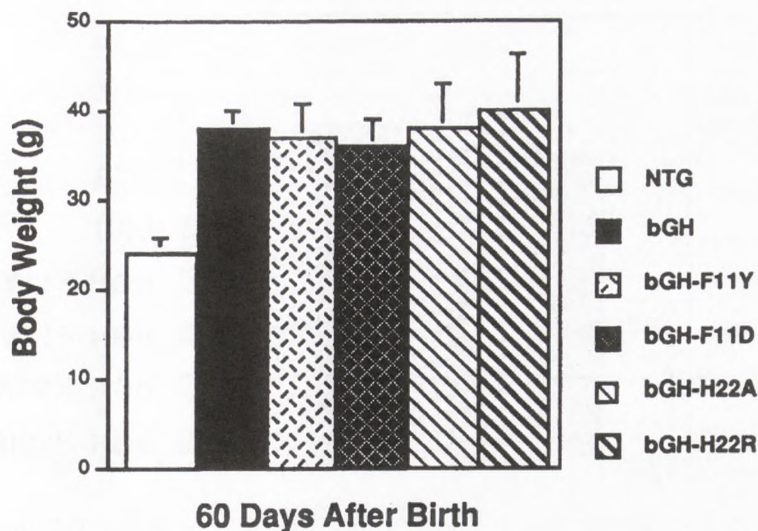

Fig. 2. Body weight (g) of bGH analog transgenic mice 60 days post-birth. There were no significant differences among the GH and bGH analog transgenic mouse lines. The body weight of the transgenic mice are significantly different from the nontransgenic mice (NTG) ($p < 0,05$).

(g) at 60 days between wild-type bGH transgenic mice and bGH-F11Y, F11D, H22R, H22A, and NTG. The transgenic mouse lines all exhibited an enhanced growth phenotype relative to their NTG littermates. Growth ratios for F11Y and F11D male TG mice at 60 days were approximately 1.5 when compared to NTG males. The growth ratios were 1.6 and 1.7 for the bGH-H22A and bGH-H22R lines, respectively. There was no significant body mass difference among the bGH and bGH analog mice at 60 days.

3.2. IGF-1

No significant differences of insulin-like growth factor 1 (IGF-1) levels were observed among the bGH and bGH analog transgenic mice (Fig. 3). As expected, the bGH and bGH analog mice exhibited significantly higher levels of IGF-1 (690-755 ng/ml) as compared to the NTG control mice (315 ng/ml). However, the NTG IGF-1 levels are significantly different ($p < 0.05$) from the other mice.

3.3. bGH and insulin

The insulin levels of the transgenic mice were elevated along with the levels of GH found in the bGH transgenic mice. RIAs for bGH showed varying levels of the proteins in the bGH lines examined. The concentrations of bGH in the sera ranged from 2 to 5 μg/ml for 4-6 month old mice (Table 1).

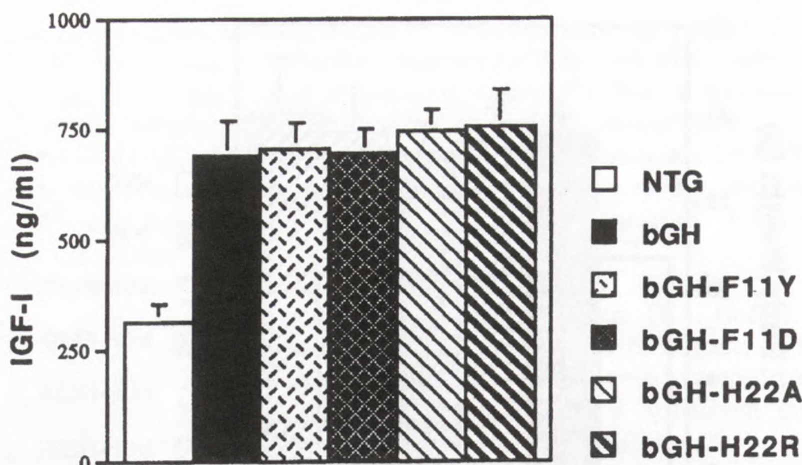

Fig. 3. Serum insulin-like growth factor-1 concentrations. Shown are NTG (n=9, 4-9 mo), transgenic bGH wild-type mice (n=8, 5-10 mo), bGHF11 D (n=5, 4-8 mo), bGH-F11Y (n=5, 3-9 mos), bGH-H22A (n=3, 4mo), bGHH22R (n=7, 4-9 mo). The NTG IGF-1 levels are significantly different from the bGH and bGH analog mice ($p<0.05$).

Insulin values for wild-type bGH and the bGH analog mice varied from approximately 5-8 ng/ml, while the values for NTGs were 2.5 ng/ml. Serum bGH or insulin values varied within the same range for the wild-type bGH mice and the bGH analog mice. For example, the bGH analog mice all had insulin levels significantly higher than NTG ($p < 0.05$).

TABLE 1

INSULIN AND bGH CONCENTRATIONS FOR bGH ANALOG TRANSGENIC MICE

Analog	bGH	Insulin
bGH	3.8 µg/ml	6.5 ng/ml
bGH-F11Y	3.3 µg/ml	5.1 ng/ml
bGH-F11D	2.2 µg/ml	6.0 ng/ml
bGH-H22A	4.5 µg/ml	6.8 ng/ml
bGH-H22R	4.9 µg/ml	8.0 ng/ml

3.4. Blood urea nitrogen and serum creatinine

Levels of BUN increased more than six-fold after 5 months of age in the bGH-H22R mice (Fig. 4), while SCR increased by about two and one half-fold in the bGH-H22R animals of that age group (Fig. 5). These elevated levels of BUN and SCR were significantly different ($p < 0.05$) as compared to levels in the other transgenic mice.

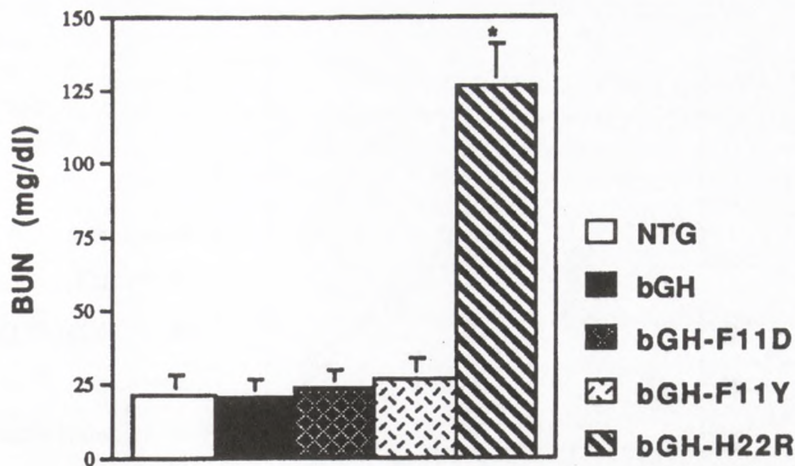

Fig. 4. BUN for bGH analog mice age 6 months and older. Shown are NTG (n=6, 6-9 mo), bGH wild-type (n=11, 6-10 mo), bGH-F11 D (n=5, 6-23 mo), bGH-F11Y (n=6, 6-14 mo), bGH-H22R (n=5, 7-9 mo). BUN values are expressed as the mean ± STD. The mean for bGH-H22R is significantly different ($p<0.05$) from the other mice tested.

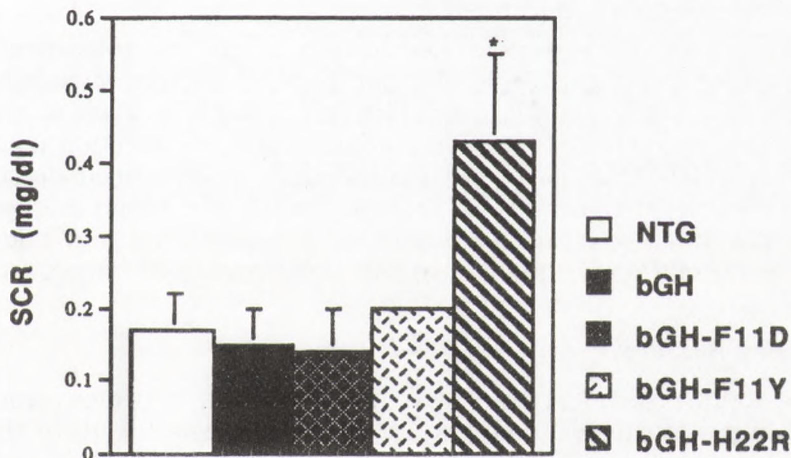

Fig. 5. SCR levels for bGH analog mice age 6 months and older. Shown are NTG (n=6, 6-9 mo), bGH (n=11, 6-10 mo), bGH-F11D (n=5, 6-23 mo), bGH-F11Y (n=6, 6-14 mo), bGH-H22R (n=5, 7-9 mo). SCR values are expressed as the mean ± STD. The mean for bGH-H22R is significantly different ($p<0.05$) from the other mice tested.

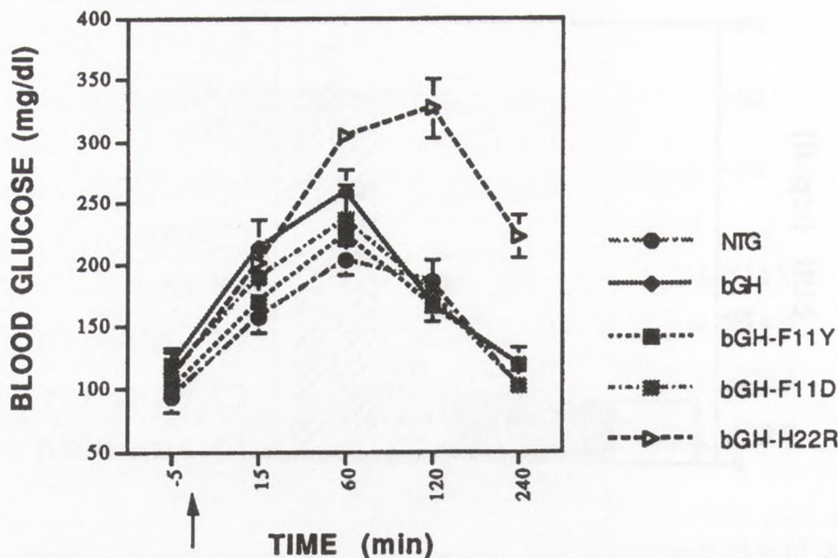

Fig. 6. *In vivo* glucose tolerance assay in bGH transgenic mice. The arrow indicates the zero time point for glucose injection. Shown are NTG (n=6, 6-9 mo), bGH (n=6, 6 mo), bGH-F11Y (n=7, 6-9 mo), bGH-F11 D (n=5, 6-11 mo), and bGH-H22R (n=8, 6-10 mo). The values are expressed as mean ± STD. Glucose levels from bGH-H22R mice differ significantly after one hour relative to other mice.

3.5. *In Vivo* Diabetogenic Activity

Diabetogenic activity was examined *in vivo* by glucose tolerance testing. A time course of blood glucose levels from bGH transgenic mice before and after glucose injection following an overnight (15-16 h) fast is shown in Figure 6. The arrow indicates the time point for glucose injection (*ip*, 2 mg/g body weight). bGH-H22R mice (6-9 mo) possess an enhanced diabetogenic activity relative to other transgenic mice and NTG. The blood glucose levels in these H22R mice were found to peak at a higher level and take longer to return to normal levels, relative to the other transgenic mice.

3.6. Kidney Histology

Coronal kidney sections from bGH analog transgenic mice which were embedded and stained demonstrated glomerulosclerosis for all of the bGH-analog kidneys examined. Glomerulosclerosis of this type has previously been reported for bGH mice (39). The sclerosis in the bGH analog mice increased in severity with age. Scores for 4-12 month old mice ranged from $2\text{-}4^{+}$ (Table 2). Five week old mice had scores of $0\text{-}1^{+}$. Similarly, nontransgenic littermates have glomerulosclerosis scores of 0^{+}. Histologically, large glomeruli were a predominant feature of the glomerulosclerosis. Proliferative

glomemlar cells, enlarged kidneys, and some tubulointerstitial lesions were also observed. Additionally, bGH-H22R mice began to develop cystic lesions between 4 and 5 months of age. bGH-H22R mice, aged 7-10 months (n=5), had cystic lesions. In contrast, cysts were not found in hGH, bGH, or bGH analog mice previously examined (39,4,5).

TABLE 2
KIDNEY HISTOLOGY IN bGH ANALOG MICE

bGH Analog	Age	Kidney Histology
H22R (n=2)	5 wks	enlarged glomeruli, no cysts minimal sclerosis +
H22R (n=5)	4-5 mos	4/5 have cystic lesions glomerulosclerosis ++/+++
H22R (n=5)	7-10 mos	numerous large cystic lesions glomerulosclerosis +++/++++
H22A (n=2)	4 mos	no cysts diffuse sclerosis ++/+++
F11Y (n=4)	6-12 mos	no cysts glomerulosclerosis ++/++++
F11D (n=2)	6-10 mos	enlarged glomeruli, no cysts glomerulosclerosis +++/++++

4. Discussion

Mice which expressed foreign GH genes were among the earliest transgenic animals developed (28,25). IGF-1, insulin, and other genes which influence the endocrine status of the animal have also been successfully introduced using transgenic technology (20). In transgenic mice which expressed either bGH-F11D, F11Y, H22A, or H22R we found that a single amino acid mutation at position 11 or 22 did not result in altered growth promotion. Correspondingly, all of the bGH analog and bGH mice exhibit significantly higher levels of IGF-1 (approximately 2.5 fold) as compared to the NTG controls (Fig. 3). None of these amino acid substitutions were predicted to disrupt the α-helical character of this region, but some did alter the amphiphilicity (Fig. 1).

Previously we reported that no significant differences exist between the binding affinities of wild-type bGH and the amino-terminal bGH analogs at positions Phe 11 or His 22 employing a cloned GH receptor (30,31). Thus, it appears that altered amphiphilicity via these substitutions is not critical for altering receptor binding or growth. Although it has not been demonstrated that His 22 interacts with the GHR, position 22 is next to 11 in the α-helix, and Phe 11 was proposed to be involved in receptor binding (9). In an alanine-scanning mutagenesis study using *E. coli* derived hGH, hGH-FlOA, which corresponds to Phe 11 in bGH Alanine-scanning studies, did not include the residue (hGH-H21) corresponding to His 22 of bGH, however, studies of Zn^{2+}:hGH dimerization and hGH:hPRLBP binding with Zn^{2+}

identified residue His 21 of hGH to be one of the putative Zn^{2+} ligands (11,10). His 22 is a highly conserved amino acid throughout the GH-PRL family of proteins and may serve a yet undefined role in GH metabolism.

The diabetogenic activity of GH has been well established using both pituitary derived and recombinant GHs for *in vivo* and *in vitro* studies (21,22,2,3). For example, an amino-terminal deletion mutant (Des-7 hGH) was found to have decreased diabetogenic activities, while retaining its lactogenic and GH binding activities (36). Although it has been demonstrated that GH has diabetogenic effects *in vivo*, bGH-H22R is unique in that it enhances the diabetogenic actions of the hormone as shown by *in vivo* glucose tolerance studies (Fig. 6). Transgenic mouse lines bGH-F11Y, bGH-F11D, bGH-H22A, and wild-type bGH all exhibited the expected diabetogenic activity. However, the glucose intolerance was significantly greater in the bGH-H22R mice relative to other animals of the same age. The elevated diabetogenic status of bGH-H22R mice is independent of the levels of IGF-1, insulin, and bGH which were elevated in all the bGH and bGH analog mice (Fig. 3, Table 1).

Overexpression of GH in mice may be used as a model system for examining conditions like acromegaly. The prevalence of insulin resistance or glucose intolerance in acromegalic patients is significantly higher than in the remainder of the population, and has been demonstrated in acromegalic animals as well (18,29). The overexpression of GH exposes peripheral tissues such as the kidney not only to excess GH, but may also expose them to conditions favoring the expression of GH dependent growth factors such as IGF-1, which may contribute to microvascular diseases by autocrine and/or paracrine effects (35).

GH causes a variety of anabolic and metabolic effects on skeletal muscle and soft tissues including the kidney. Mice transgenic for bGH have glomerular lesions that resemble diabetic nephropathy (4,24). However, transgenic mice which express GH antagonists (bGH-G199R and hGH-G120R) exhibit a dwarf phenotype and do not develop glomerular lesions (7,8,4). Even after the induction of diabetes, glomerulosclerosis is not found in these antagonist expressing mice (5). Furthermore, increased glomerular volume, which exceeded renal or body growth, was revealed by morphometric analyses of the GH transgenic mice (39,40). Interestingly, mice transgenic for IGF-1 have renal and glomerular hypertrophy and more serious renal insufficiency than their GH counterparts as indicated by higher SCR levels, although they have much less glomerulosclerosis (16,14). In order to estimate renal glomerular filtration rate (GFR), BUN and SCR levels are commonly used in clinical settings. Elevated BUN and SCR values are an indication of decreased GFR and chronic renal insufficiency. The bGH-H22R mice aged 7-9 months had BUN and SCR concentrations uncharacteristically high for bGH mice (Fig. 4, 5). BUN levels were approximately four to seven times higher than bGH mice of the same age group. BUN and SCR levels in the bGH-F11Y and F11D mice were not significantly different from the bGH mice. The BUN and SCR values in this study are consistent with the severity of the glomerulosclerosis found in the animals.

The bGH-F11Y, F11D, H22R, and H22A transgenic mouse kidneys examined had glomerular lesions normally found in GH transgenic mice. In general the kidneys contained enlarged glomeruli, some tubulointerstitial and arteriolar lesions, and severe glomerulosclerosis (Table 2). Distinctively, kidneys from mice aged 4-10 months of the bGH-H22R mouse line also contained numerous cystic lesions. These cysts, which are dilatations (or an enlargement of the lumen) of tubules, were found throughout the kidney cortex. Moreover, these bGH-H22R mice do not have increased fasting blood glucose levels, but they do attain an erihanced diabetogenic status by glucose tolerance tests. The bGH-H22R mice not only exhibit an enhancement of the diabetogenic properties of GH, but also have elevated BUN and SCR and severe glomerulosclerosis, which is partially characterized by cystic kidney lesions. The pathogenesis of this cystic dilatation in bGH-H22R is not known. Numerous kidney disorders can be characterized by cystic lesions. However, the histology of the bGH-H22R kidneys did not appear to entirely correspond to any well defined kidney disease.

The mechanism for the enhanced diabetogenic activities and renal cysts is unidentified, however several possibilities exist. It is possible that the altered amphiphilicity created by substituting the hydrophobic His with the highly hydrophilic Arg causes the changes in bioactivities. Further, bGH-H22R might interact with a different subpopulation of GHRs than does wild-type GH. Receptor heterogeneity is one of the possible explanations for the varied biological activities of the hormone (34). The highly conserved position 22 is located on the outer face of helix 1 of the GH molecule where it is accessible for binding and/or other receptor interactions. Perhaps the substitution of an Arg for His minimally alters the structure of the amino-terminal region of bGH, which in turn alters GHR interactions so that post-receptor signalling is effected.

It is not yet clear what rols GH and IGF-1 may play in the regulation of normal renal physiology and the incidence of progressive glomerulosclerosis. Accordingly, GH transgenic mice may serve as model system to increase our knowledge of glomerulosclerosis and related conditions.

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Alteration in bovine growth hormone histidine 22 results in transgenic mice with an enhanced diabetogenic profile

Summary

Transgenic mice which overexpress growth hormone (GH) may be used a model system to examine growth, kidney pathology, as well as the medical condition known as acromegaly (hyper-growth hormone secretion). GH is a pleiotropic 22 kDa polypeptide hormone which elicits body growth in jeuvenille animals and also mediates protein, carbohydrate, and lipid metabolism. The structure/function relationships of selected residues of bovine (b) GH α-helix I were approached using site-directed mutagenesis in concert with the production of bGH analog transgenic mice. Phenyalanine (Phe, F) 11 and histidine (His, H) 22 in the amino-terminus of bGH were the targeted amino acids. bGH and the bGH analog transgenic mice all exhibited the enhanced growth phenotype similar to bGH transgenic mice and had elevated IGF-1 serum concentrations. However, bGH-H22R mice demonstrated levels of blood urea nitrogen (BUN) and serum creatinine (SCR) several fold higher than the other transgenic mice. Elevated BUN and SCR are an indication of renal insufficiency in this mouse line. Glucose tolerance testing in the bGH-H22R mice revealed that they possessed a lower tolerance for glucose, or an enhancement of the diabetogenic properties of the hormone as compared to wild-type and other GH analog transgenic mice: In addition to the glomerulosclerosis found in bGH mice, histological examination of the mature bGH-H22R mice demonstrated severe glomerulosclerosis, as well as cystic kidney lesions.

Key words:
growth hormone, transgenic, site-specific mutagenesis, diabetogenic, glomerulosclerosis, kidney.

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