Effect of two human growth hormone receptor antagonists on glomerulosclerosis in streptozotocin-induced diabetic rats

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ABSTRACT

AIM: To explore the feasibility of human growth hormone (hGH) receptor antagonist in the treatment of end-stage diabetic renal complications. **METHODS:** Two hGH mutants, hGHA1 (Cys-hGH-del1-4, G120R, K168A, E174A, C182S, del186-191) and hGHA2 (hGH-H21A, G120R, E174A) were expressed in *E coli*. The IC_{50} (Mean±SD) values for the mutants for inhibiting ^{125}I-hGH binding to rabbit growth hormone receptor were (65±10) ng for hGHA1, (27±5.6) ng for hGHA2, and (10±0.6) ng for wild type hGH, respectively. **RESULTS:** After treatment for 12 weeks, the renal histology analysis showed that treatment with hGHA2 at 4 mg/kg body weight daily markedly suppressed glomerulosclerosis in streptozotocin-induced diabetic Sprague-Dawley (SD) rats; hGHA1 at the same dosage slightly increased the renal damage compared with saline; while wild type hGH at 1 U/kg body weight daily severely worsened the glomerulo-sclerosis in diabetic SD rats. **CONCLUSION:** The data indicated that hGHA2 inhibited the end-stage glomerulosclerosis in diabetic rats, but hGHA1 mildly increased the glomerulosclerosis.

INTRODUCTION

The development of growth hormone (GH) receptor antagonist and its therapeutic application in treating Gigantism have been widely known in recent years. Another exciting potential for GH receptor antagonist is its inhibitory effect on glomerulosclerosis, one of the end-stage diabetic kidney complications[1-3]. It was reported that transgenic (bGH-G119K) mice were resistant to glomerulosclerosis after treatment with streptozo-tocin (STZ), suggesting that GH receptor antagonist could be a new approach to inhibiting the end-stage diabetic kidney complications.

This exciting result reported by Chen *et al*[2] was based on the experiments using transgenic mice, in which the metallothionein I (MT-I) promoter was used to drive the expression of GH receptor antagonists. There were two facts about the GH receptor antagonist expression in their transgenic mice that did not correlated well with the clinical settings. First, the plasma concentration of the GH receptor antagonists in some transgenic mice was as high as 10 mg/L, which can not be attained in clinical treatment. Second, the expression of GH receptor antagonist driven by MT-I promoter started from as early as embryonic stage, so the high plasma concentration of GH receptor antagonist had already existed before the transgenic mice were given STZ to induce diabetes at the age of week 10. This is also markedly different from the clinical treatment. Therefore, we attempted to test the inhibi-

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tion of the recombinant hGH mutants on the diabetic glomerulosclerosis in STZ-induced diabetic Sprague-Dawley (SD) rats in conditions more similar to the clinical settings.

MATERIALS AND METHODS

Materials and reagents U-DNA Mutagenesis kit was from Boehringer Mannheim. Altered Sites II *in vitro* Mutagenesis System was from Promega. T7 Sequencing kit was from Pharmacia. Streptozotocin (STZ) was obtained from Sigma. hGH was expressed from *E coli* by recombinant DNA technology in this laboratory and ^{125}I-hGH was from Shanghai Institute of Biological Productions. Other restriction enzymes and T4 DNA ligase were from Promega. pALTER-hGH plasmid was constructed in our lab. M13BM19 vector was from Boehringer Mannheim, and OmpA3 expression plasmid was kept in this lab.

Site-directed mutagenesis pALTER-hGH plasmid was digested with Xba I/EcoR I and the hGH fragment was recovered and ligated into M13BM19 plasmid at the same cutting sites. Site-directed mutagenesis was performed using U-DNA Mutagenesis kit according to the producer's protocol using: Primer-1: 5' GAATAGACGAGATAGTGGgcAGGCCTGCGCTACGGT 3' for Cys-del1-4; Primer-2: 5' GCGTTTGGATGCgTTC-CTCTAGGTCC 3' for G120R;Primer-3: 5' GACCTTG-TCCATGTCagcCCTGAAGC-AGTAG 3' for K168A; Primer-4: 5' GCAGGAATGTCgCGACCTTGTCC 3' for E174A, Primer-5: 5' CCACAGCTGCCCtaCACA-GAGCGagAC-TGCACGATGCTC 3' for C182S-del186-191. M13BM19-hGHA1 (Cys-hGH-del1-4, G120R, K168A, E174A, C182S, del186-191) was obtained. The second hGH mutant, hGHA2 (hGH-H21A, G120R, E174A) was obtained after 3 rounds of site-directed mutagenesis using Altered Sites II *in vitro* Mutagenesis System according to the protocol of the kit with pALTER-hGH plasmid as the template. The primers used were Primer-2, Primer-4 listed above and Primer-6: 5' CAAAGGCCA-GCTGAgcAAGACGATGGGCCG 3' for H21A. All the constructs were confirmed by manual sequencing using T7 Sequencing kit.

Expression and purification of hGHA1 and hGHA2 M13BM19-hGHA1 and pALTER-hGHA2 plasmids were digested with Xba I/EcoR I. The hGHA1 and hGHA2 fragments were gel recovered and ligated into OmpA3 expression vector at the same enzyme cutting sites to obtain two expression constructs OmpA3-hGHA1 and OmpA3-hGHA2. These two expression constructs were then transformed into *E coli* BL21 competent cells respectively. Single colony of OmpA3-hGHA1/BL21 or OmpA3-hGHA2/BL21 was inoculated into 500 mL medium, shaken at 37 ºC for 12 h. Then the 500 mL culture was transferred into 10 L medium in automatic fermenter and cultured for 5 h at 37 ºC to be the seed culture. Subsequently, the seed culture was fermented in 200 L fermenter for another 14 h. The recombinant bacteria were pelleted, osmotic-shocked, and centrifuged. The supernatant, which contained the products was collected and hGHA1 or hGHA2 was further purified by isoelectric precipitation, ammonium sulfate precipitation, followed by Mono-Q ion-exchange chromatography to get the final product: hGHA1 or hGHA2. This process for production of hGHA1 and hGHA2 was the same as the protocol for recombinant human growth hormone (rhGH) production developed in our lab.

Preparation of rabbit liver membrane GH receptor The livers from two female rabbits (pregnancy day 25) were homogenized in 0.3 mol/L sucrose solution and filtered through 8 sheets of cheesecloth. The filtrate was centrifuged at 15 000×*g* at 4 ºC for 20 min. The supernatant was centrifuged again in the same condition. The final supernatant was ultracentrifuged at 100 000×*g* for 90 min at 4 ºC. The pellet was dissolved with Buffer A (Tris-HCl 25 mmol/L pH 7.6, BSA 0.1 %, merthiolate 0.6 mmol/L, $CaCl_2$ 10 mmol/L) at the ratio of 1mL Buffer A per gram of liver and stored at -80 ºC in aliquots.

Competitive inhibition of ^{125}I-hGH binding to rabbit GH receptor (GHR) with hGH, hGHA1 and hGHA2 Human GH, hGHA1 and hGHA2 were diluted with Buffer A. Twenty microliter of rabbit GH receptor preparation was mixed with 180 μL of diluted samples before 50 μL of ^{125}I-hGH was added. The mixture was incubated overnight at 4 ºC, then 1mL of Buffer B (Sodium acetate 25 mmol/L pH5.4, BSA 0.1%) was added. The samples were centrifuged at 15 000×*g* for 6 min at room temperature. The pellets were subject to scintillation counting and the competitive inhibition curves for hGH, hGHA1, hGHA2 were plotted and IC_{50} values were determined by inspection.

Treatment of the STZ-induced diabetic SD rats with hGH, hGHA1 and hGHA2 and their effects on glomerulosclerosis SD rats weighting 140-200 g were given a single dose of STZ ip at 55 mg/kg body weight after fasting for 10 h. Four days later, those rats whose

plasma glucose concentration were higher than 16.65 mmol/L[4] were taken as diabetic rats. One week later, 29 diabetic SD rats were randomized into 4 treatment groups: Group 1: saline-treated (0.3 mL, sc, daily, n=10), Group 2: hGH-treated (1 u/kg body weight, sc, daily, n=5), Group 3: hGHA1-treated (4 mg/kg body weight, sc, daily, n=7), Group 4: hGHA2-treated (4 mg/kg body weight, sc, daily, n=7). The treatment lasted for 12 weeks. Since the beginning of week 9, all the diabetic rats were given 1 unit of insulin sc every 2 d to maintain their viability. Seven normal SD rats with no STZ treatment were used as non-diabetic normal control. At the end of week 12, all the rats were sacrificed, the kidney sections were stained with periodic acid-Schiff and examined by light microscopy.

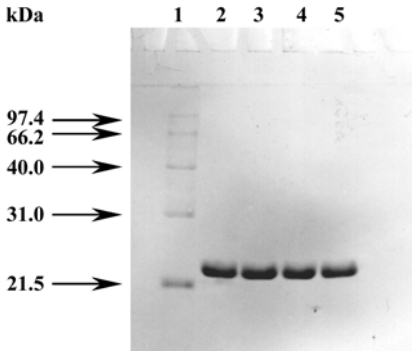

Fig 1. Reduced SDS-PAGE of rhGH, hGHA1, and hGHA2 which were obtained from 200 L fermentation of *E coli* culture. Lane 1: molecular weight marker of proteins; Lane 2: standard hGH; Lane 3: rhGH; Lane 4: hGHA1; Lane5: hGHA2.

RESULTS

hGHA1 800 mg and hGHA2 1.2 g were obtained from 200 L fermentation culture, yielding high purity products as analyzed with reductive SDS-PAGE, followed by Coomassie Blue-G250 staining (Fig 1).

The representative competition curves of hGH, hGHA1 and hGHA2 for inhibiting ^{125}I-hGH binding to rabbit liver membrane GHR were shown in Fig 2. The IC_{50} (mean±SD) for hGH, hGHA1, hGHA2 was (10±0.6) ng, (65±10) ng, and (27±5.6) ng (n=3), respectively.

The experiment was summarized in Tab 1.

At the end of week 12, all the diabetic rats and normal control rats were sacrificed; the sections of the fixed kidneys were stained with periodic acid-Schiff, and then observed under light microscope. The result of periodic acid-Schiff staining showed that: (1) in normal control SD rats, no mesangial expansion and sclerosis was found and the glormerular size is normal (Fig 3A); (2) in diabetic SD rats treated with saline for 12 weeks, mesangial expansion and glomerulosclerosis were apparent (Fig 3B); (3) in diabetic rats treated with hGH, the glomerulosclerosis were much more severe than in those rats treated with saline (Fig 3C); (4) in diabetic rats treated with hGHA1, glomerulosclerosis was observed, but was less severe than in Group 2, but slightly heavier than in Group 1 (Fig 3D); (5) in hGHA2-treated diabetic SD rats, only slight glomerulosclerosis was found (Fig 3E).

Tab 1. Summary of the experiment.

Group 1 (n=10)	Group 2 (n=5)	Group 3 (n=7)	Group 4 (n=7)	Normal non-diabetic group (n=7)	
Treatment	Saline 0.3 mL sc, daily	hGH 1u/kg body weight, sc, daily	hGHA1 4mg/kg body weight, sc, daily	hGHA2 4mg/kg body weight, sc, daily	None
At the end of week 8	4 rats died before the end of week 8, 2 were sacrificed*	1 rat died before the end of week 8	2 rats died before the end of week 8, 1 rats was sacrificed*	3 rats died before the end of week 8, 1 rats was sacrificed*	
At the end of week 10	1 rat was sacrificed*	1 rat was sacrificed*	1 rat was sacrificed*	1 rat was sacrificed*	1 rat was sacrificed**
At the end of week 12	3 rats alive	3 rats alive	3 rats alive	2 rats alive	6 rats alive

*: sacrificed due to their lethargy.

**: sacrificed for normal kidney histology control

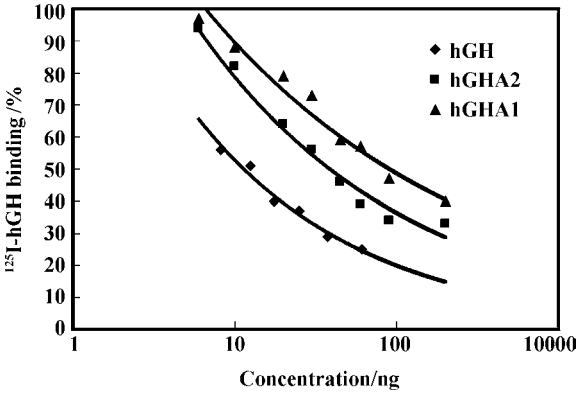

Fig 2. The competitive inhibition of ^{125}I-hGH binding to rabbit liver Growth Hormone receptor with hGH, hGHA1, and hGHA2.

DISCUSSION

In 1995, Chen *et al*[2] first reported that the bovine GH receptor antagonist (bGH-G119K) transgenic mice were resistant to glomerulosclerosis after treatment with STZ to induce diabetes. In 2000, Bellush *et al*[5] found that pathological changes such as glomerulosclerosis, increase in glomerular volume, and increased ratio of mesangial area to total glomerular area were not found in the GH receptor knockout mice. In 2002, Ana *et al*[6] reported that GH receptor antagonist suppressed the GH signal transduction observed in diabetic rats. Together with the earlier result by Quaife *et al*[7] in 1989 among others, these studies have shown clearly that GH has been implicated in diabetic end-stage organ damage, and a GH receptor antagonist was able to suppress the GH signal transduction by competitively inhibiting the wild type GH molecules from binding to the receptors and to prevent the diabetic end-stage renal pathology.

In our experiment, C182S and del186-191 were introduced in hGHA1 according to Rowlinson's report[8], which showed that these mutations increased the affinity of Site 1 of porcine Growth Hormone for rabbit GH receptor. K168A, E174A were added because they increased the binding specificity of the hGH mutant for human GH receptor versus human Prolactin receptor with which wild type hGH can also bind[9]. G120R is the most critical mutation[10] without which the GH mutant would still be an agonist. There is no unambiguous agreement for del1-4. According to Cunningham's data[11], I4 was a very important component for hGHR-binding site 2 of hGH and I4A decreased the affinity of Site 2 by 55-fold, and the 3-D structure determined by De Vos *et al* [12] also showed I4 contributed to the Site 2 binding of hGH. However, the trans-hGH-I4A mice had the same phenotype as trans-hGH mice as reported by Chen *et al*[13] in 1994. A cysteine was introduced at the N-terminal of hGHA1 by site-directed mutagenesis in order to make a sulfhydryl conjugate with PEG5000-maleimide in order to prolong the *in vivo* half life of GH receptor antagonist. Unfortunately, as

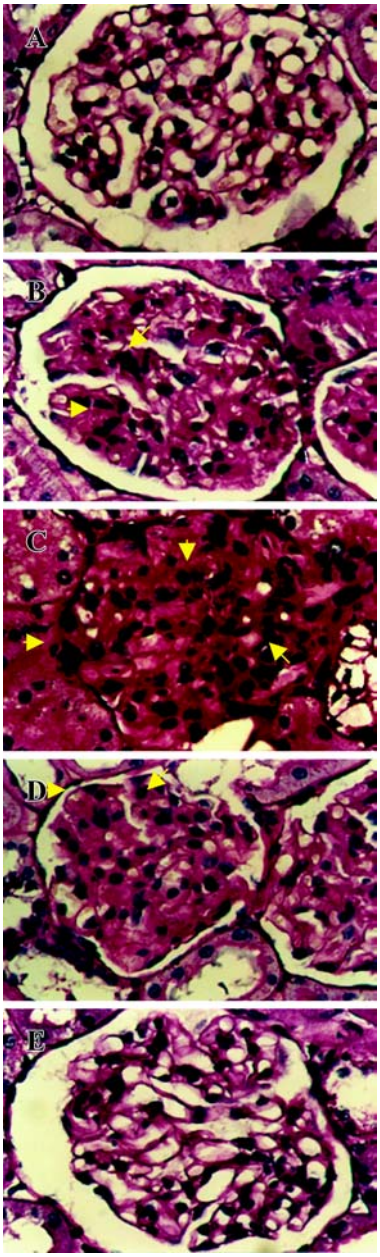

Fig 3. Representative renal histology of SD rats. A: Control normal SD rats; B: Saline-treated diabetic SD rats; C: hGH-treated diabetic SD rats; D: hGHA1-treated diabetic SD rats; E: hGHA2-treated diabetic SD rats. The arrowheads indicate the areas of mesangial expansion and sclerosis (periodic acid-Schiff staining, ×200).

the purification steps took more than one week to finish based on the current protocol, the final hGHA1 product had less than 0.1 free sulfhydryl group per molecule on average, indicating most of the sulhydryl groups have been oxidized, so the quantity of PEG5000-hGHA1 was insufficient for *in vivo* use. Therefore, hGHA1 itself was used in the *in vivo* experiment.

As for the mutations of H21A and E174A in hGHA2, Cunningham *et al*[14] in 1991 reported that these two mutations deleted two zinc-chelating sites on hGH, so the tendency for hGH to form homodimers was decreased. Fuh *et al*[15] in 1992 reported that these two mutations also increased the affinity of hGH receptor-binding site 1 of hGH.

In this experiment, the worsening of glomerulosclerosis due to hGHA1 was somewhat expected for three reasons. First, in hGHA1, the total of 15 mutations change hGH structure to some extent, as revealed by its more than 6-fold higher IC_{50} value than that of the wild type hGH, indicating hGHA1 is at most a weak GH receptor antagonist. Next, the conclusion about C182S-del 186-191, which came from the experiments studying the binding affinity of porcine GH mutants to rabbit GH receptor, might not be applicable to hGH. Finally, hGH-G120R, the widely accepted hGH receptor antagonist, increased the body weight gain in hypophysectomized SD rats, meaning that hGH-G120R functioned as GH receptor agonist, Mode *et al* 1996[16]. This "paradoxical" result, argued by the authors, might be caused by hGH-G120R binding to rat Prolactin receptor. But, this might also be due to the species difference, as hGH-G120R worked as GH receptor antagonist as expected in human, monkey and mice models (Wen Chen, personal communication). So, at present, there is no general rule to predict the *in vivo* activity of hGH mutants in rats.

In summary, two hGH mutants expressed in *E coli* were used to treat the STZ-induced diabetic rats for 12 weeks at 4 mg/kg body weight *sc* daily. At the end of the experiment, the renal histology analysis showed that hGHA2 suppressed the glomerulosclerosis, hGHA1 mildly increased the renal damage, while the wild type hGH severely worsened the glomerulosclerosis. This result concurred with the previous results obtained using GH transgenic mice[2,3] and further indicated that a GH receptor antagonist could be a potentially promising treatment for the end-stage renal damage of diabetes in clinical settings.

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