

Structure and Properties of Members of the hGH Family: A Review

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Abstract. This review will summarize the properties of five variant forms of human growth hormone: a disulfide dimer, a glycosylated form, 20 kD-hGH, and two peptides made up of portions of 22 kD-hGH. The two pituitary peptides (hGH₁₋₄₃ and hGH₄₄₋₁₉₁) have, respectively, insulin-potentiating and anti-insulin properties. Both have been detected in serum. The shorter peptide may prove to be useful in decreasing the amount of exogenous insulin required by diabetics. The larger, strongly anti-insulin peptide, may be involved in diabetic retinopathy. We believe that this peptide is the long sought after diabetogenic substance of the pituitary gland.

Key words: Growth hormone, Variants, Diabetes, Retinopathy

(Endocrine Journal 47: S1-S8, 2000)

OUR work on the variants of growth hormone began over 25 years ago when we set out to explain the multitude of activities that were known for the hormone. Many of the activities attributed to growth hormone were actually contradictory, so our thought was to try to determine if there were multiple forms, each expressing a different physiological activity. These studies began at the time that gel-electrophoresis techniques were just beginning to appear. We thought that they would be ideal for showing that multiple forms existed in pituitary extracts, and also that they could be used to follow purification. Use of bioassays, which are imprecise and time consuming, would have made the project almost impossible.

The most useful electrophoresis system for routine analyses was SDS-gel electrophoresis. Protein staining was used to detect all proteinaceous substances and immunoblotting for detecting hGH related components. We also used isoelectric focusing and disc electrophoresis but only to examine purity of an isolated substance. When a pituitary extract was exa-

mined by SDS-electrophoresis, the pattern was quite remarkable. Not just one immunoreactive component was seen, but at least 7 were visible. SDS electrophoretic patterns of individual pituitary glands can be seen in a recent publication by Sinha and Jacobsen [1]. The first component we chose to identify was one that had an apparent molecular weight of 22 kD. This was done by comparing its electrophoretic mobility in three different electrophoresis systems with that of authentic human growth hormone. Then we eluted the band and carried out a tibial-line assay on the eluted material. All tests indicated that this major component was 22 kD-hGH, the substance whose structure was known and which was considered the only form of the hormone.

Having identified the 22 kD form, we began identifying the other components. We first had to develop procedures for fractionation of extracts of pituitary glands in order to obtain sufficient material for structure and biological testing. The details of the methods we used to obtain homogenous material for each of the substances have been published. Here only a journal citation will be given during the dis-

discussion of each of the variant forms. We would, however, like to point out two things we learned from our purification work. First, it is essential during the purification procedures to use proteinase inhibitors. The pituitary gland has a high concentration of proteolytic enzymes which are particularly active in neutral or slightly alkaline media. Some of the enzymes co-fractionate with the hormones and are difficult to remove. Use of inhibitors is an easy way to eliminate this problem. The second observation is that in order to obtain a single component hormone, ion exchange steps must be used. Separations based on charge differences provide a powerful technique to effect purification.

Disulfide dimer of 22 kD-hGH

A slowly migrating component that had a molecular weight near 45 kD offered a relatively easy substance to purify because of its size. This substance was found to be a disulfide dimer of hGH [2]. Two molecules of hGH are linked in anti-parallel fashion to form the dimer. The substance is only about 10% as active as monomer hGH in an RIA that used polyclonal hGH-antibody. Of interest to us was that the substance had no growth promoting activity in the hypophysectomized rat but was active as a lactogen in the pigeon crop sac assay. These results indicate that the biological properties of variants can be different from the parent 22 kD-hGH.

Glycosylated 22 kD-hGH

A 27 kD-component that was always seen in pituitary extracts has only recently been identified by Haro and co-workers [3]. It is a glycosylated form of hGH. The first 26 amino acids were found to be identical to those in hGH. Because hGH does not have a consensus sequence for N-linked glycosylation in its structure, the carbohydrate must be by O-linkage. Biological studies and development of an RIA are eagerly awaited.

20 kD-hGH

An hGH-like substance that migrated just ahead of 22 kD-hGH during SDS-electrophoresis, 20 kD-hGH, is now the most studied variant form of human growth hormone. A procedure was devised for iso-

lation of the substance from pituitary extracts and this provided us with enough material for structure and biological studies [4]. Our analyses indicated that the substance was identical to 22 kD-hGH except that a 15 amino acid segment, residues 32-46, was missing [5]. A mechanism for the deletion of this segment was soon proposed by Dr. M. Wallis [6]. The mechanism involves alternative splicing in the processing of the precursor mRNA of hGH whereby 45 nucleotides, normally preserved in the mature mRNA of hGH, are removed. When the shortened mRNA is translated, the resulting hGH lacks residues 32-46 and has a molecular weight near 20 kD instead of 22 kD.

The growth promoting activity of 20 kD-hGH is essentially equal to that of 22 kD-hGH [4]. *In vitro* stimulation by 20 kD-hGH of CO₂ production by adipose tissue of the hypophysectomized rat was absent when tested at 0.2 µg/ml of medium, whereas 22 kD-hGH was active at that concentration [7]. The ability to lower *in vivo* plasma glucose and free fatty acids in fasted, hypophysectomized rats was absent when tested at 50 µg/rat [7]; a higher dosage (100 µg/rat) was required for the variant to show activity [8]. Production of glucose intolerance in fasted dogs was seen only at high doses and was similar for both 20 kD-hGH and 22 kD-hGH [9, 10]. This was surprising to us for we thought that because 20 kD-hGH had attenuated insulin-like properties, it would be more active than 22 kD-hGH in producing glucose intolerance. Chronic infusion into dogs of either form at a concentration of 12 µg/kg/day for 12 days produced only a small effect on fasting glucose levels but a marked insulin resistance was induced [11]. The conclusion is that the insulin-like activities of 20 kD-hGH are attenuated but not abolished. The diabetogenic properties are undiminished.

Recent studies have uncovered additional properties which definitely indicate that the physiological actions of the variant are different from 22 kD-hGH. Much of this work was made possible by preparation of a biosynthetic 20 kD that is identical to the natural material [12]. A radioimmunoassay is now available to study the action in human subjects [13]. Receptor binding properties were elegantly studied [14] with the conclusion that the variant binds differently from 22 kD-hGH. Binding to breast cancer tissue opens up a new fascinating aspect of 20 kD-hGH [15]. A

report [16] also suggests that the variant, along with IGF-2, may play a role in development of diabetes in the new-born child.

There are points of controversy relating to the biological properties of 20 kD-hGH. These are mainly matters of dosage required to observe a certain biological activity. There are some possible explanations for the conflicting data. One obvious one is that it was not always possible to use human tissues or human beings to test the bioactivity. Furthermore, there are differences in the biological activities of the growth hormones of various species, so there may also be differences in physiological response in different animals. For example, prolactin-like activity seems to be a simian characteristics of growth hormone. Rodents, especially the albino rat and mouse, are extremely resistant to the action of administered growth hormone, and at times require special surgical manipulations, such as removal of a major portion of the pancreas, to show a physiological response. Another problem is that non-physiological doses are many times used in the biological testing of the growth hormone. For example, the amount of growth hormone required to produce glucose intolerance in a 15 kg dog is equivalent to giving an adult individual about 10–20 mg of hormone. Another variable is that both naturally occurring 20 kD and biosynthetic Met-20 kD have been used. The methionyl group may have had an effect on the folding properties of the protein and thereby altered the biological response.

The peptide comprising the 32–46 sequence of 22 kD-hGH

The lower insulin-like activity of 20 kD-hGH suggested that the amino-terminal portion of 22 kD-hGH, especially the 32–46 sequence, was involved in expression of this property. We were never able to find the peptide in pituitary extracts, but being curious to see if the peptide was biologically active, we synthesized the 15 residue peptide. Many bioassays were carried out with the peptide and it soon became clear that the 32–46 peptide could indeed influence glucose utilization, even though the mechanism is not understood. Enhanced insulin activity was found when the peptide was given to rodents, dogs, and primates [17–20]. Reported effects include enhancement of clearance of glucose *in vivo*, increase in the

sensitivity of adipose tissue to insulin after *in vivo* administration of the peptide, and *in vivo* suppression of outflow of glucose from the liver with an enhancement of peripheral glucose uptake.

These studies were put aside when we discovered in pituitary extracts an even more active insulin-potentiator. This substance was a 5 kD peptide composed of residues 1–43, the properties of which are discussed in detail below in this review. It is important to stress the point that these amino terminal peptides are not active *in vitro*; they must be administered *in vivo* to be active. After treating an animal with one of the peptides, tissue can then be removed and studies carried out by an *in vitro* test, such as insulin-stimulated glucose oxidation by adipose tissue. Furthermore, there is a lag period of 30–45 min before *in vivo* or subsequent *in vitro* effects can be observed. There are at least three possibilities to explain this phenomenon: 1) the peptide must be modified *in vivo* to be active (possibly phosphorylated); 2) the peptide stimulates a cellular process needed for expression of insulin-potentiating activity; 3) the peptide stimulates production of another substance that is actually responsible for activity.

Diabetogenic activity of the pituitary gland: Discovery of 17 kD-hGH

It was suggested over 40 years ago that growth hormone was involved in diabetes but that the hormone as such was the diabetogenic substance in the pituitary gland was questioned [21–23]. By diabetogenic we mean the ability to cause glucose intolerance and insulin resistance. As a result of a project that we were doing with Dr. Gene Tutwiler of McNeil Laboratories, we were convinced that there was such a substance. We would send Dr. Tutwiler various pituitary fractions that we had produced, and he would test for their ability to produce glucose intolerance in dogs. We would then try to correlate bioactivity with the electrophoretic pattern of the samples. It soon became clear that a 17 kD substance that we saw in the SDS-electrophoretic patterns was what we were looking for. This substance proved to be a very active diabetogenic substance in the dog. Glucose tolerance tests were done by treating a dog with the test substance 10 hours before carrying out a standard glucose tolerance test. In the study we found that clinical grade pituitary hGH

produced glucose intolerance when given at a dosage of 0.3 mg/kg, whereas highly purified 22 kD-hGH had to be given at 2 mg/kg to produce an abnormal glucose tolerance curve. Further studies showed that a 17 kD substance that was removed from the clinical grade hGH was active even at a dose of 5–10 $\mu\text{g}/\text{kg}$ [24]. Purified 22 kD-hGH produced no glucose intolerance in the dogs at this dosage. We never found another pituitary fraction that was more active, so we think 17 kD-hGH is “the diabetogenic factor of the pituitary gland”. One unusual observation was that even though the peptide produced glucose intolerance, it did not produce hyperinsulinemia in the dogs, in fact many times the serum insulin was lower than the control value. Hyperinsulinemia was always observed with 22 kD-hGH.

Research directed toward understanding the role of growth hormone in producing diabetes in dogs, such as the results reported by Campbell [23], has always been controversial. We propose that many of the discrepancies can be attributed to the use of impure preparations of growth hormone. Most assuredly, the samples contained 17 kD-hGH which we know is a much more potent diabetogenic agent than is 22 kD-hGH. Importantly, 17 kD-hGH lowers serum insulin whereas 22 kD-hGH treatment is accompanied by higher insulin values.

A surprise came when we started our studies on the structure of the 17 kD peptide. The substance was made up of the portion of 22 kD-hGH from about residue 40 to 191, the carboxy-terminal portion of the hormone. The apparent proteolytic cleavage that produced the 17 kD was not totally specific. However, the most prominent cleavage point was between residues 43 and 44. Therefore, when we approached two investigators at Monsanto Chemical Co., Drs. N. R. Stanton and G. G. Krivi, to help in the project by producing a biosynthetic peptide, the structure of hGH_{44–191} was chosen [25]. The recombinant-DNA-derived peptide made possible a series of biological studies.

Even before biosynthetic 17 kD-hGH became available Dr. Luciano Frigeri and Dr. Mohammed Salem carried out bioassays on the pituitary derived peptide. Both investigators found the obese yellow mouse to be very sensitive to the anti-insulin actions of 17 kD-hGH. The albino mouse and rat had to be treated to make them obese and more diabetic-like before becoming responsive to the peptides. The

yellow mouse has impaired glucose tolerance, hyperinsulinemia, and peripheral insulin resistance. The effect of 17 kD-hGH on glucose clearance was tested. A single dose of 20 μg of 17 kD produced glucose intolerance, whereas even 200 μg of purified hGH had no effect [26]. Furthermore, *in vitro*, without addition of exogenous insulin, the 17 kD peptide inhibited glucose oxidation in adipose tissue of the obese yellow mouse [27]. The peptide also inhibited exogenous insulin-stimulated oxidation at a concentration of 10 ng/ml. Purified hGH was inactive in both situations. The 17 kD-hGH inhibited the incorporation of C¹⁴-labeled glucose into fat of the agouti mouse, a non-obese litter mate of the obese yellow mouse [27]. And finally, administration of 17 kD-hGH to obese yellow mice dramatically increased serum glucose and lowered insulin values [27]. Purified 22 kD-hGH was without effect. The decrease in serum insulin values produced by 17 kD-hGH is similar to the well known observation that prolonged treatment of dogs with growth hormone preparations (probably impure) causes a permanent diabetes as a result of destruction of the insulin-producing cells of the pancreas [23]. The growth hormone used in those early studies was most likely contaminated with 17 kD-hGH. Our view is that 17 kD-hGH is the long sought after hyperglycemic, anti-insulin agent of the pituitary gland.

The information collected on the 17 kD-peptide demonstrated its anti-insulin properties but we had no indication that the substance played a role in human physiology. We had to determine if the peptide circulated in the blood. Dr. Yagya Sinha provided this information. First, sera from 18 individuals were analyzed by SDS-electrophoresis and immunoblotting [28]. In all cases a major 17 kD-component was seen. Next an RIA for the 17 kD-hGH was developed. Biosynthetic 17 kD-hGH was used to produce an antibody for use in the assay [1]. There was little cross-reactivity with other pituitary hormones and the assay could detect less than a nanogram of 17 kD-hGH. A series of assays on serum from several human subjects were carried out. The assays indicated that the average concentration of peptide in men was about 1.2 ng/ml, and 1.4 ng/ml in women. The concentration of the peptide increased during pregnancy. Co Ng *et al.*, [29] reported that there was a large amount of the hGH-like immunoreactive material in basal serum collec-

tions from individuals with acromegaly that was not 22 kD-hGH or 20 kD-hGH. The 17 kD-peptide may be part of the unidentified immunoreactive material.

Knowing that 17 kD-hGH was a potent diabetogenic substance suggested the possibility that the peptide was in some way related to the retinopathy that is seen in diabetes. Because it has been known for many years that hypophysectomy is often beneficial in treatment of diabetic retinopathy, and because we had evidence that 17 kD-hGH circulates in blood, we wanted to know if the peptide was in higher concentration in the retinas of diabetics than in retinas of individuals without diabetes. Sections of the retina were made from tissue from diabetic individuals and from non-diabetics. The sections were first treated with anti-serum to 17 kD-hGH [1] and then with goat-anti-rabbit IgG conjugated to a fluorochrome. As shown in Fig. 1, there was significant labeling of a major retinal vessel in the retina of the diabetic but

not in retina of the non-diabetic. The normal retina showed a rather diffuse background labeling only. When the 17 kD-hGH anti-serum was treated with biosynthetic 17 kD-hGH before adding it to the tissue, binding of anti-17 kD-hGH to the retinal tissue was abolished. These results suggest that the anti-17 kD-hGH reacted with 17 kD-hGH (or a closely related substance) that was bound to retinal receptors. An additional interesting finding (data not shown) was that if the retinal sections were first treated with 17 kD-hGH, there was no detectable increase in labeling in either diabetics or the controls, an indication that all the presumed receptor sites for 17 kD-hGH were occupied. The results also suggest that there are no free binding sites for 17 kD-hGH in the retina of the normal individual. This type of labeling was not seen when a monoclonal antibody to 22 kD-hGH (Genentech) was used. Only diffuse background labeling was detected in the two kinds

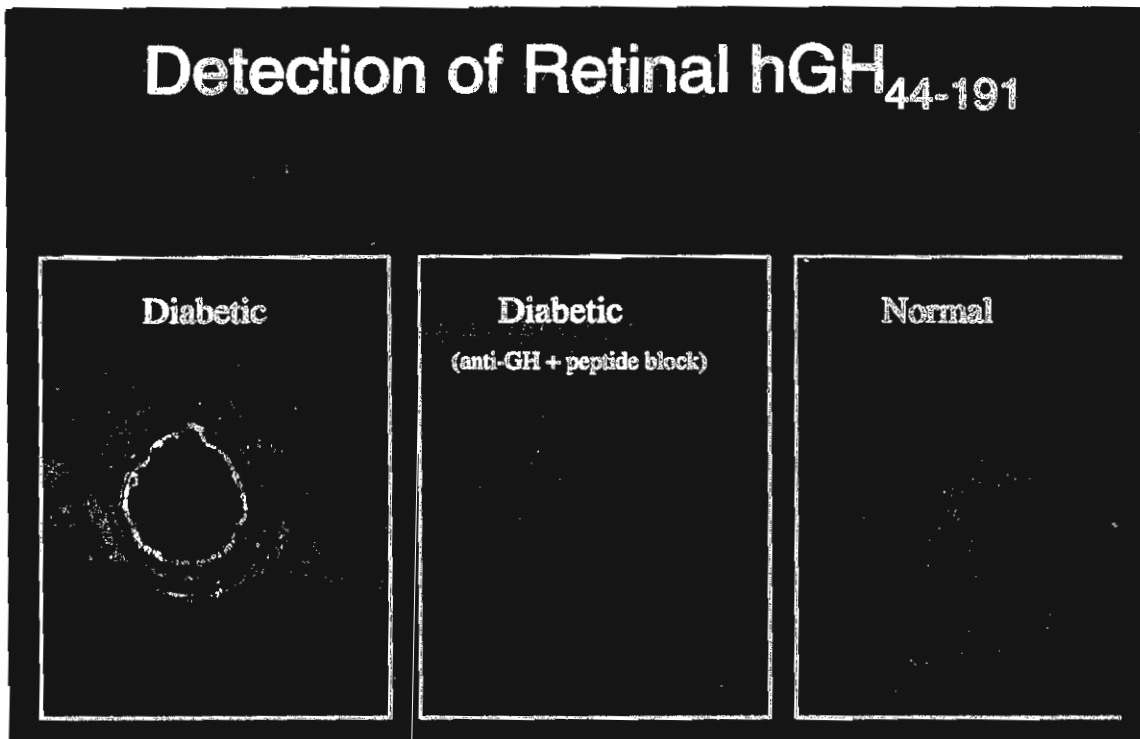


Fig. 1. Photograph of sections of retinas treated with a polyclonal antibody to 17 kD-hGH (hGH₄₄₋₁₉₁). Visualization of the binding was done by treatment with a second antibody conjugated to a fluorochrome. The first panel shows that the 17 kD-hGH-antibody reacted with 17 kD-hGH (or a closely related substance) that was present on the wall of a retinal vessel in the eye of a diabetic individual. The second panel shows that binding of 17 kD-hGH-antibody (here referred to as anti-GH) to the tissue section was abolished when the antiserum was first treated with an excess of 17 kD-hGH. The third panel demonstrates that similar retinal sections from the eye of a normal individual showed no binding of anti-17 kD-hGH.

of retinas under these conditions. One additional control was the treatment of the tissue sections with second antibody alone. No binding was detected. These studies are only preliminary and need many additional types of experiments in order to come to any definite conclusion. However, at this time the data do support the hypothesis that an hGH-like substance is involved, causative or otherwise, during the manifestation of diabetic retinopathy, and recall the studies in man and in the dog where growth hormone was implicated in retinal disease [30-31].

The 5 kD-peptide with insulin potentiating properties

We indicated above in our discussion of the insulin-like activity of 20 kD-hGH that the 32-46 sequence of 22 kD-hGH exhibited insulin potentiating properties. We abandoned additional studies with this 15-residue peptide when we detected and isolated an even more active insulin-potentiating substance that was in pituitary extracts. The fragment proved to be a amino terminal portion of 22 kD-hGH, residues 1-43 [32]. We found the fragment by accident during our work on identifying the diabetogenic substance. During the study to identify the diabetogenic substance, we learned that one of our pituitary fractions, instead of producing glucose intolerance in the dog, actually improved the ability of the dog to handle a glucose challenge. The pituitary preparation produced serum glucose values that were lower than those of the saline control. The peptide was subsequently prepared by recombinant-DNA technology, and even though the work was never reported as a separate publication, details can be found in the paper by Rowlinson *et al* [33].

The 1-43 peptide was tested for its ability to potentiate insulin-stimulated glucose clearance in rats made diabetic by alloxan [34]. The peptide by itself did not increase the rate of glucose clearance, but it did accelerate clearance when combined with insulin administration. The 5 kD-peptide also enhanced *in vitro* insulin stimulated glucose oxidation by adipose tissue of obese yellow mice after treatment of the mice with 1 μ g of hGH 1-43 [34]. The same experiment was done with tissue from ob/ob mice and obese rats. The peptide acted similarly in the tissues of these animals as it did in yellow obese mice. These experiments, together with those that were

carried out with the 32-46 peptide (discussed above), clearly show that peptides made up of portions of the amino terminal part of 22 kD-hGH are active in potentiating the action of insulin and facilitating glucose disposal. We suggest that the 5 kD-peptide may be of value in treating insulin dependent diabetics because of the decreased amount of exogenous insulin that would be required to maintain the individuals in a euglycemic state.

A study reported by Lopez-Guajardo *et al* [35] strengthens the argument that the 5 kD-hGH may have physiological relevance. These investigators reported development of a competitive ELISA immunoassay for 5 kD-hGH using recombinant-DNA-derived material. They were able to detect the peptide in both serum and pituitary extracts.

Addendum

Experimental details of the study of 17 kD-hGH and retinal binding

Normal and diabetic autopsy eyes were obtained from Dr. Robert Avery at Cottage Hospital in Santa Barbara, California. Following enucleation, the eyes were fixed in 4% paraformaldehyde in sodium cacodylate buffer (0.1 N, pH 7.4) for 2 hours. Retinal samples, approximately 2 mm square were excised and embedded in 5% agarose in phosphate buffered saline (PBS). Sections of 100 μ m thickness were cut on a vibratome and incubated in normal goat serum (1:20) overnight at 4°C on a rotator. On the following day, the blocking serum was removed and the sections were incubated (4°C, overnight on a rotator) in a 1:100 dilution of the primary antibody to 17 kD-hGH [1]. The sections were then rinsed in PBS containing bovine serum albumin (BSA) and incubated in goat-anti-rabbit IgG conjugated to the fluorochrome Cy³ (1:200; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) overnight at 4°C on a rotator. The sections were rinsed, mounted in 5% n-propyl gallate in glycerol, and viewed with a laser scanning confocal microscope (BioRad 1024, Hercules, California). All antibodies were diluted in PBS containing 0.5% BSA, 0.1% Triton X-100, and 0.1% sodium azide. For the peptide block experiment, anti-17 kD-hGH was incubated with excess 17 kD-hGH (100 ng/ml)

for 4 hours at room temperature. This solution was then allowed to react with the tissue sections overnight at 4°C on a rotator. The remainder of the blocking experiment was the same as described above, i.e., secondary antibody addition, rinsing and scanning. As a negative control, the primary antibody was omitted and only the secondary antibody was incubated with the sections.

Acknowledgement

We thank Dr. William C. Winter of Manchaca, Texas for his enthusiastic and generous support of these studies over the years.

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