Short Stature Caused by a Mutant Growth Hormone with an Antagonistic Effect

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Abstract. The molecular basis of biologically inactive GH remained unclear until recently. We have very recently reported a child with short stature and a mutant GH caused by a single missense mutation in the GH-1 gene, which itself cannot transduce the GH-signal to the cells but can blunt the action of wild-type GH by virtue of its greater affinity for the GH binding protein (GHBP)/GH receptor. Briefly the clinical features of the patient are: At the age of 4.9 years his height was 81.7 cm (−6.1 SD) and bone age was 2 years. The patient’s serum insulin-like growth factor-1 (IGF-1) concentration was 34 ng/ml. The basal serum GH concentration ranged from 7.0 to 14.0 ng/ml and peak concentrations after insulin hypoglycemia, arginine and L-dopa were 38.0, 15.0 and 35.0 ng/ml, respectively. A heterozygous single base substitution was identified in the GH-1 gene of the proband, predicted to convert codon 77 from arginine to cysteine. Isoelectric focusing revealed the presence of an abnormal GH peak in addition to a normal GH peak. The affinity of expressed mutant GH to GHBP was approximately 6 times higher than that of wild-type GH. The mutant GH not only failed to stimulate tyrosine phosphorylation by itself, but it also inhibited the activity of wild-type GH when added simultaneously even in a one tenth dose of wild-type GH. The child whom we reported is therefore the first case of short stature caused by mutant GH with an antagonistic effect.

Key words: Bioinactive GH, GH gene, Mutation, Tyrosine phosphorylation, GH binding protein

GH dependent short stature is classified into three broad categories: primary pituitary disease, pituitary deficiency secondary to hypothalamic dysfunction and syndrome of insensitivities to GH. Growth failure related to GH deficiency is more common irrespective of its cause and lesioned site. The prototype of GH insensitivities is widely known as Laron-type short stature and absent or defect GH receptors are responsible for the refractoriness to GH. As another type of GH insensitivity, Kowarski et al. reported two children with growth retardation whose GH was biologically inactive [2], and additional cases were subsequently reported [3–8]. This disorder is characterized by short stature with high serum immunoreactive GH concentrations, low serum insulin-like growth factor-1 (IGF-1) concentrations, and increases in both serum IGF-1 concentrations and somatic linear growth after exogenous GH administration. The precise molecular basis of the disorder was unknown.

Very recently, we have reported a child with short stature and a mutant GH caused by a single missense mutation in the GH gene, which itself cannot transduce the GH-signal to the cells but also can blunt the action of wild-type GH because of its greater affinity for the growth hormone binding protein (GHBP) and GH receptor [1]. Kowarski Syndrome is designated the short stature caused
by biologically inactive GH. The short child, presented by us, is not simply a proved example of Kowarski Syndrome, but rather should be classified as a new type of GH insensitivity Syndrome, that is, the short stature caused by a natural GH antagonist.

Materials and Methods

Case presentation

The proband (a boy) weighed 2250 g and was 39 cm at birth at 41 weeks of gestation. The parents were not related. At the age of 4.9 years, his height was 81.7 cm (~6.1 SD) and bone age was 2 years. His body proportions were normal and he had a prominent forehead and a saddle nose. The patient's serum IGF-1 concentration was 34 ng/ml (normal 35–293 ng/ml). The basal serum GH concentration ranged from 7.0 to 14.0 ng/ml and peak concentrations after insulin hypoglycemia, arginine, and L-dopa were 38.0, 15.0 and 35.0 ng/ml respectively. Nocturnal urinary GH excretion ranged from 58.8 to 76.7 pg/mg creatinine (normal 7.1–41.1 pg/mg creatinine). Serum IGF-1 concentrations did not change after daily subcutaneous injections of 0.1 unit per kg (0.035 mg per kg) recombinant human GH for 3 days. After treatment with GH (0.18 mg per kg per week, three subcutaneous injections per week), his linear growth increased 6.0 cm per year (vs. 3.9 cm per year before treatment). His serum GH binding protein (GHBP) level was 70 pmol/l and serum IGFBP-3 level was 1.55 μg/ml.

Hormone assays

Serum GH bioassay was performed with Nb2 rat lymphoma cells as described previously with minor modifications [9]. Serum GH concentrations were measured with a Pharmacia immunoradiometric assay kit. Serum IGF-1 [10] and IGFBP-3 [11] concentrations were determined by radioimmunoassays. Serum GHBP concentrations were determined by ligand mediated immunofunctional assay [12].

Isoelectric focusing was performed as described previously [13] with modifications.

Analysis of DNA and RNA

Genomic DNA was isolated from peripheral blood leukocytes [14]. The DNA corresponding to GH-1 gene was amplified by polymerase chain reaction (PCR), using pairs of the specific oligonucleotide primers as described previously [1]. The amplification products were extracted and sequenced using a 373A DNA Sequencer. After identification of a mutation, direct sequencing was performed.

For RNA analysis, lymphocytes were separated by a MPRM Ficoll-Hypaque (Flow Lab, Costa Mesa, CA, USA) and total RNA was isolated as described previously with slight modifications [15]. cDNA was then synthesized with total RNA [16] and used for PCR to amplify GH-1 cDNA with different sets of the oligonucleotide primers, as described previously [1]. The amplified products were analyzed as described above.

Characterization of the functional properties of wild-type and mutant GH

Wild-type and mutant GH cDNA was subcloned in pGEX-KG, and transformed into the E. coli strain DH5α. Wild-type and mutant GH were expressed and purified with a glutathione-S-transferase (GST) Gene Fusion Expression kit (Pharmacia Biotech, Uppsala, Sweden). The expression products were assayed by IRMA and Nb2 bioassay. The Nb2 bioassay was performed in the presence or absence of serum from a hypophysectomized patient as well as in the presence of 0.1, 0.5 and 1 nM recombinant human GHBP.

Competitive binding studies using [125I]-human GH were performed in the human lymphoblast IM-9 cells, which express GH receptors, as described previously [17]. Direct binding to recombinant human GHBP of wild-type and mutant GH was determined by immunoprecipitation.

GH dependent tyrosine phosphorylation in IM-9 cells was determined as described previously with modifications [18]. Anti-phosphotyrosine monoclonal antibody (RC20: Transduction Laboratories, Lexington, KY, USA) was used for both immunoprecipitation and Western blotting analysis. Antibody binding was visualized using an enhanced chemiluminescence (ECL) kit (Amerham, Buckinghamshire, England).
Results

The ratio of bioactivity to immunoreactivity of the proband’s GH, 0.6 ± 0.2 was significantly lower than those in normal subjects (1.0 ± 0.2) and other family members (father 1.0 ± 0.2; mother 0.8 ± 0.4; elder sister 1.2 ± 0.3; younger sister 1.0 ± 0.5). Isoelectric focusing analysis revealed the presence of an abnormal GH peak in addition to a normal GH peak (Fig. 1A), but his father’s serum contained only one GH peak corresponding to a normal GH (Fig. 1B). We then determined the sequence of the GH-1 gene in the proband. A heterozygous single base substitution was identified in the GH-1 gene of the proband (Fig. 2), predicted to convert codon 77 from arginine to cysteine.

To examine the bioactivity of mutant GH, the wild-type and mutant GH were expressed as GST fusion proteins. Both forms of GH were equally immunoreactive. Although the bioactivity of both proteins was similar when assayed in serum-free medium, the bioactivity of the mutant GH was less than half of that of wild-type GH in the presence of the serum from a hypophysectomized patient which contained neither GH nor prolactin (data not shown). Because of the possibility of interference by GHBP in the Nb2 bioassay system, recombinant human GHBP was added to the assay medium. The mean (± SEM) ratio of bioactivity to immunoreactivity of the mutant GH was significantly reduced to 0.45 ± 0.05 (P=0.01) and 0.22 ± 0.08 (P=0.02) in the presence of 0.5 and 1nM recombinant human GH concentrations, respectively, concentrations similar to those in the serum of normal subjects.

[125I]-human GH binding to human GH receptor in IM-9 cells was inhibited by wild-type and mutant GH in a dose-dependent manner, although the displacement curve obtained with mutant GH exhibited the presence of a shoulder in the 10^{-11}-10^{-9} M range and the mean (± SEM) concentrations at which binding was reduced by 50 percent (IC_{50}) were 0.84 ± 0.30 nM and 0.86 ± 0.41 nM, respectively (three experiments data not shown). [125I]-human GH binding to recombinant human GHBP was also inhibited by the normal and mutant protein in a dose-dependent manner, but the IC_{50} for the mutant protein (0.12 ± 0.02 nM mean ± SEM of three experiments) was significantly lower than that of wild-type GH (0.68 ± 0.08 nM), indi-
cating that the affinity of mutant GH for GHBP is approximately 6 times higher than that of wild-type GH.

The mutant GH not only failed to stimulate tyrosine phosphorylation by itself, but it also inhibited the activity of wild-type GH when added simultaneously even in one tenth dose of wild-type GH (Fig. 3).

**Discussion**

The proband was a boy with severe growth retardation (−6.1 SD) and delayed bone age who had high basal serum GH and low IGF-1 concentrations and increases in serum GH on provocation testing. These findings were consistent with those of the GH insensitivity syndrome [19]. The serum GH in this child was less bioactive than that of normal subjects in a bioassay, and the presence of an abnormal GH molecule in his serum was confirmed by isoelectric focusing.

The patient's abnormal GH resulted from replacement of arginine by cysteine at codon 77 of the GH-1 gene. This codon is located in the second α helix of the GH molecule behind the binding site 1 to GH receptor [20, 21]. The substituted cysteine probably forms new disulfide bonds, changes the charge of the GH molecule, and contributes to a conformational change, causing reduced bioactivity of the mutant GH.

GHBP is the extracellular domain of the GH receptor and serves as a GH reservoir in vivo [26-28]. The affinity of the mutant GH for GHBP was significantly higher than that of wild-type GH, and the displacement curve for mutant GH of [125I]-human GH binding to human GH receptor had a strange shape, with a shoulder around $10^{-11}$ to $10^{-9}$ M. The binding of GH to GH receptor is believed to proceed sequentially in the different portion of the GH molecule, first in site 1 and then in site 2 [20]. Our findings suggest that the properties of the mutant GH differ from those of wild-type GH with respect to these affinities in site 1 or site 2.

Dimerization of GH receptor induced by ligand binding and sequential protein phosphorylation in tyrosine residue are crucially important for GH-induced signal transduction [22-25]. Mutant GH failed to affect tyrosine phosphorylation in IM-9 cells and further inhibited tyrosine phosphorylation by wild-type GH as the antagonist possessing a dominant negative action.

Two types of GH insensitivity syndrome are known so far. The well characterized type of GH insensitivity syndrome is Laron-type short stature caused by the absent or defective GH receptors. Another type of GH insensitivity syndrome, proposed by Kowarski et al. is the short stature due to biologically inactive GH, the molecular basis of which remains unclarified. These two types of GH insensitivity syndrome could be clearly discriminated by the response after exogenous GH administration: increases in both serum IGF-1 levels and somatic linear growth in Kowarski Syndrome, but no response in Laron-type short stature. The short child, reported by us, did not show any increase in serum IGF-1 levels after 3 days sc injection of recombinant hGH although modest increases in both serum IGF-1 levels and body height curve observed during chronic GH.
MUTANT GH WAS A POTENT ANTAGONIST

administration. The growth velocity of this child to exogenous GH treatment was not so well as expected and obviously lower than those of the patient with typical Kowarski Syndrome (Our unpublished observation). The poor response of this child to exogenous GH would be explained by the presence of circulating mutant GH possessing a potent antagonistic action to the GH receptor. Therefore, we would propose that the disorder of this child should be caused by a natural GH antagonist.

The proband’s father is phenotypically normal despite having the same genetic abnormality as the proband. In an isoelectric focusing analysis, the father’s serum revealed a single GH peak corresponding to wild-type GH and no mutant GH. The mechanism which prevented the expression of the mutant GH gene in the father is not clear. It is possible that the new germline mutation might have occurred in the father and a consequent somatic mosaicism might be present in his composed tissues, but there is no evidence supporting this hypothesis.

Recently, in idiopathic short stature whose GHBP level was low, genetic abnormality of the GH receptor was present more than previously recognized. These children showed partial GH insensitivities [29]. Similarly, the number of short children caused by bioinactive GH may be more numerous than we currently estimate. A large scale study to search circulating mutant GH would be necessary for clarifying its frequency.

In conclusion, we found a heterozygous missense mutation in the GH of a child with a severe growth retardation. This mutant GH not only has a higher affinity for GHBP but also is less active in phosphorylating GH-signal transduction molecules, causing a dominant negative effect.

References


