Inhibition of Growth Hormone (GH) Secretion by a Mutant GH-I Gene Product in Neuroendocrine Cells Containing Secretory Granules: An Implication for Isolated GH Deficiency Inherited in an Autosomal Dominant Manner*

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ABSTRACT

Isolated GH deficiency (IGHD) type II is a disease inherited in an autosomal dominant manner. Although point mutations at the donor splice site of intron 3 of the GH-I gene have been identified in patients, the mechanism of how such mutations result in severe GH deficiency is unclear. Recently, we identified two mutations in Japanese patients with IGHD type II, G to A substitutions at the first (mutA) and fifth (mutE) nucleotides of intron 3. Messenger ribonucleic acids skipping exon 3 were transcribed from both mutant GH-I genes. We studied in this report the synthesis and secretion of GH encoded by the mutant GH-I genes and tested whether inhibition of wild-type GH secretion by mutant products could be demonstrated in cultured cell lines.

A metabolic labeling study in COS-1 cells revealed that a mutant messenger ribonucleic acid and retained in the cells for at least 6 h. On the other hand, the wild-type GH was rapidly secreted into the medium. Coexpression of mutant and wild-type GH did not result in any inhibition of wild-type GH secretion in COS-1 or HepG2 cells. However, coexpression of mutant GH resulted in significant inhibition of wild-type GH secretion in somatotroph-derived MtT/S cells as well as in adrenocorticotroph-derived AT-20 cells, without affecting cell viability. We conclude that the dominant negative effect of mutant GH on the secretion of wild-type GH is at least in part responsible for the pathogenesis of IGHD type II. Our results also suggest that neuroendocrine cell type-specific mechanisms, including intracellular storage of the secretory proteins, are involved in the inhibition. (J Clin Endocrinol Metab 84: 2134–2139, 1999)

GH IS ESSENTIAL for normal postnatal growth in human as well as other mammalian species, and GH deficiency causes metabolic alterations and growth failure. Familial isolated growth hormone deficiency (IGHD) has been classified into three major groups by its inheritance pattern. IGHD type I is inherited in an autosomal recessive manner, type II is inherited in an autosomal dominant, and type III is inherited in an X-linked recessive manner (1). Although genetic defects in IGHD type III have not been elucidated to date, mutations of the GH-I gene have been identified in type I and type II. In IGHD type I, deletion of the GH-I gene or point mutations result in the absence of a normal GH molecule in homozygous patients (1–5). In IGHD type II, several mutations were reported at the donor splice site of intron 3 of the GH-I gene (6–8), and the affected patients are heterozygous for the mutations. It has been demonstrated that GH messenger ribonucleic acids (mRNAs) with deleted or skipped exon 3 are transcribed from these mutant GH-I genes (6–8). Although a mutant GH with molecular mass of 17.5 kDa is deduced to be synthesized from the mutant messenger ribonucleic acid and retained in the cells for at least 6 h. On the other hand, the wild-type GH was rapidly secreted into the medium. Coexpression of mutant and wild-type GH did not result in any inhibition of wild-type GH secretion in COS-1 or HepG2 cells. However, coexpression of mutant GH resulted in significant inhibition of wild-type GH secretion in somatotroph-derived MtT/S cells as well as in adrenocorticotroph-derived AT-20 cells, without affecting cell viability. We conclude that the dominant negative effect of mutant GH on the secretion of wild-type GH is at least in part responsible for the pathogenesis of IGHD type II. Our results also suggest that neuroendocrine cell type-specific mechanisms, including intracellular storage of the secretory proteins, are involved in the inhibition. (J Clin Endocrinol Metab 84: 2134–2139, 1999)

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Subjects and Methods

Subjects and DNA analysis

Informed consent was obtained from all patients and family members for blood sampling, genomic DNA extraction, and analysis. All exons and exon-intron junctions of the GH-I gene were amplified by PCR. The amplified fragments were ligated with pGEM-T vector (Promega Corp., Madison, WI), followed by sequence determination using Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) and DNA sequencer model 373A-36 (PE Applied Biosystems). Two different mutations at the donor splice site of exon 3 in the GH-I gene (see Fig. 1B), a G to A substitution at the first nucleotide of exon 3 (referred to as mutA) and the same substitution at the fifth nucleotide in intron 3 (referred to as mutE), were identified in patients with IGHD as heterozygotes. A detailed description of the patients and these procedures will be presented elsewhere (Kamijo, T., manuscript in preparation).

Cell culture

HepG2, COS-1, and AtT-20 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 5% FBS. A rat pituitary-derived cell line, MT/T/S, was obtained from RIKEN cell bank (Tsukuba, Japan) and cultured in a half and half mixture of DMEM and F-12 medium supplemented with 2.5% FBS and 10% horse serum (10, 11). The cells were cultured at 37 °C in an atmosphere of 95% room air-5% CO₂ and 100% humidity.

Plasmid construction, transfection, and RT-PCR

PCR fragments of the GH-I gene containing mutations, mutA or mutE, were digested with ScaI present in intron 2 and BglII in exon 5. The digested fragments were ligated to corresponding sites in pTK-GH (Nichols Institute Diagnostics, Los Angeles, CA) (12), in which transcription of the wild-type GH-I gene is driven by herpes virus thymidine kinase promoter. The constructs were named pTK-GHmutA and pTK-GHmutE, respectively. HepG2 cells were cultured in 6-cm dishes (Falcon 3003, Becton Dickinson and Co., Franklin Lakes, NJ) and transfected with 3003, Becton Dickinson and Co., Franklin Lakes, NJ) and DNA sequencer model 373A-36 (PE Applied Biosystems). Two different mutations at the donor splice site of exon 3 in the GH-I gene (see Fig. 1B), a G to A substitution at the first nucleotide of exon 3 (referred to as mutA) and the same substitution at the fifth nucleotide in intron 3 (referred to as mutE), were identified in patients with IGHD as heterozygotes. A detailed description of the patients and these procedures will be presented elsewhere (Kamijo, T., manuscript in preparation).

To determine the level of human GH secreted into the medium, 200-μL aliquots of medium were collected at intervals after a medium change following the transfection procedure. The medium was diluted in 10 mM/L sodium phosphate buffer (pH 7.0) containing 0.1 mol/L NaCl and 0.1% sodium azide by more than 50-fold and directly subjected to an enzyme immunoassay using the PICOA HGH PLATE kindly provided by Sumitomo Pharmaceutical, Inc. (Tokyo, Japan). The lower

![Fig. 1. Structure of the GH-I gene, mutations used in the present study, and RT-PCR analysis of transcripts generated from the mutant GH-I gene. A, Structure of the GH-I gene. Exons are shown as boxes, and introns are shown as lines. A bold line indicates the donor splice site of exon 3, where the mutations studied in the present report are located. The primers used for RT-PCR are shown in arrowheads. B, Mutations studied in the present report. The locations of mutations used in the present study are indicated. The sequence around the donor splice site of exon 3, shown as a bold line in A, and the consensus sequence of donor splice sites are also shown. C, RT-PCR analysis of transcripts generated from mutant GH-I genes. RNA was extracted from HepG2 cells transfected with the indicated GH-I expression vector and analyzed. MW, dX174 molecular mass marker. Lane 1, pTK-GHmutA; lane 2, pTK-GHmutE; lane 3, pTK-GHwt.](https://academic.oup.com/jcem/article-lookup/doi/10.1210/jcem-84-6-2134)
The human GH-I gene consists of five exons, as depicted in Fig. 1A. Two mutations at the donor splice site of intron 3 (bold line in Fig. 1A), mutA and mutE, are depicted in Fig. 1B along with the consensus sequence for the donor splice site. At first, the structure of mRNAs transcribed from GH-I genes with these mutations was analyzed. As shown in Fig. 1C, a fragment 625 bp in length was amplified by RT-PCR using the RNA extracted from HepG2 cells transfected with pTK-GHmutA (lane 1), in agreement with exon 3 skipping as previously reported (6–8). A fragment with the same size was also amplified from cells transfected with pTK-GHmutE (lane 2), indicating that the mutation of the guanine residue in mutE affects the splicing in the same manner as mutA. Sequencing verified that the transcripts of both mutant GH-I genes lacked exon 3. It is of note that the guanine at the first and fifth nucleotides of introns are conserved 100% and 84%, respectively (19). The lack of exon 3 in the transcripts of mutE indicated the significance of the fifth residue for the splicing. Similar results were reported by Missarelli et al. (20) recently. As exon 3 of GH-I gene consists of 120 nucleic acid residues, a mutant GH protein with a deduced molecular mass of 17.5 kDa, which lacks 40 amino acid residues in-frame, was predicted to be synthesized from the two mutant GH-I genes. On the other hand, a 745-bp fragment encoding wild-type GH with a molecular mass of 22 kDa was amplified from RNA extracted from HepG2 cells transfected with pTK-GH (lane 3). Very little, if any, mRNA encoding the 20-kDa variant GH (21, 22) or other splicing variants (7) could be detected.

The synthesis and secretion of mutant and wild-type GH were then studied. It was also examined whether the mutant GH can exert dominant negative effects on the secretion of wild-type GH. COS-1 cells were transfected with simian virus 40-derived expression vectors carrying mutant or wild-type GH cDNAs for metabolic labeling. As shown in Fig. 2A, a 22-kDa protein was detected in both medium and cell lysate from the cells transfected with pHGwt after labeling for 2 h. In contrast, a protein with a smaller molecular mass, which is in agreement with a deletion of 40 amino acids encoded by exon 3, was detected in the cell lysate, but not in medium, when transfected with pHGΔEx3. In cells transfected with both pHGwt and pHGΔEx3, both peptides were detected in cell lysate, but only the 22-kDa wild-type GH was secreted into the medium. As shown in Fig. 2B, the 17-kDa mutant GH was retained in the cells, even 6 h after the chase, without significant intracellular degradation, whereas the wild-type GH was secreted into the medium with minimum retention after 1 h of chase. Secretion of wild-type GH was not affected by coexpression of mutant GH.

We then tested whether the increased ratio of pHGΔEx3 to pHGwt could result in inhibition of wild-type GH secretion. As shown in Fig. 3A, COS-1 cells transfected with pHGwt secreted GH, with the concentration reaching 150 ng/mL 5 days after transfection. Naturally, no GH was detected in the medium when pHGΔEx3 was transfected. Cotransfection of a 9-fold excess of pHGΔEx3 with pHGwt did not inhibit GH secretion. The activity of β-galactosidase, which was coexpressed to monitor the transfection efficiency, was not significantly different among the transfected cells (Fig. 3B). This result also suggests that expression of the mutant GH is not toxic for cell survival (23). Similar experiments were carried out in HepG2 cells using plasmids expressing GH cDNA (wt combined with ΔEx3) or the GH-I gene (pTK-GHwt combined with mutA or mutE). No inhibition of wild-type GH secretion by coexpression of the mutant GH-I genes or cDNA was observed (data not shown).

Failure to demonstrate the dominant negative effect by mutant GH in COS-1 or HepG2 cells led us to test this possibility in somatotroph-derived cells. MtT/S is a cell line derived from rat pituitary somatotroph, and secretory gran-
ules containing rat GH (rGH) are present in these cells (10). No GH could be detected in the medium of the MtT/S cells transfected with phGHΔEx3 or pcDNA1/Amp with no insert, indicating that PICOIA HGH PLATE, the enzyme immunoassay employed in this study, does not detect rGH (Fig. 4A). On the other hand, cells transfected with phGHwt secreted GH into the medium, with concentrations reaching 3 ng/mL after 6 days of incubation. Although cotransfection of equal amounts of phGHΔEx3 and phGHwt did not result in any inhibition of GH secretion, cotransfection of a 5-fold excess of phGHΔEx3 resulted in a significant decrease in GH secretion (Fig. 4A). Similar results were produced in the experiments using pTK-GH and pTK-GH mutants rather than GH cDNA-expressing plasmids (data not shown). This is the first demonstration of a dominant negative effect of the mutant GH on wild-type GH secretion in cultured cells. We

Fig. 3. GH secretion from COS-1 cells transfected with GH expression plasmid. COS-1 cells were transfected with 100 ng phGHwt/well together with 0, 300, or 900 ng phGHΔEx3. The control plasmid pSV-βGal was cotransfected to monitor transfection efficiency. A, GH concentration in the medium. The mean ± SD of triplicate transfections are presented without correction for transfection efficiency. B, β-Galactosidase activity in the cell lysate.

Fig. 4. Human GH secretion from MtT/S cells transfected with GH expression plasmid. MtT/S cells were transfected with 300 ng phGHwt/well and with 0, 300, or 1500 ng phGHΔEx3. Control plasmid pSV-βGal was cotransfected to monitor transfection efficiency. The mean ± SD of triplicate transfections are presented without correction for transfection efficiency. A, GH concentration in medium without dexamethasone treatment. B, GH concentration in medium with dexamethasone (100 nmol/L) treatment. C, β-Galactosidase activity of the cell lysate.
then tested the effect of dexamethasone treatment on the secretion of GH and the dominant negative effect by the mutant GH, as this treatment is known to increase the content of secretory granules in MtT/S cells (24). As shown in Fig. 4B, the concentration of GH in medium 6 days after transfection reached 15 ng/mL in cells transfected with phGHwt. The magnitude of inhibition by cotransfection of a 5-fold excess of phGHΔEx3 was 67% (Fig. 4B), which was greater than 50% inhibition in the absence of dexamethasone (Fig. 4A). As shown in Fig. 4C, the efficiency of transfection was consistent.

To test whether the dominant negative effect of the mutant GH is a phenomenon specific to the somatotroph-like cell line, we carried out similar experiments in another neuroendocrine cell line, AtT-20, which was derived from adrenocorticotroph and contains abundant secretory granules (25). As shown in Fig. 5A, AtT-20 cells transfected with phGHwt secreted GH, and the concentration of GH reached 30 ng/mL after 3 days of incubation. As observed in other cell lines, no GH in the medium could be detected when phGHΔEx3 was transfected. A metabolic labeling experiment in this cell line confirmed that mutant GH is not secreted into the medium as it is in COS-1 cells (data not shown). Cotransfection of phGHΔEx3 resulted in significant inhibition of wild-type GH secretion. The magnitude of inhibition was increased by increasing the amount of phGHΔEx3. Again, the efficiency of transfection was consistent (among the wells) (Fig. 5B).

Discussion

Dominantly inherited diseases are caused by either haploinsufficiency or dominant negative effects of the mutant molecule. In the case of IGHD type II, the former possibility is easily excluded, as deletion of one of the two copies does not produce GH deficiency in IGHD type I. Thus, a dominant negative mechanism was suggested to be the cause of the GH deficiency in IGHD type II when the patients were demonstrated to be heterozygous for the mutations in GH-I gene (6–8). Although it has been demonstrated that a mutant GH mRNA with a deleted exon 3 is transcribed from the mutant GH-I genes, no experimental evidence for the dominant negative effect by the mutant product has been demonstrated to date. Binder et al. suggested overexpression of the mutant GH mRNA in lymphoblasts of the patients (7); however, no significant differences in the level of GH secreted from the lymphoblasts was observed between the patients with IGHD type II and normal subjects (9). Moreover, no protein expression study of the mutant GH has been reported, leaving the synthesis and secretion of the mutant GH unclear.

In the present study, we showed by metabolic labeling experiments using COS-1 cells that mutant GH is not secreted into the medium, but is retained in the cells. We also demonstrated that mutant GH can inhibit the secretion of wild-type GH in two neuroendocrine cell lines containing abundant secretory granules, namely AtT-20 and MtT/S (10, 25). As the inhibition was not observed in COS-1 or HepG2 cells, it was suggested that neuroendocrine cell-specific mechanisms, probably involving intracellular storage of secretory proteins, are required for the inhibition of wild-type GH secretion by mutant GH. The magnitude of inhibition in MtT/S cells was enhanced by dexamethasone treatment, which increases the content of secretory granules (24). This finding supports the hypothesis that intracellular storage is involved in the inhibition. This is the first experimental demonstration of the inhibition of wild-type GH secretion by mutant GH, which accounts at least in part for the pathogenesis of IGHD type II.

It should be noted that the inhibition of wild-type GH secretion observed in the present study was partial and required an excess of mutant GH-expressing plasmids. In contrast, serum GH levels remains as low as the lower limit of detection even after the administration of GHRH in patients with IGHD type II (6, 8). These discrepancies may be due to the relatively low efficiency of GH production in AtT-20 or MtT/S in this study. Pituitary somatotrophs produce huge amounts of GH, with the contents of GH reaching nearly 3% of the weight of the pituitary gland (26). We speculate that expression of large amounts of mutant GH could result in more prominent inhibition of wild-type GH secretion due to an increased chance of intermolecular interaction.

The normal 22-kDa GH molecule contains four cysteine residues, and these residues form two pairs of intramolecular disulfide bonds. As the mutant GH lacks 40 amino acid

Fig. 5. GH secretion in AtT-20 cells. A, GH concentration in medium. B, β-Galactosidase activity of the cell lysate. Transfected AtT-20 cells were transfected with 100 ng phGHwt/well and the indicated amounts of phGHΔEx3. Control plasmid pSV-βGal was cotransfected to monitor transfection efficiency. The mean ± SD of triplicate transfections are presented without correction for transfection efficiency.
residues, including 1 cysteine residue, it has been previously speculated that uncoupled cysteine residues in the mutant GH molecule lead to the formation of intermolecular disulfide bonds, resulting in multimeric GH aggregates containing both mutant and wild-type molecules (6). Recently, a biologically inactive mutant GH that competes with wild-type GH for its receptor binding was reported (27). Interestingly, this mutant GH also has an uncoupled cysteine residue resulting from substitution of an arginine residue at codon 77 to cysteine. Yet, the heterozygous patient has high serum GH levels, indicating that the presence of an uncoupled cysteine residue is not sufficient to inhibit the secretion of wild-type GH.

Beside inhibition of wild-type GH secretion, cell toxicity by the accumulation of mutant GH should be considered as a mechanism of IGHD type II. The present study also provides an opportunity to test this possibility by monitoring β-galactosidase activity, as it has been demonstrated that the reduction of reporter gene activity is linked to cell loss in transient transfection studies (23). The activity of β-galactosidase in cells transfected with the mutant GH-expressing plasmid was not significantly lower than that in cells transfected with the wild-type GH-expressing plasmid. Thus, it is unlikely that the accumulation of mutant GH causes cell loss. However, we cannot exclude the possibility that the expression of mutant GH may lead to different consequences in premature somatotrophs during development. In familial central diabetes insipidus, the secretion of arginine vasopresin is not impaired in early life. The affected patients who are heterozygous for point mutations in the arginine vasopressin precursor manifest symptoms long after birth (28). In such a disease with delayed onset, a reduction of the number of cells due to the accumulation of the mutant protein is more likely to be involved in the pathogenesis. Indeed, cell loss by accumulation of mutant vasopressin precursors was recently demonstrated by experiments using cultured cells (29).

There are few dominantly inherited diseases caused by mutations in the genes encoding secretory proteins. Moreover, as far as we know, almost complete inhibition of secretion of the wild-type molecule by the mutant molecule is unique to IGHD type II. The present study for the first time demonstrated that mutant GH synthesized from IGHD type II mutant GH-I genes can inhibit wild-type GH secretion, and a neuroendocrine cell-specific mechanism and/or cosegregation of wild-type and mutant GH in the secretory granules were suggested to be involved in this mechanism.

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