A Circulating, Biologically Inactive Thyrotropin Caused by a Mutation in the Beta Subunit Gene

Geraldo Medeiros-Neto,§ Demetrios T. Herodotou,* Sabitha Rajan,‡ Sitara Kommareddi,§ Luiz de Lacerda,‡ Romolo Sandrini,‡ Margaret C.S. Boguszewski,§ Anthony N. Hollenberg,* Sally Radovick,‡ and Fredric E. Wondisford*§

*Thyroid Unit, Beth Israel Hospital, Boston, Massachusetts 02215; ‡Endocrinology Division, Children's Hospital, Boston, Massachusetts 02115; §Thyroid Laboratory, University of São Paulo Medical School, São Paulo 0891, Brazil; †Endocrinology Division, Department of Pediatrics, UF Parana §1660, Brazil; and ¶Medical School, Case Western Reserve University, Cleveland, Ohio 44106

Abstract

Mutation of a critical carboxy-terminal cysteine residue (C105V) in the thyrotropin-β (TSH-β) subunit gene was found in two related families with central hypothyroidism. Affected patients had low thyroid hormone levels and radioactive iodine uptake in the thyroid gland associated with measurable serum TSH. Thyrotropin-releasing hormone-stimulated TSH secretion did not increase thyroid hormone production in these patients as compared to their unaffected siblings, suggesting that the mutant TSH was biologically inactive in vivo. Recombinant TSH harboring this mutation was confirmed to be biologically inactive in an in vitro bioassay. Based on crystallographic structure of choriionic gonadotropin, a disulfide bond between C19 and C105 in the TSH-β subunit is predicted to form the “buckle” of a “seat belt” that surrounds the common α subunit and maintains the conformation and bioactivity of the hormone. This natural mutation of the TSH-β subunit confirms the importance of the seat belt in the family of pituitary and placental glycoprotein hormones. (J. Clin. Invest. 1996. 97:1250–1256.)

Key words: thyrotropin β subunit • central hypothyroidism • gene mutation • pituitary

Introduction

The etiologies of anterior pituitary hormone deficiency are numerous, and include vascular, neoplastic, immunologic, developmental, and genetic abnormalities of the pituitary and hypothalamus. β subunit gene mutations of hormones within the pituitary glycoprotein family, comprised of luteinizing hormone (LH), follicle-stimulating hormone, and thyroid-stimulating hormone (TSH), cause anterior pituitary hormone deficiency. For example, gonadotroph function is impaired by β subunit gene mutations in LH (1) and follicle-stimulating hormone (2) resulting in infertility and amenorrhea. In the thyrotroph, mutations within a pituitary-specific transcription factor, Pit-1, and the β subunit of TSH cause central hypothyroidism (3–8). Central hypothyroidism due to point mutations in the second exon of the TSH β subunit gene has been reported in two families (7–9). These mutations, located near the NH₂-terminus of the β subunit peptide, interfere with either the synthesis of β subunit or its combination with the α subunit in the thyrotroph. Pituitary TSH secretion is undetectable in affected family members and does not increase in response to either hypothyroidism or thyrotropin-releasing hormone (TRH) stimulation (9, 10). We describe two related Brazilian families with central hypothyroidism due to a circulating form of biologically inactive TSH containing a mutation in the TSH-β subunit peptide.

Methods

Subjects. Family A lives in a remote village in Brazil; a partial pedigree of the entire kindred is shown in Fig. 1. The mother (IV.15) and father (IV.16) of Family A are second degree cousins and have six children. The mother has a large goiter and takes thyroid hormone replacement for a presumed iodine-deficient goiter. The father is euthyroid and does not have a goiter. Three of their children (V.3, V.4, and V.6) had congenital hypothyroidism without a goiter and are on thyroid hormone replacement (Table I). Two of her children are euthyroid; one child died at age 2 mo and was also apparently euthyroid. The father (IV.22) and mother (IV.23) of Family B are first-degree cousins and had two children (V.8, and V.9). One child has congenital hypothyroidism (V.8) without a goiter and is on thyroid hormone replacement; the other child is euthyroid (V.9).

Table I lists selected thyroid function tests from these families. Congenital hypothyroidism was diagnosed in patients (V.3, V.4, V.6, and V.8) on the basis of clinical findings and low serum T₄ and T₃ in the absence of serum thyroid hormone–binding abnormalities (data not shown). Other pituitary hormone levels were normal in affected family members. Serum TSH levels were determined before and after TRH administration (200 μg TRH i.v.), at 15, 30, 45, and 60 min. Serum-free α subunit levels were determined before and 15 and 30 min after TRH administration. Normal serum-free α subunit levels are < 1 μg/liter, except in postmenopausal and premenopausal women during the midcycle LH surge, and < 2.5 μg/liter in patients with primary hypothyroidism. Patients receiving thyroid hormone replacement were withdrawn from their replacement for at least 4 wk before TRH testing was performed; and TRH testing was not performed during the midcycle LH surge in affected family members. Radioactive iodine uptake (RAIU) at 2 and 24 h (values at 24 h are shown) and thyroid scans were obtained in most family members. Affected family members were also given bovine TSH 10 U i.m. daily for 3 d in an attempt to normalize thyroid uptake; and thyroid scans were obtained after exogenous TSH stimulation.

Genetic analysis. Genomic DNA was isolated from peripheral blood leukocytes using a phenol-chloroform extraction method (11). The second and third exons of the human TSH-β gene were amplified.
using PCR (12). The strategy and oligonucleotide primers used in the PCR reaction are shown in Fig. 2 A. Both the 5' (A, 5' TT-
TAAGCTTTAACAATAGGTTC 3') and 3' primers (B, 5' GGCAGCTTATTAAACCAATGTCAAATTATA 3') contained HindIII restriction sites (underlined) to facilitate their cloning into pGEM4Z (Promega Biotech, Madison, WI). Successful PCR amplification resulted in a 900-bp DNA fragment, containing both the second and third exons and second intron. This PCR was performed on patients IV.15, IV.16, V.2, V.3, V.4, V.5, and V.6; six to ten different subclones of the PCR product from each patient were sequenced using the chain termination method of Sanger et al. (13). Allele-specific PCR was used to confirm a mutation in codon 105 of the human TSH-β gene (14). Fig. 2 A illustrates the primers used in this method. Two separate reactions were performed on each genomic DNA sample. The same 5' primer (C, 5' TTTCCCAGGATATCAATG-GCAAC 5') was used in both reactions, but the 3' primer contained either a normal (D, 5' TTCTGAGTTTGGTACA 3') or mutant 105 (E, 5' TTCTGAGTTTGGTACG 3') codon; codon 105 is underlined. A 235-bp fragment should only be obtained with the mutant 3' primer (E) when at least one allele contains a codon 105 mutation.

Expression of recombinant TSH in mammalian cell culture. To determine the functional properties of the mutant TSH, we synthesized wild-type and mutant TSH in a mammalian expression system. The Rous sarcoma virus promoter (pRSV) was placed upstream of a human TSH-β minigene containing both coding exons or a human common α subunit cDNA in the vector, pREP-9, (In vitro Corp., San Diego, CA). The minigene was obtained from a PCR and contained first exon sequences (+1 to +37 bp) fused directly to second exon sequences (15). The plasmid also confers neomycin resistance. The

### Table I. Serum Hormone Levels and Radioactive Iodine Uptakes in a Kindred with a TSH-β Subunit Gene Mutation

<table>
<thead>
<tr>
<th>Family Member</th>
<th>TSH Basal</th>
<th>TRH-Stim</th>
<th>RAIU Basal</th>
<th>TSH-Stim</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV-15</td>
<td>1.0</td>
<td>6.4</td>
<td>26.9</td>
<td>ND</td>
</tr>
<tr>
<td>IV-16</td>
<td>1.0</td>
<td>5.0</td>
<td>19.8</td>
<td>ND</td>
</tr>
<tr>
<td>IV-22</td>
<td>1.3</td>
<td>7.1</td>
<td>24.1</td>
<td>ND</td>
</tr>
<tr>
<td>IV-23</td>
<td>2.9</td>
<td>19.3</td>
<td>33.3</td>
<td>ND</td>
</tr>
<tr>
<td>V-2</td>
<td>2.0</td>
<td>7.6</td>
<td>16.5</td>
<td>ND</td>
</tr>
<tr>
<td>V-3</td>
<td>0.8</td>
<td>1.7</td>
<td>0.9</td>
<td>5.8</td>
</tr>
<tr>
<td>V-4</td>
<td>0.02</td>
<td>0.8</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td>V-5</td>
<td>3.0</td>
<td>15.0</td>
<td>32.2</td>
<td>ND</td>
</tr>
<tr>
<td>V-6</td>
<td>0.3</td>
<td>0.9</td>
<td>1.4</td>
<td>4.2</td>
</tr>
<tr>
<td>V-7</td>
<td>0.1</td>
<td>1.6</td>
<td>1.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Normal range</td>
<td>0.4–5.0</td>
<td>5–20</td>
<td>12–35</td>
<td>18–48</td>
</tr>
</tbody>
</table>

* Peak TSH after administration of 200 μg i.v. TRH. † 131I uptake after bovine TSH administration, 10 U i.m. every day for 3 d. Measurements of thyroid function after withdrawal of thyroid hormone treatment for at least 4 wk. To convert T2 and T3 to SI units multiply by 12.87 (nM) or 0.15 (nM), respectively. Affecte family members are shown in bold type; carriers of the mutation are underlined. ND, not determined; Stim., stimulation.
say (Nicholas Institute, San Juan Capistrano, CA). The sensitivity of this assay is 0.01 mU/liter for recombinant TSH synthesized in tissue culture medium. The in vitro biological activity of mutant TSH was determined from a standard curve for wild-type recombinant TSH and a biologic to immunologic ratio (B/I) was determined.

**Results**

Congenital hypothyroidism without goiter was diagnosed in three members of Family A and one member of Family B. Affected patients (V.3, V.4, V.6, and V.8) all had low serum thyroid hormone levels and inappropriately low or normal serum TSH levels (Table I). Serum TSH increased in all affected patients in response to TRH treatment, although peak values were significantly lower than expected for hypothyroid individuals. Basal RAIU was also low in these patients but increased in response to bovine TSH treatment. Thyroid scans before and after bTSH administration confirmed the presence of functional thyroid tissue in the neck in patients V.3, V.4, V.6, and V.8.

Fig. 3 A demonstrates that three of the four affected patients (V.3, V.6, and V.8) had elevated serum α subunit levels when compared to patients with primary hypothyroidism whose α subunit levels should be < 2.5 μg/liter (arrowhead). Note that α subunit levels increased 2- to 3.4-fold in response to TRH stimulation in all affected patients. As expected, two normal individuals (C.1 and C.2) had low α subunit levels, which increased ~ 30% with TRH stimulation. The serum α subunit/TSH molar ratio was also markedly elevated in all affected patients as compared to controls (Fig. 3 B), indicating that free α subunit was secreted in high amount in these patients (18, 19). Moreover, this ratio increased in patients, but not controls, after TRH stimulation, suggesting that the thyrotroph was the source of the free α subunit in affected patients.

Since a partial deficiency of TSH with preservation of other pituitary hormone function was observed in affected patients, we investigated TSH-β gene structure using PCR. Primers complementary to the 3′ end of the first intron (A) and the 3′ end of exon 3 (B) were synthesized (Fig. 2 A) (13). Exon 2
contains the leader peptide and first 34 amino acids of the mature peptide. Exon 3 encodes amino acids 35 through 118 of the mature peptide. Successful PCR amplification resulted in a band of ~900 bp. PCR products were cloned and individual subclones were subjected to DNA sequencing. Both coding exons from an unaffected child (V.5), one parent (IV.16), and control DNA had a normal DNA sequence (Fig. 2B). Approximately half of the clones from the mother and patient V.2 and all of the clones from affected children (V.3, V.4, and V.6) had a single base deletion in codon 105, resulting in a shift in the peptide reading frame (Fig. 2B). This mutation resulted in a loss of a cysteine residue at position 105, and the addition of a nonhomologous 9 amino acid extension (partial predicted peptide sequence shown). Cysteine 105 is 1 of 12 cysteine residues conserved among the pituitary and placental glycoprotein β subunits contained in follicle-stimulating hormone, LH, and TSH, and CG (18).

To confirm the C105V mutation and to determine carriers of this mutation in Family A and B, allele-specific PCR was performed (12). Fig. 2C shows the results of this analysis. The predicted 235-bp product was observed in the mutant but not wild-type amplification of affected patients V.3, V.4, V.6, and V.8, indicating that both TSH-β alleles contained this mutation. The predicted 236-bp product was observed in only the wild-type amplification of unaffected family members IV.16, V.1, and V.5, indicating that these patients do not carry the C105V mutation. A PCR product was observed in both the wild-type and mutant amplifications of unaffected family members IV.15, IV.22, IV.23, V.2, and V.9, indicating that they are carriers of the C105V mutation. Curiously, the father (IV.16) of Family A does not carry the C105V mutation. Analysis of nine independent dinucleotide repeat microsatellite loci on different chromosomes confirmed that IV.16 was the biological father of V.2, V.3, V.4, V.5, and V.6 with a 99.99% probability (data not shown). Since the mother (IV.15) is heterozygous for the 105 mutation, the father’s germ line must contain either a deleted TSH-β allele (affecting individuals are compound heterozygotes) or a mutant TSH-β allele not found in leukocyte genomic DNA.

To determine the functional properties of TSH, we synthesized wild type and mutant TSH in a mammalian expression system. Rous sarcoma expression constructs containing either the common α or TSH-β subunits and a neomycin gene driven by the thymidine kinase promoter were transfected into a human kidney cell line (293), and pools of neomycin-resistant clones were expanded. Transfection efficiency was similar between wild-type and mutant TSH-β transfections as measure by growth hormone levels in cell culture medium (data not shown). Medium of cell cultures transfected with the α construct and either the wild-type TSH-β, mutant TSH-β, or “empty vector” construct was assayed for immunoreactive TSH. Immunoreactive TSH in medium from either the human α subunit and wild type or Y112 mutant TSH-β transfections was ~20 mU/liter and was significantly higher (three to fourfold higher) than cotransfection of the human α subunit and either the C105 or C105V mutant TSH-β subunit. A significantly lower amount of immunoreactive TSH in the medium from the later two mutant constructs could be due to either a lack of synthesis, combination, and/or secretion of TSH from transfected cell cultures, or to a lack of detection of the mutant TSH in the chemiluminescent immunoassay. The latter possibility is unlikely, since similar results were obtained in two different immunometric TSH assays (data not shown). These in vitro data correlated with the impaired TSH secretion levels in affected children.

Data from the family, however, suggest that the mutant TSH is also biologically inactive. Twin sisters from Family B had TRH testing performed, and their serum TSH and T₄ were measured. As shown in Table II, patient V.9, a carrier of the mutation, had a normal increase in serum TSH and T₄ after TRH administration. In contrast her affected twin sister (V.8) had an impaired TSH and T₄ response to TRH. This patient has functional thyroid tissue (Table I) as indicated by a signifi-
Table II. Serum Hormone Levels after a TRH Stimulation Test in Sisters from Family B

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>V.8 TSH (mU/liter)</th>
<th>V.8 T₃ (ng/dl)</th>
<th>V.9 TSH (mU/liter)</th>
<th>V.9 T₃ (ng/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.11</td>
<td>79</td>
<td>2.64</td>
<td>180</td>
</tr>
<tr>
<td>30</td>
<td>1.62</td>
<td>81</td>
<td>19.81</td>
<td>211</td>
</tr>
<tr>
<td>60</td>
<td>1.52</td>
<td>89</td>
<td>16.62</td>
<td>212</td>
</tr>
<tr>
<td>120</td>
<td>0.14</td>
<td>79</td>
<td>12.04</td>
<td>242</td>
</tr>
<tr>
<td>180</td>
<td>0.09</td>
<td>81</td>
<td>9.77</td>
<td>280</td>
</tr>
</tbody>
</table>

Discussion

Congenital hypothyroidism in this kindred is associated with a frameshift deletion in codon 105 of the TSH-β subunit gene C105V. Cysteine 105 is one of 12 cysteines conserved among all dimeric pituitary and placental glycoprotein hormone β subunits. Data from chemical modification of the TSH-β subunit peptide suggest that cysteine 105 forms a disulfide bond with cysteine 19 during the folding of this subunit (22). Recent crystallographic structure of CG indicates that this bond forms the “buckle” of a “seat belt” that holds the α subunit in place (23). Based on this structure, a model of TSH can be developed where a seat belt from the β subunit surrounds the α subunit and holds it in place with a buckle formed between C19 and C105 (Fig. 5). Thus, the C105V mutation would be predicted to affect the subunit conformation, and either enhance TSH-β subunit intracellular degradation or diminish its ability to interact with the α subunit. Data from in vitro expression of this mutant confirm this prediction. TSH expression from cell cultures transfected with the C105V mutant β subunit was significantly reduced relative to cell cultures transfected with the wild-type β subunit.
Impaired TSH subunit combination was also demonstrated by Hayashizaki et al. for a mutation in the CAGYC region of the TSH-β subunit gene (G29R) (7). The CAGYC region is named for its one letter amino acid codes, is located near NH2 terminus of all pituitary and placental glycoprotein β subunits, and is necessary for α-β subunit combination (22). Affected family members with the G29R mutation had no measurable TSH in the serum even after TRH stimulation in the hypothyroid state. TSH levels in affected patients with the C105V mutation, however, were low-normal in the hypothyroid state and increased in response to TRH stimulation. Synthesis of TSH, therefore, is completely abolished with the G29R mutation but only partially impaired with the C105V mutation. As such, this is the first report of a genetic abnormality in the TSH-β subunit gene where immunoreactive mutant TSH is measurable in the serum.

Clinical data in this family also indicate that an impairment in TSH subunit combination exists. Fig. 3 demonstrates elevated basal and TRH-stimulated glycoprotein-free α subunit levels in affected family members. Since the origin of TRH-stimulated α subunit is the thyrotroph, in the absence of a pituitary adenoma (19), these data indicate the lack of efficient TSH-β subunit combination in affected patients. This is reflected in low serum TSH levels in affected family members, which are 10- to 100-fold lower than expected for hypothyroid individuals (Table I). As noted in Table I, however, affected children did have a 3- to 15-fold rise in serum TSH levels after TRH stimulation, confirming that some TSH subunit combination must occur with the mutant TSH-β subunit molecule.

In addition to impaired secretion of mutant TSH in affected family members, the mutant TSH has reduced biological activity. Two separate lines of evidence support this conclusion. First, when hypothyroidism was induced in affected family members by stopping their hormone replacement, mutual TSH serum levels increased to a low-normal range, but radioactive iodine uptake was negligible (Table I). For example, the father of Family A (IV.16) had a serum TSH of 1.0 mU/l and a 24 h RAIU of 19.8%, but his daughter (V.3) with a similar serum TSH of 0.8 mU/liter had a 24 h RAIU of only 0.9%. In addition, patient V.8 had no increase in TSH levels even though her TSH level increased 15-fold (Table II). Of course, the impaired RAIU response to mutant TSH molecule may be due to chronic TSH deficiency in affected family members. Bovine TSH administration, however, increased RAIU in the thyroid bed of affected individuals, V.3, V.4, V.6, and V.8 indicating functional thyroid tissue. Second, recombinant TSH harboring the C105V mutation in the TSH-β subunit had reduced bioactivity compared to wild-type TSH. The B/I could be even lower for the C105V mutant TSH, since the immunoassay may underestimate the amount of mutant TSH due to epitope loss. In contrast, the artificial carboxy-terminal truncations of the TSH-β subunit demonstrate that the mutation of cysteine at position 105 and not the absence of the remaining 13 amino acids is responsible for the impaired bioactivity of mutant TSH in this family.

Measurement of TSH is a valuable tool for evaluating thyroid function in individuals and in the differential diagnosis of thyroid disorders. This kindred, however, points out the difficulties of using a TSH measurement alone to evaluate thyroid disorders in the absence of a goiter. For example, if TSH was used as the primary screen for hypothyroidism in this kindred, hypothyroidism may have been missed, since TSH levels were normal or low in affected individuals. Moreover, since TSH levels were normal in many of the affected patients, central hypothyroidism might not have been considered in the differential diagnosis unless thyroid hormone levels were also determined. Unlike previous reports of unmeasurable TSH due to TSH-β subunit gene mutations (7, 8), a circulating form of mutant TSH exists in these patients, suggesting that other biologically inactive forms of TSH due to TSH-β subunit gene mutations remain to be discovered.

Acknowledgments

This work was supported by Public Health Service grants (DK-43653 and DK-49126) from the National Institutes of Health and Knoll Pharmaceuticals Co. (Whippany, NJ).

References


