Biochemical and Biophysical Research Communications **275,** 33–36 (2000)
doi:10.1006/bbrc.2000.3247, available online at http://www.idealibrary.com on IDEAL®

A Polymorphism in the Growth Hormone (GH)-Releasing Hormone (GHRH) Receptor Gene Is Associated with Elevated Response to GHRH by Human Pituitary Somatotrophinomas *in Vitro*

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Received July 13, 2000

A previous study has suggested that a G to A base change at position 169 of the GHRH-receptor gene in human somatotrophinomas is a mutation and confers hypersensitivity to GHRH. The alternative base converts codon 57 from GCG to AGC, resulting in replacement of alanine (Ala) with threonine (Thr). In the present study, two of five human GH-secreting somatotrophinomas were found to possess the codon 57 AGC sequence. The GCG allele was also detected, indicating heterozygosity. However, the patients' normal blood-derived DNA also yielded the same sequence pattern, indicating that the Ala ⇒ Thr amino acid change is a normal polymorphism, and not a somatic mutation. Nevertheless, *in vitro*, the tumors possessing the Ala ⇒ Thr amino acid change responded very strongly to GHRH in terms of cAMP formation, being increased 40- and 200-fold, in comparison to the 2-fold increases by tumors without the alternative GHRH-receptor sequence. Likewise, the *in vitro* response of GH secretion to GHRH was elevated. One of the two tumors with the alternative Thr residue, and the highest responder to GHRH, possessed a *gsp* mutation, despite the fact that these defects are thought to reduce responsiveness to GHRH. These results fail to confirm that the GCG ⇒ AGC at codon 57 of the GHRH-receptor gene is a mutation, but do support the concept that the alternative form with Thr confers increased sensitivity to GHRH.

There is compelling evidence that the vast majority of human pituitary tumors are monoclonal in origin, suggesting that they arise as a consequence of somatic mutation (1, 2). Many researchers are now searching for the types of defect which may be the cause of tumorigenesis (2). At least some secretory tumors are associated with activating mutations in genes controlling intracellular transduction pathways. The most understood are *gsp* oncogenes in GH-secreting somatotrophinomas, whereby somatic point mutations in the $G_s\alpha$ gene lead to amino-acid substitutions which abolish the intrinsic GTPase activity of this G-protein subunit, and hence result in increased intracellular cAMP formation (3). These oncogenes are found in about 40% of human pituitary somatotrophinomas and the resultant elevated cAMP production is believed to be the cause of excessive cell proliferation and GH secretion in this subset of tumors. The molecular etiology of the remaining 60% of somatotrophinomas remains unknown, but it might be anticipated that defects elsewhere in the GHRH-receptor-signaling cascade may well prove to have similar consequences as $G_s\alpha$ mutations, and thus be the cause of tumorigenesis. In apparent support of this concept, it has recently been reported that exon 3 of the GHRH-receptor gene possesses a GCG (Ala) ⇒ ACG (Thr) point mutation at codon 57 (4). Moreover, transfection studies demonstrated increased sensitivity to GHRH of the alternative form of the GHRH-receptor and it was suggested that it may play a role in tumorigenesis. We report here that the alternative form of the GHRH-receptor is, in fact, a normal polymorphism. Nevertheless, somatotrophinomas expressing this form do appear to exhibit increased sensitivity to GHRH.

MATERIALS AND METHODS

Mutational analysis of exon 3 of the GHRH-receptor gene. Studies were performed on DNA extracted from pituitary somatotrophinomas removed from 5 patients with acromegaly. The polymerase chain reaction (PCR) was used to amplify a 265 base pair region of the GHRH-receptor gene, encompassing the whole of exon 3. The forward and reverse primer sequences, targeted to intronic regions flanking exon 3, were as follows: 5′-GTGGTGGCTTCTCGATT-

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0006-291X/00 $35.00

CCTTTAT-3′, and 5′-TTCCAGATGAAAGCACCTCCCTTTC-3′. PCR was carried out on 5 μg DNA using a Gibco (Paisley, Scotland) amplification kit, as described (5). PCR DNAs were salt-ethanol precipitated, re-dissolved in 20 μL water and run on 1% agarose gels from which the DNA bands were excised and purified with a Qiaex gel extraction kit (Qiagen, Dusseldorf, Germany). Each purified PCR DNA was sequenced from the forward primer using the di-deoxy method and running the reactions on a Perkin-Elmer automated analyser. When an apparent base alteration was observed, an identical procedure was performed on the patients' blood-derived genomic DNA.

In vitro cell culture. At operation, a portion of each somatotrophinoma was placed into culture medium, transported to the laboratory and processed for cell culture as previously described in detail (6). Briefly, tissues were divided into small pieces with scalpels and incubated with collagenase (1 mg/mL) for 1–2 hours, with gentle shaking. Dispersed tissues were washed with fresh medium and equal aliquots of 1–2 $\times$ 10^5 cells distributed into at least 6 glass culture tubes, together with 2 mL medium. The pituitary cells were then allowed to equilibrate and attach to the glass tubes during the following 24 hours, after which the media were removed and replaced with fresh medium with no additive (control) or containing GHRH (2 nmol/L). At least three cultures were used for controls or GHRH treatment. After a 30-minute incubation at 37 C, the cells were extracted with ice-cold acidified ethanol and cAMP in the extracts assessed by radioimmunoassay, as described (7). For three of the tumors, the effect of GHRH on GH secretion was also determined. For these studies, cultures were incubated in the absence (controls) or presence of GHRH (2 nmol/L) for 2 hours, and media collected prior to measurement of GH by an ELISA technique (6). Results are expressed as mean cAMP or GH production $\pm$ SD per 30 minutes or 2 hours, respectively, and Student's t-test used to determine statistical significance.

Gsp oncogenes. Each of the five somatotrophinomas was tested for presence of *gsp* oncogenes, as previously described (6).

RESULTS

Sequencing of the PCR product yielded by 2 of the 5 tumor-derived samples revealed a double peak corresponding to base number 169 within codon 57 of the coding region of the GHRH gene (Fig. 1). These peaks represent G and A and indicate presence of the published (8) GCG (Ala) and an alternative ACG (Thr) codon 57 in the somatotrophinoma DNA. An example is shown in Fig. 1A. This G $\Rightarrow$ A base alteration represents a normal polymorphism, since the double peak was also observed in the patients' corresponding blood-derived genomic DNA (Fig. 1B). The remaining three somatotrophinoma-derived DNA samples yielded only the GCG sequence at codon 57 (data not shown). Table 1 summarises the basic clinical information on the 5 acromegalic patients (Patients A–E), their *gsp* oncogene status and the GHRH-receptor codon 57 sequence observed in the DNA extracted from the corresponding somatotrophinoma. Based on this very small series, no obvious difference in clinical characteristics were observed between patients with and without the alternative GHRH-receptor. Tumor D was found to possess a *gsp* oncogene, since codon 201 of the $G_s\alpha$ gene was altered from CGT to TGT, as described (3, 6).

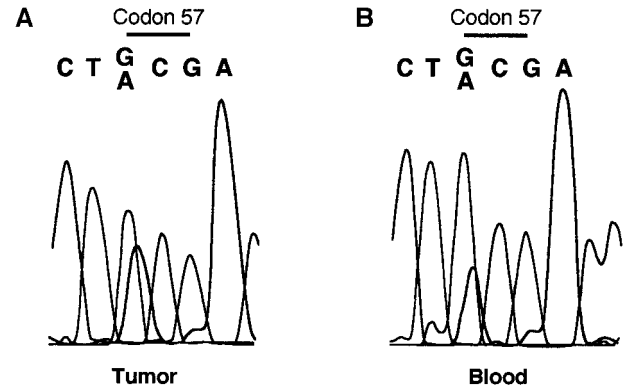

FIG. 1. Sequence of codon 57 of the GHRH-receptor gene in DNA derived from a human pituitary somatotrophinoma (left panel) and the same patient's corresponding blood-derived DNA (right panel). The double peak in the 5′ base of this codon observed in the somatotrophinoma-derived DNA represents two allelic forms, GCG (alanine) and AGC (threonine). The AGC codon results from a G $\Rightarrow$ A normal polymorphism, since this alternative base is also present in the blood-derived DNA.

The effect of GHRH on cAMP production by 4 of the tumors (Tumors A–D, corresponding to Patients A–D in Table 1) was examined *in vitro* (Fig. 2). Tumors A and B (Fig. 2, upper panel) did not possess the alternative codon 57 and exhibited a 2-fold increase in cAMP production in response to GHRH. In marked contrast, cAMP production was very greatly stimulated (40 and 200-fold) by GHRH when added to cultures of Tumors C and D (Fig. 2, lower panel). Both these tumors were shown to possess the Ala $\Rightarrow$ Thr alternative form of codon 57. Interestingly, although Tumor D exhibited the highest, and very powerful, response to GHRH, it possessed a *gsp* oncogene (Table 1). GH secretion in response to GHRH was also determined in Tumors A–C (Fig. 3). After a 2-hour exposure, GHRH stimulated GH secretion by Tumors A and B by a moderate amount (about 70–80% increases), whereas a much larger 650% increase was found with Tumor C.

DISCUSSION

The GHRH receptor is a typical G_s-protein coupled, 7-transmembrane receptor consisting of 423 amino acids (8). Binding of its ligand, GHRH, leads to displacement of GDP from the α-subunit of the G_s-protein ($G_s\alpha$) and its replacement with GTP (9). Subsequently, adenylyl cyclase is activated and the resultant increase in intracellular cAMP production leads to GH secretion. Tight control of these events is exerted by the intrinsic GTPase activity of the $G_s\alpha$ subunit, which soon results in removal of a phosphate group from the bound GTP and a return to the basal state of the GHRH-adenylyl cyclase-cAMP transduction cascade. Since experimentally-induced constitutive activation

TABLE 1

Characteristics of Acromegalic Patients A–E and Sequence of Codon 57 of the GHRH-Receptor Gene

Patient	Age	Sex	Pre-operative serum GH level (ng/mL)	Tumor diameter (mm)	Codon 57 sequence*	*gsp* oncogene status[+]
A	33	M	93	16	GCG/GCG	−ve
B	59	M	13	16	GCG/GCG	−ve
C	61	F	16	12	GCG/**AGC**	−ve
D	51	M	157	24	GCG/**AGC**	**+ve**
E	60	F	20	25	GCG/GCG	−ve

* Both alleles, alteration highlighted in bold.
[+] −ve/+ve = absence/presence of oncogene, respectively.

of this pathway leads not only to excessive GH secretion, but also pituitary somatotroph hyperplasia and tumor development in animals (8, 10), it is not surprising that activating mutations with similar effects are associated with somatotrophinomas in humans (3). Specifically, point mutations which abolish the GTPase activity of $G_s\alpha$-GTP subunits (*gsp* oncogenes) are the presumed major etiological factor in about 40% of these pituitary tumors (3). Biochemically, *gsp* oncogenes lead to constitutive adenylyl cyclase activity and excessive cAMP production, properties which originally led to the suspicion of, and search for, a defect in the G_s-protein (3, 11). Because of these finding, it is logical to suspect that defects elsewhere in the GHRH-adenylyl cyclase-cAMP pathway would be another molecular factor in the development of at least some somatotrophinomas. An obvious target for investigation is the GHRH-receptor, particularly since activating mutations in other G-protein-coupled receptors have been described (12). Because of these concepts, Petersenn *et al.* (4) investigated the GHRH-receptor gene structure in a series of human somatotrophinomas, and observed the G ⇒ A alternative form at position 169, as described in this paper. Transfection of constructs containing the GCG (Ala) and AGC (Thr) forms under the control of a CMV promoter into COS-7 cells revealed that the GHRH-receptor with threonine at position 57 responded more powerfully to GHRH in terms of cAMP accumulation. Although parallel studies on the patients' blood-derived genomic DNA were not performed, the compelling evidence of the transfection studies led to the conclusion that the alternative form of the GHRH-receptor gene, containing AGC instead of GCG at codon 57, represents a mutation in human somatotrophinomas and may contribute to tumorigenesis. The present results, however, have failed to confirm this concept, and indicate that the threonine substitution at residue 57 is a normal polymorphism. It should also be noted that this amino acid position is neither conserved nor semi-conserved between species (8). In rats and mice, glycine is found at residue 57, whereas it is arginine in the porcine and bovine receptors. It would thus be surprising if a somatic mutation at this position would yield a protein product with sufficiently altered activity leading to tumorigenesis. Nevertheless, our results do support the idea that presence of threonine at position 57 confers increased sensitivity to GHRH. Thus, GHRH stimulated cAMP production by remarkably high amounts

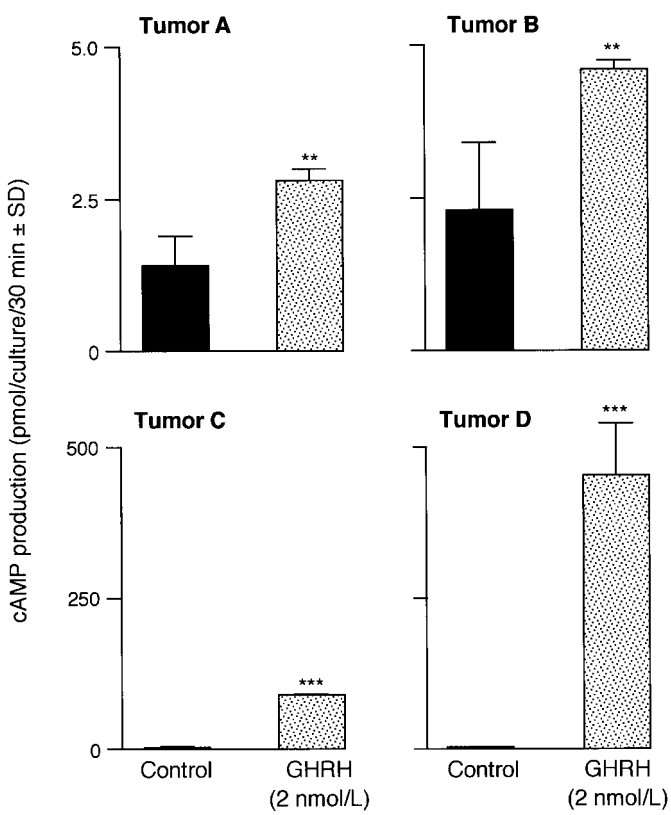

FIG. 2. Stimulatory effect of GHRH on intracellular cAMP production by *in vitro* cultures of human pituitary somatotrophinomas without (Tumor A and B) and with (Tumors C and D) the Ala ⇒ Thr alteration in residue 57 of the GHRH-receptor gene. **$P < 0.01$; ***$P < 0.001$ v. control.

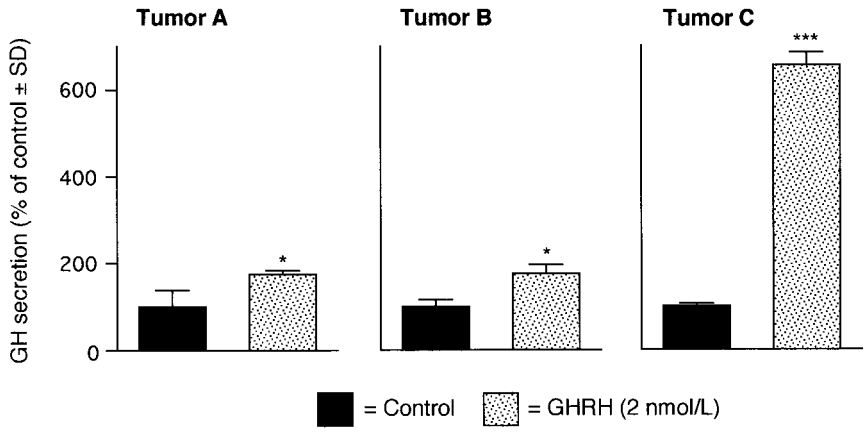

FIG. 3. Effect of a 2-hour incubation with GHRH on GH secretion by *in vitro* cultures of three somatotrophinomas, without (Tumors A and B) and with (Tumor C) the Ala ⇒ Thr alteration in residue 57 of the GHRH-receptor gene. *P < 0.05; ***P < 0.001 v. control.

in the two tumors possessing the alternative form of the GHRH-receptor. Of added significance, one of these tumors possessed a *gsp* oncogene, and thus constitutive adenylyl cyclase activity (3, 11). Since *gsp* oncogenes act at a site distal to the GHRH-receptor, it might be anticipated that somatotrophinomas possessing these mutations would be resistant to further stimulation with GHRH (11). Quite clearly, the present finding shows that this is not necessarily the case, perhaps due to parallel presence of a slightly different GHRH-receptor with increased sensitivity to GHRH. Related to this phenomenon, it is well established that GHRH exerts highly variable effects on GH secretion in normal and acromegalic subjects, both *in vivo* and *in vitro* (6, 13). Indeed, our previous studies demonstrated that a sub-group of human somatotrophinomas responds very strongly to GHRH, whereas others show minimal sensitivity. The present findings, and those of Petersenn *et al.* (4), indicate that this might be due to presence of different forms of the GHRH-receptor. How the presence of threonine confers increased sensitivity to GHRH remains to be determined. Position 57 corresponds to an amino acid with the N-terminal extracellular region of the receptor and might be involved in ligand binding (7). It will therefore be of interest to establish the ligand binding characteristics of GHRH-receptors with alanine or threonine at residue 57.

The clinical significance of the alternative GHRH-receptor remains to be determined. However, it seems unlikely that subjects possessing the codon 57 ACG form have increased susceptibility to developing pituitary somatotrophinomas since it appears to be quite a common allele and there would be far more cases of genetic predisposition to developing acromegaly than currently reported. Studies on the incidence of the different allelic forms in acromegalic and normal populations are required to more fully answer this question.

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