Growth hormone gene structure in human pituitary somatotrophinomas: promoter region sequence and methylation studies

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ABSTRACT

The structure of the GH gene in human somatotrophinomas was examined in terms of promoter region sequence and degree of methylation. In six tumours, the promoter sequence did not differ from that observed in the corresponding genomic (white blood cell-derived) DNA, suggesting that it is unlikely that excessive GH production is due to a point mutation within this region. In contrast, Southern blot analysis using the methylation-sensitive restriction enzymes HpaII and HhaI revealed lower levels of methylation of the GH gene in somatotrophinomas when compared with that found in DNA derived from normal pituitary glands. We conclude that hypomethylation of the GH gene in human somatotrophinomas may play at least a partial role in excessive GH production. Journal of Molecular Endocrinology (1994) 12, 167–172

INTRODUCTION

The application of DNA technology to the study of human pituitary tumours has proved fruitful in the elucidation of the aetiology and functioning of somatotrophinomas. Two recent discoveries exemplify the value of this approach. Using Southern blot analysis of restriction fragment length polymorphisms (RFLPs) and exploiting the phenomenon of X chromosome inactivation, human pituitary tumours, including somatotrophinomas, have been shown to be monoclonal in origin (Alexander et al. 1990; Herman et al. 1990). The implication of this finding is that mutations at the DNA level confer the property of excessive proliferation which, in turn, leads to tumour formation. The earlier discovery of gsp oncogenes in somatotrophinomas concurs with this concept (Landis et al. 1989). Approximately 40% of human pituitary somatotrophinomas harbour single base missense mutations in the α subunit gene of the Gs protein which regulates adenyl cyclase. These mutations lead to amino acid substitutions resulting in constitutive adenyl cyclase activity (Vallar et al. 1987). The resultant abnormally elevated intracellular cyclic AMP levels are thought to be the cause of uncontrolled somatotroph proliferation and excessive growth hormone (GH) secretion.

Despite these important findings, the cause of GH overproduction in the remaining 60% of somatotrophinomas is unknown and requires further investigation. A potentially important concept is that the GH gene itself may be defective, as suggested by U et al. (1988). These authors found a lesser degree of GH gene methylation in DNA extracted from a somatotrophinoma when compared with the GH gene in normal pituitary tissue. Since there is a negative correlation between gene activity and the amount of methylation (Razin & Riggs, 1980), this finding may explain excessive GH production.

U et al. (1988) also found that the GH gene showed no gross structural abnormalities but suggested that an activating point mutation within the GH gene may be present, although no experimental evidence in support of this was presented. Nevertheless, this is an important concept, particularly with respect to the promoter region of the GH gene. Control of GH gene transcription is exerted through the binding of a variety of nuclear proteins, such as Pit-1 (Bodner & Karin, 1987), Sp1 (Lemaigre et al. 1989) and thyroid hormone receptor (DeGroot et al. 1988), to the promoter region. Both Pit-1 and Sp1 stimulate transcriptional activity, but strong evidence exists for negative controlling elements also within the
GH gene promoter region (Peritz et al. 1988). Interestingly, the genes for human chorionic somatomammotrophin (hCS) and GH-variant (GH-V) have very high degrees of similarity to the GH gene, including the promoter regions (Seeburg, 1982). Despite this similarity, the expression of these genes is tissue-specific, with GH produced in the pituitary and hCS and GH-V expressed in placental tissue (Lefevre et al. 1987). This observation demonstrates that small differences within the promoter region can dramatically alter transcriptional activity. It is conceivable, therefore, that somatic mutations within the promoter region of the GH gene in somatotrophinomas could account for abnormally increased rates of transcription.

The purpose of the present study was to confirm and extend the findings of U et al. (1988) by examining for GH gene hypomethylation in several human pituitary somatotrophinomas, and to use sequence analysis to determine whether a point mutation in the controlling promoter region may occur.

**MATERIALS AND METHODS**

**GH gene promoter sequence**

DNA extraction was performed as previously described (Davis et al. 1986) and was carried out on blood and pituitary tumour tissue removed from six patients with acromegaly. Studies were performed with the written informed consent of the patients. All had elevated preoperative serum GH levels, ranging from 14.5 to 125 μg/l, which did not suppress to below 2 μg/l during an oral glucose tolerance test. None of the patients received preoperative medical therapy. A portion of each tumour specimen was placed in in vitro explant culture and secretion of pituitary hormones assessed. All the tissues secreted large amounts of GH and two also secreted prolactin, suggesting that these were mixed somatotrophic–lactotrophic pituitary tumours. Using previously described methods (Adams et al. 1993), two of the six tumours were found to harbour gsp mutations.

A 1.2 kb fragment encompassing bases −497 to +686 of the GH gene was synthesized by PCR. To facilitate cloning, each PCR amplifier contained a restriction enzyme sequence: EcoRI in the A amplifier and XbaI in the B amplifier. The full sequence of the A amplifier was 5’-GAAATTCAG GACTGAATCGTGCTCAC-3’ and that of the B amplifier was 5’-TCTAGAGACCGCCACCAAA GGTCTGAGCTGA-3’. The A amplifier was complementary to the anti-sense strand at the 5’ end of the GH gene. The B amplifier was complemen-

**FIGURE 1.** Sequence found in an AG-rich region within the first intron of the cloned 1-2 kb gene fragments. The sequence is specific to GH and reads ATGGAGAGAGAAAAACAAAA. The corresponding sequences for the hCS and human GH-V genes are AGAGAGAGAGAAAAACAAAA and AGAGAGAGAGAGAGAGAGAGAAAAACAAAA respectively (Seeburg, 1982).
reactions were loaded onto 8% polyacrylamide gels (Sequagel; National Diagnostics, Stuttgart, Germany) prepared in a Model S2 sequencing cell (Gibco-BRL, Eggenstein, Germany) and electrophoresed for 3 and 6 h at 60 W, using 0·1 mol Tris/l, 0·1 mol borate/l, 2 mmol EDTA/l (pH 8·3) as running buffer. After electrophoresis, the gels were transferred to filter paper (Bio-Rad, Munich, Germany), dried and autoradiographed for 1–3 days using Kodak X-OMAT film (Sigma, Deisenhofen, Germany).

To determine whether somatic mutations may occur in the GH gene promoter region, the sequences obtained from DNA derived from the pituitary tumour and the corresponding patient's blood were compared.

**GH gene methylation studies**

To determine whether a change in the tissue-specific methylation pattern of the GH gene in pituitary cells may occur in somatotrophinomas, DNA derived from both normal and tumorous tissues was subjected to Southern blot analysis using methylation-sensitive and methylation-resistant restriction enzymes. DNA from five of the six somatotrophinomas and from two normal pituitaries, obtained at post-mortem from a female subject aged 66 years and a male subject aged 80 years, was used in the studies. For the normal pituitaries, the time between death and tissue removal was 21 and 30 h respectively, and neither subject showed any signs of an endocrine defect. Written informed consent was obtained from the next-of-kin to perform post-mortem studies.

DNA (20–40 μg) was restriction-digested overnight with at least a fivefold excess of one of MspI, HpaII or HhaI (Boehringer, Mannheim, Germany). The digested DNA was electrophoresed through a 0·8% agarose gel for approximately 20 h at 35 V using TBE buffer (50 mmol Tris/l, 50 mmol borate/l, 1 mmol EDTA/l, pH 8·3). Following electrophoresis, the separated DNA fragments were denatured by immersion of the gel in 0·5 mol NaOH/l, 1·5 mol NaCl/l for 1 h at room temperature. After neutralization of the gel by immersion for 1 h in 1 mol Tris/l, 1·5 mol NaCl/l (pH 8·0), the denatured DNA bands were transferred to a nitrocellulose membrane as described by Davis *et al.* (1986). The nitrocellulose membrane blots were then air-dried and baked at 80 °C for 2 h.

Blots were subjected to hybridization with a GH probe (100 ng) consisting of the 500 bp EcoRI–BamHI promoter region, labelled with [32P]dATP (Amersham-Buchler) by the random primer method (Gibco-BRL). Each blot was prehybridized for 1 h at 42 °C in hybridization buffer N (Davis *et al.* 1986), followed by the addition of denatured probe and incubation overnight at 42 °C. Following hybridization, blots were sequentially washed in 6 × SSC/0·5% SDS (20 × SSC is 3 mol NaCl/l, 0·3 mol sodium citrate/l, pH 7·0), 2 × SSC/0·5% SDS and finally in 0·1 × SSC/0·5% SDS. Each wash was for 20 min and at 50 °C. Washed blots were air-dried and autoradiographed for 7 days.

**RESULTS**

**GH gene promoter**

To prove that the PCR strategy used amplified only the GH gene, each cloned 1·2 kb insert was sequenced between bases +150 and +200, in the first intron of the gene. This area contains a sequence of 5'-AGAGAGAAAACAAA-3' in the GH gene, but differs significantly in the number of AG and A repeats in both the GH-V and hCS genes (Seeburg, 1982). In all of the clones studied, the sequence unique to the GH gene was found (Fig. 1), indicating that only the GH gene was amplified during the PCR.

The sequence of the promoter region in all of the DNA samples was four bases shorter than that previously described by Seeburg (1982). Specifically, bases assigned by Seeburg (1982) to be at positions −75 (G), −276 (C), −283 (C) and −327 (C) were not found in any of the DNA samples, and the sequence probably represents an additional allelic form of the GH gene.

Analysis of the 500 bp GH gene promoter sequence revealed no differences between tumour- and blood-derived genomic DNA from the six acromegalic patients. Although an apparent base alteration was occasionally observed, these were attributed to Taq DNA polymerase errors since, when found, they occurred in only one clone in the set of ten studied, and the same base changes were not found in a second round of PCR-cloning–sequencing. These Taq DNA polymerase errors occurred with a frequency of about 1 in 2500 bp. In contrast, an allelic variant, in which G was replaced by T at position −57, was found in clones of both tumour and blood DNA from two of the patients at a ratio of approximately 50:50, indicating the validity of the cloning procedure used.

**GH gene methylation**

Figure 2 shows Southern analysis of the GH gene in tumour-derived (lanes 1–5) and normal (lanes 6 and 7) DNA after digestion with either MspI (Fig. 2a) or HpaII (Fig. 2b). Both these enzymes recognize
Figure 2. Southern blot analysis for the human GH gene of (a) MspI- and (b) HpaII-digested DNA obtained from normal pituitaries (lanes 6 and 7) and somatotrophinomas (lanes 1–5). Lambda phage DNA digested with EcoRI and HindIII was used as molecular size markers.

and cut the sequence CCGG; HpaII, however, is methylation-sensitive and will fail to cut at these sites if one or both of the cytosine residues are methylated. When digested with HpaII, the majority of the GH gene-hybridizing sequences in the DNA derived from normal pituitary tissue were found to have a relatively large molecular size. In contrast, HpaII digestion of tumour-derived DNA yielded GH gene-hybridizing bands of, on average, shorter length. This indicates that the tumour-derived DNA was more fully digested than the normal DNA, and thus demonstrates a lower degree of methylation of the GH gene in DNA derived from somatotrophinomas.

Apart from an additional band in one tumour sample (Fig. 2a, lane 5), MspI digestion of tumour-derived and normal DNA yielded essentially identical band patterns. This finding excludes the possibility that the band differences observed with HpaII digestion were due to polymorphisms in terms of the sites and frequency of CCGG sequences.

Figure 3 shows the result of a similar study using the methylation-sensitive enzyme HhaI which cuts DNA at GCGC sites provided the cytosine residues are not methylated. Again, GH gene-hybridizing bands obtained from the normal pituitary DNA (lanes 6 and 7) migrated, on the whole, to positions corresponding to large molecular sizes during electrophoresis, whereas the tumour-derived DNA was clearly more fully digested and yielded GH gene bands of lower molecular size. This finding further indicates a lesser degree of GH gene methylation in human somatotrophinomas.

DISCUSSION

Despite the theoretical possibility that promoter region alterations may result in abnormal gene expression, our results suggest that this is an unlikely explanation for excessive GH production in acromegaly. Using a similar strategy, Mönig et al. (1992) examined the pro-opiomelanocortin gene promoter region in 11 human corticotrophin
pituitary tumours and also found no difference in nucleotide sequence compared with that found in normal genomic DNA. It is becoming increasingly clear, therefore, that excessive hormone secretion in pituitary tumours is not due to promoter region defects, although the rare occurrence of such defects cannot be excluded.

Southern blot analysis of DNA derived from normal pituitaries and somatotrophinomas did, however, reveal structural differences in the GH gene. The greater degree of GH gene digestion by the restriction enzyme HpaII indicates lower levels of methylation. This may be of significance in terms of GH overproduction, since there is indirect and direct evidence of an inverse relationship between levels of DNA methylation and gene activity (reviewed by Razin & Riggs, 1980). DNA methylation is achieved by the addition of a methyl group to position 5 of cytosine residues. This methyl group protrudes into the major groove of the double helix and may modify the binding of regulatory proteins. Furthermore, there is a correlation between gene activity and hypomethylation. For example, the human globin gene is less methylated in tissues in which it is expressed than in non-expressing tissues. Direct evidence of a relationship between levels of methylation and GH gene activity has also accumulated. Thus, the GH gene shows tissue-specific methylation patterns, exhibiting lower levels in pituitary cells where it is expressed than in white blood cells in which the GH gene is not transcribed (Hjelle et al. 1982). Additionally, treatment of GH-secreting cells derived from the pituitary with the DNA methylation-inhibiting agent 5-azacytidine induces increased GH synthesis and secretion (Lan, 1984).

Because of these observations, U et al. (1988) investigated whether GH overproduction in acromegaly may be at least partially explained by hypomethylation of the GH gene within the pituitary tumour. In the single somatotrophinoma examined, the authors did indeed observe hypomethylation of the GH gene, based on restriction fragment lengths obtained after HpaII digestion. The present results confirm and extend these findings. First, we have examined five somatotrophinomas, all of which were found to exhibit GH gene hypomethylation. Secondly, we used another methylation-sensitive enzyme, HhaI, which recognizes a different sequence (GCGC) from HpaII (CCGG). The pattern of GH gene fragments obtained with this second enzyme again indicated hypomethylation in tumour-derived DNA. In support of these findings, and the concept that decreased methylation may induce excessive GH production, GH gene hypomethylation was also found to occur in an ectopic GH-secreting tumour (Ezzat et al. 1992). It should be noted, however, that the probes used in all three studies will also bind to the GH-related genes, hCS and GH-V. Consequently, the band patterns observed on Southern analysis will also include hCS and GH-V fragments, suggesting that these genes also are hypomethylated in somatotrophinomas. Since somatotrophinomas do not produce hCS or GH-V, this finding suggests that other factors besides hypomethylation are required for excessive GH production.

A potential problem with the present study arises from the use of post-mortem normal pituitary as a control. Whole pituitary tissue consists of other cell types besides somatotrophs, in contrast to the relatively homogenous pituitary tumours. Since the non-somatotrophs are non-expressing in terms of GH, some of the high molecular weight bands found on Southern analysis after HpaII digestion may be the result of DNA derived from these cells. Nevertheless, the majority of secretory cells in the normal pituitary gland are somatotrophs (Kovacs & Horvath, 1986) and it is likely that the GH gene band patterns observed by Southern analysis are a fairly accurate representation of somatotroph-derived DNA.

The control enzyme, Mspl, resulted in virtually identical band patterns in both normal and tumour-derived DNA, indicating identical positions and numbers of CCGG sites in the GH gene in the two DNA sources. An extra band was found in one tumour sample, and this probably represents an Mspl RFLP. An Mspl RFLP was also shown by U et al. (1988).

In summary, no alterations were found in the GH gene promoter region in DNA of human somatotrophinomas, but the gene appears to be hypomethylated compared with that found in normal tissue.

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REFERENCES


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