Reduced expression of the growth hormone and type 1 insulin-like growth factor receptors in human somatotroph tumours and an analysis of possible mutations of the growth hormone receptor

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Summary

OBJECTIVE Clinical acromegaly is characterized by elevated GH secretion in the presence of high circulating IGF-I levels. We hypothesized that the physiological IGF-I/GH negative feedback loop may be reset in somatotroph adenomas, specifically in terms of the level of expression of these receptors or mutations of the GH receptor (GH-R) in such tumours.

METHODS We therefore investigated the full coding sequence of the GH-R in a series of somatotroph and other pituitary adenomas. We also investigated the mRNA expression of these putative feedback receptors in a series of pituitary adenomas and normal pituitary tissue, and their protein expression by immunostaining. Real-time RT–PCR assay was used for the quantification of the type 1 IGF receptor (IGF-R) and GH receptor (GH-R) mRNA, and sequence analysis was performed on the coding region of the GH-R gene.

RESULTS No somatic mutations of the GH-R mRNA were detected in 18 GH-secreting tumours or two non-functioning pituitary adenomas (NFPAs). However, the levels of GH-R mRNA were significantly lower in both somatotroph tumours and NFPAs compared to the normal pituitary (P < 0.05 for both). Immunostaining for GH-R also showed significantly less GH-R expression in somatotroph adenomas compared to normal pituitary tissue (P < 0.0001). IGF-R mRNA levels were significantly lower in somatotroph tumours compared to normal pituitary (P = 0.005), and trended lower in corticotroph tumours (P = 0.07), while the other tumour types showed no significant difference from normal pituitary. Immunostaining for IGF-R also showed less IGF-R protein in the somatotroph adenomas compared to the normal pituitary tissue (P < 0.01).

CONCLUSIONS Our findings suggest that decreased feedback inhibition of GH because of somatic mutations of the coding region of the GH-R are unlikely to be a common factor in the pathogenesis of these tumours. Nevertheless, decreased expression of the GH-R and of IGF-R in somatotroph tumours (both at the mRNA and protein level) may, at least in part, help explain the continuous secretion of GH from the tumour despite the high circulating levels of IGF-I and GH.
et al., 1983; Billestrup et al., 1986), ghrelin (Bowers et al., 1984; Kojima et al., 1999; Hosoda et al., 2000) and somatostatin (Lamberts, 1988), but also involves feedback regulation by GH and IGF-I. IGF-I is the most important peripheral inhibitory factor regulating GH secretion, acting at both hypothalamic and direct pituitary levels to inhibit GH release (Berelowitz et al., 1981; Tannenbaum et al., 1983; Yamashita et al., 1986; Yamashita & Melmed, 1987). There is also evidence for a paracrine/autocrine role of IGF-I on the pituitary. Binding sites for IGF-I and later the presence of mRNA coding for the type 1 IGF receptor (IGF-R) and its binding proteins were found throughout the anterior lobe of the rat pituitary (Goodyer et al., 1981; Melmed, 1987). There is also evidence for a paracrine/autocrine role in place of, or in addition to, any regulation not only by somatotrophs, suggesting that it may have a growth factor-related role in place of, or in addition to, any regulation of somatotroph secretion (Bach & Bondy, 1992).

There is also considerable evidence that GH can regulate its own secretion via a negative feedback mechanism (Fagin et al., 1988; Pontiroli et al., 1991; Rosenthal et al., 1991; Lanzi & Tannenbaum, 1992; Asa et al., 2000). Given either centrally or peripherally, GH causes a marked suppression of the spontaneous bursts of GH secretion in the male rat (Tannenbaum, 1980; Willoughby et al., 1980; Abe et al., 1983; Clark et al., 1988). In humans, GH pretreatment attenuates the GH response to various pharmacological (hypoglycaemia, arginine, clonidine or GHRH) or physiological (sleep, exercise) stimuli (Abrams et al., 1971; Hagen et al., 1972; Mendelson et al., 1983; Nakamoto et al., 1986; Ross et al., 1987a). These effects occur prior to the rise in circulating IGF-I levels, suggesting a direct role of GH per se. These effects are mediated, at least in part, by hypothalamic pathways, including the stimulated release of somatostatin (Ross et al., 1987b). However, there may be direct modulation of pituitary function: GH receptor (GH-R) mRNA is found ubiquitously distributed throughout the pituitary gland (Frazier & Harvey, 1992; Harvey & Fraser, 1992; Hull et al., 1992; Harvey et al., 1993), and GH is also involved in the synthesis and regulation of pituitary IGF-I (Fagin et al., 1988, 1989). GH might therefore act directly as a regulator of GH synthesis and secretion (Ezzat & Melmed, 1990; Mertani et al., 1994).

Acromegaly is characterized by alterations in the physiological negative feedback regulation of GH and IGF-I on their own secretion, with coexistent elevations in both GH and IGF-I simultaneously. We have therefore speculated that the physiological IGF-I/GH negative feedback loop may be altered or set to a higher point in this disease as a consequence of either qualitative or quantitative changes in GH-R and IGF-R expression. The aim of this study was to investigate the possible presence of GH-R mutations in somatotroph tumours, and to quantify and compare the level of expression of the GH-R and IGF-R mRNA in somatotroph tumours compared to other pituitary tumours and to normal pituitary tissue.

Materials and methods

Tumour specimens

Fifty-eight human pituitary adenomas, 25 GH-secreting tumours, 21 nonfunctioning pituitary adenomas (NFPAs), five ACTH-secreting tumours, four prolactin-secreting tumours and three FSH-secreting tumours were obtained at the time of transsphenoidal surgery (Table 1). Tumours were collected in liquid nitrogen and then stored at −80°C. The tumour type was determined on the basis of clinical and biochemical findings before surgery, and by morphological and immunocytochemical data of the removed tissue sample. Normal human pituitary autopsy specimens (six samples), obtained within 24 h of death, were obtained from patients with no evidence of any endocrine abnormality. Informed consent was obtained from all patients, and the study was approved by the local Ethics Committee.

RNA preparation

Total RNA was prepared using the Promega SV isolation kit (Promega, Southampton, UK), which includes a DNase step. RNA was quantified by spectrophotometry (Cecil CE Computing Double Beam UV Spectrophotometer, Cecil Instruments Ltd, Cambridge, UK) and by using RiboGreen™ RNA Quantification Reagent Kit (Molecular Probes Europe BV, Leiden, the Netherlands) in a 1420 Multilabel Counter Wallac Victor 2. RNA was diluted to 50 ng/μl for use in the RT–PCR assay and stored at −80°C.

Primers and probes

Real-time RT–PCR primers and probes for GH-R and IGF-R (Table 2) were designed using Primer Express software [PE-Applied Biosystems (PE-ABI), Warrington, UK] based on the published sequence data of the two genes (GH-R: NM_000163, IGF-R: NM_000875). The TaqMan™ probes were labelled with a reporter dye (6-carboxy-fluorescein, FAM) at the 5′ end and a quencher dye (6-carboxy-tetramethylrodamine, TAMRA) at the 3′ end. Seven sets of primers were designed for amplifying and sequencing the whole coding region of the GH-R mRNA (Table 3).

RT and PCR for sequencing

Total RNA was reverse-transcribed into complementary DNA (cDNA) using standard procedures as described before (Korbonits et al., 2001). PCR reactions run with a control tube containing the PCR reaction mixture but instead of cDNA, water as a template. For a 50-μl reaction, we used 5 μl of cDNA, deoxynucleotides at 200 μM concentration, primers at 1 μM concentration, MgCl2 at 1·5 mM concentration and 0·125 U Taq (Promega) according to the manufacturers’ guidelines. Forty cycles were
Table 1 Clinical characteristics of the patients

| Sample no. | Diagnosis     | Age (years) | Sex | Size of tumor | Immunohistochemistry |
|------------|---------------|-------------|-----|---------------|----------------------|
| 1          | Acromegaly    | 35          | M   | Macro         | GH, αSU              |
| 2          | Acromegaly    | 53          | M   | Macro         | GH                   |
| 3          | Acromegaly    | 26          | M   | Macro         | LH, FSH, TSH, GH, PRL|
| 4          | Acromegaly    | 29          | F   | Macro         | GH                   |
| 5          | Acromegaly    | 44          | M   | Macro         | GH, PRL, TSH         |
| 6          | Acromegaly    | 22          | M   | Macro with SSE| GH, αSU              |
| 7          | Acromegaly    | 60          | M   | Macro         | GH                   |
| 8          | Acromegaly    | 50          | M   | Macro         | GH, PRL              |
| 9          | Acromegaly    | 61          | M   | Macro with SSE| GH                   |
| 10         | Acromegaly    | 47          | M   | Macro with SSE| GH                   |
| 11         | Acromegaly    | 35          | M   | Macro with SSE| GH                   |
| 12         | Acromegaly    | 41          | M   | Micro         | GH                   |
| 13         | Acromegaly    | 69          | M   | Macro         | GH                   |
| 14         | Acromegaly    | NA          | F   | Macro         | GH                   |
| 15         | Acromegaly    | 28          | F   | Macro         | GH                   |
| 16         | Acromegaly    | 66          | M   | Micro         | GH                   |
| 17         | Acromegaly    | 44          | M   | Macro         | GH                   |
| 18         | Acromegaly    | 66          | M   | Macro         | GH                   |
| 19         | Acromegaly    | 44          | F   | Macro         | GH                   |
| 20         | Acromegaly    | 48          | M   | Macro         | GH                   |
| 21         | Acromegaly    | 52          | F   | Macro         | GH                   |
| 22         | Acromegaly    | 30          | M   | Macro with SSE| GH, PRL              |
| 23         | Acromegaly    | 65          | F   | Micro         | GH                   |
| 24         | Acromegaly    | 44          | F   | Macro         | GH                   |
| 25         | Acromegaly    | 30          | F   | Macro         | GH, PRL              |
| 26         | Cushing’s     | 53          | F   | Micro         | ACTH                 |
| 27         | Cushing’s     | 28          | F   | Micro         | ACTH                 |
| 28         | Cushing’s     | NA          | F   | NA            | ACTH                 |
| 29         | Cushing’s     | 66          | F   | Micro         | ACTH                 |
| 30         | Cushing’s     | 25          | F   | Micro         | ACTH                 |
| 31         | FSHoma        | 48          | M   | Macro         | FSH                  |
| 32         | FSHoma        | 59          | M   | Macro         | FSH, αSU, αHCG       |
| 33         | NFPA          | 59          | M   | Macro         | all neg              |
| 34         | NFPA          | 38          | M   | NA            | NA                   |
| 35         | NFPA          | 80          | F   | Macro with SSE| αSU                 |
| 36         | NFPA          | 82          | M   | Macro         | all neg              |
| 37         | NFPA          | 68          | M   | Macro with SSE| all neg              |
| 38         | NFPA          | 41          | M   | Macro with SSE| all neg              |
| 39         | NFPA          | 66          | F   | Macro         | LH, αHCG             |
| 40         | NFPA          | 53          | F   | Macro with SSE| all neg              |
| 41         | NFPA          | 50          | F   | Micro         | all neg              |
| 42         | NFPA          | 61          | M   | NA            | all neg              |
| 43         | NFPA          | 72          | F   | Macro         | all neg              |
| 44         | NFPA          | 58          | M   | NA            | all neg              |
| 45         | NFPA          | 59          | M   | Macro with SSE| FSH                  |
| 46         | NFPA          | 68          | M   | NA            | all neg              |
| 47         | NFPA          | 65          | M   | NA            | all neg              |
| 48         | NFPA          | 45          | M   | Macro         | all neg              |
| 49         | NFPA          | 55          | M   | NA            | all neg              |
| 50         | NFPA          | 61          | F   | Macro         | all neg              |
| 51         | NFPA          | 60          | F   | Macro         | all neg              |
| 52         | NFPA          | 50          | M   | Macro         | all neg              |
| 53         | NFPA          | 51          | M   | Macro         | all neg              |
| 54         | NFPA          | 30          | F   | Macro         | PRL                  |
| 55         | PRLoma        | 36          | M   | Macro         | PRL, TSH, GH         |
| 56         | PRLoma        | NA          | M   | Macro         | PRL                  |
| 57         | PRLoma        | 29          | F   | Macro         | PRL                  |
| 58         | Normal pituitary| NA          | NA  | NA            | NA                   |
| 59         | Normal pituitary| NA          | NA  | NA            | NA                   |
| 60         | Normal pituitary| NA          | NA  | NA            | NA                   |
| 61         | Normal pituitary| NA          | NA  | NA            | NA                   |
| 62         | Normal pituitary| NA          | NA  | NA            | NA                   |
| 63         | Normal pituitary| NA          | NA  | NA            | NA                   |
| 64         | Normal pituitary| NA          | NA  | NA            | NA                   |

αSU, α-subunit; Macro > 10 mm, SSE, suprasellar extension; NA, not available.
performed at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, after a first denaturing cycle at 95°C for 5 min. A final extension cycle of 10 min at 72°C was used. PCR products were run on 2% ethidium bromide-stained agarose gels.

**Sequencing**

Direct sequencing was performed for the GH-R gene on 18 somatotroph tumours and two NFPAs as follows: PCR products were purified by using a QIAquick PCR purification kit protocol (Qiagen, Crawley, West Sussex, UK) or by using ExoSAP-IT kit protocol (USB Corporation, Cleveland, Ohio, USA). A 10-µl cycle sequencing reaction was prepared by using 5·5 µl of the purified PCR product, 4 µl of Terminator Ready Reaction Mix (ABI PRISM BigDye™ Terminator version 2.0, PE-ABI) and 0·5 µl of the primer. Each PCR product was sequenced in both the forward and the reverse direction. The same set of primers used for the PCR amplification was used in this case as well. The cycle sequencing was performed on the GeneAmp 9700 with 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min a rapid thermal ramp was used each time. The extensions products were consequently purified in order to remove the excess of dye terminators, which can interfere with base calling. This step was performed either by ethanol precipitation or by using Sephadex column precipitation plates (Amersham Life Science Ltd, Buckinghamshire, UK). Electrophoresis of the samples was performed on the ABI Prism 3700 DNA Analyser (PE-ABI). The samples were resuspended in 6 µl of formaldehyde before being loaded into the analyser. The obtained sequences were compared to the published GH-R sequence.

**Determination of the ‘gsp’ status**

The 18 somatotroph adenomas, which were sequenced for GH-R, were also tested for the gsp mutations of the α-subunit of the G protein (codons 201 and 227). The oligonucleotides used for amplification were 2F: ACCATTGTGAAAGCAGATGAGGAT and 10R: CACGGATGATGATGGCAGTCAC (Hayward et al., 2001). For a 25-µl reaction, we used 2·5 µl of cDNA, deoxynucleotides at 200 µM concentration, primers at 0·4 µM concentration, MgCl₂ at 1·5 mM concentration and 0·125 U Taq polymerase. Thirty cycles were performed at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, after a first denaturing cycle at 94°C for 5 min. A final extension cycle of 7 min at 72°C was used. Direct sequencing was performed in both directions using primers 7F: CCTGGACAAGATCGACGTGA and 10R: CACGGATGATGATGGCAGTCAC and the same protocol as described above for the GH-R gene.

**Real-time PCR**

The RT–PCR reactions were performed, recorded, and analysed by using the ABI PRISM 7700 Sequence Detection system (PE-ABI). Absolute mRNA copy numbers were determined from a standard curve, which was obtained by serially diluting single stranded sense oligodeoxynucleotides, GH-R and IGF-R specific amplicons. A strong linear relationship between the threshold cycle (Ct) and the log of the starting RNA-copy number was always found (r = 0·99). Absolute mRNA levels are expressed as mRNA copy numbers/µg total RNA (Bustin, 2000). There are conflicting data regarding the best method of RNA expression with some authors suggesting the use of housekeeping genes while others advocate the use of absolute RNA quantification. In the current study we have followed the latter possibility, particularly as we have shown earlier the unreliable nature of the former approach (Tricarico et al., 2002). Real-time RT–PCR were performed in duplicate as previously described (Bustin, 2000) using TaqMan™

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**Table 2** Primers and probes for GH-R and IGF-R for real-time RT–PCR

| Gene | Forward primer | Reverse primer | TaqMan probe |
|------|----------------|----------------|--------------|
| GH-R | TTTGGAATATTTGGG CTAACAGGT | TCACCTCCCTAAATT TT CTTCCCTT | AAGGATTAATAGCTG ATTCTGCCCCAGT |
| IGF-R | CTCCGTGTCTCTCGCCG | CTCCGCTGTGGTGG ATGAGTGTG | TGGGCCGAGAATTCTC CACTGTC |

**Table 3** GH-R gene primers for conventional PCR and sequencing (base-pair numbers refer to NM_000163)

| Primer | Primer sequence | Base-pairs |
|--------|-----------------|------------|
| F1     | GCTCGGAAGTCTCTACAGGTAT | 25–518    |
| R1     | CATTGCCCTCAACTGGAGCTT |          |
| F2     | GGCACCTGGACAGATGGAGTT | 240–741   |
| R2     | GAAGTGTGTTGGATGCCCAA |          |
| F3     | AGTGAGATGGGAGAACCAC | 559–1059  |
| R3     | AAACCCGAATTTCCAGTG |           |
| F4     | GAAGTGTGAGATGGACCACC | 722–1208  |
| R4     | CGACTCTGGGAGTACCACC |           |
| F5     | CAAAAGATTAAAGGATCATGCACC | 954–1462 |
| R5     | CTACCAAGGCTCCCATT |            |
| F6     | AACCTAGGGGTTAGAGATGG | 1169–1676 |
| R6     | AGATGCCAAAAGGTGACCC |          |
| F8     | CGACAGGTAGTGGTGGCTC | 1526–2025 |
| R8     | TGACTGGGGCAAATAACGTTT |          |
Immunostaining

A sample of six somatotroph tumours and five samples of normal pituitary were subjected to immunocytochemical staining with antibodies to the GH-R and the IGF-R according to standard techniques. The normal pituitary was collected at transsphenoidal surgery from patients thought to harbour tumours but in whom no tumour was identified: we have previously found that such tissue provides a good control for tumour specimens, and by being collected in exactly the same manner as the pituitary tissue does not suffer from problems with postmortem degradation seen with autopsy material (Lidhar et al., 1999; Korbonits et al., 2002). In brief, the tissue was collected into 4% formalin and processed for paraffin embedding and avidin–biotin–peroxidase staining (Mertani et al., 1995). The GH-R antibody was the murine monoclonal mAb 263, raised against the extracellular domain of the GH-R and supplied by Agen (Brisbane, Australia). The specific lot designation was D7·18·3D5/131. This was used at a final dilution of 1 : 100, and without pretreatment. Control slides with the addition of nonspecific immune serum were run to control for nonspecific staining. The IGF-R antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; product number SC-712) was used in a similar manner at a dilution of 1 : 50. Slides were assessed by a histopathologist and the extension of the immunostaining was morphometrically evaluated blind in 10 high-power fields, counting positively stained cells according to a 100-point graticule. The results were expressed as the percentage of positive tumour cells. Standard western blotting was attempted for both antibodies, but neither demonstrated a band of the requisite size under a variety of different conditions (data not shown). It is well known that it is difficult to generate antibodies to the extra-cellular domain of cytokine receptors, and our results are compatible with data from the originators of the GH-R antibody (M. J. Waters, personal communication).

Photographs of slides were taken using a Leica Corporation DMR microscope and a Leica Corporation DC200 digital camera at a magnification of × 630, and printed by an Epson 880 Stylus Color printer on HP PremiumPhoto paper.

Statistical analysis

Comparisons between mRNA expression of normal pituitary and each type of tumour were carried out with a nonparametric analysis of variance test, the Kruskal–Wallis test followed by the Dwass–Steel–Christchlow–Fligner multiple comparison test (StatsDirect, I. Buchan, Addison Wesley Longman Ltd. Cambridge, UK). Spearman’s rank order correlation tests were performed to see whether there was any correlation between GH-R mRNA level/IGF-R mRNA level and size of tumour or GH and IGF-I levels. Immunostaining data were calculated using the unpaired student’s t-test as these data were normally distributed. Significance was taken at \( P < 0.05 \).

Results

GH-R mRNA was detected and quantified by using real-time RT–PCR in the following pituitary tumours: 25 GH-secreting tumours, 21 NFPAs, five ACTH-secreting tumours, four prolactin-secreting tumours and three FSH-secreting tumours, and in six autopsy normal pituitary samples. The level of GH-R mRNA expressed as median copy number/µg RNA was: somatotroph tumours \( 1.47 \times 10^5 \), ACTH-secreting tumours \( 7.10 \times 10^5 \), FSHomas \( 3.06 \times 10^5 \), NFPAs \( 1.50 \times 10^7 \), PRLomas \( 2.50 \times 10^7 \) and normal pituitary \( 1.31 \times 10^6 \). The levels of GH-R mRNA were significantly lower in somatotroph tumours compared to normal pituitary (\( P = 0.013 \)), and NFPAs also showed significantly lower GH-R mRNA expression compared to normal pituitary (\( P = 0.0292 \); Fig. 1). There was no difference in GH-R mRNA levels between micro- and macroadenomas.

The presence of GH-R protein was identified by immunohistochemistry using a specific human GH-R antibody; the staining was blocked when the antibody was replaced by a nonimmune serum (Fig. 2). Somatotroph tumours showed significantly less GH-R immunostaining compared to normal pituitaries (\( 11.7 \pm 0.8 \text{ vs. } 30.4 \pm 1.1\% ; P < 0.0001 \), Fig. 3a).

IGF-R mRNA was detected and quantified by using real-time RT–PCR. The level of IGF-R mRNA expressed as median copy number/µg RNA was: somatotroph tumours \( 3.86 \times 10^5 \), ACTH-secreting tumours \( 2.34 \times 10^6 \), FSHomas \( 8.30 \times 10^7 \), NFPAs

![Fig. 1](image-url) GH-R mRNA expression in pituitary tumours (GH = somatotroph adenoma, \( n = 25 \); NFP = nonfunctioning pituitary adenoma, \( n = 21 \); ACTH = corticotrophinoma, \( n = 5 \); FSH = FSH-secreting tumour, \( n = 3 \); PRL = prolactinoma, \( n = 4 \); NP = normal pituitary, \( n = 6 \)). Inter-quartile ranges, maxima and minima are shown. \( *P < 0.05 \) vs. normal pituitary.
GH and IGF-I receptor in pituitary tumours

7.97 x 10^7, PRLomas 5.7 x 10^7 and normal pituitary 4.42 x 10^7. IGF-R mRNA levels were significantly lower in somatotroph tumours compared to normal pituitary (P = 0.005), while the other tumour types showed no significant difference from normal pituitary, although there was a trend (P = 0.07) for the corticotroph tumours to also express less IGF-R (Fig. 4). There was no difference in IGF-R mRNA levels between micro- and macro-adenomas. IGF-R immunostaining showed significantly less positive cells in somatotroph adenomas than in normal pituitary tissue (15.7 ± 1.4 vs. 29.9 ± 2.1%; P < 0.01, Figs 3b and 5).

Direct sequencing of the coding region of (60–1960 bp) of the GH-R was performed in 18 GH-secreting tumours and two NFPAs. No alterations of the GH-R mRNA sequence were detected in the samples apart from four different and known polymorphisms of the GH-R gene (Table 4 and NCBI SNP Database; Edens & Talamantes, 1998). Mutations of the Gsα gene were identified in 4/18 samples, three samples with the R201C mutation and one sample with the Q227R mutation; there was no correlation with GH-R or IGF-R status and the gsp status of the adenomas.

Discussion

This study showed that GH-R and IGF-R are expressed in both normal and adenomatous human pituitary tissue. We used real-time RT–PCR to detect and quantify GH-R and IGF-R levels.
All the samples, normal pituitaries and tumours arising from somatotroph, corticotroph, lactotroph and gonadotroph cells, as well as nonfunctioning adenomas, expressed both the GH-R and IGF-R. The level of GH-R mRNA was significantly lower in somatotroph tumours compared to normal pituitary; NFPA also showed lower GH-R mRNA expression while the other adenoma types were not significantly different to normals. We demonstrated that IGF-R mRNA levels were also significantly lower in somatotroph (and possibly corticotroph) tumours compared to normal pituitary. For the RNA studies we used autopsy normal pituitaries. In these samples a higher degree of RNA degradation and therefore lower level of specific RNA expression might be expected, while we found a higher level of GH-R RNA expression, suggesting that the use of autopsy tissue would not be likely to cause these results. Our immunostaining data suggest that the reduced expression of mRNA for both the GH-R and IGF-R in somatotroph tumours is also reflected in lower protein expression. In some of the tumour samples normal tissue was present adjacent to adenoma tissue, and the obvious difference of protein staining was clearly demonstrated in these slides as well.

The sequence analysis of the coding region of the GH-R gene did not reveal any abnormalities in 18 GH-secreting tumours. The relatively low frequency of gsp mutations in our population might be explained by the mixed ethnic background of our patients, as certain racial groups have a lower level of gsp mutations than patients of European origin (Harris, 1996).

Defects in the hormonal regulatory pathways are attractive candidates for causal factors in pituitary pathogenesis (Ezzat, 2001). Increased secretion of GH/IGF-I would suppress the surrounding normal somatotrophs, and the clonal expansion could re-establish feedback at a higher reset level. In favour of this is the report that showed that the negative feedback exerted by IGF-I on GH secretion is retained, but is markedly attenuated (Jaffe et al., 2001). However, somatic activating mutations of the GHRH-R (Salvatori et al., 2001) and ghrelin receptors (Petersenn et al., 2001) have not been identified in somatotroph tumours, and neither have inactivating mutations of the principal somatotroph somatostatin receptor, SSTR-2 (Petersenn et al., 2000). GH-R-deficient mice demonstrate mild somatotroph hyperplasia (an increase in proliferating cell nuclear antigen of 10–14%), disruption of the reticular fibre network and the presence of sparsely granulated cells, typical of pituitary GH-producing adenomas, suggesting a direct GH inhibitory feedback on the pituitary (Asa et al., 2000). There is also evidence for a ubiquitous distribution of the GH-R throughout the pituitary gland. GH-R mRNA and GH binding protein mRNA have been located in the cytoplasm and in the nucleus of somatotroph, lactotroph and gonadotroph rat pituitary cells (Mertani et al., 1994, 1995; Mertani & Morel, 1995). We have now carefully looked for mutations of the coding region of the GH-R, but found no somatic mutations in the coding regions of any of 18 somatotroph tumours or two NFPA. This suggests that an abnormal form of the GH-R is unlikely to be causal in satmotroph pathogenesis. However, we did note that the GH-R is expressed by normal pituitary tissue, as well as in all the adenomas assessed. It is currently unclear as to the precise cell types which express the GH-R, but studies on GH feedback suggest that it is functionally present on at least the somatotrophs. Immunocytochemical staining indicates that the GH-R may also be expressed in lactotrophs and gonadotrophs, but is absent from corticotrophs and thyrotrophs (Mertani et al., 1995). Because the somatotrophs constitute some 50% of the total normal pituitary population, the four-fold lower expression in somatotroph tumours is highly suggestive that GH-R mRNA is indeed underexpressed by individual somatotroph adenoma cells.

We attempted to demonstrate the presence of GH-R protein in our samples. Western blotting proved to be unsuccessful due to lack of suitable antibody against the extracellular part of the GH-R. However, immunostaining demonstrated the presence of...
the GH-R in many cells of the normal pituitary, compatible with a previous report using the same antibody (Mertani et al., 1995). In quantitative terms, the staining was significantly less than in the small series of somatotroph tumours stained in an identical manner and at the same time. Our results suggest that the decrease in GH-R mRNA is paralleled by a fall in receptor protein expression in somatotroph tumours.

Our results also showed a lower expression of IGF-R mRNA in somatotroph tumours compared to normal pituitary tissue. There is one previous report of a relatively low expression of IGF-R mRNA in a small number of pituitary GH-producing adenomas, quantified by competitive RT–PCR reactions (Otsuka et al., 1999). We also noted that the normal pituitaries showed cytoplasmic staining for the IGF-R which was significantly decreased in the somatotroph tumours. In another study, the type 1 IGF receptor was screened for mutations of the submembrane domain of the β subunit of the IGF-R, which was found to be intact in 19 somatotroph adenomas, studied initially by SSCP and consequently confirmed by direct DNA sequencing (Greenman et al., 1995). Nevertheless, as IGF-1 acts as a growth factor in

Fig. 5 A series of five somatotroph tumours (left panel) and three normal pituitaries (right panel) immunostained for IGF-R. One somatotroph tumour and one normal pituitary show tissue sections where the IGF-R antibody was replaced by nonspecific immune serum (‘negative control’). All photomicrographs are shown at a magnification of ×630.
addition to any specific role it may have in terms of growth axis feedback, it is difficult to be certain that any changes seen in somatotroph tumours are pathogenetic.

It is difficult to establish from the present studies whether the changes in GH-R and IGF-R expression in somatotroph tumours, or indeed in other types of pituitary tumours, are a cause or a consequence of the tumorous state. In the rat, GH appears to suppress or indeed in other types of pituitary tumours, are a cause or a consequence of the tumorous state. In the rat, GH appears to suppress the expression of the GH-R, at least in the hypothalamus and pituitary adenomas, and have excluded such mutations in the coding region as a significant causal factor in the majority of somatotroph adenomas. We also quantified the GH-R and type 1 IGF-R mRNA in a series of somatotroph tumours, and related the levels to normal pituitary and other types of pituitary adenoma. The GH-R appears to be significantly lowered in somatotroph adenomas compared to the normal pituitary, where somatotrophs are the predominant cell type. The levels of IGF-R are also lower in somatotroph tumours, compared to the normal pituitary. Our immunostaining data are compatible with the diminution in GH-R and IGF-R mRNA leading to decreased protein expression.

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