Free alpha-subunit and intact TSH secretion in vitro are closely associated in human somatotroph adenomas

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Abstract

Objective: GH-secreting pituitary adenomas frequently co-secrete prolactin and glycoprotein hormone alpha-subunit (αSU), but expression of additional hormones is considered unusual. The aim of this study was to establish the frequency with which acromegalic tumours secrete intact glycoprotein hormones LH, FSH and TSH, in comparison with other types of pituitary adenoma.

Design and methods: Pituitary tumours were studied by cell culture, measuring the basal secretion of anterior pituitary hormones in vitro. Light microscopy was used to exclude tumours where normal pituitary tissue was present, and immunocytochemistry was employed to confirm the clinical diagnosis and for comparison with tissue culture data.

Results: TSH secretion was observed in vitro in 15/23 somatotroph adenomas, but from only 1/8 lactotroph, 4/29 null cell, 2/12 gonadotroph and 1/10 corticotroph adenomas; moreover, somatotroph adenomas secreted the largest amounts of TSH (P < 0.001). Somatotroph adenomas also secreted LH (7/23) and FSH (2/23) but less frequently than gonadotroph adenomas. Immunocytochemistry demonstrated glycoprotein expression in somatotroph adenomas (LHβ: 13%, FSHβ: 26%, TSHβ: 30%, αSU: 46%) more frequently than in lactotroph, corticotroph and null cell adenomas. A strong correlation was found between αSU secretion and TSH secretion in somatotroph adenomas (rho = 0.683, P < 0.001).

Conclusions: TSHβ is frequently expressed by somatotroph adenomas, often associated with αSU expression. Both GH and TSHβ are dependent on the transcription factor, Pit-1, which is frequently expressed in somatotroph adenomas, although the expression of αSU requires an alternative explanation. Increased expression of αSU compared with TSHβ may account for the secretion of free αSU by somatotroph adenomas.

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Introduction

Human pituitary adenomas that cause acromegaly may co-express other pituitary hormones in addition to growth hormone (GH), and it has long been established that prolactin (PRL) hypersecretion occurs in vivo (1, 2) and in vitro (3, 4). In addition to PRL, secretion of glycoprotein hormone alpha-subunit (αSU) occurs in a proportion of acromegalic tumours as evidenced by in vivo studies (5, 6), immunocytochemistry (7) and in vitro tissue culture (8–10). Multiple hormone secretion by a proportion of acromegalic tumours has led to the description of a ‘plurihormonal’ tumour type, and in some instances such plurihormonal tumours have been shown by immunocytochemistry to express the β-subunits of glycoprotein hormones, most commonly that of thyroid-stimulating hormone (TSHβ), but also those of luteinising hormone (LHβ) and follicle-stimulating hormone (FSHβ) (7, 11).

Elevated in vivo levels of αSU have been demonstrated in 32% of patients with prolactinomas (12) and 64% of those with thyrotroph adenomas (13), and there is immunocytochemical evidence of α-subunit co-expression in corticotrophinomas (14, 15). Glycoprotein hormone secretion is known to occur in approximately 25% of clinically non-functioning pituitary adenomas (NFPAs) (16, 17). NFPAs themselves probably represent a heterogeneous group of tumours including null cell adenomas, oncocytomas, true gonadotrophinomas, and ‘silent’ somatotroph and corticotroph adenomas. Null cell adenomas and oncocytomas are distinguishable principally by electron microscopy, and have also been shown to contain immunoreactive intact glycoproteins or their α- and β-subunits, suggesting that they may also arise from gonadotroph cells (18, 19).

To date, no studies have compared quantitatively the in vitro secretion of intact glycoproteins between...
different tumour types, and few have matched *in vitro* secretion and immunocytochemistry. Using highly specific two-site IRMAs for intact glycoprotein heterodimers (LH, FSH, and TSH) and a sensitive and specific RIA for αSU, we have examined the *in vitro* secretion of intact LH, FSH, TSH and αSU from a large series of human pituitary tumours, comparing the levels of secretion between different tumour types. Here we report that a high proportion of tumours from acromegalic patients release intact glycoproteins in addition to αSU, most frequently TSH. *In vitro* TSH secretion occurred almost exclusively in acromegalic tumours, where it was strongly correlated with the secretion of αSU.

**Subjects and methods**

**Clinical details and patient selection**

Pituitary tumours were collected at the time of transsphenoidal adenomectomy. Tissues were divided at the time of surgery for diagnostic histological studies and for tissue culture. Acromegaly was diagnosed on the basis of persistently measurable GH and inadequate suppression on administration of an oral glucose load. Patients with corticotroph adenomas presented with typical clinical and biochemical features of cortisol excess, a pituitary source of adrenocorticotrophin (ACTH) hypersecretion was determined by magnetic resonance imaging and on the basis of inferior petrosal venous sampling; data are reported only from patients with macroadenomas. Those patients with presumed lactotroph macroadenomas represented a subset of patients with hyperprolactinaemia who had demonstrated a suboptimal response to treatment with dopamine agonist therapy, and who required debulking surgery prior to external beam radiotherapy. Patients who presented due to mass effect, without clinical features of anterior pituitary hormone excess, and whose serum PRL was less than 3500 mU/l, were classified initially as having clinically NFPAs. Preoperative serum free thyroxine (T4), total T4 and buffered with Hepes (0.02 mol/l), hereafter referred to as culture medium. Tumour tissue was dispersed enzymatically with trypsin as described previously (15). Dispersed cells were harvested by centrifugation, washed once and subsequently resuspended in culture medium. Cell viability was assessed using trypan blue exclusion and was more than 90% of cells in all of the tumours studied after cell dispersion. The yield from each tumour varied from 1 to 15 × 10^6 cells. The cells were plated in six-well plates at approximately 0.5–1 × 10^6 cells per well in 4 ml medium. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ for 48 h to allow cell attachment to occur, after which time the medium was collected and centrifuged to remove detached cells and debris prior to hormone assays. Approximately three to five individual cultures were obtained from each tumour.

**Hormone assays**

GH, PRL, LH, FSH and TSH were measured using two-site chemiluminescent enzyme immunometric assays on the Immulite autoanalyser (Euro/DPC Ltd, Gwynedd, UK). The inter- and intra-assay coefficients of variation for all these assays are less than 6 and 10% respectively.

NFPAs were subsequently classified as being gonadotrophinomas if they demonstrated strong immunocytochemical staining for LHβ and/or FSHβ, the remainder of NFPAs were classified as being null cell adenomas, including those that demonstrated positivity for αSU alone.

**Morphology and immunocytochemistry**

All tumours were examined by standard haematoxylin and eosin, reticulin and periodic acid Schiff stains and by routine immunostaining performed for GH, PRL, ACTH, TSHβ, LHβ, FSHβ (antibodies obtained from Biogenex Ltd, Berks, UK) and αSU (rabbit polyclonal, UCB Bioproducts, Braine-L’alleud, Belgium) using a standard streptavidin–biotin horseradish peroxidase method. The distinction between normal pituitary gland and tumour was confirmed by reticulin staining in all cases. Microwave pretreatment was used routinely for immunocytochemistry as previously described (15). The amount of positive staining in all tumours was initially assessed semi-quantitatively by means of a four point scale (−, negative; ±, scattered cells representing less than 10%; +, 10–50% positive; ++, >50% positive).

**Pituitary tumour cell culture**

Pituitary adenoma tissue was transported to the laboratory in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal calf serum, 0.06 g/l penicillin, 0.1 g/l streptomycin and 2.5 g/l fungizone (Gibco-BRL, Paisley, Strathclyde, UK) and buffered with Heps (0.02 mol/l), hereafter referred to as culture medium. Tumour tissue was dispersed enzymatically with trypsin as described previously (15). Dispersed cells were harvested by centrifugation, washed once and subsequently resuspended in culture medium. Cell viability was assessed using trypan blue exclusion and was more than 90% of cells in all of the tumours studied after cell dispersion. The yield from each tumour varied from 1 to 15 × 10^6 cells. The cells were plated in six-well plates at approximately 0.5–1 × 10^6 cells per well in 4 ml medium. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ for 48 h to allow cell attachment to occur, after which time the medium was collected and centrifuged to remove detached cells and debris prior to hormone assays. Approximately three to five individual cultures were obtained from each tumour.
ACTH was measured by a specific double antibody RIA (Euro/DPC Ltd) with inter- and intra-assay coefficients of variation of less than 10%. αSU concentrations were measured by a direct double antibody RIA using antibodies purchased from UCB Bioproducts (Brussels, Belgium), chloramine-T-iodinated antigen (National Institute for Biological Standards and Controls reagent 76/508, Potters Bar, Herts, UK) and calibrated against the first international reference preparation 75/569 (National Institute for Biological Standards and Controls). Inter- and intra-assay coefficients of variation were less than 6 and 11% respectively. Cross-reactivities (in ng per ng) with purified LH, FSH and TSH were 3.6, 1.9 and 1.3% respectively. The detection limits of the above assays, defined as the concentration two standard deviations above the response at zero dose, were as follows: GH: 0.5 mU/l, PRL: 10 mU/l, LH: 0.4 IU/l, FSH: 0.6 IU/l, TSH: 0.008 mU/l, ACTH: 4 pmol/l, αSU: 0.1 μg/l. All samples from each individual tumour were analysed in the same assay. Hormone data were initially obtained as concentrations but were then corrected for cell number and incubation time. The data presented are therefore expressed as amount of hormone secreted per 24 h per 10⁶ cells. The reported detection limits per 10⁶ cells per 24 h, after this normalisation, were as follows: GH: 2.0 μU, PRL: 50 μU, LH: 2.0 mU, FSH: 3.0 mU, TSH: 0.1 μU, ACTH: 20 fmol, αSU: 0.5 ng.

Statistical analysis

Data from in vitro hormone secretion did not conform to a normal distribution, and was not amenable to transformation given a high proportion of data points that fell below a detection threshold. Non-parametric analysis was therefore used throughout. For a given pituitary hormone, differences between adenoma types was determined in each case by the Kruskall–Wallis test, which tests the null hypothesis that all groups belong to the same population – a non-parametric equivalent to the F-statistic in analysis of variance. Differences between two groups was performed using the Mann–Whitney U test for continuous variables. Spearman rank correlation coefficients (ρ) were calculated to examine the correlations between the secretion of αSU and the other glycoprotein hormones. In all cases, P < 0.05 was considered a statistically significant difference. The above analyses were performed using Statview software (Abacus Concepts, Inc., Berkeley, CA, USA). Fisher’s exact test (STATXACT-Turbo, Cytel Software Corporation, Cambridge, MA, USA) was used to calculate exact two-tailed P values and odds ratios for the association between TSHomas and gonadotrophin immunohistochemical positivity.

Results

Pituitary tissue was obtained from a total of 137 patients. Following histological examination, 49 tumours were excluded because of the presence of normal pituitary, necrotic tissue, an alternative diagnosis, discrepancy or non-availability of immunocytochemical data. The final series of 90 fully characterised adenomas comprised 23 somatotroph adenomas, including those co-expressing PRL, 10 corticotroph macroadenomas, 8 lactotroph macroadenomas, 14 gonadotroph adenomas, 2 TSH-secreting adenomas (TSHomas) and 29 null cell adenomas.

In vitro data

Secretion of GH, PRL and ACTH from each dispersed adenoma is shown in Fig. 1. The pattern of secretion of ACTH, GH and PRL paralleled clinical and immunocytochemical diagnosis. GH secretion was observed at moderately high levels in 4/8 lactotroph adenomas in addition to the somatotroph adenomas. In the majority (87%) of corticotroph, null cell and gonadotroph adenomas GH was not detected. PRL was secreted by lactotroph adenomas and was also observed in adenomas from 70% of patients with acromegaly (Fig. 1).

Figure 2 shows the amounts of intact glycoprotein hormones and αSU secreted by each tumour type. Table 1 shows the median and range for each tumour type, all values represent the amount of hormone per 10⁶ cells per 24 h. The in vitro secretion (and immunocytochemistry) is also detailed in Table 2 for somatotroph adenomas and in Table 3 for gonadotroph adenomas. TSH secretion was observed most frequently (68%) and at much higher levels in somatotroph adenomas compared with other tumour types (P < 0.001), being detected in only 13% of the remaining tumours where concentrations were close to the detection limit. TSH secretion in two TSHomas was 7.8 and 48 μU, levels that are comparable to some of the somatotroph adenomas. Figure 2a demonstrates the marked preponderance of TSH secretion by somatotroph adenomas. LH (71%) and FSH (57%) were detected in the media most frequently and at the highest levels from gonadotroph adenomas. TSHomas was 7.8 and 48 μU, levels that are comparable to some of the somatotroph adenomas. Figure 2a demonstrates the marked preponderance of TSH secretion by somatotroph adenomas. LH (71%) and FSH (57%) were detected in the media most frequently and at the highest levels from gonadotroph adenomas (P < 0.01), whereas αSU was secreted by a wide variety of tumour types. It is interesting to note that somatotroph adenomas showed the highest median secretion rate of αSU (5.9 ng/10⁶ cells per 24 h, P < 0.001), 4-fold higher than in gonadotroph (median 1.4 ng) and 7-fold higher than in lactotroph (0.8 ng) adenomas, and more than 11-fold higher than in null cell and corticotroph adenomas (medians both <0.5 ng). αSU secretion by the two TSHomas in this series was 0.5 and 5.2 ng/10⁶ cells per 24 h.

Spearman rank correlation coefficients were calculated in order to demonstrate the interrelationships between the secretion of intact glycoproteins (LH, FSH, and TSH) and free αSU, and these are shown in Table 4. The strongest correlations are shown between αSU and LH and FSH in gonadotroph and null cell adenomas, and between αSU and TSH in somatotroph
adenomas. These correlation coefficients show that whilst gonadotroph adenomas secreted αSU in association with LH and FSH, somatotroph adenomas secreted αSU principally in association with intact TSH.

**Immunohistochemistry**

Immunohistochemical data for LH, FSH TSH, ACTH, GH and PRL were obtained for all tumours reported; immunostaining for αSU was carried out on 57/84 adenomas. Table 2 shows in detail the glycoprotein immunocytochemical data from 23 acromegalic tumours, Table 3 shows data from gonadotroph adenomas and Table 5 summarises the percentage of adenomas in each category in which >10% of the section was positive (i.e. ‘+’ or ‘++’, see Methods section). αSU was demonstrated in each tumour type, being most frequently identified in gonadotroph (82%) and somatotroph (46%) adenomas. Somatotroph adenomas were most frequently seen to stain positively for TSHβ (30%), and also showed positivity for FSHβ (26%) and LHβ (13%). Within the 23 somatotroph adenomas, 4 demonstrated strong TSHβ reactivity (‘++’), and 3 weaker reactivity (‘+’) (see Table 2). TSHβ was also

![Figure 1: Scattergrams illustrating the hormones secreted by each adenoma grouped according to the histological diagnosis. Each data point represents the amount of hormone per 10^6 cells per 24 h secreted by each adenoma. Detection limits are indicated by a dotted line for each hormone, numbers on the abscissa indicate the number of adenomas of each type in which that hormone was not detected. Note that the ordinate values are on a logarithmic scale for GH, PRL and ACTH. All P values represent the probability that all groups come from the same distribution (Kruskall–Wallis).](image)
observed in 3/14 gonadotroph adenomas, all ‘+’ (Table 3). β-Subunits were not identified by immunocytochemistry in any of the lactotroph or corticotroph adenomas.

When the in vitro TSH secretion was compared with the immunocytochemical data, as shown in Table 2, the amount of TSH secreted by somatotroph adenomas that were immunoreactive for TSHβ, either ‘++’ or ‘+’ (median 5.2, range 0.2–16.8 mU) was substantially higher than in those where TSHβ was either negative or seen in scattered cells alone (median <0.1, range <0.1–5.9 mU; P < 0.05, Mann–Whitney U). Thus the two modalities can be seen as broadly concordant, although in our system in vitro tissue culture was a more sensitive technique for the detection of TSH. These TSHβ-positive somatotroph adenomas also secreted more αSU (P < 0.05) but there was no difference in the in vitro secretion of other hormones. The amount of αSU secreted by tumours that were positive for αSU on immunocytochemistry (median 1.8 ng) was also

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**Figure 2** Scattergrams illustrating the hormones secreted by each adenoma included in the series, grouped according to the histological diagnosis. Each data point represents the amount of hormone per 10^6 cells per 24 h secreted by each adenoma. Detection limits are indicated by a dotted line for each hormone, numbers on the abscissa indicate the number of adenomas of each type in which that hormone was not detected. All P values represent the probability that all groups come from the same distribution (Kruskal–Wallis).
greater than in αSU negative tumours (median 0.6 ng; 
P < 0.05, Mann–Whitney U). It is noteworthy, as shown 
in Table 2, that those somatotroph adenomas that were 
positive for TSHβ stained positively for LH and/or FSH 
significantly more frequently than the remaining 
somatotroph adenomas (odds ratio = 90, P < 0.001).

Discussion

In this study we have shown that 68% of tumours from 
patients with acromegaly secreted TSH in addition to 
GH in vitro, and that the amount of TSH secreted 
by these somatotroph adenomas was substantially 
higher than by the other tumour types (P < 0.001). 
Immunocytochemical data from these tumours demon-
strated the highest incidence of TSHβ positivity in 
somatotroph adenomas (30%), and those adenomas 
immunoreactive for TSHβ secreted the highest quanti-
ties of intact TSH in vitro, demonstrating concordance 
between immunocytochemical and tissue culture data.

Although our patients did not show clinical evidence 
of hyperthyroidism, occasional patients with clinical 
thyrotoxicosis due to TSH hypersecretion and coexistent 
acromegaly have been described (20, 21). Only 3/24 of 
the somatotroph adenomas in this study secreted 
amounts of TSH in vitro (median 0.4 range <0.1– 
16.8 mU) that were similar to two thyrotroph adenomas 
(7.8–48 mU). Thus, in the majority of cases, it is likely 
that the co-secretion of TSH remained subclinical 
because the quantity of TSH was not sufficient to 
cause a clinical syndrome. Of the remaining three 
tumours which exhibited the highest levels of secretion
in vitro, each demonstrated TSHβ immunopositivity and it may be that in vivo TSH was synthesised but not released; alternatively, TSH secreted in vivo may not have been biologically active.

Subclinical TSH co-expression has been noted previously in GH-secreting adenomas, although its occurrence has been considered unusual. In vivo, GH-releasing hormone has been shown to cause a rise in TSHβ in 26% of acromegalic patients (22), whilst in vitro TSH co-secretion has been reported only infrequently (23, 24). Terada and co-workers reported 10 plurihormonal somatotroph adenomas that we have observed has several possible explanations. First, we have used a somatotroph adenomas which will have increased the sensitivity of our study.

**Table 3.** Immunocytochemistry and in vitro hormone secretion by gonadotroph adenomas G1–G14. All secretion data refer to amount per 10⁶ cells per 24 h.

<table>
<thead>
<tr>
<th>Tumour number</th>
<th>Immunocytochemistry</th>
<th>In vitro secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LHβ</td>
<td>FSHβ</td>
</tr>
<tr>
<td>G1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>G4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G6</td>
<td>+</td>
<td>+</td>
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<td>G7</td>
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<td>G10</td>
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<td>G11</td>
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<td>+</td>
</tr>
<tr>
<td>G13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G14</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+++*: >50% positive; +*: 10–50% positive; +/−*: <10% positive; ‘−’: negative; nd: not done.

**Table 4.** Spearman rank correlation coefficients (rho) between αSU and the glycoprotein hormones for each tumour histological type.

<table>
<thead>
<tr>
<th>Adenoma type</th>
<th>αSU vs LH</th>
<th>αSU vs FSH</th>
<th>αSU vs TSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatotroph</td>
<td>0.416</td>
<td>−0.119</td>
<td>0.683***</td>
</tr>
<tr>
<td>Lactotroph</td>
<td>0.466</td>
<td>0.415</td>
<td>0.415</td>
</tr>
<tr>
<td>Corticotroph</td>
<td>0.433</td>
<td>N/A</td>
<td>0.309</td>
</tr>
<tr>
<td>Null cell</td>
<td>0.642***</td>
<td>0.546**</td>
<td>0.101</td>
</tr>
<tr>
<td>Gonadotroph</td>
<td>0.659*</td>
<td>0.777**</td>
<td>−0.123</td>
</tr>
</tbody>
</table>

*** p < 0.001; ** p < 0.01; * p < 0.05; N/A not appropriate.

**Table 5.** Percentage of adenomas with positive immunocytochemistry for each anterior pituitary hormone (>10% of cells positive).

<table>
<thead>
<tr>
<th>Adenoma type</th>
<th>LHβ</th>
<th>FSHβ</th>
<th>TSHβ</th>
<th>αSU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatotroph</td>
<td>13</td>
<td>26</td>
<td>30</td>
<td>46a</td>
</tr>
<tr>
<td>Lactotroph</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20a</td>
</tr>
<tr>
<td>Corticotroph</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17a</td>
</tr>
<tr>
<td>Null cell</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9a</td>
</tr>
<tr>
<td>Gonadotroph</td>
<td>93</td>
<td>93</td>
<td>21</td>
<td>82a</td>
</tr>
</tbody>
</table>

a n = 13; b n = 5; c n = 6; d n = 22; e n = 11.
and TSHβ genes (29, 30), although a recent study has demonstrated that these Pit-1 isoforms were similarly expressed in prolactinomas, GH-secreting adenomas and TSHomas (31). That somatotroph adenomas should express TSHβ, as shown in this study, is therefore to be anticipated; conversely, TSH-secreting pituitary adenomas frequently immunostain for GH and PRL (13).

In the somatotroph adenomas we demonstrated a strong correlation between secretion of intact TSH and αSU (ρ = 0.683, P < 0.001). However, shared activation by Pit-1 cannot account for the co-ordinated expression of both α- and β-subunits of TSH that we have demonstrated, as Pit-1 itself does not have a role in the expression of the αSU gene (32). Recent studies have shown that Ilx3, a homeobox gene expressed at a very early stage in pituitary ontogeny, is involved in the activation of both Pit-1 and αSU genes (33). The expression of early differentiation factors by pituitary adenomas undoubtedly warrants further study.

Amongst somatotroph adenomas, in vitro αSU secretion rarely occurred in isolation, being almost always accompanied by synthesis and/or secretion of TSHβ. However, as has been documented in TSHomas (13), αSU was synthesised in molar excess compared with the TSH β-subunit, which could account for the well described in vivo and in vitro co-secretion of free αSU by somatotroph adenomas.

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References


