Production of alpha-subunit of glycoprotein hormones by pituitary somatotroph adenomas in vitro

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Somatotroph adenomas of the pituitary secrete growth hormone in excess and are associated with acromegaly. Morphologically, they can be separated into two entities, densely and sparsely granulated variants. It has been shown that a number of somatotroph adenomas produce \( \alpha \)-subunit of glycoprotein hormones; however, it is not clear whether \( \alpha \)-subunit production correlates with tumor cell morphology. We studied 32 surgically removed pituitary somatotroph adenomas in tissue culture to determine structure-function correlations of growth hormone and \( \alpha \)-subunit production. All tumors were classified on the basis of detailed histological, immunocytochemical and electron-microscopic studies. Fifteen tumors were densely granulated and 17 were sparsely granulated. In addition to growth hormone, all 15 densely granulated tumors released \( \alpha \)-subunit in vitro, whereas of the 17 sparsely granulated tumors only 4 released \( \alpha \)-subunit; moreover, the mean baseline levels of \( \alpha \)-subunit were significantly higher in densely granulated adenomas than in sparsely granulated adenomas. Parallel response of release of both hormones was found during stimulation with growth hormone-releasing hormone or thyrotropin-releasing hormone and during suppression with somatostatin or bromocriptine in densely granulated tumors. \( \alpha \)-subunit response to stimulation or suppression could not be determined with significance in sparsely granulated tumors because of low basal levels. The results indicate that \( \alpha \)-subunit production and release is characteristic of densely granulated somatotroph adenomas and that \( \alpha \)-subunit is coregulated with growth hormone by adenohypophysiotropic substances; in contrast, \( \alpha \)-subunit production by sparsely granulated somatotroph adenomas is rare and, when present, much lower in quantity. Our studies confirm that densely and sparsely granulated somatotroph adenomas represent separate entities.

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Clinical studies have documented elevated blood levels of \( \alpha \)-subunit of glycoprotein hormones (\( \alpha \)-SU) as well as growth hormone (GH) in a subset of acromegalic patients (1, 2). Some have shown a parallel response of GH and \( \alpha \)-SU secretion during stimulation with GRH-releasing hormone (GRH) or thyrotropin-releasing hormone (TRH) or suppression by somatostatin (SRIH) or bromocriptine (1–4).

Morphologic studies of tumors in patients with acromegaly have revealed that monomorphic somatotroph adenomas represent the largest group; by electron-microscopy they are separated into two structurally distinct types: densely granulated and sparsely granulated somatotroph adenomas (5). In addition to GH immunoreactivity, somatotroph adenomas often contain variable numbers of cells immunopositive for \( \alpha \)-SU (6–9); electron-microscopy occasionally discloses focal lactotroph or mammosomatotroph differentiation but provides no evidence of glycoprotein differentiation (5, 9). Coexistence of GH and \( \alpha \)-SU in the same cell and colocalization in the same secretory granule have been demonstrated by immunoelectron-microscopy (1, 10).

Release of \( \alpha \)-SU in association with GH has been documented in vitro (1, 4, 11–13). Some investigators have reported response of GH and \( \alpha \)-SU to GRH or TRH stimulation and SRIH or bromocriptine suppression in culture (1, 4, 11–14). However, to our knowledge, the morphologic features of GH-secreting adenomas with \( \alpha \)-SU release in vitro have only been analyzed in a group of plurihormonal adenomas of the acidophil cell line (13).

To clarify structure–function correlations in somatotroph adenomas, we evaluated \( \alpha \)-SU release by densely and sparsely granulated somatotroph adenomas and its response to GRH, TRH, SRIH and bromocriptine in vitro.
and correlated the findings with the histological, immunocytochemical and ultrastructural features in a large series of tumors.

Materials and methods

Patients

In a series of 380 surgically removed pituitary adenomas, 32 were morphologically classified as somatotroph adenomas; the remainder of acromegalic patients had tumors with features of mammosomatotroph/mixed somatotroph-lactotroph (24 cases) or unclassified plurihormonal adenomas (4 cases). Fifteen were classified as densely granulated and 17 as sparsely granulated somatotroph adenomas. All patients presented with active acromegaly and all had elevated serum GH levels preoperatively; serum z-SU levels were available only in 4 cases. Eighteen patients were males and 14 were females. Eleven males and 4 females had densely granulated adenomas (mean age ± SEM: 44 ± 2.5 years); 7 male and 10 female patients had sparsely granulated tumors (mean age ± SEM: 46 ± 3.2 years). No significant sex and age differences were noted. The grade of adenomas, in terms of their size, was estimated according to Hardy’s method (15).

Morphologic methods

For light microscopy, surgically resected tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections 4–6 μm thick were stained with hematoxylin and eosin (H&E) and the periodic acid-Schiff (PAS) technique. For immunocytochemistry, the avidin-biotin-peroxidase complex technique was employed. Primary antisera were directed against the following pituitary hormones or subunits of glycoprotein hormones: GH (DAKO, Santa Barbara, CA); PRL (donated by Dr H Friesen, Winnipeg, Manitoba); adrenocorticotrophic hormone (ACTH 1–39) (donated by Dr S Raiti, NIDDK, Bethesda, MD); β-follicle stimulating hormone (β-FSH) and β-luteinizing hormone (β-LH) (both donated by Dr S Raiti, NIDDK, Bethesda, MD); primary monoclonal antibodies were used to detect β-thyroid stimulating hormone (β-TSH) (Chemicon Inc., El Segundo, CA) and α-SU (Biogenex Laboratories, Dublin, CA). Working dilutions of primary antibodies and antisera ranged from 1:200 to 1:2000. The specificity of immunostaining was tested using appropriate positive and negative controls.

For electron-microscopy, surgically resected tissue specimens were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate were studied using a Philips 300 or 410-LS electron-microscope.

Culture methods

For tissue culture, fresh tissue specimens were collected under sterile conditions in a solution of collagenase in medium (CMRL-1969 Connaught; Willowdale, Ontario, Canada) as described previously (16). Dispersed cells were counted, suspended in CMRL-1969 supplemented with 10% fetal calf serum (Gibco; Grand Island, NY) and plated in plastic multiwell culture dishes (Linbro, Flow Laboratories Inc; McLean, VI) coated with collagen or with Millicell-CM inserts (Millipore; Belford, MA) at a concentration of 2–8 × 10⁴ cells/500 μl/well. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Basal hormone release was estimated in media collected after 24, 48 and 72 h.

After measuring basal hormone levels, cells were incubated with the following substances for two consecutive 2 and 24 h periods: GRH (1–40 NH₃) and SRIH at a concentration of 8.5 × 10⁻⁷ mol/l and 5 × 10⁻⁷ mol/l respectively (both donated by Dr W Vale, Salk Institute, San Diego, CA); TRH at a concentration of 2 × 10⁻⁷ mol/l (Hoechst Canada, Montreal, Quebec) and bromocriptine at a concentration of 600 ng/ml (donated by Sandoz Pharmaceuticals, Dorval, Quebec). The experiments and controls were performed in duplicate or triplicate; control dishes received vehicle alone. Sufficient wells for experiments with GRH, TRH, SRIH and bromocriptine were available for 15, 9, 5 and 5 tumors, respectively.

To characterize the population of cells studied, at the end of each experiment cells were trypsinized (Worthington, Freehold, NJ), harvested, washed and collected into pellets by centrifugation. Pellets were appropriately fixed and embedded for electron-microscopy as described above.

Collected media were stored in polyethylene vials at −20°C until processed for radioimmunoassay.

Hormone assays

For measurement of culture media hormone content, the standard double antibody radioimmunoassay technique was employed to detect GH, PRL, ACTH, α-SU, TSH, FSH and LH as described previously (16). Antibodies directed against GH were obtained from Bio Ria, Montreal, Quebec and those against α-SU were donated by Dr S Raiti, NIDDK, Bethesda, MD. All hormone data were expressed as amount per 24 h per 2 × 10⁴ cells.

Statistics

To determine if there were differences between the two groups in the immunocytochemical reactions, the Cochran–Mantel–Haenszel mean score test was used. To simplify the analysis, reactions were also classified as positive or negative and a chi-squared test computed. Comparisons of hormone release in vitro by the two
tumor types utilized the non-parametric Kruskal-Wallis test.

Results

Morphologic findings

By light microscopy the densely granulated tumors were predominantly acidophilic, whereas the sparsely granulated tumors were composed of chromophobic cells. Many cells of the latter contained pale cytoplasmic fibrous bodies. All tumors were PAS negative.

Immunocytochemistry revealed the presence of GH in all adenomas. The immunoreactivity in densely granulated tumors was primarily diffuse throughout the cytoplasm of many cells (Fig. 1a), whereas it was often juxtanuclear and less intense in the sparsely granulated adenoma cells (Fig. 2a). Immunocytochemical studies to localize α-SU were not performed in two densely and one sparsely granulated adenomas (Tables 1 and 2). In addition to the immunopositivity for GH, 12 of 13

Fig. 1. (a) Immunocytochemistry localizes GH throughout the cytoplasm of the majority of tumor cells in a densely granulated somatotroph adenoma. (b) A large number of tumor cells in the same lesion are strongly positive for α-SU. (Avidin-biotin peroxidase complex; magnification ×110.)

Fig. 2. (a) Immunocytochemistry localizes GH focally and in a juxtanuclear location in a sparsely granulated somatotroph adenoma. (b) Only a small number of tumor cells in the same lesion contain immunoreactivity for α-SU and the staining is weak. (Avidin-biotin peroxidase complex; magnification ×110.)
Table 1. Clinical features, biochemical data, in vitro, immunocytochemical and ultrastructural findings of densely granulated somatotroph adenomas.

<table>
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<tr>
<th>Patient</th>
<th>Tumor grade (Hardy's)</th>
<th>Serum hormone levels*</th>
<th>Hormone release in vitro</th>
<th>Immunocytochemistry</th>
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Mean ± SE (ng/24 h/2 × 10⁴ cells) 7569 ± 2400 198 ± 108

*ng/ml; SC somatomedin C; MSM mammosomatotroph differentiation; LTR lactotroph differentiation; 3+ many; 2+ few; + scattered occasional (<5%) cells; − negative; blank—data not available.

Table 2. Clinical features, biochemical data, in vitro, immunocytochemical and ultrastructural findings of sparsely granulated somatotroph adenomas.

<table>
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<th>Serum hormone levels*</th>
<th>Hormone release in vitro</th>
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<td>M-68</td>
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<td>23</td>
<td>77</td>
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Mean ± SE (ng/24 h/2 × 10⁴ cells) 1217 ± 498 9 ± 7

*ng/ml; SC somatomedin C; MSM mammosomatotroph differentiation; LTR lactotroph differentiation; 3+ many; 2+ few; + scattered occasional (<5%) cells; − negative; blank—data not available.

densely granulated and 7 of 16 sparsely granulated adenomas exhibited varying numbers of cells immuno-positive for α-SU. Immunoreactivity for α-SU was usually more extensive in densely than in sparsely granulated adenomas (Figs. 1b, 2b). Overall, 12 tumors contained more than 5% positive cells; these were 8 densely granulated and 4 sparsely granulated adenomas. The mean scores of positivity were significantly different in the two groups with p = 0.010. When tumors were classified as either positive or negative for α-SU, a summary statistic had a p-value of 0.007. In 7 densely granulated and 4 sparsely granulated adenomas, occa-
Fig. 3. Electron-micrograph of a densely granulated somatotroph adenoma showing numerous electron-dense secretory granules within the cytoplasm. (Magnification × 6,900.)

Fig. 4. Electron-micrograph of a sparsely granulated somatotroph adenoma with few secretory granules and conspicuous juxtanuclear fibrous bodies. (Magnification × 8,400.)
sional cells contained β-subunits of TSH, FSH or LH, but in all instances with the exception of one sparsely granulated adenoma which, interestingly, did not exhibit α-SU staining, the positivity involved less than 1% of cells in the specimen.

By electron-microscopy, cells of densely granulated adenomas were polyhedral with round, euchromatic nuclei and contained well-developed rough endoplasmic reticulum and extensive Golgi apparatus. The secretory granules measured approximately 250–500 nm (Fig. 3). The cells of sparsely granulated adenomas had characteristic morphology: they harbored irregularly ovoid nuclei with conspicuous nucleoli, well developed membranous cytoplasmic organelles and fibrous bodies consisting of spherically arranged filaments. The secretory granules were sparse, unevenly distributed and small, measuring 100–250 nm (Fig. 4).

Three tumors (1 densely and 2 sparsely granulated) included occasional adenoma cells exhibiting features of mammosomatotroph differentiation containing larger secretory granules and showing secretory granule extrusions. One of these sparsely granulated adenomas presented additional signs of focal lactotroph differentiation: there were small, sparse secretory granules and secretory granule exocytosis. In another densely granulated tumor, similar signs of lactotroph differentiation were focally observed. One densely granulated adenoma was admixed with a lesser component of sparsely granulated cells.

In vitro findings
All tumors released GH in tissue culture (Tables 1 and 2). The GH release showed considerable variations and there was a marked overlap in baseline levels among tumors of the two types. Overall, basal GH release by densely granulated adenomas was greater than by sparsely granulated adenomas, and this difference was statistically significant (p = 0.0024).

All densely granulated adenomas released variable quantities of α-SU. It was detected in much lower amounts in media of only 4 of 17 sparsely granulated adenomas (1.15, 1.96, 2.15 and 31.2 ng/2 × 10⁴ cells/24 h). Even considering this small number of cases in the sparsely granulated adenomas, the difference in α-SU release between the two groups was statistically significant (p = 0.0455). Release of GH and α-SU was examined during incubation with GRH, TRH, SRH and bromocriptine. The values were expressed as a percentage of levels released by untreated control wells of the same tumor. All densely granulated adenomas showed a parallel and almost equal response of GH and α-SU to both stimulation and suppression (Fig. 5). The effect of GRH on GH and α-SU was more prominent than that of TRH, whereas the effect of SRH was comparable to that of bromocriptine.

While GH release by densely granulated adenomas responded to these compounds, changes in α-SU release could not be evaluated in 3 of 4 sparsely granulated adenomas owing to very low basal levels.

Other pituitary hormones, including PRL, TSH, FSH and LH, were detected at low levels in the initial culture media of 10 densely and 5 sparsely granulated adenomas; in all but 2 densely granulated adenomas, the levels of these hormones fell rapidly and they were undetectable after less than one week in culture.

Electron-microscopic studies of cultured cells yielded results comparable to those of the initial surgical specimens. No non-tumorous elements were identified to account for the persistent α-SU release.

Discussion
From the clinical point of view, the two variants of somatotroph adenomas exhibit some differences. It appears that densely granulated tumors may represent the group of somatotrophs which contain the Gₛ protein mutations and exhibit high adenylate cyclase activity (17). Sparsely granulated adenomas in comparison to densely granulated adenomas tend to occur in younger patients, have a faster growth rate, often become large, produce visual abnormalities, are difficult to operate and have a higher rate of recurrence after surgical excision (5, 18). The reason for the marked similarity in age in our study is unclear.

Elevated serum α-SU levels have been reported in 9–28% of acromegalic patients (1, 3, 4) and were found in up to 37% of acromegaly patients in a recent study employing a sensitive monoclonal antibody radioimmunoassay (2). These data are comparable with the results of immunocytochemical studies, wherein a frequency as high as 55% of tumors contained α-SU in addition to GH immunoreactivity (6–9).

Previous in vitro studies based on limited numbers of tumors from patients with active acromegaly proved α-
SU release in culture media (1, 4, 11–13). White et al. (11) analyzed 32 adenomas; 22 were found to release various amounts of α-SU, but the detected quantities of α-SU in 10 tumors were very small. In another similar series of 28 adenomas, 11 tumors showed a close correlation between GH and α-SU secretion (12). These reports did not separate densely from sparsely granulated adenomas, since no morphologic investigations were reported.

Our work provides evidence that the amounts of α-SU release in culture media are greater and more consistent in densely than in sparsely granulated somatotroph adenomas; thus, they correlate with the higher incidence of immunopositivity and the greater numbers of α-SU positive cells in densely than in sparsely granulated tumors. These data correlate well with the recent paper reporting higher α-subunit levels in somatotroph tumors with G_i mutations (19).

Immunopositivity for α-SU in more than 5% positive cells was detected in 12 of 29 tumors (45%); it was demonstrated in 8 of 13 densely granulated adenomas (62%) and 4 of 16 sparsely granulated adenomas (25%). Lack of immunoreactivity can be due to antigen alteration or loss during tissue fixation and processing. This may explain the discrepant findings in densely granulated adenomas and tissue culture may be more useful in some instances to detect α-SU release by these adenomas. Similarly, focal α-SU immunoreactivity in scattered cells detected by immunocytochemistry may reflect trapped non-tumorous elements; this may explain the greater incidence of α-SU immunoreactivity in sparsely granulated adenomas by immunocytochemistry than in tissue culture. In addition, tissue sampling may reflect the features of a small area only. Also, GH and α-SU identified by immunocytochemistry may reflect storage. Lack of immunoreactivity in sparsely granulated adenomas may be due simply to reduced storage just as GH immunoreactivity is lower. Thus, to detect α-SU in culture media of sparsely granulated adenomas confirms lack of secretion by these tumors.

All adenomas were associated with GH release in culture, however, GH release was significantly greater in densely than in sparsely granulated tumors. These findings were not clearly evident in our previous studies of somatotroph adenomas, although the heterogeneity of these tumors was emphasized (20, 21). Given the wide range of GH baseline levels in densely and sparsely granulated tumors, the difference in GH release between the two variants requires cautious interpretation. It is known that there are no differences between serum levels of patients with densely and sparsely granulated adenomas (5, 18). Nevertheless, it has been shown that GH mRNA is more abundant in densely than in sparsely granulated adenomas (22), a feature which is consistent with our findings.

Cosecretion of GH and α-SU and similar parallel response to various adrenohypophysiotropic factors represent an additional distinguishing feature between densely and sparsely granulated somatotroph adenomas. This provides further evidence for cosecretion by a single population of tumor cells; if α-SU had been released by non-tumorous glycoprotein producing cells, it would not have been expected to respond in this fashion. A similar pattern of GH and α-SU release has also been observed in mammosomatotroph and mixed somatotroph–lactotroph tumors (13). A similar response or absence of response of GH and α-SU to both stimulation and suppression by four adrenohypophysiotropic substances (GRH, TRH, SRH and bromocriptine) was reported by Hofland et al. (12).

Our data indicate that densely and sparsely granulated adenomas differ in hormone release in vitro and predict similarities between densely granulated and mammosomatotroph adenomas (13). It is known that somatotroph adenomas can be heterogeneous; classification is based on the predominating cell type (5). In our series, three tumors exhibited signs of mammosomatotroph differentiation and two had focal lactotroph differentiation by electron-microscopy. No ultrastructural features of glycoprotein hormone-producing cells were observed to explain α-SU release.

Even though GH and α-SU represent structurally different molecules, normally originating from ostensibly distinct adrenohypophysial cell lines, they may both be released by adenomas of acromegalic patients. Interestingly, α-SU is also known to be localized in non-tumorous somatotrophs (23, 24). It has been shown that the transcription factor Pit-1 is required for activation of GH and PRL genes (25, 26) and this factor is also localized in thyrotroph cells. Pit-1 expression may play a role in the regulation of α-SU production by somatotroph adenomas (9, 13). However, it is conceivable that additional factors may be implicated in cytodifferentiation that would explain the expression of α-SU by GH producing cells.

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