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Case report

Somatostatin cells in human somatotropic adenomas*

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Summary. Data from our group have shown that the human adenomatous and normal anterior pituitary may be the source of somatostatin (SRIH). SRIH-producing cells were identified in two somatotropic adenomas. Immunoreactive SRIH cells were present in both cases. In case 2, material was available for RNA studies, in situ hybridization and electron microscopy. The size of the transcript identified by Northern blot analysis was identical to that of hypothalamic SRIH mRNA. In situ hybridization showed that the SRIH gene was expressed in a cell subset superimposable to that identified by immunocytochemistry. Co-localization studies revealed that SRIH and growth hormone (GH) immunoreactivities were not present in the same cells. Ultrastructural immunogold labelling showed that SRIH cells had features distinct from those of the somatotropes. The results confirm that the somatotropic adenomas have the ability to synthesize SRIH, indicate that SRIH expression is restricted to a subset of adenoma cells different from GH-producing cells, and imply that SRIH cells are involved in paracrine regulation of neighbouring somatotropes.

Key words: Somatostatin – Adenohypophysis – Somatotropic adenoma – Paracrine control

Introduction

In addition to the hypothalamus and the gastroenteropancreatic diffuse endocrine system, somatostatin (SRIH) gene expression is widely distributed in the body. It occurs in the cerebral cortex, sensory neurons, thyroid C-cells, the adrenal medulla and tumours arising from these tissues (Wass 1989).

A consistent body of evidence from our group has suggested that the normal and neoplastic human anterior pituitary gland is the unforeseen site of SRIH synthesis. Pre-pro-SRIH mRNA has been detected in human normal anterior pituitaries and secreting or non-secreting pituitary adenomas (Pagesy et al. 1989). Substantial amounts of high-molecular-weight forms of SRIH-like immunoreactivity (SLI) present in a somatotropic adenoma have been identified as pro-SRIH (Levy et al. 1991). Lastly, both human normal pituitaries and somatotropic adenomas release large amounts of SRIH-28 in vitro in a perifusion system (Joubert-Bression et al. 1989).

Hypothalamic SRIH is the physiological inhibitor of growth hormone (GH) secretion. Treatment with long-acting SRIH analogues causes GH plasma level reduction in 80–90% of acromegalic patients and tumour shrinkage in half of them (Frohman 1991). SRIH production by the adenoma itself may thus be of potential importance in intra-adenomatous regulatory mechanisms of GH secretion and tumour growth.

Morphological evidence of the cells that synthesize SRIH in somatotropic adenomas is lacking. Their characterization might be indicative of the type of local interaction in which SRIH is involved. The aim of the present study was to identify the cells responsible for the synthesis of SRIH in somatotropic adenomas.

Case reports

Case 1, a 41-year-old man, consulted for joint complaints. Examination disclosed a typical acral dysmorphia. Visual fields were normal. Elevated basal GH plasma levels were found on two occasions at 22 and 29 μg/l (normal values <5 μg/l). Insulin-like growth factor-I (IGF-I) plasma level was increased (3.80 units/ml; normal values <1.41 units/ml). Other laboratory data were in the normal range. Enlargement of the sella turcica was seen on skull radiography and CT scan showed a sellar mass, 20 mm in diameter, surmounted by a discrete suprasellar extension. Ablation of the tumour was performed by the trans-sphenoidal approach.

Case 2, a 35-year-old man, had characteristic features of acral and facial outgrowth. He complained of rheumatic pains and suf-

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fered from bilateral carpal tunnel syndrome. There were no visual disturbances. On radiological examination, the sella turcica was enlarged. CT scan revealed a pituitary mass, 15–17 mm in diameter, mostly intrasellar with a discrete suprasellar extension. Basal GH plasma level was 39 μg/l. IGF-I plasma level was high (2.50 units/ml). Attempts were made, over an 8-month period, to cure the patient by medical treatments. At first, he received somatuline (BIM 23014, Ipsen International, Paris), a long-acting octapeptide somatostatin analogue (Sassolas et al. 1989). Then somatuline was associated with the dopamine agonist bromocriptine. This treatment resulted in only partial clinical improvement and his plasma GH and IGF-I levels were decreased but not normalized. The patient underwent surgery 2 weeks after discontinuation of the treatment and the sellar mass was resected by the trans-sphenoidal route.

Materials and methods

Tumour fragments obtained in the operating room were fixed for light microscopy, immunocytochemistry and in situ hybridization. Other fragments were immediately frozen in liquid nitrogen until use for hormonal tissue content and RNA studies.

For light microscopy, specimens were fixed in Gérard's fluid consisting of a mixture of Bouin-Hollande fixative and saturated aqueous mercuric chloride (9:1, v/v), embedded in Paraplast and stained using Herlant's tetrachrome (Herlant 1960).

Indirect immunoperoxidase (Mazzuca and Dubois 1974) was performed with the following antisera, raised in rabbits, which were used at the specified dilutions: anti-hGH (batch 5976) 1:2000, anti-SRIH-14 (batch 19608) 1:2000. The two antisera were donated by Dr. Y. Tillet (Nouzilly, France).

For electron microscopy, small tissue fragments from case 2 were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon 812. Light microscopic immunocytochemistry, using the same antisera listed above, was performed on serial 1-μm-thick sections after removal of the epoxy resin by alcoholic sodium hydroxide (Lane and Europa 1965) and bleaching of the sections with saturated aqueous sodium metaperiodate (Bendayan and Zollinger 1983). Immunoreactive (IR) SRIH was localized by post-embedding ultrastructural immunogold labelling (De Mey 1983). Ultrathin sections were etched with sodium metaperiodate (Bendayan and Zollinger 1983) and incubated with the anti-SRIH-14 antiserum at 1:1000 or 1:2000 dilutions. The antigen-antibody complexes were visualized using immunogold conjugates (Amersham, Les Ulis, France). The sections were contrasted with uranyl acetate and lead citrate and then examined with a Siemens Elmiskop 1 electron microscope.

Immunostaining specificity was checked by replacing the antisera by non-immune rabbit serum or by the specific antisera absorbed with an excess of purified or synthetic homologous antigens (200 nmol of hGH and 250–1000 nmol of SRIH-14 per ml of pure antiserum).

In situ hybridization of SRIH mRNA was performed with the same SRIH oligonucleotide probe that was used for Northern blot analysis (see below). Adenoma tissue from case 2 was fixed in 4% paraformaldehyde and 0.5% glutaraldehyde and embedded in Paraplast. Sections were mounted on gelatine-chrome alum-subbed slides. The embedding medium was removed with xylene and the sections rehydrated. In situ hybridization was carried out according to protocol of Bloch et al. (1986). To demonstrate the specificity of the signal, a 20-fold excess of non-labelled probe was added to the labelled probe at the hybridization step.

Total RNA was extracted by the guanidine isothiocyanate caesium chloride procedure (Chirgwin et al. 1979) from the tumour of case 2 and from two autopsy specimens, one human hypothalamus and fragments of human muscle. A 45 base SRIH oligonucleotide probe was ^{32}P-labelled at its 3′-end and used in Northern blot analysis as described previously (Thomas 1980).

Results

The two adenomas consisted of a diffuse, monotonous population of polygonal cells with round nuclei and prominent nucleoli. Clusters of cells with orangeophilic cytoplasm were seen in case 2. Mitoses were scarce. Arterioles were observed in the two tumours.

In paraffin sections, most of the cells in the two tumours appeared to be adenomatous somatotropes (Figs. 1a, 2a). Cells immunoreactive with the anti-SRIH-14 antiserum were found in both cases. They were few but strongly immunostained (Figs. 1b, 2b–e). The SRIH staining pattern slightly differed in the two cases. In case 1, SRIH-IR cells were polygonal and mainly gathered in an area of the tumour specimen (Fig. 1b). In case 2, the cells, single or in small groups, were randomly interspersed between the somatotropic cells (Fig. 2b–e). In this latter case, the cells displayed various shapes. Some presented slender processes (Fig. 2b) which, at times, seemed to surround adjacent adenoma cells (Fig. 2c). Multinucleated SRIH-IR cells were also observed (Fig. 2d).

In case 2, light microscopic immunocytochemistry was performed on Epon-embedded tissue samples. Adjacent sections were serially immunostained for GH and

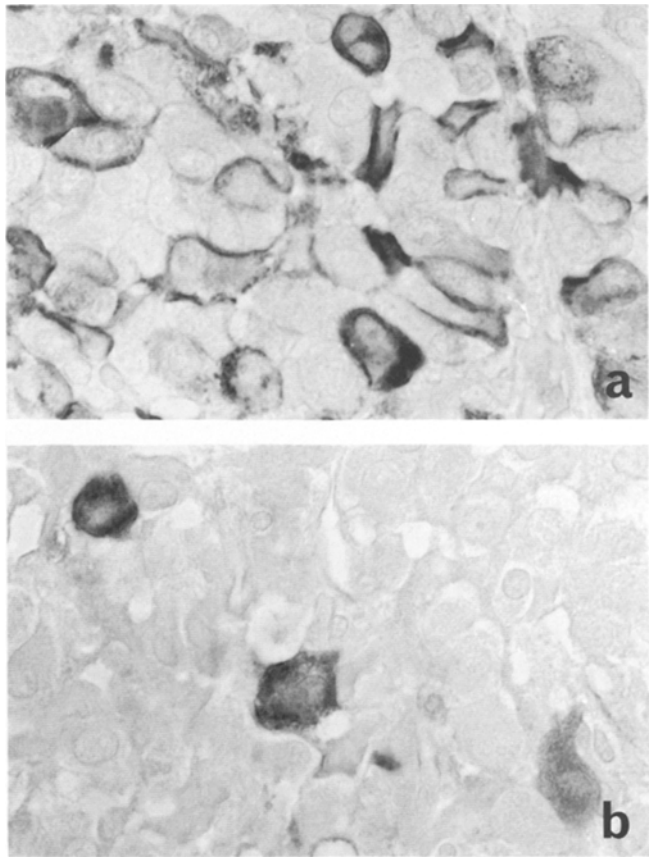

Fig. 1a, b. Somatotropic adenoma of case 1. Paraffin sections, immunoperoxidase, ×700. **a** The majority of adenoma cells are somatotropes (anti-GH antiserum). **b** A few polygonal somatostatin cells are observed in the tumour (anti-SRIH-14 antiserum)

SRIH. The comparison of homologous fields of serial 1-μm-thick sections showed that SRIH-IR cells were distinct from GH-producing cells (Fig. 3a, b).

The ultrastructure of the SRIH-IR cells in case 2 was analysed by immunogold labelling (Fig. 4a, b). In the cytoplasm of the immunolabelled cells, the rough endoplasmic reticulum was organized in stacks of short saccules. The Golgi complexes were not very prominent. Gold particles gathered on the sections of secretory granules that varied in size and electron density. Some SRIH-IR cells contained numerous and relatively small granules (150–250 nm in diameter), all of which had an electron-dense core. In other SRIH-IR cells (Fig. 4a), the secretory granules were larger (200–350 nm in diameter). The texture and the electron density of the granule content varied from one granule to another. Some granules had a homogeneous and highly electron-dense core. This type of granule was seen in association with the Golgi apparatus (Fig. 4b). The other granules had a lucent and fibrillar content. Regardless of their morphology, all the granules were equally immunoreactive to the anti-SRIH antiserum. Lysosomes were commonly seen in SRIH-IR cells.

The remainder of the tumoural parenchyma consisted mainly of adenomatous somatotropes. Their rough endoplasmic reticulum had a typical lamellar organization. The Golgi complexes were prominent. The secretory granules, 250–500 nm in diameter, had a homogeneous electron-dense content. Small bundles of microfilaments and giant mitochondria were frequently seen in these cells. Spaces between the adenomatous secretory cells were occupied by numerous interdigitating cell processes. These processes resembled those commonly seen in somatotropic adenomas and were not remarkable except for very rare but typical synapse-like structures which occurred between two adjacent processes and in which a presynaptic compartment, a postsynaptic compartment and a synaptic cleft could be clearly individualized (Fig. 5). The small clear vesicles that were present in the presynaptic element were not stained by the anti-SRIH-14 antiserum.

By applying the same probe used in Northern blot analysis to sections of the tumour of case 2, dense collections of silver grains were observed over cells scattered throughout the adenoma tissue (Fig. 6a). Treatment of sections with an excess of non-radioactive probe at the

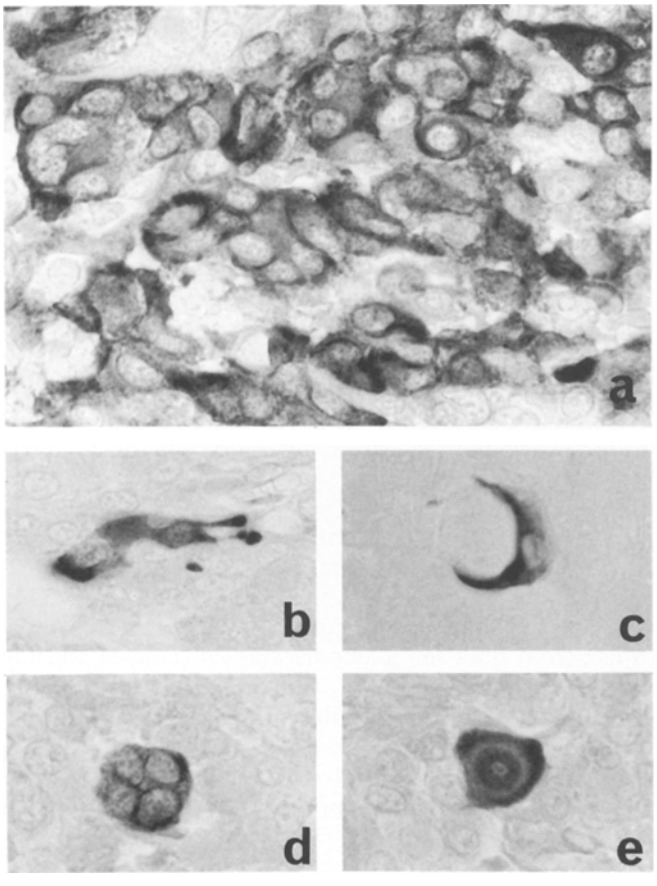

Fig. 2a–e. Somatotropic adenoma of case 2. Paraffin sections, immunoperoxidase, × 700. **a** Most adenoma cells are somatotropes (anti-GH antiserum). **b–e** The somatostatin cells in this case have different shapes. They may have slender processes (**b**, **c**). Some are multinucleated (**d**)

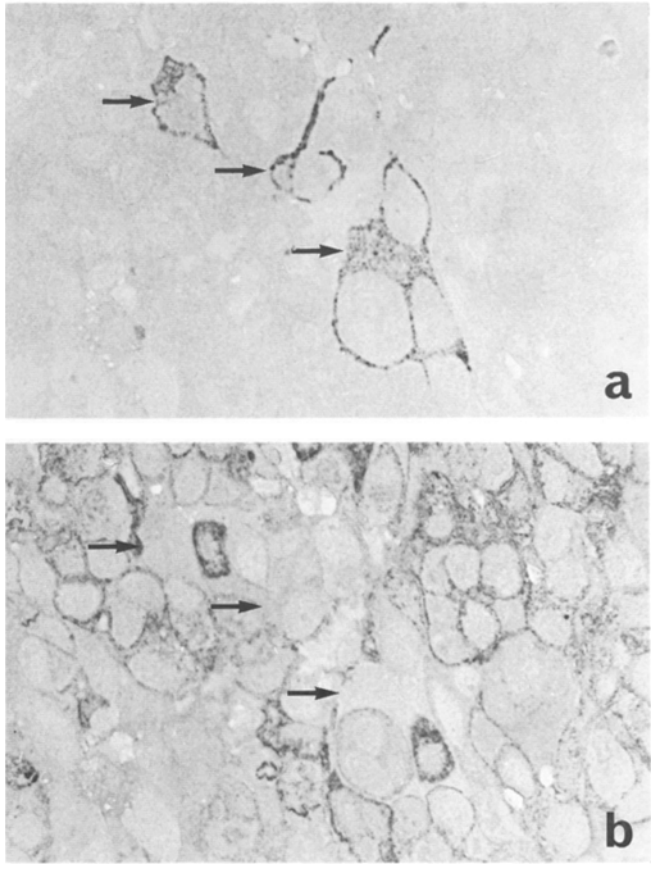

Fig. 3a, b. Somatotropic adenoma of case 2. Homologous fields of serial 1-μm sections, immunoperoxidase, × 700. **a** Three groups of somatostatin cells (*arrows*) are immunostained by the anti-SRIH-14 antiserum. **b** On the homologous field of the serial section stained by the anti-GH antiserum, the same groups of cells (*arrows*) are not stained and are surrounded by somatotropes

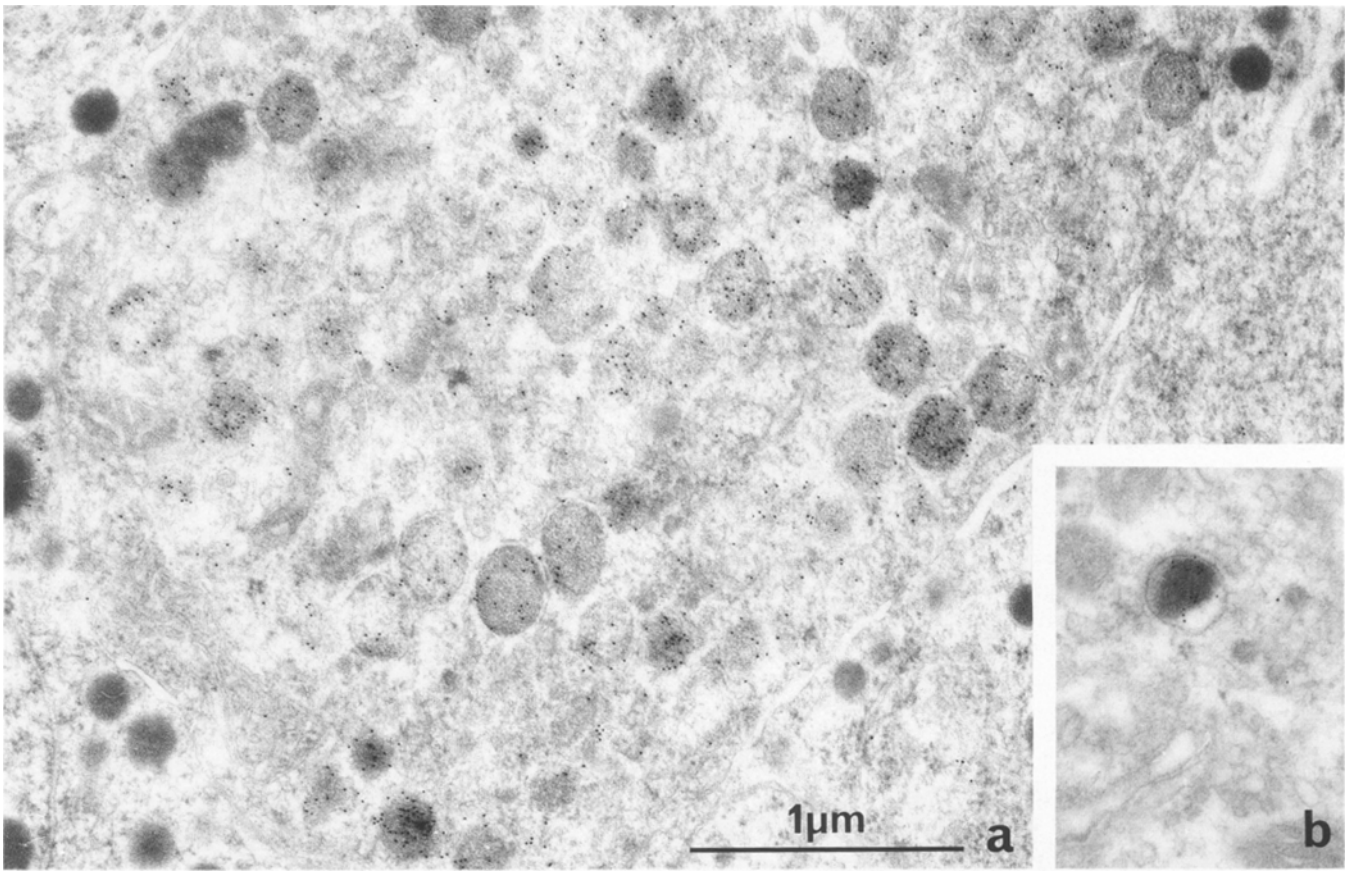

Fig. 4a, b. Somatotropic adenoma of case 2. Ultrastructural immunogold labelling. Anti-SRIH-14 antiserum, ×36,000. **a** Gold particles (10 nm) are gathered over the secretory granules of a somatostatin cell. The contents of the secretory granules have different electron densities. **b** The electron-dense granules are often associated with the Golgi complex

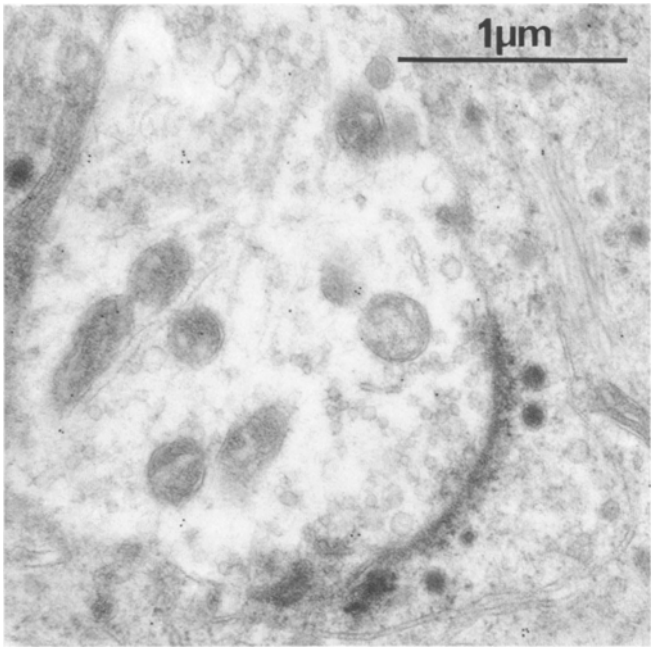

Fig. 5. Somatotropic adenoma of case 2. Ultrastructural immunogold labelling. Anti-SRIH-14 antiserum, ×36,000. A synapse-like structure between two adenoma cell processes. Neither the clear vesicles in the presynaptic compartment nor the dense core vesicles in the postsynaptic compartment are immunostained

hybridization step prevented the binding of the labelled probe.

In some experiments, SRIH immunostaining was performed on one section and SRIH mRNA hybridization on the adjacent section. On homologous fields of serial sections, immunopositivity and collection of silver grains were superimposable when the cytoplasm of a SRIH-producing cell was split between the two sections (Fig. 6a, b).

Autoradiograms of Northern blots hybridized under stringent conditions with the ^{32}P-labelled probe complementary to pre-pro-SRIH mRNA displayed a readily detectable signal of comparable intensity in RNA extracts from both the adenoma studied (case 2) and the human hypothalamus (Fig. 7). The signal was absent in human muscle RNA. In the two positive tissues, the RNA transcripts were approximately 600 nucleotides long, which corresponded to the size of human pre-pro-SRIH mRNA that was reported in the literature (Shen et al. 1982).

Discussion

The present data confirm the previous biochemical demonstration of SRIH synthesis in human somatotropic

adenomas and strongly suggest that a peculiar cell type in the tissue is responsible for this synthesis. Expression of the SRIH gene in the tumour of one of the two cases was shown by Northern analysis. In situ hybridization located this expression in a subset of adenoma cells. Immunocytochemistry and in situ hybridization independently showed that SRIH expression occurred in a discrete cell population of the adenoma tissue. In the case in which a precise co-localization study was possible, SRIH-IR cells appeared to be distinct from GH-producing cells.

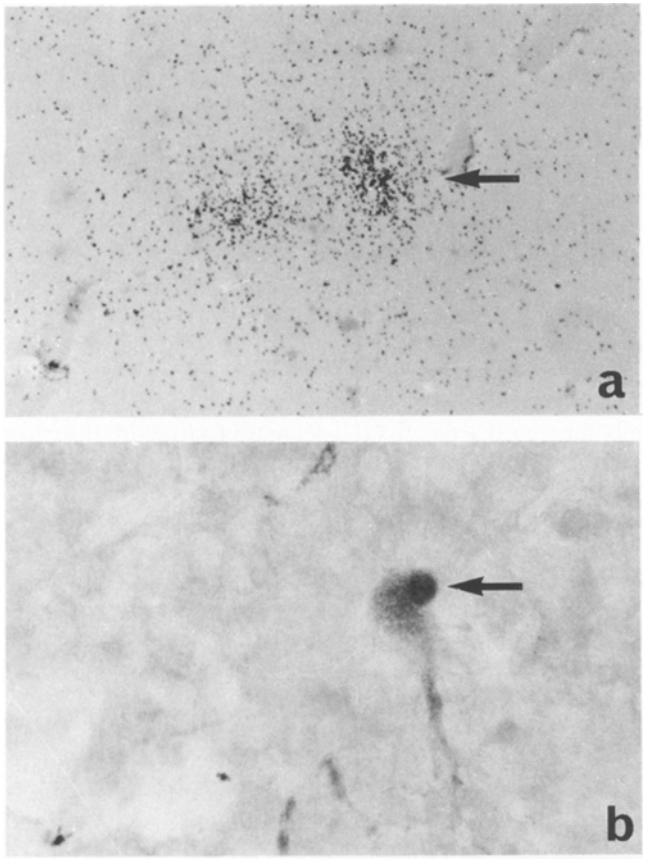

Fig. 6a, b. Somatotropic adenoma of case 2. Homologous fields of serial paraffin sections, ×700. **a** In situ hybridization of a ^{32}P-labelled, 45 base probe complementary to pre-pro-SRIH mRNA. After 10 days of autoradiography, dense collections of silver grains are seen over two somatostatin cells. **b** On the homologous field of the serial section, immunoperoxidase was performed with the anti-SRIH-14 antiserum. The hybridization and the immunoperoxidase signals are superimposable in the cell shown by an *arrow*

Immunocytochemical characterization of SRIH cells was made possible in these two cases due to the large amounts of SLI in the tissues but, in both cases, this SLI was shown to exclusively consist of SRIH precursor (pro-SRIH) (Levy et al. 1991 and personal communication). This does not imply that the ability of SRIH synthesis is limited to these few cases. In somatotropic adenomas, pre-pro-SRIH mRNA has been consistently detected (Pagesy et al. 1989) but it is likely not translated in vivo since most of them do not contain any SLI (Benlot et al. 1991). A few acromegalic patients whose tumours cause only a minimal GH plasma elevation (Daughaday et al. 1987) represent another situation. In contrast with other somatotropic adenomas, these tumours actively expressed the SRIH gene and synthesized SRIH-28 in vivo (Benlot et al. 1991; Pagesy et al. 1990). These data suggest that specific patterns of SRIH expression occur in different subgroups of somatotropic adenomas.

Ultrastructurally, the adenomatous SRIH cells individualized themselves from GH-producing cells and shared certain similarities with gastroenteropancreatic D-cells, particularly in relation to the features of their secretory granules. In D-cells, the electron-dense core granules and the electron-lucent core granules have been regarded, respectively, as maturing and mature granules according to the degree of intragranular processing of SRIH-28 to SRIH-14 (Ravazolla et al. 1983). In the two somatotropic adenomas studied here, despite the similarities of the secretory granules with those of D-cells, all the secretory granules contained pro-SRIH whatever their type.

Our results demonstrate the existence of peculiar SRIH-producing cells in a subgroup of human somatotropic adenomas. These findings raise the question of the origin of these cells and that of their possible role in the tumoral tissue.

Endocrine tumours can express phenotypes which are different from those of the parent cell type (Mendelsohn and Baylin 1988). The synthesis of SRIH by somatotropic adenomas might be another illustration of an ectopic

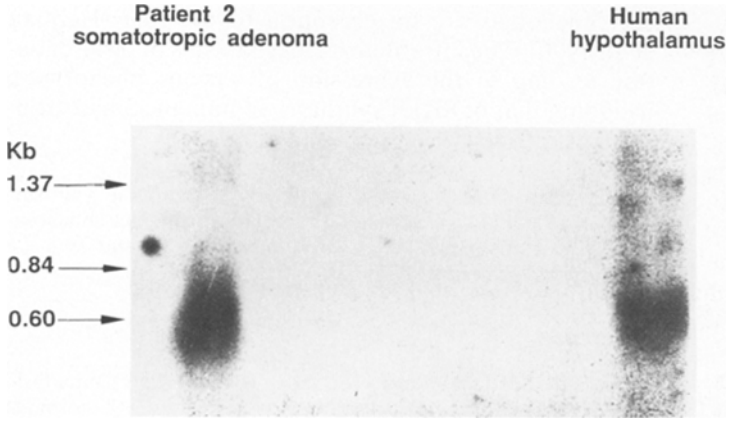

Fig. 7. Autoradiogram of a Northern blot of total RNA from the adenoma of case 2 and from a human hypothalamus. Samples of 40 µg RNA were electrophoresed, transferred to a nylon membrane and hybridized to a ^{32}P-labelled, 45 base probe complementary to pre-pro-SRIH mRNA. After 7 days of autoradiography, a readily detectable signal is present in the two samples at a position corresponding to the expected size of human pre-pro-SRIH mRNA

tumoral production of neuropeptide. However, SRIH synthesis seems also to take place in the human normal anterior pituitary in which SRIH mRNA and SRIH-28 have been demonstrated and which release substantial amounts of the mature peptide in perifusion (Joubert-Bression et al. 1989; Pagesy et al. 1989). The high SLI contents in the two somatotropic adenomas studied here could represent a neoplastic emphasis of a synthesis that occurs in the normal gland, rather than an ectopic production of the neuropeptide. A similar situation has been found in thyroid C-cells and the adrenal medulla, since SRIH synthesis can be detected both in the normal tissues and in medullary thyroid cancers or phaeochromocytomas (Sasaki et al. 1990; Scopsi et al. 1990).

Insofar as the synthesis of SRIH by the anterior pituitary is the expression of a neuroendocrine phenotype, it is tempting to explain this in the light of recent embryological findings. Experimental data have challenged the classical opposition between the ectodermal origin of the adenohypophysis and the neurectodermal origin of the hypothalamus by showing that both tissues derive from the neural primordium (Couly and Le Douarin 1985). The synapse-like structures that were found in one of the two cases were another unexpected sign of neural differentiation consistent with the neuroectodermal origin of the anterior lobe. Up to now, the presence of synapses in intrasellar tumours involving the anterior lobe has only been observed in rare cases of gangliocytomas and mixed gangliocytoma-adenomas. These tumours consist of large ganglion cells resembling neurosecretory hypothalamic neurones which may or may not be associated with an adenomatous proliferation of anterior pituitary cells (Kamel et al. 1989; Kovacs and Horvath 1986; Li et al. 1989). On pathological examination, neither of the two somatotropic adenomas studied here displayed any such ganglion cells.

The synthesis of SRIH by somatotropic adenomas is in keeping with the steady accumulation of data suggesting the existence of a control exerted within the anterior lobe by regulatory peptides which are endogenously produced (Houben and Denef 1990; Jones et al. 1990). By comparison with GH, the expression of SRIH in somatotropic adenomas is exceedingly low. The presence of SRIH mRNA in somatotropic adenomas has been confirmed by another group and its level of transcription quantified (Levy and Lightman 1990). The study showed that SRIH mRNA levels were approximately 300-fold less than GH mRNA levels. Similarly, SLI tissue contents, when they were detectable, were several orders of magnitude lower than those of GH (Benlot et al. 1991). Such minute amounts preclude any systemic effect of SRIH originating from anterior pituitary tissues. However, by comparison with the hypothalamus, the level of SRIH expression by somatotropic adenomas in perifusion matched that of the hypothalamus in vitro (Joubert-Bression et al. 1989), suggesting that adenomatous SRIH cells may play a role in the local regulation of GH secretion and tumour growth. Evidence for the synthesis of neuropeptides, like SRIH, supports the idea that pituitary adenomas may function as independent secretory units with regard to hypothalamic control. The potential importance of local regulatory mechanisms in pituitary adenomas has been stressed by the discovery that most if not all somatotropic adenomas were rich in arterial vessels (Racadot et al. 1986). Arteriogenesis probably results in the escape of adenoma tissue from hypothalamic regulation, the concentrations of hypothalamic factors in systemic blood, compared to those in portal blood, being too low to regulate anterior pituitary hormone secretion.

The absence of SRIH and GH co-localization favours the involvement of adenomatous SRIH cells in paracrine regulatory mechanisms. Although it could not be ruled out that a small amount of GH remained undetected in these cells, our results strongly support the view of a discrete adenoma cell-type specifically expressing SRIH and acting upon neighbouring somatotropes via paracrine regulatory mechanisms mediated by SRIH receptors which are present in somatotropic adenomas (Moyse et al. 1985; Reubi and Landolt 1984). The present morphological data suggesting a paracrine interaction between SRIH- and GH-producing cells are in agreement with previous functional results showing that, in perifusion, when the release of SRIH by adenomatous cells increased that of GH decreased (Joubert-Bression et al. 1989). These findings could not be explained if GH and SRIH were stored in the same cellular compartment. Pro-SRIH has no known biological activity but the release of SRIH-28 by somatotropic adenomas in perifusion is an indication of the ability of the tissue to process pro-SRIH to mature end-products at least in vitro (Joubert-Bression et al. 1989). The possibility that adenomatous SRIH production plays a role in vivo might be inferred from the study of somatotropic adenomas from patients with minimal plasma GH elevation. The biologically active form of SRIH present in vivo in the adenoma might participate in the local inhibition of GH expression and secretion which characterizes these peculiar tumours (Benlot et al. 1991; Pagesy et al. 1990).

Somatotropic adenomas are often plurihormonal. A large proportion of them have been shown to synthesize, in addition to GH, prolactin and/or the alpha-subunit common to the glycoprotein hormones (Bassetti et al. 1986; Beck-Peccoz et al. 1985; Kovacs and Horvath 1986). The presence of specific SRIH-producing cells in this type of tumours adds to their cellular heterogeneity. However, most tumours have a clonal origin and pituitary adenomas are no exception to the rule (Herman et al. 1990). The intratumoral mechanisms of differentiation leading to the expression of various phenotypes, including that of SRIH synthesis, in human somatotropic pituitary adenomas, remain a puzzle.

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