

Co-secretion of calcitonin gene-related peptide, gastrin-releasing peptide and ACTH by a carcinoid tumour metastasizing to the cerebellum

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Summary: A carcinoid tumour presenting as Cushing's syndrome is reported. Although no tumour mass could be initially identified the patient returned with first a liver and subsequently a cerebellar mass both of which were resected. Only at post-mortem was the lung primary discovered. ACTH, gastrin-releasing peptide (GRP) and calcitonin gene-related peptide were elevated in plasma before resection of the hepatic tumour. These peptides were demonstrated in both the hepatic and cerebellar tumours by immunocytochemistry and radioimmunoassay. This case illustrates the occasional tendency of primary lung carcinoids to remain small and clinically undetectable while generating secondary tumours which are symptomatic. It is suggested that immunological demonstration of GRP may be diagnostically helpful in directing attention to the lung as a primary site in neuroendocrine tumours which present in this fashion.

Introduction

Gastrin-releasing peptide (GRP), a peptide of 27 amino acid residues,¹ and its C-terminal decapeptide^{2,3} (neuromedin C), are now considered to be the mammalian forms of bombesin.⁴ Both amphibian bombesin and its mammalian counterparts have potent pharmacological effects in the gastrointestinal tract and the central nervous system. Infusion in man has shown that both bombesin and GRP release gastrointestinal and pancreatic hormones.^{5,6} Bombesin and GRP-like immunoreactivity have been reported in oat cell carcinoma of the bronchus^{7,8} where production of these peptides is frequently associated with calcitonin^{9,10} and ACTH.^{11,12}

Recently, a new peptide, 'calcitonin gene-related peptide' (CGRP), has been isolated. This 37 amino acid residue peptide is a product of mRNA formed by alternative tissue-specific RNA splicing after transcription of the calcitonin gene.¹³ Human CGRP has been isolated from medullary carcinoma of the thyroid¹⁴ and from a cell line derived from small cell carcinoma of the bronchus,¹⁵ but its secretion from other neuroendocrine tumours has not previously been reported.

We report here a patient who presented with ectopic

ACTH secretion syndrome resulting from a metastatic carcinoid tumour of pulmonary origin, which was found in addition to contain and secrete three forms of gastrin-releasing peptide (bombesin) and CGRP.

Case report

A 49 year old Caucasian male presented in September 1980 with clinical and biochemical features of ectopic ACTH secretion syndrome. Despite extensive radiological investigation including ultrasonography, computerized tomographic (CT) scanning and selective venous sampling, the source of ACTH secretion was not identified. He was treated with potassium supplements and metyrapone, but his symptoms became increasingly difficult to control and he therefore underwent bilateral adrenalectomy by the posterior route. The adrenals were hyperplastic (left adrenal 15 g; right adrenal 16 g). Post-operative steroid replacement therapy was started, but over the next two years the patient became hyperpigmented. Further investigations were negative until, in May 1982, a technetium-99m liver scan revealed a large filling defect in the right lobe of the liver. Angiography and CT scan confirmed the presence of a mass, and in June 1982, a malignant hepatic tumour 9 cm in

diameter was removed by right lobectomy. No other tumour was found at laparotomy. Postoperatively, the pigmentation faded. The patient remained well until October 1983 when he presented with ataxia and dysarthria. CT scan showed a cystic right cerebellar tumour, and vertebral angiography demonstrated an intrinsic avascular mass in the right cerebellar hemisphere which was subsequently resected. Postoperatively his ataxia and dysarthria improved considerably.

Eighteen months later he again deteriorated with re-emergence of neurological symptoms and the onset of obstructive jaundice leading to his death in April 1985.

At post-mortem there was a tumour in the porta hepatis invading the portal vein and occluding the common bile duct. A single nodule was present in the liver and dissection of the brain revealed deposits in the left parietal area and pineal gland. An ill defined 1 cm diameter firm area was noted in the upper lobe of the right lung. Histological examination confirmed this to be a tumour similar in appearance to that resected previously from liver and brain.

Materials and methods

Radioimmunoassay

During the course of his illness, fasting blood samples (10 ml) were obtained from the patient, and placed in chilled heparinized tubes containing 200 μ l aprotinin (Trasylol – Bayer). Plasma was separated within 20 minutes and stored frozen for measurement of concentration of ACTH, GRP and CGRP.

Peptides were extracted from tumour tissue by boiling in 0.5 M acetic acid (10% weight/volume) for 10 minutes. The concentration of GRP was determined by radioimmunoassay as described previously.¹⁶ CGRP concentration were measured using a rabbit antiserum (MJ-2) raised against synthetic human CGRP (Bachem Laboratories) conjugated to bovine serum albumin (BSA) with carbodiimide and used at the final dilution of 1:320,000. This antiserum does not cross react with calcitonin or other known

regulatory peptides. Radiolabelled CGRP was prepared by a chloramine-T iodination of the histidine residue in synthetic human CGRP and purified by reverse-phase high pressure liquid chromatography (HPLC) on a μ Bondapak C-18 column. The assay was performed in a total volume of 0.8 ml of phosphate buffer (0.06 M, pH 7.4) containing 0.01 M EDTA and 3% BSA. For assays of plasma GRP and CGRP, 100 μ l samples were used and all standard and assay control tubes contained 100 μ l pooled human time expired plasma from which peptide immunoreactivities had been removed by charcoal absorption. Chromatography of these plasma samples on Sephadex G-50 column revealed no peaks of GRP or CGRP-like immunoreactivity. After 5 days' incubation at 4°C, free label was separated from antibody-bound label by adding 8 mg dextran-coated charcoal per tube. The sensitivity of the assay was 1.5 fmol/tube (95% confidence limit). Plasma ACTH-immunoreactivity was kindly measured by Professor L.H. Rees (Department of Chemical Endocrinology, St Bartholomews Hospital, London).¹⁷

Chromatography

Tissue extracts and plasma were loaded onto a Pharmacia column (1.5 $\times$ 100 cm) containing Sephadex G-50 Superfine, which was eluted at 4°C with 0.06 M phosphate buffer, pH 7.4, containing 0.01 mol/l EDTA, 0.3% BSA, and 0.2 mol/l sodium chloride at a flow rate of 3 ml/h. The column was recalibrated between each series of runs with dextran blue (molecular weight 2×10^6 , elution coefficient, Kav 0), horse heart cytochrome C (molecular weight 12,384, Kav 0.23) and Na ¹²⁵I (Kav 1) as molecular size markers. Kav was calculated according to Laurent & Killander.¹⁸ GRP and CGRP immunoreactivities detected in the tumour extracts were further analysed by HPLC on a Techsil C-18 (Water Associates Inc.) reverse phase column (3.9 $\times$ 300 mm). A linear gradient elution system was used from 15 to 40% acetonitrile in water containing 0.05% trifluoroacetic acid over 75 minutes. The flow rate was 1 ml/min and fractions of 1 ml were collected. Fractions from the gel

Table 1 Characterization of antibodies for immunocytochemistry

Antiserum	Method of conjugation		Titre	Antigen concentration*
NSE (human)	—	—	1/4000	1.0
ACTH	CDI	BSA	1/2000	10.0
Bombesin	BDB	BSA	1/4000	10.0
CGRP	CDI	BSA	1/2000	10.0

* nmol of antigen required per ml of diluted antiserum to prevent immunostaining.
CDI, carbodiimide; BDB, bid-diazotized benzidine; BSA, bovine serum albumin.

columns and HPLC were assayed at different dilutions for both GRP and CGRP-like immunoreactivities.

Immunocytochemistry

Surgically resected hepatic and cerebellar tumour tissue were separately fixed in 10% buffered formalin and in Bouin's solution. Other fragments were immediately frozen and subsequently freeze-dried and vapour-fixed in benzoquinone.¹⁹ Tissue obtained at post-mortem was fixed in 10% formaldehyde solution. All tissues, whether fixed in solution or vapour-fixed, were embedded in paraffin wax. Sections were stained with haematoxylin and eosin, Grimelius silver impreg-

nation technique²⁰ and by immunocytochemistry,²¹ using antibodies to neurone-specific enolase,²² a marker for neuroendocrine differentiation, and peptide hormones including ACTH, GRP/bombesin and CGRP (see Table I for antibody characterization). In order to demonstrate the presence of more than one peptide within a single cell, pairs of 3 μ m serial sections were cut. The first section of each pair was inverted before being picked up on a glass slide. Pairs of sections were stained for ACTH and CGRP, CGRP and GRP and GRP and ACTH. For electron microscopy, small fragments of tumour were fixed in 2.5% glutaraldehyde and subsequently post-fixed in osmium tetroxide.

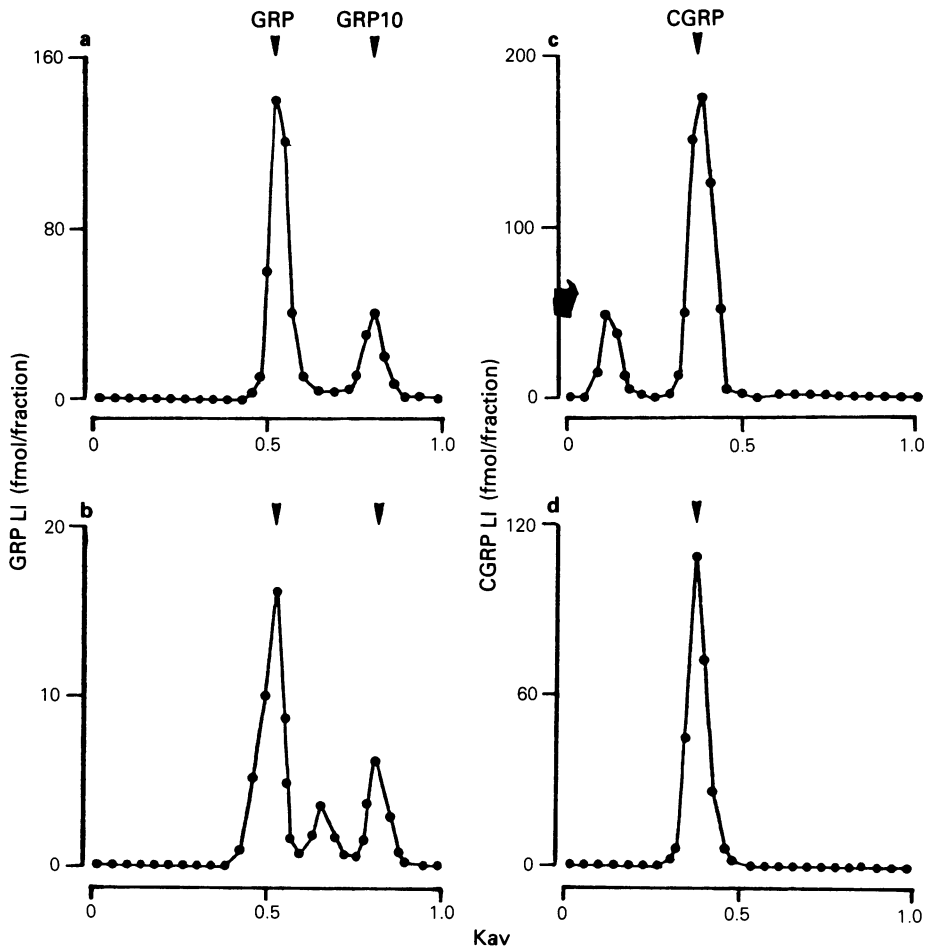


Figure 1 Gel permeation chromatographic profiles of GRP (GRPLI) and CGRP-like (CGRPLI) immunoreactivities of tumour extract (a,c), and pre-operative plasma (b,d) on a Sephadex G-50 superfine column (1.5 $\times$ 100 cm). GRP, synthetic porcine gastrin-releasing peptide; GRP10, synthetic porcine C-terminal decapeptide of gastrin-releasing peptide (neuromedin C); CGRP, synthetic human calcitonin gene-related peptide.

Results

Radioimmunoassay of plasma

Analysis of plasma samples taken prior to partial hepatectomy showed a raised level of ACTH at 539 ng/l, GRP 180 pmol/l (normally undetectable, i.e. <10 pmol/l) and CGRP 420 pmol/l (normally undetectable, i.e. <20 pmol/l). After right hepatectomy plasma levels of ACTH fell to 63 ng/l while CGRP and GRP became undetectable.

Radioimmunoassay of tumour

Extracts from the tumour resected from the right hepatic lobe contained 2.4 nmol/g of GRP and 130 pmol/g of CGRP-like immunoreactivity. The cerebellar tumour contained 1.2 nmol/g GRP and 96 pmol/g CGRP.

Chromatographic analysis

Gel permeation chromatography of the preoperative plasma and the tumour extracts demonstrated two major GRP-like immunoreactive peaks (Figure 1a,b). The earlier peak (K_{av} 0.54) emerged in the position of synthetic porcine gastrin-releasing peptide

(GRP), while the second minor peak was co-eluted with C-terminal decapeptide of GRP (K_{av} 0.81) just after amphibian bombesin (K_{av} 0.78). Analysis of the tumour extracts on reverse phase HPLC revealed three major peaks of GRP-like immunoreactivity (Figure 2), in which only a proportion of the immunoreactivity had an identical retention time to that of C-terminal decapeptide of GRP (Figure 2). Neither of the other two immunoreactive peaks corresponded to either pure synthetic porcine GRP or synthetic amphibian bombesin.

Analysis of the tumour extracts on the gel column revealed two major CGRP immunoreactive peaks (Figure 1c). The earlier peak of K_{av} 0.17 was eluted between the dextran blue and cytochrome C, indicating a larger molecular form, whereas the second peak was co-eluted with synthetic human CGRP (K_{av} 0.37). In contrast, however, the pre-operative plasma contained only one major molecular form of CGRP-like immunoreactivity which emerged in the position of synthetic human CGRP (Figure 1d).

Histology of tumour tissue

The hepatic, cerebellar and pulmonary masses showed similar histological features. They were composed of ribbons, trabeculae, acini and irregular masses of

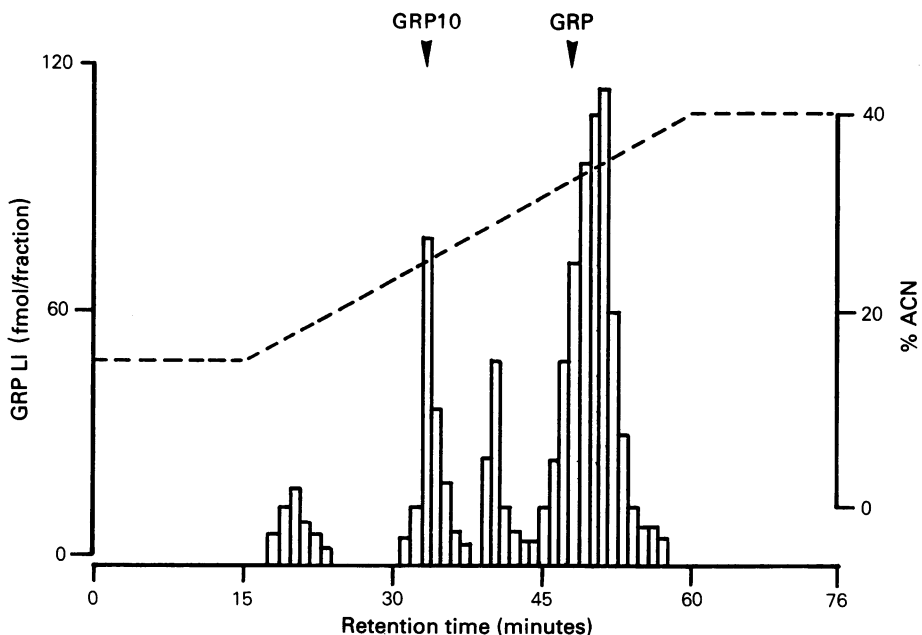


Figure 2 Reverse phase high pressure liquid chromatography analysis of GRP-like (GRPLI) immunoreactivity of the tumour tissue extract. The column was eluted with a gradient (dotted line) between two acetonitrile (ACN) 0.05% aqueous TFA solvent system. From 15% to 40% by volume acetonitrile.

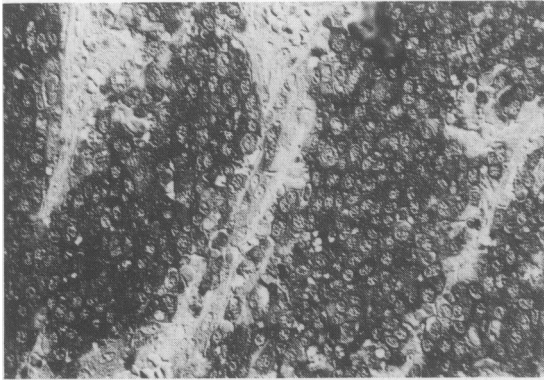


Figure 3 NSE immunoreactivity in the cells of the liver tumour. Freeze-dried, benzoquinone vapour-fixed tissue; 5 μ m wax-section. Photographed using Normarski interference contrast; $\times 700$.

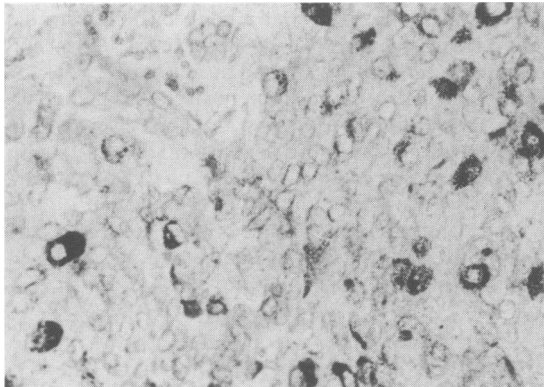


Figure 4 GRP-immunoreactivity in the cells of liver tumour. Freeze-dried, benzoquinone vapour-fixed tissue; 5 μ m wax-section; $\times 900$.

monomorphic polygonal cells with high nuclear: cytoplasmic ratio, eosinophilic granular cytoplasm and slightly irregular hyperchromatic nuclei showing normal and abnormal mitoses. The lung tumour occupied the lumen of a bronchus and had infiltrated through its wall to invade adjacent lung parenchyma.

Immunocytochemistry with antiserum to neurone-specific enolase, showed reactivity in all tumour cells, albeit of variable intensity (Figure 3). Numerous diffusely spread tumour cells throughout the tumours reacted also to antibodies to ACTH, GRP/bombesin (Figure 4) and CGRP.

Co-localization studies using serial, mirror image sections demonstrated the co-existence of ACTH and CGRP, CGRP and GRP and ACTH and GRP within

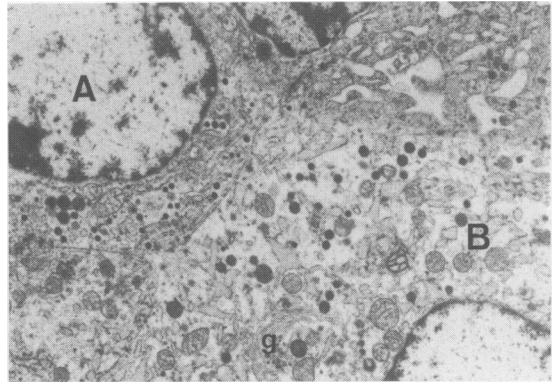


Figure 5 Electron micrograph of two distinct endocrine cell types from ACTH-producing tumour-cell type A exhibits the conventional ultrastructural appearance of ACTH-containing secretory granules ($D_{\max} = 160$ nm) whereas cell B contains much larger ($D_{\max} = 220$ nm) electron-dense secretory granules. Both cell types were immunoreactive against ACTH antisera. A prominent Golgi apparatus (g) is present in cell B. Gluteraldehyde fixation/osmium tetroxide/araldite/uranyl acetate and lead citrate counterstain, $\times 13,200$.

single cells. Electron microscopy revealed the presence of two endocrine cell types containing dense core secretory granules measuring approximately 230 nm and 160 nm in diameter (Figure 5).

Discussion

Cushing's syndrome due to ectopic production of ACTH is a well recognized complication of neuroendocrine tumours, in particular small cell carcinoma of the bronchus. When tumor mass is small it may be the only manifestation and thus the presenting feature. Indeed the source of ACTH in this case eluded imaging techniques until 2 years after presentation when a hepatic mass and subsequently a posterior fossa deposit were detected. Nevertheless during life the tumour primary site remained unclear. Although primary neuroendocrine tumours of the liver have been reported,²³ these are uncommon and at the present limit of resolution of imaging techniques the diagnosis may only be confidently made at post-mortem when alternative more likely sites can be excluded.

When this patient came to autopsy a small pulmonary nodule was discovered. We consider this to be the primary for several reasons. These include the relative frequency of bronchial carcinoids, the presence of cranial metastases (common in pulmonary tumours) and the histological demonstration that the tumour

was present within a bronchus. Although a satisfactory retrospective diagnosis was possible, were there any other clues to the origin of the primary available during life?

Analysis of plasma and tumour extracts together with immunohistochemical examination of tissue sections suggested that three peptides were being synthesized and secreted by the carcinoid; ACTH, responsible for the Cushing's syndrome, gastrin-releasing peptide (GRP, bombesin) and calcitonin gene-related peptide (CGRP). In normal human tissues GRP-like immunoreactivity has been demonstrated predominantly in endocrine cells of the fetal and neonatal lung.^{24,25,26} A number of common neoplasms also synthesize GRP, which occurs in two major molecular forms distinguishable by gel chromatography, small cell carcinoma of the bronchus,^{7,8,9,27,28} bronchial carcinoids^{29,30,31,32} and medullary carcinoma of the thyroid.³²⁻³⁶ A thyroid neoplasm is usually easily excluded clinically and therefore we suggest that GRP synthesis is a strong indicator of pulmonary origin and should direct attention to the lung in cases such as this in which the primary site is obscure.

GRP is thought to have a number of biological effects including release of many gastrointestinal hormones. This may be demonstrated by infusion of GRP into healthy volunteers at lower levels than were present in this patient.^{5,6,37} Nevertheless no other gastrointestinal hormone was elevated in this case, a finding which is generally consistent with reports of patients with elevated plasma GRP immunoreactivities. The lack of stimulatory effect is unlikely to be due to the production of biologically inactive forms of the molecule as the antiserum used for radioimmunoassay reacts with the active site of GRP. It is conceivable, however, that chronic GRP secretion from a slowly developing tumour may be associated with down regulation of GRP receptors and consequent lack of effect. This is similar to the lack of effect of elevated plasma somatostatin concentrations in patients with somatostatinomas.^{38,49}

Carcinoid tumours of the bronchus are in general

slow growing neoplasms with high 5 year survival rates. This patient's neoplasm is therefore one of the minority which behave aggressively and metastasize. This unusual behaviour may be associated with the autocrine growth regulatory activity or trophic effect ascribed to GRP.^{40,41} This has been demonstrated particularly convincingly on small cell carcinoma lines which synthesize GRP⁴² and confirmed by showing that antibodies which inhibit the binding of GRP receptors inhibit growth *in vitro* and tumour xenografts *in vivo*.⁴² It is conceivable that some bronchial carcinoids are under GRP autocrine control and that occasional aggressive tumours such as the one reported here synthesize and secrete particularly large amounts, accounting for their behaviour. Manipulation of GRP autocrine control by use of antagonists or antibodies may provide future approaches to therapy of such tumours.^{42,43}

The final unusual feature of this case is the synthesis and secretion of CGRP. Initially characterized by isolation of its mRNA from cells of medullary carcinoma of the rat thyroid^{13,44} and it has been demonstrated immunohistochemically in neurones of the central and peripheral nervous system and in thyroid C cells.^{13,45,46} Among neoplasms it has been restricted to medullary carcinoma of the thyroid¹⁴ and a cell line derived from small carcinoma of the bronchus¹⁵ until demonstration in this case. The biological effects of CGRP are still being characterized although potent effects on the cardiovascular system^{47,48} have been described. Of the clinical features present in this case none could be ascribed to raised plasma CGRP levels.

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