A phaeochromocytoma presenting with Cushing’s syndrome associated with increased concentrations of circulating corticotrophin-releasing factor

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ABSTRACT

The case is described of a 61-year-old male who presented with hypertension and Cushing’s syndrome which resolved on excision of a unilateral adrenal mass. Histology of the tumour revealed a benign phaeochromocytoma which immunostained for corticotrophin-releasing factor (CRF-41) but not for ACTH. Preoperative plasma concentrations of immunoreactive CRF-41 were increased, and gradients for both CRF-41 and ACTH were demonstrated across the tumour.

Post-operatively, CRF-41 was undetectable in plasma. The tumour contained high concentrations of immunoreactive CRF-41 which co-eluted with synthetic human CRF-41 on reversed-phase high-performance liquid chromatography. Tumour CRF-41 stimulated the release of ACTH in a dose-dependent manner from isolated rat anterior pituitary cells. We conclude that this tumour secreted CRF-41 and ACTH and had the capacity to produce ACTH-dependent Cushing’s syndrome directly by secreting ACTH and indirectly by secreting CRF-41 to stimulate ACTH secretion from the anterior pituitary.


INTRODUCTION

Cushing’s syndrome can be defined as the symptoms and signs associated with prolonged exposure to inappropriately increased plasma corticosteroid concentrations. Whilst the majority of patients suffering from Cushing’s syndrome have a basophil adenoma of the pituitary secreting adrenocorticotrophin (ACTH), in about 20% of cases the syndrome results from secretion of ACTH from a wide variety of non-pituitary tumours (Howlett, Drury, Perry et al. 1987). Although it has been shown that corticotrophin-releasing factor (CRF-41) can stimulate the release of ACTH in man (Grossman, Perry, Schally et al. 1982), there is as yet no direct evidence for the involvement of CRF-41 in the genesis or maintenance of Cushing’s syndrome. Corticotrophin-releasing factor-like activity has been reported in a number of tumours not associated with Cushing’s syndrome (Yamamoto, Hirata, Matsukura et al. 1976; Suda, Demura, Demura et al. 1977; Kirkland, Lumsden & Ellison, 1984; Suda, Tomori, Tozawa et al. 1984) but reports relating CRF-41 production to Cushing’s syndrome are rare. Upton & Amatruda (1971) demonstrated CRF-like bioactivity in extracts of pancreatic and small-cell lung tumours, and proposed that tumour CRF-like peptides may contribute to Cushing’s syndrome. Corticotrophin-releasing factor-like bioactivity has been found in blood draining a medullary thyroid carcinoma (Birkenhager, Upton, Seldenrath et al. 1976) and a nephroblastoma (Hashimoto, Takahara, Ogawa et al. 1980) in patients whose Cushing’s syndrome was cured by tumour excision. In all of these cases, however, precise characterization of this CRF-like bioactivity was limited by the non-availability of purified standard and it is now known that tumour peptides other than CRF-41, such as bombesin (Howlett, Price, Hale et al. 1985), may

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stimulate the release of ACTH. A more recent study (Carey, Varma, Drake et al. 1984) demonstrated immunoreactive (ir) and bioactive CRF-41 in metastatic deposits of a prostatic carcinoma, adjacent to the pituitary, invading the median eminence and pituitary stalk of a patient with high concentrations of circulating ACTH and cortisol. Negative tumour immunostaining for ACTH together with pituitary corticotroph hyperplasia led the authors to conclude that the secretion of tumour CRF-41 was responsible for the Cushing’s syndrome. In one other report of ectopic CRF-41 secretion associated with Cushing’s syndrome (Belsky, Cuello, Swanson et al. 1985) a medullary thyroid carcinoma stained positively for CRF-41 but negatively for ACTH.

In this study of a phaeochromocytoma in a patient with Cushing’s syndrome, we show that the tumour was secreting both CRF-41 and ACTH and appeared to be responsible for the syndrome. The report also discusses the problems inherent in employing only immunocytochemistry to locate tumour peptides.

MATERIALS AND METHODS

Case report

A 61-year-old male was admitted with severe labile hypertension. Investigation suggested the diagnosis of a phaeochromocytoma with raised concentrations of plasma catecholamines and urinary vanillyl mandelic acid. Hypertension was controlled by α-adrenoceptor blockade with phenoxybenzamine. An [131I]meta iodo benzyl guanidine scan was consistent with a tumour of the left adrenal gland. Shortly afterwards, with the development of diabetes mellitus, Cushing’s syndrome was diagnosed on the basis of high non-suppressible concentrations of plasma cortisol (> 2000 nmol/l) and ACTH (350 ng/l). A computerized tomography scan revealed a large left adrenal mass together with marked enlargement of the right adrenal. Control of the Cushing’s syndrome and diabetes was achieved by metyrapone until after removal of the tumour. There was complete post-operative resolution of the Cushing’s syndrome.

Immunocytochemistry

Tumour tissue was surgically excised and rapidly fixed in 10% (v/v) formaldehyde saline before processing to paraffin wax. Consecutive 4 μm thick sections from two samples of tissue were stained with haematoxylin/eosin by the Grimelius method for argyrophil granules. Tissue was immunostained by an indirect immunoperoxidase method (Coates, 1985) using primary antisera raised against synthetic ovine (o) CRF-41, and the human forms of ACTH(1–39), N-pro-opiomelanocortin-28, β-endorphin, Met-enkephalin, α-melanocyte-stimulating hormone, arginine vasopressin and bombesin. An antiserum to neuroenest-specific enolase was used as a marker of neuroendocrine differentiation. Immunostaining controls were conducted using negative control sections, each primary antiserum being replaced by non-immune serum from the same species in which the primary antiserum had been raised. Positive control sections consisted of simultaneous staining of sections of human tissue which contained each antigen. Liquid-phase absorption tests were performed on the anti-oCRF serum by adding synthetic oCRF-41 and incubating for 24 h at 4 °C before immunostaining.

Tissue extraction

Tumour tissue, stored at -70 °C, was homogenized in 10 volumes of ice-cold HCl (0·1 mol/l) containing 0·1% (w/v) ascorbic acid and the extract heated at 85 °C for 10 min. The extract was then centrifuged at 3300 g for 30 min at 4 °C and the supernatant stored at -20 °C. Samples were diluted 1:50 with assay buffer for radioimmunoassay (RIA).

Measurements of CRF-41 and ACTH

Corticotrophin-releasing factor was detected in tissue and plasma by extraction and RIA as described by Cunnah, Jessop, Besser & Rees (1987). Adrenocorticotrophin was measured by the method of Rees, Cook, Kendall et al. (1971) using antiserum raised against synthetic ACTH(1–24) (Ciba Laboratories, Horsham, Sussex).

Gel filtration chromatography

Tumour extract (0·5 ml) was chromatographed on a 100 × 1·5 cm column of Sephadex G-75 Superfine (Pharmacia, Uppsala, Sweden) eluted with 1% (v/v) aqueous formic acid, containing 0·1% (w/v) Polyacrylamide (Sigma, St Louis, MO, U.S.A.), at a flow rate of 3 ml/h at 4 °C. Fractions (1·4 ml) were collected, desiccated under vacuum and reconstituted in assay buffer for RIA.

Reversed-phase high-performance liquid chromatography (RPHPLC)

The HPLC system (Altex, Berkeley, CA, U.S.A.) comprised two 100A pumps controlled by a 421 controller, a 210 loop injector and an Ultrapore RPSC C3 column of pore size 30 nm (0·46 cm internal diameter × 7·5 cm). Water was distilled, deionized and further purified through a Milli-Q four-cartridge system (Millipore, Horrow, Middx). All other reagents used were HPLC grade. Solvent A was aqueous 0·1% (v/v) trifluoroacetic acid (TFA) and solvent B
was 1-propanol (Rathburn Chemicals, Walkburn, Borders) containing 0·1% TFA.

All samples and standards (synthetic human CRF-41, 2 ng aliquots) were injected in a mixture of solvent A containing 0·1% (v/v) Triton X-100, as it was found that CRF-41 adsorbed onto the injection syringe unless the detergent was present. Samples were eluted at a flow rate of 1 ml/min and fractions (1 ml) collected, desiccated and reconstituted in assay buffer for RIA.

Bioassay

The ACTH-releasing potential of tumour CRF-41 was studied using the isolated rat anterior pituitary cell column method of Gillies & Lowry (1978) in which tissue was dispersed in Earle’s medium containing trypsin (2·5 mg/ml) and cells were suspended in Bio-Gel P2 (Bio-Rad, Richmond, CA, U.S.A.) and perfused with medium equilibrated with 95% O₂/5% CO₂. Synthetic human CRF-41 was used as internal standard at concentrations of 0·4, 1·2 and 3·6 μg/l. The flow rate of medium through the column was 0·6 ml/min and cells were stimulated every 10-min with 3-min pulses of standard or sample. Tumour CRF-41 was prepared for the column by chromatography of extracts on RPHPLC, collecting those fractions which contained CRF-41. This enabled CRF-41 to be separated from tumour ACTH, which eluted at a lower percentage of 1-propanol. Fractions containing CRF-41 were pooled in 2 ml phosphate buffer (50 mmol/l; pH 7·4) containing 0·5% (w/v) human serum albumin and divided into equal aliquots. To one was added 200 μl antiserum specific for CRF-41 (final dilution 1:1000) and both aliquots were incubated for 18 h at 4°C. For application to the column, these aliquots were diluted in 4 ml medium and the aliquot without antiserum was further diluted. Fractions of tumour extract, after RPHPLC, which contained no CRF-41 were reconstituted in 5 ml medium and used to determine non-specific cell stimulation. The cells were initially equilibrated for 90 min with medium and all standards and samples added during the next 3 h. Column fractions (1 ml) were collected and assayed for ACTH by RIA.

RESULTS

Tissue staining

Morphologically, the tumour was a typical phaeochromocytoma consisting of a mixed alveolar and trabecular arrangement of mature, finely granulated phaeochromocytes. Mitoses were seen infrequently. Many cells contained argyrophil granules, and neurone-specific enolase immunoreactivity was detected in the majority of cells. The tumour stained positively for Met-enkephalin. Small groups of cells stained strongly positive for CRF-41 (Plate) and this staining was abolished by preincubation of antiserum with oCRF-41 but not by preincubation with other peptides. Replacement of primary antiserum with non-immunized serum from the same species resulted in an absence of staining. Tumour sections were negative for all other peptides tested.

Measurements of CRF-41 and ACTH

Corticotrophin-releasing factor and ACTH in both tumour and plasma diluted in parallel to standard human CRF-41 and ACTH(1–39) respectively (Text-fig. 1 for CRF-41 data, ACTH data not shown). Circulating concentrations of CRF-41 before and after removal of the tumour were 270 and <20 ng/l respectively, and the concentration of CRF-41 in a sample of adrenal vein plasma was 190 compared with 44 ng/l in a sample of plasma taken simultaneously from the superior vena cava (SVC). Circulating concentrations of ACTH before and after removal of the tumour were 399 and 24 ng/l respectively, and the concentration of ACTH in adrenal vein plasma was 150 compared with 73 ng/l in a sample of plasma taken simultaneously from the SVC. Tumour extracts contained high concentrations of CRF-41 (3–8 μg/g wet weight) compared with those in a phaeochromocytoma not associated with Cushing’s syndrome (31 ng/g) and in a normal adrenal gland (14 ng/g). Concentrations of tumour ACTH were 0·6–0·7 μg/g.
Chromatography of plasma and tumour CRF-41 and ACTH

Tumour extract chromatographed on Sephadex G-75 and RPHPLC contained a single peak of CRF-41 which eluted in the position of human CRF-41 (Text-figs 2a and 3a). When a preoperative plasma sample was chromatographed on RPHPLC, 90% of irCRF eluted in the position of CRF-41 (Text-fig. 3b). Tumour ACTH co-eluted with ACTH(1–39) on Sephadex G-75 (Text-fig. 2b).

![Text-figure 2](image)

TEXT-Figure 2. Sephadex G-75 chromatograms of (a) corticotrophin-releasing factor (CRF-41) and (b) ACTH in an extract of phaeochromocytoma tissue (90 mg). Fractions (1.4 ml) were collected and measured for immunoreactivity by radioimmunoassay. The void volume ($V_0$), salt peak ($V_s$) and elution positions of synthetic human CRF-41 and ACTH(1–39) are arrowed.

![Text-figure 3](image)

TEXT-Figure 3. Reversed-phase high-performance liquid chromatograms of immunoreactive corticotrophin-releasing factor (CRF) in (a) an extract of phaeochromocytoma tissue (1.3 mg) and (b) a sample of plasma (10 ml) taken before the phaeochromocytoma was removed. The plasma was extracted using Vycor glass and the extract reconstituted in 0.1% (v/v) trifluoroacetic acid (0.3 ml) containing 0.1% (v/v) Triton X-100. Tumour extract (10 µl) and plasma extract (200 µl) were eluted on a gradient of 1-propanol (broken line) and fractions collected and assayed for CRF-41 by radioimmunoassay. The elution position of synthetic human CRF-41 is arrowed.

Bioassay

The index of precision ($\lambda$) of the assay was 0.11 ± 0.05 (mean ± s.d., $n = 10$). The ratio of bioactivity to immunoreactivity of CRF-41 was 0.9 ± 0.4 (12 dilutions of standard in four assays) and 0.7 for a sample of CRF-41 after RPHPLC. The ratio of biological to immunological activity for CRF-41 was calculated by measuring the amount of CRF-41 in the sample perfused through the cell column and the amount of ACTH released in response, and dividing the latter amount by the former. The cells released ACTH in response to CRF-41 and tumour CRF-41 in a dose-dependent manner, with a ratio of bioactivity to immunoreactivity of 1.3 ± 0.7 (three dilutions; Text-fig. 4).

Tumour CRF-41 incubated with antiserum lost 72% of its ACTH-releasing potential. Adrenocorticotrophin was not released above basal secretion in response to RPHPLC tumour fractions containing no CRF-41, and no ACTH was detected in samples applied to the column. Thus the release of ACTH from pituitary cells was a specific and dose-dependent
DISCUSSION

We have described a patient whose Cushing’s syndrome was resolved after excision of an adrenal pheochromocytoma. Increased concentrations of circulating CRF-41 and ACTH returned to normal after tumour excision, suggesting that the tumour was responsible for the secretion of both these peptides. Both CRF-41 and ACTH were found in large amounts in tumour extracts, and ACTH concentrations were within the range observed for tumours associated with the ectopic ACTH syndrome (Rees & Ratcliffe, 1974). However, because tumours may contain CRF-41 and ACTH and yet not be associated with Cushing’s syndrome (Bloomfield, Holdaway, Corrin et al. 1977; Suda et al. 1984), the detection of circulating bioactive forms of these peptides is a necessary prerequisite of any proposal for their role as etiological agents in Cushing’s syndrome. We found that this tumour secreted a compound indistinguishable from synthetic human CRF-41 which had the potential to stimulate the release of pituitary ACTH and thus cause Cushing’s syndrome. The tumour also contained ACTH which was chromatographically identical to human ACTH(1–39), and the gradient measured in blood across the tumour showed that this ACTH was secreted. No high molecular weight forms of ACTH were detected, despite the use of an antiserum which has detected such forms in other tumours. This tumour is therefore an exception to those described by Hale, Besser & Rees (1986), in which high molecular weight forms of ACTH are very commonly secreted by ectopic tumours.

The presence of ACTH in this tumour was established by RIA and chromatography but the tumour stained negatively for ACTH, despite using antiserum raised against synthetic ACTH(1–24) in both techniques. Inability of immunocytochemistry to stain peptides in tumours which secrete those peptides has been reported (Heitz, Kloppel, Polak & Staub, 1981; Coates, Doniach, Howlett et al. 1986) and is more pronounced in malignant tumours such as the small-cell lung carcinomas commonly associated with the ectopic ACTH syndrome. It has been proposed that failure to stain for ACTH in these tumours may be related to reduced storage concentrations of ACTH due to a high secretion rate (Coates et al. 1986). It appears, therefore, that negative staining for ACTH in a tumour is insufficient evidence to rule out ACTH biosynthesis. Two recent reports of tumours secreting CRF-41 in ectopic Cushing’s syndrome (Carey et al. 1984; Belsky et al. 1985) eliminated tumour ACTH as a contributory cause on the basis of negative tumour staining. Both these tumours were metastatic carcinomas and one report (Yamamoto et al. 1976) suggested that the existence of ACTH in carcinomas containing CRF-like bioactivity may be the rule rather than the exception. Our observations lead us to believe that no case of ectopic Cushing’s syndrome caused solely by the secretion of CRF-41 has yet been substantiated.

This patient fulfils all the main criteria for ectopic hormone production associated with a clinical syndrome: increased concentrations of CRF-41 in peripheral plasma and in the blood draining the tumour, with a return to normal concentrations following tumour excision; high concentrations of CRF-41 contained within the tumour tissue and secretion of CRF-41 from the tumour in a bioactive form. There are two principal mechanisms by which the release of CRF-41 and ACTH from this tumour may cause Cushing’s syndrome. Tumour CRF-41 may stimulate the release of pituitary ACTH, and/or tumour ACTH may act directly on the adrenal cortex. A less likely
but still plausible scenario is that tumour CRF-41 stimulates the release of tumour ACTH in an autocrine or paracrine manner. Secretion of ACTH from tumours obtained from patients with the ectopic ACTH syndrome was stimulated by crude extract of rat median eminence, suggesting that the mechanism of release of ACTH from these tumours is similar to that from the pituitary (Hirata, Yamamoto, Matsukura & Imura, 1975).

The presence of ACTH within this phaeochromocytoma means that this cannot be regarded as an unequivocal example of Cushing's syndrome due to ectopic CRF-41 production, but we have demonstrated that tumour CRF-41 has the capacity to at least contribute to the syndrome. With the availability of methods for more detailed biochemical analysis of CRF-41, this peptide may be detected more frequently in the ectopic ACTH syndrome.

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REFERENCES


DESCRIPTION OF PLATE

Section of tissue from a phaeochromocytoma immunohistochemically stained to demonstrate the presence of immunoreactive corticotrophin-releasing factor-41 as a dark peroxidase reaction product (× 100).