Pancreatic endocrine tumour producing growth hormone-releasing hormone associated with multiple endocrine neoplasia type I syndrome

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Abstract. We report the first documentation of GHRH production by a tumour associated with proven multiple endocrine neoplasia (MEN). A 30-year-old woman had hypoglycaemia, hyperparathyroidism, and pituitary adenoma with hyperprolactinaemia. Serum growth hormone elevation was attributed to hypoglycaemia but plasma GHRH was elevated. Subtotal pancreatectomy revealed multiple endocrine tumours and nesidioblastosis. Immunohistochemistry demonstrated insulin, glucagon, and somatostatin in several tumours. GHRH was localized in the largest one and was released from that tumour in vitro. Post-operative plasma GH returned to normal. Excess secretion of humoral factors by one tumour may stimulate growth of other tumours in MEN syndromes. The prevalence of GHRH in MEN-I tumours remains to be established.


GHRH-producing tumours have been associated with pituitary somatotrope hyperplasia and adenoma (Asa et al. 1984; Thorner et al. 1982), and it has been suggested that GHRH excess may play a role in somatotrope proliferation (Asa et al. 1984, 1985).

We report the immunohistochemical localization of GHRH in a pancreatic tumour of a patient with multiple endocrine neoplasia type I (MEN-I) syndrome, and the documentation of GHRH production by that tumour in vitro.

Materials and Methods

Case report

A 30-year-old woman had a 5-year history of episodes of lightheadedness and perspiration, occasionally associated with lapses of consciousness. The symptoms were precipitated by physical exertion or missing meals and responded promptly to food. The patient's weight had increased from 45 kg to 125.9 kg during the 5 years.
She also complained of episodes of amenorrhoea; she had two successful pregnancies 10 and 6 years ago. Family history was negative.

On physical examination, she was obese without features of hypercortisolism or hypersomatotropism. She was normotensive but had a tachycardia of 120/min associated with a blood sugar of 2.8 mmol/l documented 3.5 h after a meal. There were no corneal calcifications, neck masses, hepatomegaly or galactorrhoea. Visual fields were normal.

A diagnosis of insulin-producing tumour was made on the basis of the clinical data. Investigations revealed insulin/glucose ratios ranging from 0.52 to 2.94. Haemoglobin A1C was low at 3.2% (normal 4.5–6.5%). Abdominal computerized tomographic (CT) scans and coeliac angiography showed a 4.5 × 2.5 cm mass in the pancreas.

Other investigations showed elevated serum prolactin levels at 75.6–79.4 µg/l (normal < 20 µg/l) with blunted peak response to TRH of only 89.9 µg/l. Gonadotropins were normal but showed an exaggerated response to GnRH (LH 8.4 to 113 mIU/l, FSH 5.8 to 29.4 mIU/l). Blood GH was 32.0 µg/l (normal < 5.0 µg/l) during an episode of hypoglycaemia and 1.8 µg/l during normoglycaemia. The degree of glycaemia did not alter serum prolactin levels significantly. Thyroid indices and TSH were normal, as was the TSH response to TRH. A CT scan of the pituitary showed a tumour with a maximum height of 1.1 cm. The serum calcium was elevated at 2.90 mmol/l (normal 2.20–2.60 mmol/l) with a low phosphorus of 0.78 mmol/l (normal 0.85–1.45 mmol/l), albumin of 47 g/l (normal 35–50 g/l) and inappropriate PTH level of 5.7 pmol/l (normal with normal calcium 3.1–25.1 pmol/l). Urinary calcium was 6.2 mmol/day (normal < 6.2 mmol/day). Abdominal imaging studies showed an asymptomatic large right renal calculus.

The diagnosis was MEN-I syndrome with insulin-producing pancreatic tumour, hyperparathyroidism and pituitary tumour associated with hyperprolactinaemia.

The patient underwent 85%pancreatectomy. Post-operatively the blood glucose, insulin, and GH levels have remained normal. The patient was given a daily dose of 5 mg of bromocriptine post-operatively; the serum prolactin fell to within the normal range, and the pituitary tumour shrank markedly within 1 month. Subsequently, parathyroid exploration revealed hyperplasia of two parathyroid glands which were resected.

Morphologic studies

For light microscopy, tissue was fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Sections, 5 µm thick, were stained with haematoxylin and eosin (H&E), the periodic acid-Schiff (PAS) technique, and Grimelius silver method. For immunohistochemistry, the avidin-biotin-peroxidase complex (ABC) technique was used as described elsewhere (Asa et al. 1985).

Primary antisera were directed against the following antigens and were used in the dilutions specified: neuron specific enolase (NSE) (lot 015, Dako Corp, Santa Barbara, CA) 1:1000; chromogranin (lot LK2H10, prepared and donated by Dr R. V. Lloyd, University of Michigan, Ann Arbor, MI) 1:20; insulin and glucagon (lots 022 and 013-P, respectively, Dako Corp, Santa Barbara, CA) 1:1500 and 1:200 respectively; somatostatin (SR1H) (lot 655, prepared and donated by Dr S. Reichlin, Tufts University, Boston, MA) 1:2000; gastrin (lot 142068, Calbiochem-Behring, La Jolla, CA) 1:10 000; vasoactive intestinal peptide (VIP) (lot 028, prepared and donated by Dr J. Polak, Hammersmith Hospital, London UK) 1:10 000; growth hormone (GH) (lot 083-P, Dako Corp) 1:1000; prolactin (PRL) (lot AR-1-7, donated by Dr H. Friesen, University of Manitoba, Winnipeg, Manitoba) 1:2000; adrenocorticotropic hormone (ACTH) 1–39 (lot AFP-173P, prepared and donated by Dr A. F. Parlow, Pituitary Hormone Distribution Program, NIADDK, Bethesda, MD) 1:1000; α-subunit of pituitary glycoprotein hormones (lot 3/29/73, prepared and donated by Dr I. Kourides, Memorial Sloan-Kettering Cancer Center, New York, NY) 1:2000; growth hormone-releasing hormone 1–40 (GHRH) (batch G60, donated by Dr W. Vale, Salk Institute, San Diego, CA) 1:600; and corticotropin-releasing factor (CRF) (batch rC70, donated by Dr W. Vale, Salk Institute, San Diego, CA) 1:600. The immunological reaction was visualized with a solution of 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide. The specificity of the reactions was verified by replacing primary antisera with appropriate substitutes.

For electron microscopy, small pieces of tissue were fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and embedded in an Epon-Araldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and investigated with a Philips-410-LS electron microscope.

In vitro studies

Tissue for culture was placed aseptically in 10 ml of culture medium (CMRL-1969, Connaught, Willowdale, ONT) containing penicillin, 165 KU/l, and streptomycin, 200 mg/l (Sigma, St. Louis, MO). Fresh tissue was minced into small pieces and cell dispersion was performed by gentle agitation in an enzyme solution of collagenase (Worthington Biochemical Corp, Freehold, NJ), 1 g/l CMRL-1969, incubated at 37°C for 30–45 min with periodic trituration. Dispersed cells were harvested by centrifugation, resuspended in CMRL-1969 supplemented with 10% foetal calf serum (Gibco, Grand Island, NY). Viability was determined by trypan blue exclusion and was greater than 95%. Plastic multiwell incubation chambers (Linbro, Flow Laboratories Inc, McLean, VI) were coated with a thin layer of purified collagen (Vitrogen 100, Flow Laboratories Inc)
and air dried at room temperature. Dispersed cells were plated onto collagen-coated dishes, $10^4$ cells/2 ml per well. Cultures were maintained in a CO$_2$ incubator (Napco, Portland, OR) at 37°C in a humidified atmosphere of 95% air/5% CO$_2$. Cells were allowed to attach for 2–3 days. Subsequently, media were collected every 24 h. All media were stored in polyethylene vials at −20°C until processed for radioimmunoassay. At the termination of cultures, monolayers were lifted with a trypsin-EDTA solution (Gibco) 0.05% in saline for 10 min and harvested by centrifugation into pellets. An aliquot of the cell suspension was used to determine cell number using a haemocytometer; the proportion of cells collected was at least 90% of the number plated. The remaining tissue was prepared for electron microscopic examination as described above.

**Hormone assays**

GHRH was measured in unextracted plasma and tissue culture media by radioimmunoassay. The technique has been described previously as the 'Charlottesville assay' by Thorner et al. (1984). The antibody was directed against GHRH-(1-40)-horshoe crab haemocyanin conjugate (Leong/Thorner no. 716) and was used at a concentration of 1:200 000. Cross-reactivity of this antibody with GHRH-(1-44)-NH$_2$ is 90% with parallel displacement; no cross-reaction is detected with SRIH, substance P, cholecystokinin, secretin, VIP, gastric inhibiting peptide, glucagon, bradykinin, angiotensin I, TRH, oCRH, GnRH or neurotensin up to a concentration of 1000 µg/l. The intra-assay coefficient of variation at a level of 1 µg/l was 8.5% and at a level of 2 µg/l was 5.3. The sensitivity of the assay was 600 ng/l.

**Results**

**Morphologic findings**

On gross examination of the surgically removed pancreas, multiple well-demarcated tumours were identified; the largest measured $1.7 \times 2.0 \times 2.3$ cm. Histologic examination revealed numerous pancreatic endocrine tumours of variable size (Fig. 1a). Endocrine cells were identified with

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**Fig. 1.**

Photomicrographs of surgically resected pancreas containing (a) an endocrine tumour and (b) nesidioblastosis. (Haematoxylin-eosin stain; original magnification $\times 25$).
Photomicrographs of the largest endocrine tumour which contains immunoreactive GHRH (a) and insulin (b) in the cytoplasm of tumour cells. Amyloid deposition is prominent and is negative for both hormones. (Immunoperoxidase technique for GHRH (a) and insulin (b); original magnification × 64).

H&E and Grimelius silver, as well as NSE and chromogranin immunostains; there was widespread nesidioblastosis, proliferation of endocrine cells in small clusters, in peri-ductular connective tissue, budding off ducts, and interspersed in exocrine pancreas (Fig. 1b). Islets of Langerhans were large and irregular in contour; septal islets were also found. Approximately 10–20% of islets contained peliotic sacs, blood-filled cavities lacking endothelium; the features of peliosis in this case are described elsewhere (Kovacs et al. 1986).

Immunohistologic localization of hormones was performed on 12 tumours, all of which contained argyrophilic granules and were positive for NSE. Chromogranin was not demonstrated in any of the neoplasms. The largest tumour contained insulin and GHRH in the majority of tumour cells (Fig. 2); occasional cells were positive for glucagon and somatostatin. Nine tumours contained mainly glucagon and scattered cells which were positive for insulin and/or somatostatin; one small tumour contained only insulin and one contained insulin and glucagon. Amyloid deposition was prominent in the largest tumour and in the small insulin-containing adenoma. Immunostains were negative for gastrin, VIP, GH, PRL, ACTH, α-subunit and CRH.

Four tumours were examined by electron microscopy. They were composed of polygonal cells with well developed rough endoplasmic reticulum (RER), Golgi complexes and secretory granules of variable shape and electron density measuring 50–300 nm. The majority of cells comprising the largest tumour resembled B cells; the granules were membrane bound with needle-shaped or rectangular crystalloids. In addition, amyloid fibrils were deposited in the extracellular matrix. Two other tumours showed features character-
Ultrastructure of the largest pancreatic endocrine tumour. a) The majority of tumour cells have granules with crystalline electron dense cores (arrows), characteristic of B cells (electron micrograph; magnification × 10 200). b) Some tumour cells have secretory granules of variable size, shape and electron density which cannot be classified (electron micrograph; magnification × 7900).

Fig. 3.

istic of A cells and one small B cell tumour was identified. In all four neoplasms, occasional cells were not characteristic enough to classify them into distinct cell types.

In vitro findings
Portions of the two largest tumours were maintained in culture for four weeks. GHRH was detectable in culture media from the tumour which contained immunohistochemical positivity for GHRH; the values ranged from 1.62 to 3.08 ng/10⁴ cells per 24 h. GHRH was not detected in media from the other tumour in culture nor in control media which were not incubated with tissue.

Morphologic investigation of cultured tissues revealed endocrine cells resembling those seen in surgically resected tumours. Tissue from the largest tumour was composed of polygonal cells with scattered RER profiles and small Golgi complexes; some cells had characteristic crystalline granules of B cells (Fig. 3a), whereas others had granules of variable size (50–300 nm) and electron density, occasionally with small, eccentric, dense cores and peripheral lucent halos (Fig. 3b). Cells of the second tumour resembled A cells.

In vivo plasma GHRH assays
Plasma immunoreactive GHRH levels ranged from 1.03 to 1.28 µg/l. Normal levels are reported to be less than 100 ng/l (Thorner et al. 1984), a value which would have been undetectable in our assay. There was no correlation between GH and GHRH levels, and plasma GHRH showed no response to insulin-induced hypoglycaemia during triple-bolus dynamic testing.
Discussion

The patient reported here has evidence of the classical MEN-1 syndrome with multiple pancreatic endocrine tumours, hyperparathyroidism, and a pituitary adenoma. In addition, the surgically resected pancreas contained nesidioblastosis. The proliferation of pancreatic endocrine cells in this case is consistent with that previously described in association with MEN-1 (Thompson et al. 1984).

Immunoreactive GHRH was documented in the largest pancreatic tumour using immunohistochemistry. Release of GHRH by this tumour was confirmed by documentation of hormone in tissue culture medium. Morphologic examination of cultured tissues showed that cells containing secretory granules and resembling those in the surgical tissue were maintained in vitro.

The clinical significance of GHRH release by the pancreatic tumour is unclear. Elevated blood levels of GHRH provide evidence for secretion of that hormone by the tumour in vivo. However, our patient had no clinical stigmata of acromegaly. Her serum GH levels were elevated on several occasions, but this was attributed to marked hypoglycaemia. Thus it is uncertain whether hypoglycaemia or GHRH released from the pancreatic tumour in excess was responsible for GH hypersecretion in this case. Her GHRH levels were lower than those found previously in patients with acromegaly due to GHRH production by extracranial tumours (Ch’ng et al. 1985; Thorner et al. 1984) with the exception of one patient in whom peripheral blood contained values similar to those found in our patient (Schulte et al. 1985); it may be that the amount of GHRH secreted was insufficient to affect GH release. Alternatively, the immunoreactive GHRH may have been biologically inactive. A third possibility is that production of somatostatin by several pancreatic tumours may have inhibited GHRH-induced stimulation of somatotropes and prevented the development of acromegaly. This suggestion has been put forth previously to explain the lack of clinical evidence of GH excess associated with GHRH-containing tumours (Asa et al. 1984, 1985).

This represents the first report of GHRH production by a tumour associated with a fully developed MEN-1 syndrome. Some patients with GHRH-secreting tumours have had evidence of hyperfunction of several endocrine tissues (Berger et al. 1984; Ch’ng et al. 1985); other patients with GHRH-containing neoplasms may also represent undiagnosed cases of MEN in whom multiple lesions have not yet become evident. Hormone production by one tumour may play a role in the growth and secretion of other neoplasms in syndromes of MEN; GHRH excess may be important in the development of pituitary adenomas in patients with MEN-1 (Asa et al. 1985; Sönksen et al. 1976). Tumours containing GHRH have been associated with pituitary somatotrope hyperplasia and adenoma (Asa et al. 1984; Thorner et al. 1982). The factors underlying pituitary adenomas producing PRL are unknown. It has been shown that GHRH increases both GH and PRL secretion by bihormonal pituitary adenomas in vitro (Ishibashi & Yamaji 1985) and in patients with ectopic GHRH secretion, removal of the GHRH-secreting tumour restores prolactin to normal (Scheithauer et al. 1984; Thorner et al. 1982); it is therefore possible that GHRH excess may play a role in the development of several types of pituitary adenomas. The relationship between GHRH production and the pituitary tumour in our patient remains to be clarified.

GHRH production by tumours in patients with MEN-I may be a frequent phenomenon which has remained unrecognized; moreover, its association with pituitary adenomas may help to clarify one of the problems this syndrome presents. It is therefore important to determine the prevalence of GHRH-containing tumours in the MEN-I syndrome, and their association with pituitary adenomas.

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