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CLINICAL CASE SEMINAR

Prolonged Gastrointestinal Transit in a Patient with a Glucagon-Like Peptide (GLP)-1- and -2-Producing Neuroendocrine Tumor

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Neuroendocrine tumors overexpressing the proglucagon-derived peptides have been associated with severe constipation. The relationship between two of the intestinal proglucagon-derived peptides, glucagon-like peptide (GLP)-1 and -2, and delayed gastrointestinal transit, was characterized in a patient with a neuroendocrine proglucagon-derived peptide tumor. A 60-yr-old female presented with intractable constipation and intermittent vomiting. Gastric, oral-ileal and colonic transit times, and plasma hormone levels were determined before tumor resection. Expression of the proglucagon-derived peptides by the tumor was determined by immunohistochemistry, Northern blot analysis, HPLC, and RIA. Oral-cecal transit was more than 3 h, and a barium follow-through study showed dilated and thickened folds with most of the barium concentrated in the ileum at 24 h; residual barium was identified in the colon at 14 d post ingestion. Circulating levels of GLP-1 and -2 were 300- to 400-fold elevated compared with levels in normal human subjects. Normal bowel function was restored by tumor resection. Consistent with the elevated plasma hormone levels, the tumor was found to express the proglucagon gene, and immunoreactive GLP-1 and -2 were detected by both immunohistochemistry and RIA. Overexpression of glucagon-like peptide-1 and -2 is associated with markedly prolonged gastrointestinal transit in humans. These findings are consistent with a role for these peptides in the regulation of gastrointestinal motility. (*J Clin Endocrinol Metab* 87: 3078–3083, 2002)

THE ILEAL BRAKE is a neurohormonal feedback mechanism that delays gastric and intestinal transit time, thereby enhancing nutrient digestion and absorption in the proximal small intestine and preventing nutrient overflow into the distal gut (1). A number of gut peptides have been identified as possible effectors of intestinal motility (2), including the glucagon-like peptides (GLP), GLP-1 and GLP-2, and peptide YY (PYY). All three of these hormones are synthesized in the intestinal L cell from their prohormone precursors, proglucagon and proPYY, respectively (3, 4). Ingestion of nutrients, and of fat and carbohydrates in particular, increases the release of GLP-1, GLP-2, and PYY into the circulation (5–8). Infusion of GLP-1 and GLP-2 has also been shown to prolong the rate of gastric emptying and delay intestinal transit time (9–11). Additionally, antagonism of the GLP-1 receptor increases gastric emptying, further suggesting a physiological role for GLP-1 in the ileal brake (12, 13). Injection of PYY also potently inhibits gastrointestinal motility, whereas immunoneutralization of PYY causes acceleration of intestinal transit (7, 14, 15). Other biological actions of these peptides include stimulation of glucose-dependent insulin secretion and inhibition of glucagon release by GLP-1 (16–18), and stimulation of intestinal growth by GLP-2 (17–19).

An association between overexpression of the GLPs and the ileal brake was first made in a patient with severe constipation and the presence of an ectopic enteroglucagon-producing tumor (20, 21). It is now recognized that enteroglucagon is comprised of two proglucagon-derived peptides, glicentin and oxyntomodulin, and that these peptides are co-synthesized with GLP-1 and GLP-2 in the intestinal L cell (Fig. 1). However, the existence and functions of GLP-1 and GLP-2 were not known at the time of this original case report. In the present report, we describe the presence of markedly delayed gastrointestinal transit in a patient with an ectopic tumor producing both GLP-1 and GLP-2.

Patients and Methods

Patient

A 60-yr-old white female presented for assessment of progressive intractable constipation of 5 yr duration. Bowel actions occurred every 10–14 d but only with ingestion of magnesium citrate or after an enema. The patient also experienced vomiting of solid food about three times weekly, but her weight remained stable. Physical examination was unremarkable. Investigations after referral, undertaken after the patient provided informed consent, included normal hematology, biochemistry, and a normal colonoscopy with the exception of a few diverticula in the sigmoid colon. A barium upper gastrointestinal and follow-through study showed dilated and thickened mucosal folds; progression of the

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Abbreviations: GLI, Glucagon-like immunoreactivity; GLP, glucagon-like peptide; GLUTag, glucagon-SV40-large T antigen cells; IRG, immunoreactive glucagon; PYY, peptide YY; STC-1, secretin tumor cells.

barium column was also delayed and at 24 h post ingestion most of the barium remained concentrated in the ileum with residual barium identified in the colon at 14 d post ingestion. A lactulose breath hydrogen study indicated delayed oral-cecal transit with the breath hydrogen peak observed more than 3 h post ingestion. A 99^{M} technetium-sulfur colloid gastric emptying study showed more than 20% residual activity after 4 h. The patient refused a gastroscopy. A diagnosis of pseudo-obstruction was made; treatment with cisapride (40 mg three times daily) and subsequently clarithroymcin (250 mg three times daily) was without effect.

To further characterize the constipation, investigations after referral included a colonoscopy, which was normal with the exception of sigmoid diverticula; the cecum and ileum were not visualized because of the presence of impacted stool, notwithstanding extensive preparation. Biopsies of the colon showed crypts with epithelial atypica, mild crypt distortion, and moderate fibrosis of the lamina propria. Crypt epithelial atypia consisted of some nuclear enlargement with mild stratification and mucous depletion; there was no evidence of crypt budding or proliferation. The epithelial changes showed no evidence of dysplasia and were consistent with reactive changes occurring secondary to chronic colonic stasis.

An abdominal CT scan identified a 6.5 × 5.5 × 7.3 cm solid mass in the left abdomen, just to the left of the aorta with the center at the level of the inferior pole of the left kidney; the liver was normal. An indium^{111}-labeled octreotide scan demonstrated a large focus of activity corresponding to the lesion identified on the CT scan; there was no evidence of metastasis. A needle aspirate of the mass was consistent with a neuroendocrine neoplasm. The patient underwent resection of the tumor with an uneventful postoperative course. The bowel habit normalized after surgery and at 2 yr follow-up, the patient remained asymptomatic with one formed bowel motion daily, a normal CT scan of the abdomen and negative octreotide scan. No changes in body weight or height after surgery were noted (weight, 69 kg; height, 1.65 m). Random blood glucose levels before and after surgery were also not different (6.2 and 5.4 mM, respectively).

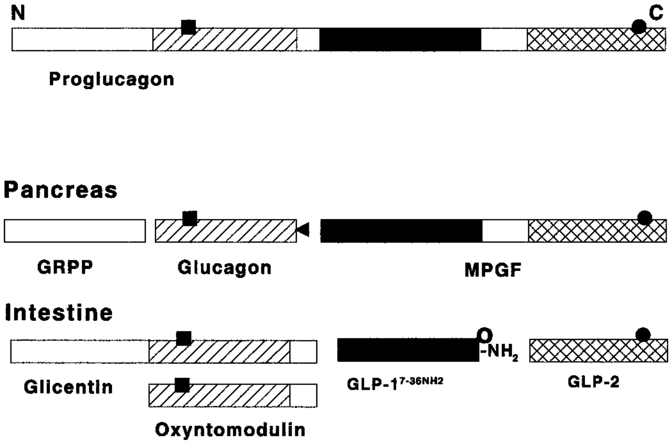

FIG. 1. Schematic of proglucagon and the proglucagon-derived peptides. Posttranslational processing in the pancreas liberates glucagon, whereas intestinal-specific processing results in the biosynthesis of glicentin, oxyntomodulin, GLP-1, and GLP-2 (GRPP, glicentin-related pancreatic peptide; MPGF, major proglucagon fragment). Recognition sites for antisera used in RIAs are indicated: ■, GLI; ◀, IRG; ○, GLP-1^{36NH2}; ●, GLP-2.

Sample collection

Blood samples from the patient were collected by venipuncture into a 10% volume of Trasylol:EDTA:Diprotin A [5000 kallikrein-inhibitory units/ml (a general protease inhibitor; Miles Canada, Etobicoke, Canada): 1.2 mg/ml: 0.1 mM [an inhibitor of dipeptidylpeptidase activity (6); (Sigma, St. Louis, MO)]. Plasma was collected by centrifugation, and stored at −70 C. After resection, the tumor was also stored at −70 C.

Immunohistochemistry

For electron microscopy, small pieces of the tumor were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded ethanols, processed through propylene oxide, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a Philips CM 100 electron microscope, as previously described (22).

For immunohistochemistry, tumor pieces were fixed in formalin and embedded in paraffin. Sections were immunostained for adrenocorticotropic hormone, α-subunit, calcitonin, calcitonin gene-related peptide, cholecystokinin, chromogranin, CRH, β-endorphin, enkephalin, gastrin, glial fibrillary acidic protein, GLP-1, GLP-2, glucagon, GHRH, insulin, neurofilaments, pancreatic polypeptide, PYY, S100, secretin, serotonin, somatostatin, synaptophysin, tyrosine hydroxylase and vasoactive intestinal peptide, as previously described (23). The immunostaining was detected with the streptavidin-biotin-peroxidase complex technique.

Northern blot analysis

Pieces of tumor from opposite ends of the mass were extracted in guanidium isothiocyanate for Northern blot analysis. Blots were probed using cDNA probes for proglucagon, proPYY, pro-cholecystokinin and 18S rRNA, as described (24–27). As positive controls, RNA from secretin tumor cell (STC-1) and glucagon-SV40-large T antigen (GLUTag) cells (both derived from mouse enteroendocrine tumors), and InR1-G9 cells (a hamster pancreatic islet A cell line), was also analyzed by Northern blot, as previously reported (26, 27).

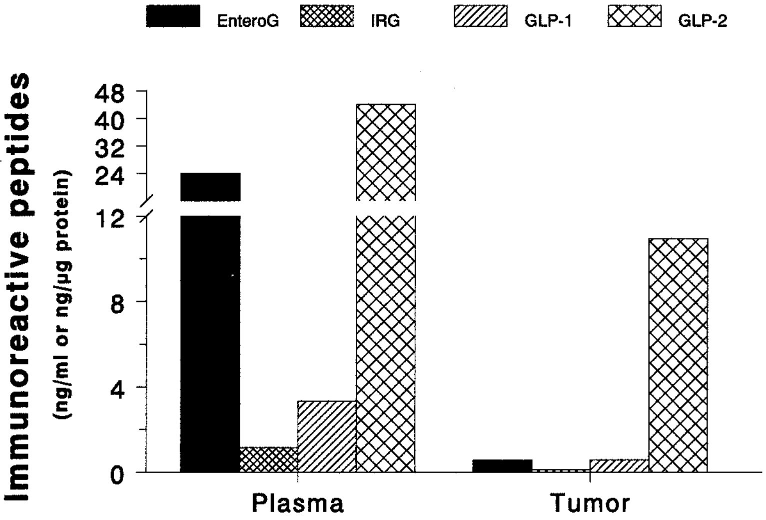

FIG. 2. Levels of immunoreactive proglucagon-derived peptides in plasma and the tumor. Samples were assayed for enteroglucagon (glicentin + oxyntomodulin), IRG (pancreatic glucagon), GLP-1^{36NH2} and mid-sequence GLP-2, and data are expressed as nanograms per milliliter for plasma and nanograms per microgram protein for the tumor.

Peptide analysis

Peptides in plasma and in a section of tumor were extracted by reversed-phase adsorption to a C18 silica Sep Pak (Waters Associates, Milford, MA), as previously reported (3, 6). Glicentin, oxyntomodulin, and glucagon were separated by reversed-phase HPLC on a C18 μBondaPak column (Waters Associates), using a gradient of 25–62.5% solvent B [solvent A = 1% TFA (pH adjusted to 2.5 with diethylamine); solvent B = 80% acetonitrile] (3, 27, 28). Different forms of GLP-1 were separated by HPLC using a gradient of 45–68% solvent A (solvent A = 0.1% H_3PO_4 and 0.3% triethylamine; solvent B = 40% solvent A and 60% acetonitrile) (3, 27, 28). GLP-2 and related peptides were separated by HPLC using a gradient of 30–60% solvent B (solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in acetonitrile) (3, 6). All fractions were dried *in vacuo* before RIA.

Peptides were measured by RIA, as previously described (3, 6, 27, 28). The antigenic sites recognized by each antiserum are indicated in Fig. 1. In brief, RIA for glucagon-like immunoreactivity (GLI; glicentin, oxyntomodulin and glucagon) was carried out using antisera K4023 (Biospacific, Emeryville, CA), whereas RIA for immunoreactive glucagon (IRG) was conducted using antiserum 04A (Dr. R. Unger, Dallas, TX); synthetic glucagon$^{1-29}$ was used as the standard for both RIAs. In the same plasma samples, the difference between GLI and IRG represents enteroglucagon (*e.g.* glicentin + oxyntomodulin). RIA for GLP-1 was conducted using antiserum GLP-1$^{7-36NH2}$ (Affinity Research Products Ltd., Mamhead, UK), which recognizes the C-terminal sequence of GLP-1, including both biologically active and inactive forms of the peptide, and synthetic GLP-1$^{7-36NH2}$ as the standard. RIA for GLP-2 was conducted using an antiserum (UTTH-7) that recognizes the mid-sequence of both biologically active and inactive forms of GLP-2, and synthetic GLP-2$^{1-33}$ was used as the standard.

Results

Characterization of plasma proglucagon-derived peptides

Plasma levels of enteroglucagon (glicentin + oxyntomodulin), glucagon, GLP-1 and GLP-2 were found to be 6.87, 0.33, 1.01, and 11.23 nmol/liter, respectively, in the patient (Fig. 2). These levels were 10- to 400-fold greater than those found in normal fasting humans (range = 7–35 pmol/liter (5, 6). HPLC analysis of the plasma (Fig. 3) demonstrated the presence of oxyntomodulin and glucagon, as well as the biologically active GLP-1$^{7-36NH2}$ and GLP-1$^{1-33}$. An inactive metabolite of GLP-2, GLP-2$^{3-33}$, was also detected in the plasma, consistent with studies in normal humans (6). No immunoreactive peptide could be found eluting in the position as glicentin, although a number of unidentified peaks of GLI were observed with retention times shorter than those of the known proglucagon-derived peptides that are detectable with this assay. No immunoreactive peptide was detected eluting with the same retention time as proglucagon.

Tumor histology and characterization of proglucagon-derived peptides

The tumor was highly vascularized with widespread angioinvasion. The cells were epithelial in morphology, with moderate-to-abundant pink cytoplasm and bland nuclei with finely dispersed chromatin and occasional inconspicuous nucleoli and areas of differentiation with small cell morphology (Fig. 4A). Electron microscopy revealed moderately to well differentiated endocrine cells with well-developed endoplasmic reticulum and Golgi complexes, as well as numerous round secretory granules of variable size and electron density. Immunohistochemical analysis revealed the presence of immunoreactivity for synaptophysin (Fig. 4B), GLP-1 (Fig. 4C) and GLP-2 (Fig. 4D), as well as PYY and pancreatic polypeptide (not shown). The cells were negative for all other markers tested.

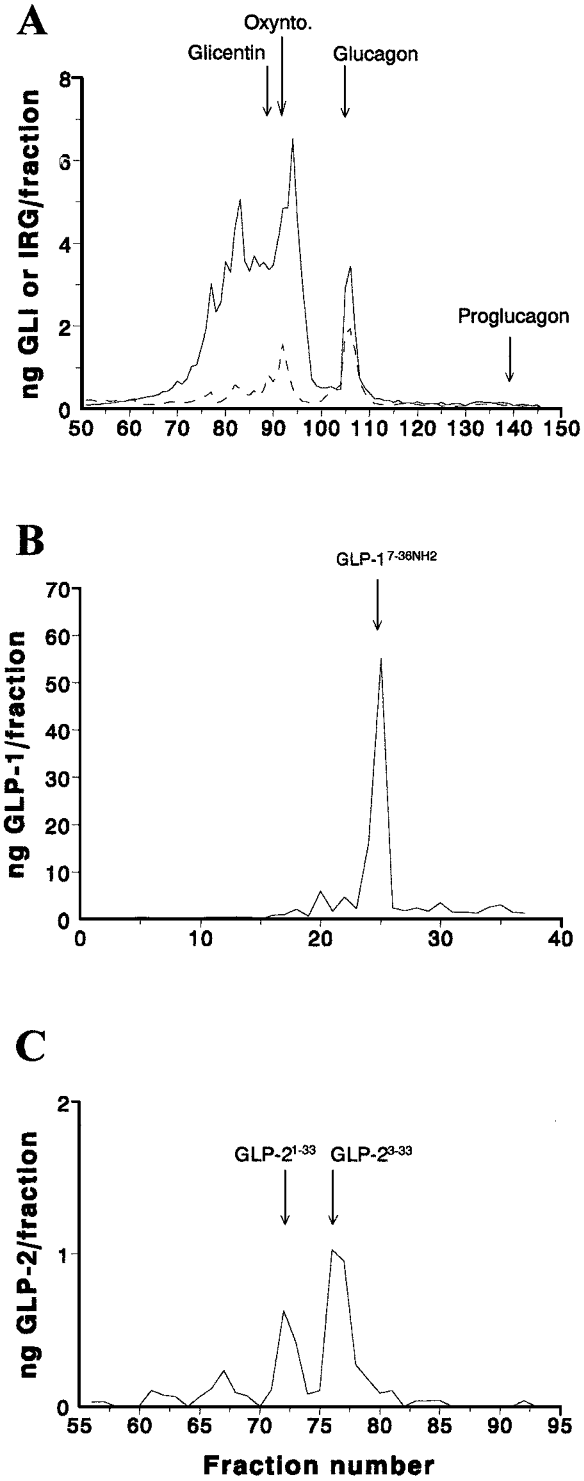

FIG. 3. HPLC and RIA analyses of plasma for: A, GLI (glicentin + oxyntomodulin + glucagon; *solid line*) and IRG (glucagon; *dashed line*); B, GLP-1; and C, GLP-2. The known elution positions of glicentin, oxyntomodulin, glucagon, proglucagon, GLP-1$^{7-36NH2}$, GLP-2$^{1-33}$, and GLP-2$^{3-33}$ are indicated by the *arrows* (3, 6).

Northern blot analysis of the tumor revealed the presence of mRNA transcripts for proglucagon and PYY, but not cho-

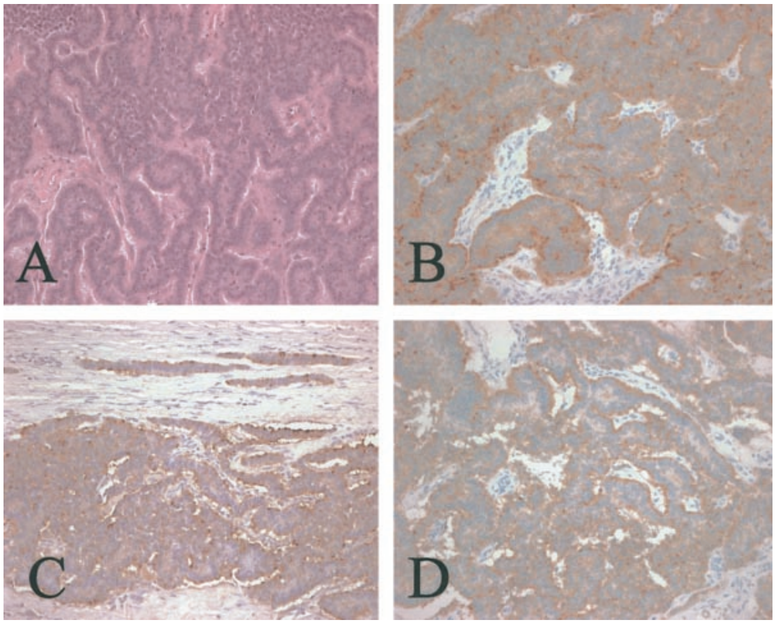

FIG. 4. Immunochemistry of tumor sections, showing (A) H&E staining, as well as positive staining (*brown*) for (B) synaptophysin, (C) GLP-1, and (D) GLP-2.

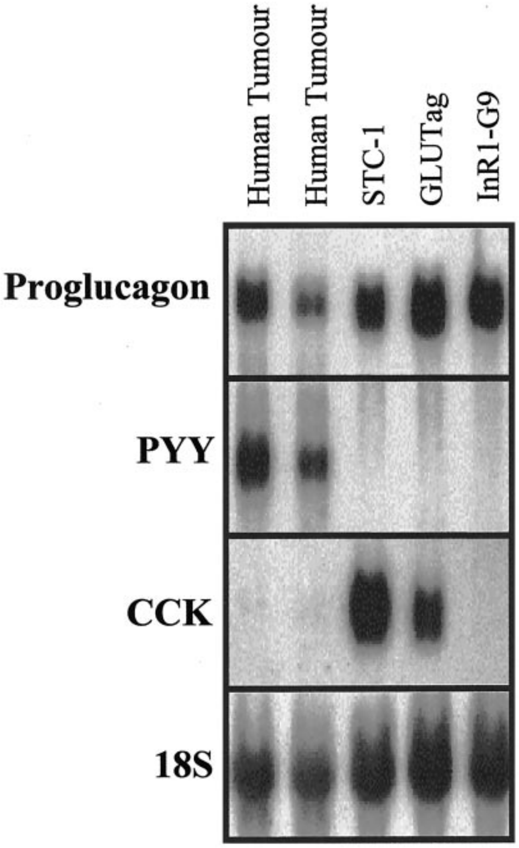

FIG. 5. Northern blot analysis of RNA from two pieces of the tumor, and from STC-1, GLUTag, and InR1-G9 cells (controls). 18S RNA was determined as a control for equal loading of the gel.

lecystokinin (Fig. 5). Proglucagon mRNA transcripts were also detected in STC-1, GLUTag, and InR1-G9 cells, and cholecystokinin, but not PYY, mRNA was found in the STC-1 and GLUTag enteroendocrine cell lines, consistent with previous reports (27, 28).

Consistent with the elevated plasma levels of proglucagon-derived peptides in the patient, enteroglucagon (glicentin + oxyntomodulin), IRG, GLP-1, and GLP-2 were all detected in a sample of the tumor (0.16, 0.04, 0.18, and 2.79 nmol/mg protein, respectively; Fig. 2). HPLC analysis of the tumor sample revealed an intestinal profile of proglucagon processing, with significant peaks of immunoreactivity eluting with the same retention times as oxyntomodulin and GLP-$1^{7-36NH2}$; no peaks of glicentin or glucagon were detected (Fig. 6). HPLC analysis of the GLP-2-related peptides revealed the presence of three main peaks, the first of which corresponded to the elution position of synthetic GLP-2^{1-33}; the identity of the other two peaks remains to be established.

Discussion

This report describes a patient with a proglucagon-expressing tumor that secreted an intestinal profile of proglucagon-derived peptides in association with prolonged gastric emptying, delayed small intestinal transit and intractable constipation; these abnormalities were fully reversed after complete resection of the tumor. These findings are consistent with and extend an earlier report describing a patient with an enteroglucagonoma and severe constipation (20, 21) and are in accord with the delayed intestinal transit described recently in one additional patient, who succumbed to a metastatic neuroendocrine tumor also producing GLP-1, GLP-2, and PYY (29). Thus, elevated circulating levels of GLP-1, GLP-2, and/or PYY appear to be associated with marked prolongation of gastric emptying and small and large intestinal motor activity. These findings are also consistent with studies conducted in experimental animal models demonstrating roles for all three of these peptides in the regulation of gastrointestinal motility (1, 7, 9–15).

The levels of both GLP-1 and GLP-2 were elevated by 300- to 400-fold in the patient compared with normal fasting humans (5, 6). However, plasma GLP-2 concentrations were higher than those of GLP-1 in the patient, which may reflect

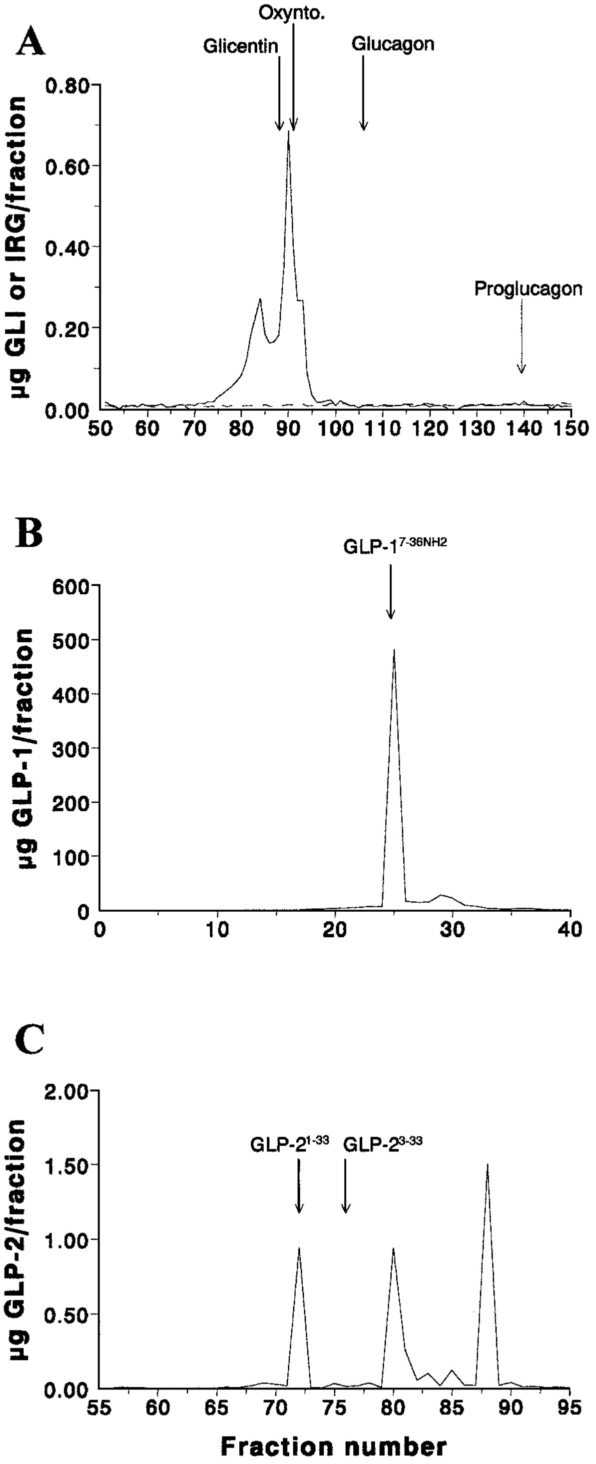

FIG. 6. HPLC and RIA analyses of the tumor for: A, GLI (glicentin + oxyntomodulin + glucagon; *solid line*) and IRG (glucagon; *dashed line*); B, GLP-1; and C, GLP-2. The known elution positions of glicentin, oxyntomodulin, glucagon, proglucagon, GLP-1$^{7-36NH2}$, GLP-2$^{1-33}$, and GLP-2$^{3-33}$ are indicated by the *arrows* (3, 6).

either the relatively greater levels of GLP-2 contained within the tumor, or different rates of clearance of the two peptides in the circulation (30, 31). It has been reported that GLP-2 has similar potency to GLP-1 in the inhibition of hypoglycemia-induced antral motility in pigs (11). However, in humans, administration of identical doses (4–5 nmol/kg body weight) of GLP-1 (16) or GLP-2 (32) has been reported to cause nausea and gastrointestinal discomfort only in the patients receiving GLP-1. These results therefore suggest that GLP-1 may be more potent than GLP-2 in the suppression of gastrointestinal motility in humans.

The processing of proglucagon in the tumor was found to be similar to that of the normal intestinal L cell, leading to production of oxyntomodulin, GLP-1 and GLP-2. It was somewhat unexpected, however, that the tumor did not contain any glicentin. Proglucagon processing in the L cell is mediated by the enzyme prohormone convertase 1 (3). As oxyntomodulin may be synthesized by secondary processing of glicentin (Fig. 1), the lack of tumor-associated glicentin suggests that either glicentin was rapidly generated and secreted, or proglucagon processing in this tumor by PC1 was more efficient than that normally found in the L cell, resulting in the cleavage of glicentin to form oxyntomodulin. Altered proglucagon processing in endocrine L cell tumors has also previously been noted in transgenic mice harboring proglucagon-producing intestinal tumors (27, 33), although in those tumors, aberrant production of pancreatic-type glucagon was found to occur.

Although the present tumor did not contain any glucagon, slightly elevated levels of glucagon were found in the circulation. Similarly, the proportion of circulating oxyntomodulin was also markedly elevated compared with that in the tumor (Fig. 2). These findings suggest that the secretion of glucagon and oxyntomodulin was increased in this patient and/or their clearance from the circulation may have been reduced. Inappropriately elevated plasma levels of glucagon were also noted in the first reported patient with an enteroglucagonoma (20, 21), indicating that this may be a common feature of such tumors.

The tumor was found to contain mRNA transcripts for PYY, and immunoreactive PYY was detected by immunohistochemical analysis of tumor sections. These findings were not unexpected, as PYY has been colocalized to the same intestinal L cells that produce GLP-1 and GLP-2 (34). Furthermore, these peptides appear to be cosecreted by the normal L cell (35). Additionally, it is well known that dedifferentiated gastroenteropancreatic endocrine cell tumors are often plurihormonal in nature (36).

In addition to its effects on intestinal transit, GLP-2 has been found to be an intestinal growth factor (17–19). Administration of GLP-2 to normal rodents for 10 d stimulates marked increases in small and large bowel wet weight. This growth occurs concomitant to increased crypt cell proliferation and decreased villus cell apoptosis (19, 37) and leads to an increased capacity for nutrient digestion and absorption (38). Consistent with these findings, small intestinal biopsies from other patients with enteroglucagonomas have shown the presence of elongated villi (20, 21, 29, 39). It was not possible to obtain small intestinal biopsies from the present patient; however, enlarged mucosal folds were noted during a small bowel follow-through. In contrast, the epithelial hyperplasia found in colonic biopsies were consistent with reactive changes secondary to colonic stasis; hence, it is difficult to ascribe a specific role to one or more of the tumor-derived peptides in the regulation of large bowel mucosal epithelial proliferation.

Finally, the recent report of another patient with an unresectable GLP-1- and GLP-2-producing tumor (29) provides some opportunity for comparison with the present patient. In the other patient, circulating levels of GLP-1 and GLP-2 were found to be 0.24 and 0.82 nmol/liter, respectively, and the patient was reported to move his bowels weekly, unaided. By contrast, in our patient, plasma levels of these peptides were 4- to 14-fold greater (1.01 and 11.23 nmol/liter, respectively), and the patient moved her bowels bimonthly, and then, only with the use of potent laxatives or enemas. Because the tumor was amenable to complete resection in our patient, her symptoms fully abated postoperatively. Thus, in these two patients, there appears to be a relationship between the magnitude of circulating levels of the GLPs and the severity of the motility impairment. Consistent with this suggestion, a dose-dependent relationship has been reported between GLP-1 and gastric symptoms (nausea and vomiting) in normal human volunteers (16).

In summary, the results of the present study provide evidence of roles for GLP-1 and GLP-2 in the regulation of the ileal brake and in colonic motor activity, whereby overexpression of these intestinal hormones leads to marked gastrointestinal stasis. It remains to be established whether these peptides may also contribute to other clinical conditions associated with either impaired or enhanced intestinal motility.

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