Large Glucagon-Like Immunoreactivity in a Primary Ovarian Carcinoid

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A primary ovarian carcinoid composed of both trabecular and strumal types was studied by histochemical, immunocytochemical, and biochemical techniques. High contents of glucagon, secretin, and calcitonin were demonstrated in the tumor homogenate. All of the tumor cells, irrespective of histologic type, showed properties of argyrophilia and neurosecretory granules on electron microscopy. Glucagon-producing cells were positive in trabecular carcinoid by immunoperoxidase techniques. Bio-Gel P10 gel filtration showed that the molecular weight of major immunoreactive glucagon in tumor was 20,000. It migrated faster than true glucagon after polyacrylamide gel electrophoresis. No clinical symptoms of glucagonoma developed.

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OVARIAN CARCINOID is a germ cell tumor,[1] and three types, *i.e.*, insular, strumal, and trabecular carcinoids, were recognized from microscopic analyses.[2–4] Strumal carcinoid is composed of thyroid parenchyma together with a carcinoid component, which is usually of the trabecular type.[4–6] Neuroendocrine cells in pituitary, thyroid, pancreas, lung, adrenal, and gastrointestinal tract have common features of argyrophilia and secretion of polypeptide hormones or amines, and are regarded as an amine precursor uptake and decarboxylation (APUD) cell system.[7] Neuropeptide hormone production by ovarian carcinoids has been examined by the immunocytologic methods.[8] This report describes primary ovarian carcinoid of trabecular and strumal types producing glucagon, secretin, and calcitonin by radioimmunoassay of the tumor homogenates. Glucagon was also demonstrated immunocytologically and further characterized by gel filtration and polyacrylamide gel electrophoresis. The case presented here has provided interesting information, in that high-molecular-weight immunoreactive glucagon was produced in the carcinoid tissue without any clinical syndrome.

Case Report

A 35-year-old woman, gravida 5, para 2, was hospitalized because of acute lower abdominal pain, nausea, and vomiting. Her menstruation was irregular, and amenorrhea occurred 2 months before hospitalization. No signs of carcinoid syndrome, such as flushing, diarrhea, cardiac murmur, hypertension, and pedal edema, and glucagonoma syndrome, such as necrolytic migratory erythema, weight loss, anemia, and stomatitis, had developed. Physical examination revealed a right pelvic mass. A solid mass, 7 × 5 × 4 cm, was found in the right ovary at laparotomy under the diagnosis of torsion of the ovarian tumor. There was no ascites or adhesion, nor was there evidence of tumor spread. The uterus, left ovary, and gastrointestinal tract were normal. Right salpingo-oophorectomy was performed. The postoperative course was uneventful. Tumor markers, such as alpha-fetoprotein and carcinoembryonic antigen (CEA) in serum and human chorionic gonadotropin (HCG) in urine, were all within normal limits. No preoperative glucagon determination in plasma nor glucose tolerance test was made.

Materials

The tumor tissue was fixed in 10% formalin and embedded in paraffin. Others were stored at −20°C. Specimens for electron microscopic study were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4; 0.15 mol/l) and postfixed in 1% osmium tetroxide. After dehydration in ethanol of increasing concentrations, they were embedded in epoxy resin (Quetol 812, Prom Nisshin EM, Tokyo). Thick and ultrathin sections were prepared by an LKB ultratome. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and examined with a JEOL 100C electron microscope. The tumor was homogenized with polytron in 2 volumes of 0.9% saline. After centrifugation at 10,000 × *g* for 30 minutes, the supernatant was assayed for various neuropeptide hormones. Glucagon radioimmunoassay kit

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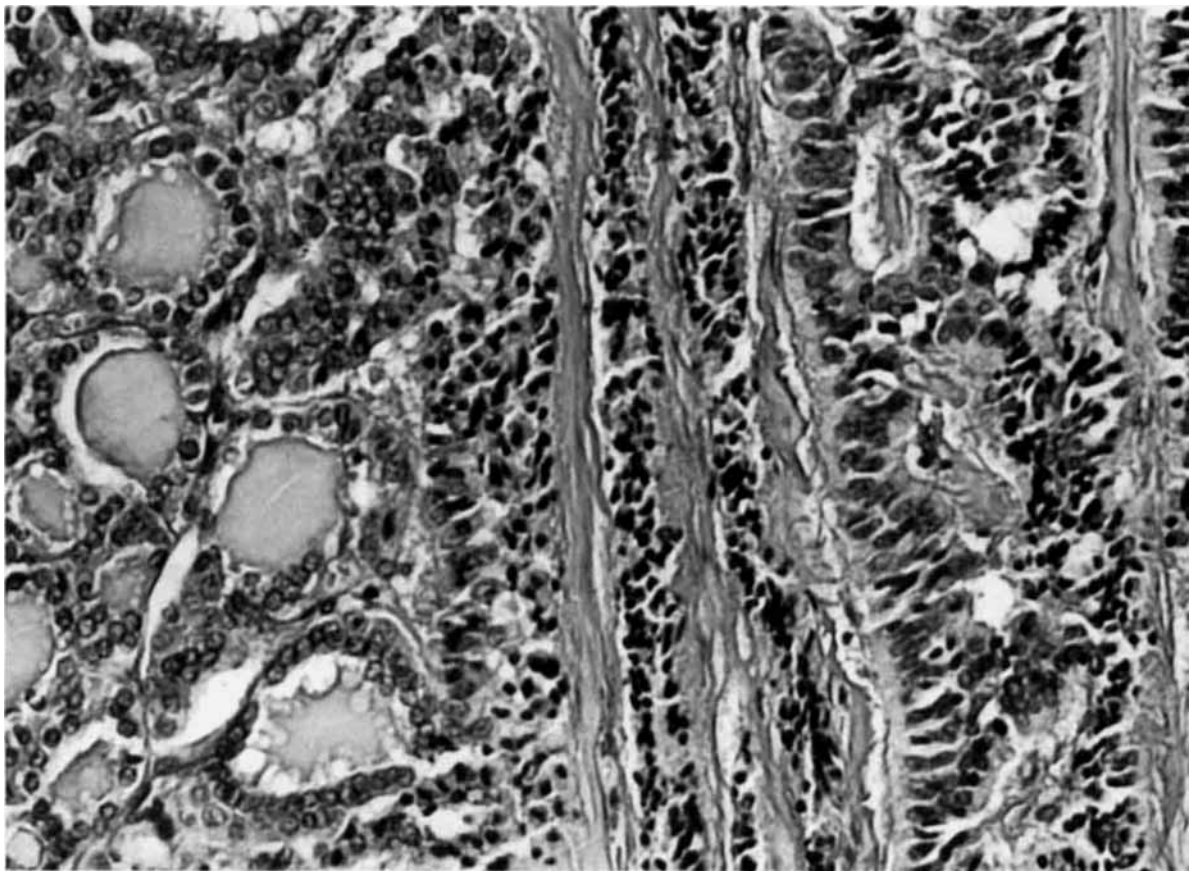

FIG. 1. Strumal and trabecular patterns in carcinoid (H & E, ×40).

was obtained from Daichiradioisotope (Tokyo, Japan). Bio-Gel P10 was obtained from Bio-Rad (Richmond, VA), polyacrylamide from Seikagaku Kogyo (Tokyo), marker proteins for calibration of the molecular weight from Schwarz/Mann (Orangeburg, NY), and adrenocorticotropic hormone (ACTH)$^{1-24}$ as marker protein from Organon (Oss, Holland). Glucagon as marker protein for gel electrophoresis was obtained from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Methods

Glucagon, secretin, gastrin, calcitonin, somatostatin, ACTH, thyroglobulin, and thyroxine were determined by radioimmunoassay. Antiserum OAL-123, which recognize the 19-29 sequence of glucagon, was used in glucogan radioimmunoassay.[9] The specificity of antiserum OAL-123 has been shown to be almost identical to glucagon specific antiserum 30K.[9] Benzamidine was used as a protease inhibitor in radioimmunoassay. The degradation of the tracer by acid ethanol extract was not observed. Serotonin was determined by high-performance liquid chromatography (HPLC).

Glucagon Extraction

The crude glucagon extract was prepared by homogenizing 5 g carcinoid tissue in 25 ml acid alcohol at 0°C in polytron, and the supernatant was precipitated in ether alcohol according to the method of Kenny.[10]

Gel Filtration

The acid alcohol fraction was dissolved in 2.5 ml glycine buffer pH 8.8. The solution was applied to a column (2 × 77 cm) of Bio-Gel P10 equilibrated with the same buffer. Fractions of 3 ml were collected, and immunoreactive glucagon was determined by radioimmunoassay. The column was calibrated with chymotrypsinogen, cytochrome c, ^{125}I glucagon, ACTH$^{1-24}$, and bacitracin.

Electrophoresis

Disc polyacrylamide gel electrophoresis was carried out with 6.5% polyacrylamide gel in Tris-glycine buffer pH 8.3 as described by Smith and Rutenburg.[11] Gels were either stained with Amido black or divided into

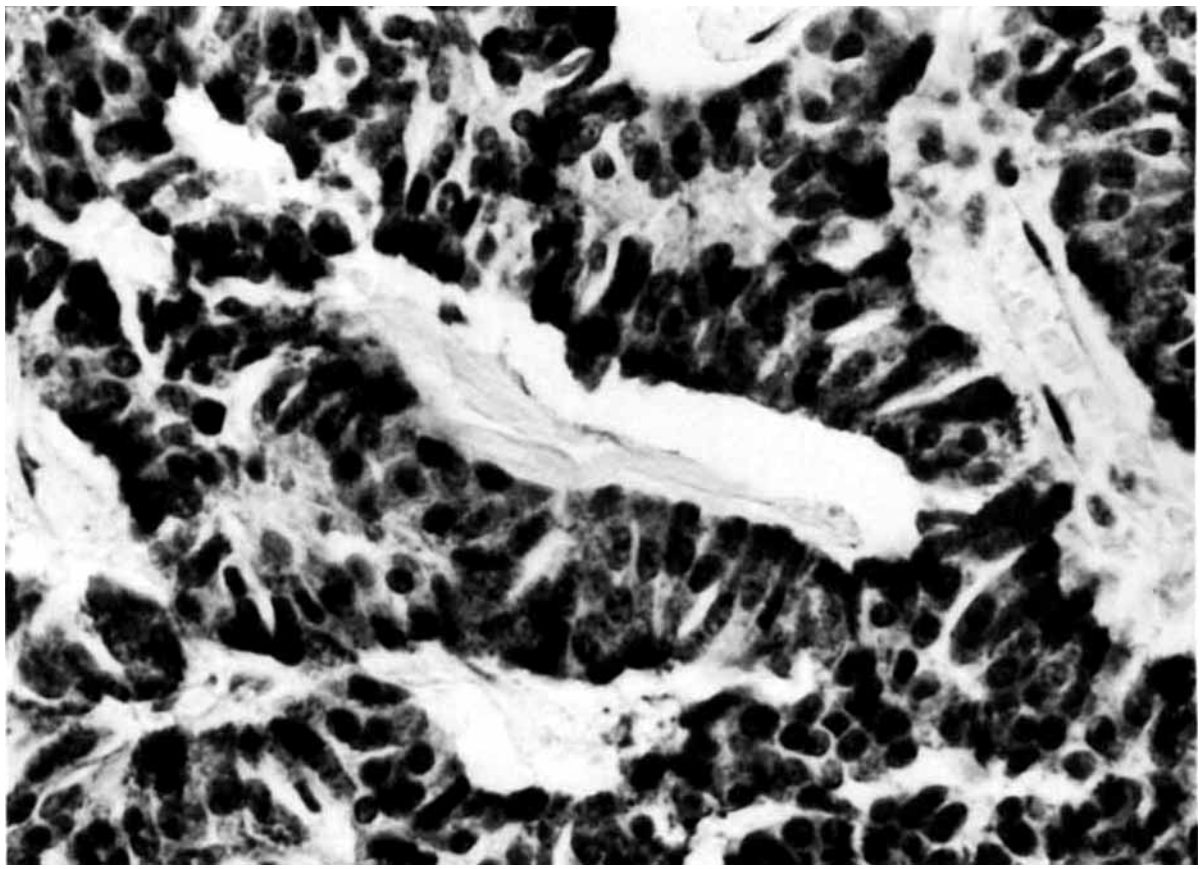

FIG. 2. Argyrophil granules within the cytoplasms of both trabecular and strumal carcinoid (Glimelius, ×100).

0.3-cm sections for glucagon assay. Each section was placed in a tube containing 0.5 ml of 0.2 mol/l glycine buffer pH 8.8 in 0.1% bovine serum albumin (BSA) for 12 hours. Immunoreactive glucagon in the extract was assayed for each fraction.

Staining

The histologic sections were stained for argyrophil granules by the Glimelius argyrophil silver stain method[12] and for glucagon by the immunoperoxidase technique using PAP Kit K512, DAKO (Santa Barbara, CA). As a negative control, nonimmunized rabbit serum was used instead of antiglucagon antibody.

Results

Pathologic Findings

The tumor was smooth on the external surface and firm, homogenous, and yellow on the cross-section. It was composed of an intimate mixture of trabecular and strumal types of carcinoid as shown in Figure 1. The cytoplasm of both types was eosinophilic, and argyrophil granules were conspicuous in the cytoplasmic rims of cells in either types of carcinoid (Fig. 2). The nuclei were typically oblong. Prominent nucleoli and mitosis were rare. The immunoperoxidase reaction test for glucagon was performed. A positive reaction was obtained from the brown granules in the trabecular type (Fig. 3). Electron microscopic examination of the sections from carcinoid showed that both types of cells were rich in electron-dense secretory granules (Fig. 4). Location of the endosecretory granules was usually observed at the end of the cell adjacent to the capillary. The granules were 160 to 320 nm in size and contained a dense core within the limiting membrane. Some of the granules showed a clear halo around the dense core.

Neuropeptide Hormones in Tumor

As shown in Table 1, high levels of glucagon, secretin, and calcitonin were observed in the homogenate fraction. Thyroglobulin and thyroxine were not observed in spite of the presence of follicular structure. Serotonin, which is a cause of carcinoid syndrome, was also not observed.

Gel Filtration of Carcinoid Extract

To confirm the presence of glucagon in carcinoid, a glucagon-rich fraction was obtained by the method of

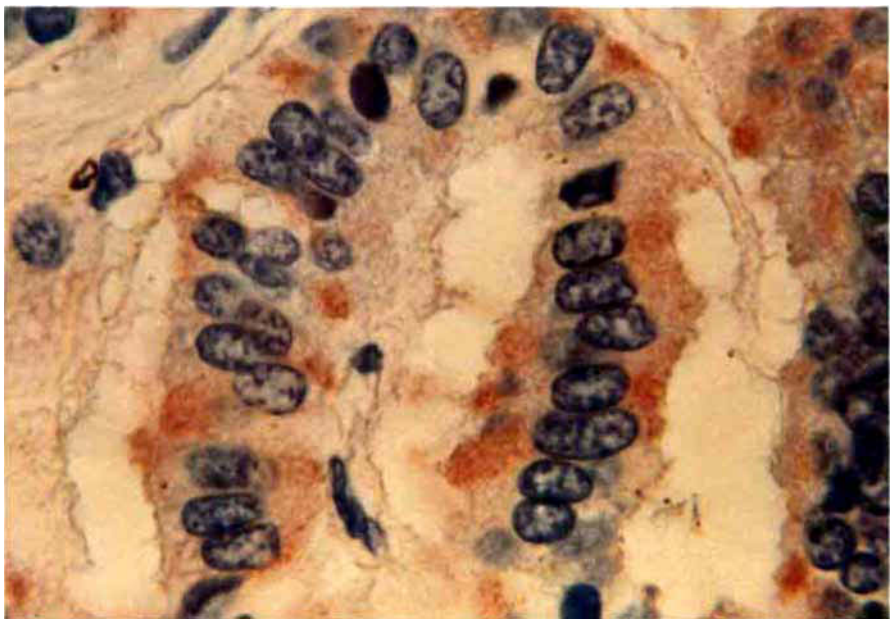

FIG. 3. Intracytoplasmic granules of glucagon in trabecular carcinoid (immunoperoxidase, ×100).

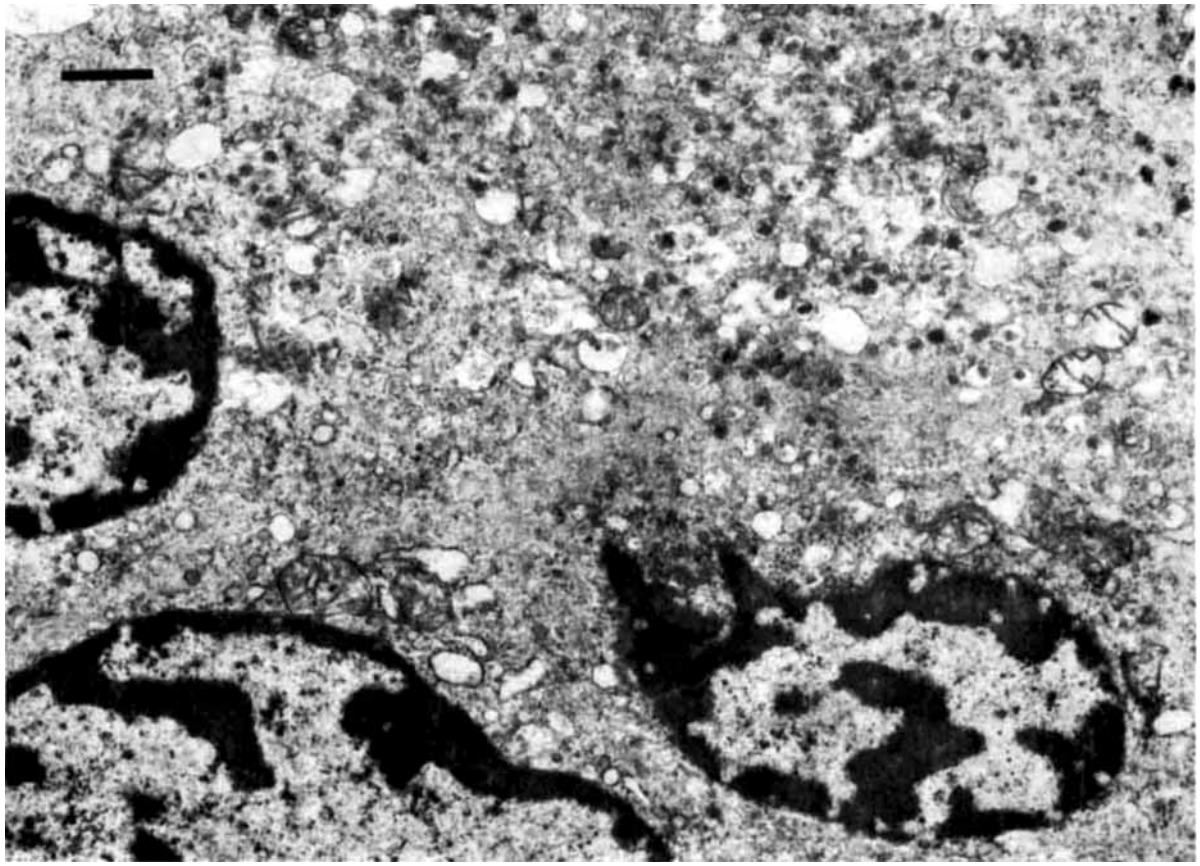

FIG. 4. Electron micrograph of cells having dense-core secretory granules. Bar indicates 1 μm.

Kenny[10] and subjected to Bio-Gel P10 gel filtration. The elution pattern of immunoreactive glucagon is shown in Figure 5. Two peaks of immunoreactive glucagon were observed. More than 80% of immunoreactive glucagon (large glucagon-like immunoreactivity) appeared in the molecular weight of 20,000. The residual fraction

of immunoreactive glucagon appeared in the zone of ^{124}I glucagon. A proglucagon peak of molecular weight (MW) 9000 was not found in the tumor extracts.

Electrophoresis

To determine whether self-aggregation of glucagon during Bio-Gel P10 gel filtration might account for the high molecular weight fraction of immunoreactive glucagon, polyacrylamide gel electrophoresis was attempted. Two major peaks of immunoreactive glucagon were also observed, as shown in Figure 6. The major fast-moving fraction constituted approximately 70% of total immunoreactive glucagon. The minor slowly moving fraction corresponded to true glucagon and constituted 30% of total immunoreactive glucagon. The major fast-moving fraction was supposed to be large glucagon-like immunoreactivity.

Discussion

The presence of argyrophil granules by Glimelius staining and neurosecretory granules by electron microscopy clearly indicates the neuroendocrine nature of strumal carcinoid. The presence of glucagon was shown by radioimmunoassay of the homogenate fraction and by the immunocytologic method. Sporrong *et al.*[8] examined the incidence of various polypeptide hormone production in carcinoid by the immunocytologic method, and reported the presence of glucagon in 9 cases from 81 primary ovarian carcinoids. However, the character of glucagon produced by carcinoid has never been examined previously. We partially purified glucagon by the method of Kenny[10] and examined the molecular weight and electrophoretic pattern of glucagon. In our case, 80% of total immunoreactive glucagon in the carcinoid extracts was Mr 20,000 after Bio-Gel P10 gel filtration. Proglucagon of Mr 9000 was not found. Approximately the same amount of immunoreactive glucagon migrated faster than true glucagon in polyacrylamide gel electrophoresis. Big plasma glucagon also migrated faster than true glucagon on celluluse acetate electrophoresis.[13] These observations show the predominance of large glucagon-like immunoreactivity in ovarian carcinoid compared to pancreas and pancreatic glucagonoma. Patients with glucagonoma usually present a distinctive clinical syndrome and elevation of both Mr 9000 and Mr 3500 glucagon in plasma or tumor extracts.[13-17] Most of glucagon immunoreactivity of dog, rat, bovine, and human pancreas was true glucagon.[18]

TABLE 1. Hormone Contents in Carcinoid Tissue

Hormones	Contents (ng/g weight)
Glucagon	10,390
Gastrin	26
Secretin	2078
Somatostatin	4.7
Calcitonin	2130
Thyroxine	260
Thyroglobulin	Negative
ACTH	65
Serotonin	1.9

ACTH: adrenocorticotropic hormone.

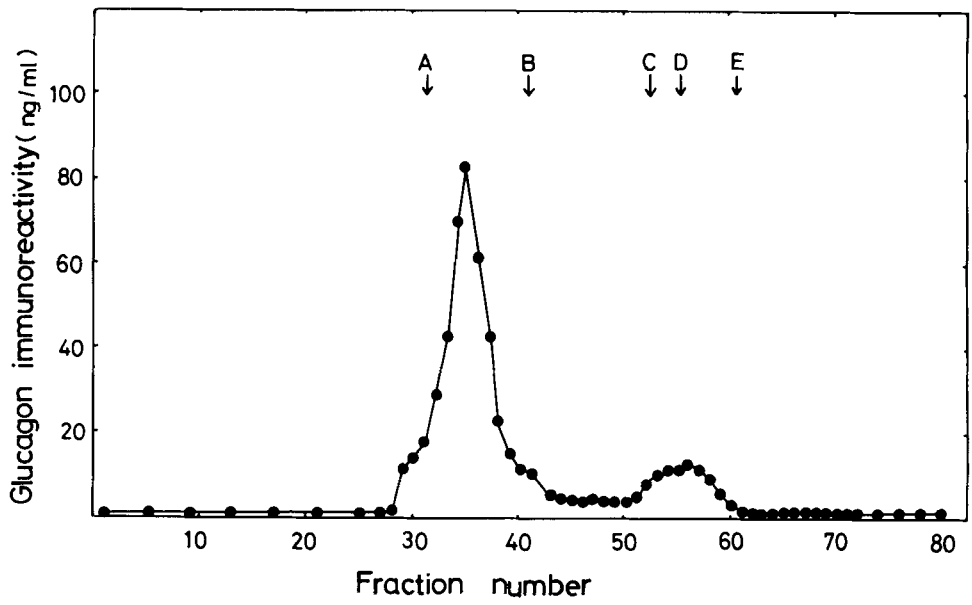

FIG. 5. Bio-Gel P10 gel filtration. Experimental conditions are described in the Materials and Methods section. Acid alcohol fraction containing 2042 ng immunoreactive glucagon was applied on a column. Immunoreactive glucagon recovered was 1752 ng. Elution positions of calibration materials: (A) chymotripsinogen (25,000); (B) cytochrome c (12,400); (C) ^{125}I-glucagon (3550); (D) $ACTH^{1-24}$ (2930); and (E) bacitracine (1450).

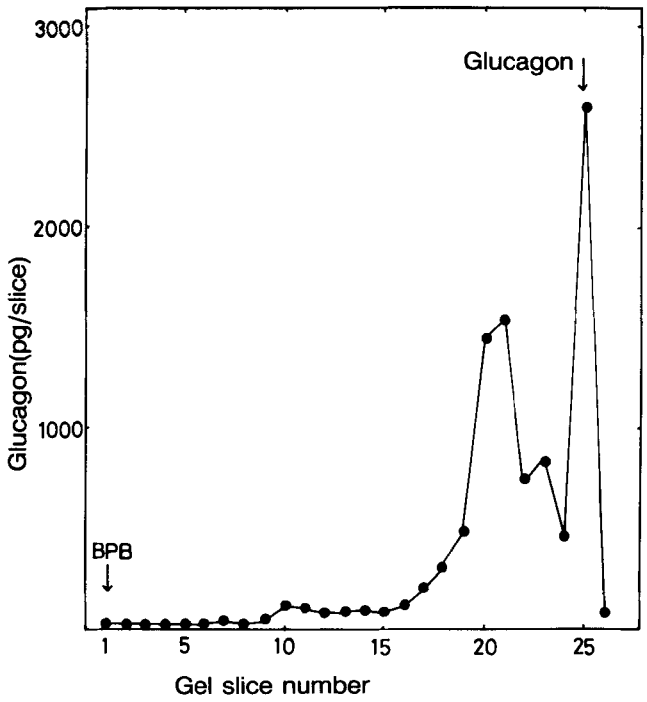

FIG. 6. Polyacrylamide gel electrophoresis. Experimental conditions are described in the Materials and Methods section. Acid alcohol fraction containing 10,200 pg immunoreactive glucagon was applied on polyacrylamide gel; 9800 pg (96%) were recovered after extraction from gel slices. True glucagon of 5 μg was also electrophoresed in the same conditions as marker protein and stained by Amido black. Arrow indicates the position of electrophoretic mobility of true glucagon.

Then the defect of processing enzyme from proglucagon to glucagon was suggested in ectopic glucagon-producing tumor compared to pancreas or pancreatic glucagonoma.

Large glucagon-like immunoreactivity, Mr 20,000 in our case nearly corresponds to preproglucagon of Mr 18,000, which has been determined from either pulse-chase experiment[19] or glucagon gene analysis.[20] The homogenates of carcinoid also contained secretin and calcitonin. There are several regions of a high degree of amino acid sequence homology between glucagon and secretin. The content of secretin in carcinoid may then be overestimated by the cross-reaction of glucagon after radioimmunoassay of secretin. We did not purify and characterize calcitonin and secretin from carcinoid because of the limited amount of carcinoid tissue. The presence of calcitonin and the absence of thyroxine and thyroglobulin in our case suggest that the follicular structure of carcinoid is not a true thyroidal differentiation but, rather, of C-cell origin. However, the production of thyroglobulin in strumal carcinoid has also been reported.[21,22] Further characterization of large glucagon-like immunoreactivity and processing enzyme to yield bioactive glucagon in the ovarian carcinoid tissue remains to be elucidated.

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