A New Bioactive Form of Human Calcitonin

Paul H. Tobler, Maximilian A. Dambacher, Walter Born, Philipp U. Heitz, René Maier, and Jan A. Fischer

Research Laboratory for Calcium Metabolism, Departments of Orthopedic Surgery (Balgrist) and Medicine, University of Zurich, 8008 Zurich, Switzerland [P. H. T., M. A. D., W. B., J. A. F.]; and Department of Pathology, University of Basel [P. U. H.] and Pharmaceuticals Division, Ciba-Geigy, Ltd., 4002 Basel, Switzerland [R. M.]

ABSTRACT

Distinct immunoreactive forms of calcitonin (CT), extracted with 2 M acetic acid from two pancreatic tumors, were characterized and identified by gel permeation chromatography and by reverse-phase high-performance liquid chromatography. The extracted CT forms were compared to CT obtained from medullary thyroid carcinoma and from normal thyroid glands, and were, furthermore, analyzed in a rat hypocalcemic bioassay. On gel filtration analysis, two broad peaks coeluting with synthetic human CT-(1-32) and extracted dimeric CT, respectively, were found in variable amounts. An acetonitrile gradient high-performance liquid chromatography system revealed two to three predominant CT peaks. Biologically active monomeric and dimeric CT and the biologically inactive sulfonamide form of human CT-(1-32) have been identified. Moreover, we have detected for the first time a new biologically active CT-like component which was most prominently recognized in a benign pancreatic tumor.

INTRODUCTION

hCT is a 32-amino acid polypeptide hormone (M, 3418) of the thyroid gland that causes hypocalcemia by inhibition of the release of calcium from bone (3, 23). Increased production of CT by medullary carcinoma of the thyroid has been amply documented. In 1973, Sizemore et al. (31) detected raised concentrations of immunoreactive CT in the plasma of 6 of 8 patients with the Zollinger-Ellison syndrome and mentioned the possibility of ectopic CT secretion from non-β-islet cell tumors of the pancreas. One of their patients showed no postmortem evidence of medullary carcinoma of the thyroid. Schwartz et al. (30) have found elevated plasma levels of CT in 42% of the patients with pancreatic tumors. Immunocytochemical examination of endocrine pancreatic tumors revealed the presence of CT among other polypeptide hormones such as adrenocorticotropic hormone, gastrin, β-endorphin, and somatostatin (25). The findings have been confirmed by other groups of investigators (2, 8, 9, 16, 19). Abe et al. (1) demonstrated superimposable immunodiffusion curves of extracts of pancreatic tumors and of synthetic hCT-(1-32) suggesting immunological similarities. On gel permeation chromatography of tumor extracts (15) and plasma (10, 26, 29) of patients with pancreatic tumors, several immunoreactive CT components have been recognized. In view of the limited resolution of peptides on gel filtration analysis, different CT forms cannot be identified.

With the advent of HPLC analysis, hCT-(1-32) and its sulfonamide form have been recognized in thyroid extracts and in the plasma of normal subjects and of patients with MTC (17, 35, 36). Moreover, large molecular weight (12 to 25 k dalton) components of CT have been detected in cell extracts and in the incubation medium of a CT-producing epidermoid bronchial carcinoma cell line (21, 24).

In the present report, we have for the first time recognized different CT forms in tissue extracts of patients with endocrine pancreatic tumors and MTC by HPLC analysis, and have, furthermore, detected a new biologically active CT-like peptide.

MATERIALS AND METHODS

Patients. Two female patients with endocrine pancreatic tumors secreting CT ectopically, a 52-year-old with a benign tumor and a 74-year-old with a malignant tumor, were compared to 2 patients (a 32-year-old female and a 38-year-old male) with CT producing MTC. Plasma CT levels were raised in the patients with pancreatic tumors (27.0 and 97.4 ng equivalent/ml; normal range, <0.05 ng equivalent/ml) and with MTC (2.0 and 2000 ng equivalent/ml). In the pancreatic tumor patients, plasma levels of calcium were increased (3.44 and 2.89 mmol/liter; normal range, 2.07 to 2.42 mmol/liter) and those of parathyroid hormone, 5 and 18 ng equivalent/ml, were in the normal range (6 to 40 ng equivalent/ml). The benign pancreatic tumor was surgically removed, plasma levels of CT became undetectable, and plasma calcium was normalized. The patient with the malignant pancreatic tumor died of pulmonary embolism, and the diagnosis was established at autopsy.

In one of the MTC patients, plasma calcium (2.37 mmol/liter) was in the upper normal range, and parathyroid hormone (62 ng equivalent/ml) was increased. This patient belonged to a kindred with multiple endocrine adenomatosis type 2 (32) and, moreover, presented hyperplastic parathyroid glands and pheochromocytomas. The other MTC patient was inoperable because of generalized metastases as confirmed at autopsy.

Peptides. Synthetic hCT-(1-32) (monomeric form), extracted purified dimeric hCT from MTC, and synthetic human parathyroid hormone-(1-34) have been donated by W. Rittel, Ciba-Geigy AG, Basel, Switzerland; [3H]hCT-(1-32), by R. Wade, Ciba-Geigy Ltd., Horsham, Great Britain (6); and extracted bovine parathyroid hormone-(1-84), by the Medical Research Council, Great Britain. Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO, and human serum albumin was from Behring Werke, Hoechst Pharma AG, Zurich, Switzerland.

Preparation of Tissues and Extraction. Tumor tissue obtained at surgery was, after determination of wet weight, snap-frozen in liquid nitrogen shortly after excision and stored at −70° until extraction. The frozen tissues were homogenized in 2 M acetic acid, and the clear supernatant of the centrifuged homogenates were extracted by adsorption of the peptides on octadecasily silica (C18-Sep-Pak cartridges; Waters Assoc., Milford, Mass.) according to a method described previously (36). Lyophilized extracts were dissolved in 1 ml 0.1 M acetic acid (pH 2.6) and applied to different columns of Bio-Gel P-150 (100 to 200 mesh; Bio-Rad Laboratories, Richmond, Calif.), 1.6 × 100 cm, have been used by ascending flow (flow rate, 3.8 to 5.2 ml/hr)
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at 4°C in 0.2 M ammonium acetate, pH 4.7, containing 0.5 g bovine serum albumin per liter, and fractions were collected in 1.9- to 2.6-ml volumes. The void volume (V₀) was estimated by the elution position of the largest-molecular-weight proteins determined spectrophotometrically at 280 nm and the salt volume (Vₛ) was estimated with Na⁺⁺. The elution position of CT components, designated kₑ, is defined as:

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\frac{(\text{elution volume of the substance} - V₀)}{(Vₛ - V₀)}
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Tracer amounts of radioiodinated human serum albumin, bovine parathyroid hormone-(1-84), human parathyroid hormone-(1-34), hCT-(1-32), and Na⁺⁺ were added to each extract as calibrating substances. Radioactivity was measured in an automatic γ-well spectrometer (Model MR 252; Kontron AG, Zurich, Switzerland). Recovery of immunoreactive CT ranged from 60 to 80%.

HPLC. The HPLC system consisted of a programmer (Model 420; Altex, Berkeley, Calif.) and 2 pumps (Model 110 A; Altex). Samples were injected via a septumless valve (Model 7125; Rheodyne, Berkeley, Calif.) fitted with a 0.5-μl injector loop. Acetonitrile (HPLC-grade S), methanol (HPLC-grade), and trifluoroacetic and heptafluorobutyric acid (both sequencer grade) were obtained from Rathburn Chemicals (Walkerburn, Great Britain). Doubly glass-distilled water from the deionized laboratory water supply was pumped through a 0.22-μm filter (Millipore/Continental Water Systems, Bedford, Mass.) and subsequently through a C₁₈-Radial-PAK cartridge (Waters Assoc.) to remove traces of organic impurities. Reverse-phase HPLC was performed on C₁₈-Nucleosil (10 μm, 250 x 4.6 mm; Macherey-Nagel GmbH, Düren, West Germany) columns under either isocratic conditions or by gradient elution as described previously (13, 36). Extracts containing 150 ng to 10 μg immunoreactive CT and tracer amounts of [³²P]hCT-(1-32) and its sulfoxide (30,000 to 60,000 dpm) not interfering in the radioimmunological determinations were injected in a volume of 0.5 ml. Fractions were collected in siliconized tubes; 0.2 to 0.3-ml aliquots were analyzed for ³²P radioactivity by liquid scintillation spectroscopy (Model MR 300; Kontron AG) in Rotiszint 22 (Carl Roth KG, Karlsruhe, West Germany). The remaining fractions were analyzed radioimmunologically. Peak fractions of several extractions of all numbered CT components (Charts 2 and 3) were pooled and recirculated in the same systems. The purified CT peptides were analyzed in the hypocalcemic rat bioassay. The recoveries of immunoreactive hCT on HPLC were 63.7 ± 7.2% (range, 36.7 to 105.0%) under isocratic conditions and 80.9 ± 4.2% (range, 62.3 to 95.0%) in the gradient elution system.

Radioimmunoassays. CT was determined in a homologous hCT-(1-32) assay described previously (12, 13). The antibodies (goat-6A obtained on Day 143) were used at a dilution of 1/100,000. They are predominantly directed to determinants located in the COOH-terminal parts of the hCT-(1-32) molecule. The specificity of the antibodies used has been studied previously (13). The CT-like structure of the corticotropin-β-lipotropin precursor (13, 22) and salmon CT-(1-32) (18) were not recognized radioimmunologically. Of several polypeptide hormones tested, only the corticotropins, -(1-13), -(1-24), and -(1-39), and β-endorphin, oxytocin, and vasopressin inhibited the immunological reaction in at least 105-fold higher concentrations than those of hCT-(1-32); all of

Chart 1. Fractionation of immunoreactive hCT of tissue extracts by gel permeation chromatography on Bio-Gel P-150. Radioiodinated human serum albumin ([¹³¹]HSA), bovine parathyroid hormone - (1 - 84) ([¹³¹]bPTH - (1-84)), synthetic human parathyroid hormone-(1-34) ([¹³¹]hPTH-(1-34)), [¹³¹]HCT-(1-32), and Na⁺⁺ were added as calibrating substances to extracts containing 150 ng to 10 μg immunoreactive CT (hCT-D) and of hCT-(1-32); 1.6 x 100-cm column; 0.2 M ammonium acetate (pH 4.7) and bovine serum albumin (0.5 mg/ml) as eluent; reversed flow (flow rate, 4.2 ml/hr; 2.1-ml fractions). A, benign pancreatic tumor; B, malignant pancreatic tumor; C, medullary carcinoma of the thyroid; D, lymph node metastasis of a medullary carcinoma of the thyroid; E, normal thyroid; F, extracted dimeric CT from MTC before (•) and after (○) incubation with 1 M ammonia at 45°C for 1 hr.
these peptides had retention times of less than 4 min in the isocratic HPLC system (Chart 3) used in the present study and, therefore, did not interfere with our radioimmunological CT measurements. All of the CT components analyzed and plasma showed parallel immunodilution curves to hCT-(1-32) (11) (not shown).

Immunoreactive parathyroid hormone was estimated as described previously (34).

Immunohistochemistry. Tumor tissue was fixed in liquid formaldehyde or Bouin’s fluid. Sections (5 μm) were stained with hematoxylin and eosin, van Gieson’s stain, and the periodic acid-Schiff reaction. Samples of the tumors were immediately quenched in melting isopentane at −180°, lyophilized overnight in a thermolectric freeze-dryer at −40°, vapor-fixed at 60° for 3 hr with formaldehyde vapor, and embedded in paraffin. Deparaffinized sections (5 μm) were incubated with the antibodies to hCT used in the radioimmunological measurements of CT, but at a dilution of 1/100 using the unlabeled antibody enzyme method (33). For immunocytochemical controls, adsorption of the primary antibody with 10−12 to 10−4 μ hCT-(1-32) was carried out at 4° for 24 hr.

Bioassay. The hypocalcemic rat bioassay was performed according to the method of Kumar et al. (20) in female rats.

RESULTS

Gel Filtration. Analyses of tumor extracts by gel permeation chromatography revealed a broad peak coeluting with synthetic hCT-(1-32) (0.74 kDa) (Chart 1). 131I-labeled hCT-(1-32) always eluted 0.1 kDa units after the peak of immunoreactive synthetic hCT-(1-32). A second peak eluting in the position of dimeric CT (0.60 kDa) was also seen. In the extract of the benign pancreatic tumor (Chart 1A), the earlier eluting component was hardly visible, whereas, in the malignant pancreatic tumor and MTC, this peak was clearly recognized (Chart 1, B to D). In extracts of a normal human thyroid, dimeric CT was only visible as a shoulder (Chart 1E) (36). The peak coeluting with dimeric CT from MTC was, in part, converted into the peak coeluting with monomeric hCT-(1-32) by incubation in 1 M ammonia for 1 hr at 45° (Chart 1F) (28). Moreover, in the extract of the malignant pancreatic tumor, a broad peak of nonidentified CT-like components having apparent molecular weights greater than that of dimeric CT was seen.

HPLC. Analysis by reverse-phase HPLC with an acetonitrile gradient system revealed 2 to 3 predominant peaks of immunoreactive CT (Chart 2). Peak 1 had the retention time of synthetic methionine8-hCT-(1-32) sulfoxide (Chart 3F). In our extractions, CT was almost fully recovered in peaks coeluting with the nonoxidized biologically active monomeric (Peak 2) and dimeric (Peak 3) CT forms. HPLC revealed a similar variation of the amounts of presumed dimeric CT in the different tumors examined as noted previously on gel filtration analyses (Charts 1 and 2). A shoulder designated as 2a eluting 2 min earlier than hCT-(1-32) was primarily visible in the extract of the benign pancreatic tumor; Peak 2b represents a CT form coeluting with hCT-(1-32) with low biological activity (Chart 2A). An additional small peak, noted in the malignant pancreatic tumor and in one of the MTC extracts with a retention time of 2 to 3 min shorter than that of dimeric CT, was not identified (Chart 2, B, C, and F).

The region between Peaks 1 and 3 was examined in more detail by HPLC under isocratic conditions (Chart 3). Excellent resolution of 12 hCT analogues differing in only one or 2 amino acids from hCT-(1-32) has been achieved (not shown). The 2 CT forms, designated Peaks 2a and 2b, in 5 different extracts of the
Charts. Reverse-phase HPLC profiles of hCT of tissue extracts under isocratic conditions. Column (Cia-Nucleosil) was used with methanol/water/trifluoroacetic acid (65/34/1, v/v/v) at a flow rate of 1 ml/min. Effluent fractions were analyzed for immunoreactive CT (●) and for 3H radioactivity (○ and arrows). Arrows, elution positions of [3H]hCT-(1-32) sulfoxide ([3H]hCT-(1-32)·, retention time, 9 min; range, 7.8 to 10.2 min) and [3H]hCT-(1-32) (retention time, 21.2 min; range, 20 to 22.9 min). Ciphers, immunoreactive CT forms coeluting with hCT-(1-32) sulfoxide (Peak 1) and hCT-(1-32) (Peak 2). Peak 2a, a new CT-like peptide. Peak 2b elutes in the position of hCT-(1-32). A, benign pancreatic tumor; B, malignant pancreatic tumor; C, medullary carcinoma of the thyroid; D, lymph node metastasis of a medullary carcinoma of the thyroid; E, normal thyroid; F, calibration with tritiated (○) and immunoreactive (●) hCT-(1-32) and its sulfoxide form.

benign pancreatic tumor were now clearly separated (Chart 3A). In all of the other tumor extracts examined, Peak 2a was absent or visible as a shoulder only (Chart 3, B to D). In normal thyroid glands, a small peak with the same retention time was also noted (Chart 3E). Tritiated and synthetic hCT-(1-32) and its sulfoxide form have been used as calibrating substances (Chart 3F). Peaks 1 and 2 correspond to hCT-(1-32) sulfoxide and to monomeric hCT-(1-32), respectively. Peaks 2a and 2b, and an immunoreactive peak in the dead volume have not been identified.

Bioassay. Hypocalcemic activity of the immunoreactive Peaks 2 and 3 extracted from MTC representing monomeric and dimeric CT was comparable to that of synthetic hCT-(1-32) (Chart 4). Peak 1, corresponding to the methionine8-sulfoxide form of hCT-(1-32), was biologically inactive in amounts as high as 100 ng. Similar findings have been obtained in extracts of normal human thyroid glands (36). Assuming that the affinity of the antibodies to Peaks 1, 2a, and 2b of the benign pancreatic tumor extract and to hCT-(1-32) is the same, Peak 2a from the benign pancreatic tumor had twice the biological potency of synthetic hCT-(1-32), and Peaks 1 and 2b had low biological activity.

Immunocytochemistry. CT-like immunoreactivity was detected in many but not all cells of the pancreatic tumors, and of the MTC and a metastasis thereof (Fig. 1). Staining was confined to the cytoplasm of the tumor cells with the nuclei remaining clear. The CT-specific immunoreactivity was similarly reduced in the pancreatic tumors and MTC after preadsorption of the hCT antiserum with 10⁻⁸ and 10⁻⁵ M hCT-(1-32) and equally blocked with 10⁻⁴ M hCT-(1-32).
DISCUSSION

Ectopic production of CT by tumors has been widely recognized (4, 14, 27). In the present report, we have characterized different CT forms extracted from pancreatic tumors secreting CT ectopically and from MTC. The fact that CT was localized immunocytochemically in tumor cells suggests that hCT or CT-like components have been formed in the tumor tissue. Moreover, the pancreatic tumors produced hypercalcemic factors unrelated to parathyroid hormone secreted in primary hyperparathyroidism. Demonstration of the specific mRNA coding for the factors would have been required for more definitive evidence that indeed the biosynthesis took place in the tumors (7). Proof of the expression of the translation products, however, can only be obtained by identification of the different CT forms generated by the tumor cells. Sufficient amounts of tumor material have not been available for an analysis of the sequence of the amino acid residues. Nonetheless, we and others have attempted to identify different immunologically and biologically active CT forms in crude extracts of pancreatic tumors (10, 15, 26, 29). We realize that CT-like peptides might cross-react in our systems.

Different CT forms have been characterized by gel permeation chromatography and reverse-phase HPLC. Peaks coeluting with biologically active hCT-(1-32) and its biologically inert methionine^3-hCT-(1-32) sulf oxide form have been detected in 2 HPLC systems. Moreover, the peak coeluting with synthetic methionine^3-hCT-(1-32) sulf oxide was in part converted into the nonoxidized monomeric hCT-(1-32) by a methionine sulf oxide reductase obtained from Escherichia coli (5, 7, 35). A hypocalcemic peak on HPLC with the retention time of dimeric hCT has also been recognized; its conversion into the monomer during incubation with ammonia (28) is further evidence that the peak probably corresponds to the dimeric form of the hormone. Similarly, monomeric hCT-(1-32) and its sulf oxide form and dimeric CT have been recognized in extracts of thyroid glands from normal subjects (36).

In some tissue extracts, a new peak (2a), eluting 3 min earlier than hCT-(1-32), was, moreover, detected in our isotropic HPLC system. This CT-like form was primarily visible in the extract of the benign pancreatic tumor, and it was biologically active in the rat hypocalcemic assay. Another peak (2b) of the same extract coeluted with hCT-(1-32), but showed low biological activity; it probably does not correspond to the native hormone. On gel permeation chromatography, a single immunoreactive peak eluting in the region of monomeric hCT-(1-32) has been detected in extracts of the benign pancreatic tumor. In extracts of normal thyroid glands, gel permeation chromatography under non-denaturing (Chart 1) and denaturing conditions (using a column equilibrated with 6 M guanidine- HC1) yielded essentially the same results (not shown). The new CT-like form is related in molecular weight to monomeric hCT-(1-32). The structure of this immunoreactive CT-like component, which is also recognized in extracts of normal thyroid glands and possibly in MTC tissue, remains to be elucidated.

In conclusion, CT components extracted from pancreatic tumors, from MTC, and from normal thyroid glands have been characterized by gel permeation chromatography and by HPLC as well as in a hypocalcemic bioassay. Three predominant components, corresponding to biologically active dimeric and monomeric CT and to the biologically virtually inactive sulf oxide form of hCT-(1-32) have been identified. The retention behavior of tritiated hCT-(1-32) and of its sulf oxide form added to the frozen tissues prior to the extractions was identical to the nonlabeled hormones. Moreover, we have detected for the first time a new biologically active CT-like form. The question as to whether this CT-like component was formed pre- or posttranslationally remains to be elucidated.

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REFERENCES

Fig. 1. Immunocytochemical localization of CT-like peptides in tumor cells, x 333. Unlabeled antibody enzyme method (for details, see “Materials and Methods”). A, benign pancreatic tumor; B, malignant pancreatic tumor; C, medullary carcinoma of the thyroid; D, bone marrow metastasis of a medullary carcinoma of the thyroid.
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