Tumor and Plasma Somatostatin-like Immunoreactivity in Transplantable Rat Medullary Thyroid Carcinoma

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ABSTRACT

We have recently reported the establishment of 16 series of calcitonin-producing transplantable rat medullary thyroid carcinoma. In the present study, these tumor series have been evaluated for the presence of somatostatin-like immunoreactivity. Each of the series contained detectable levels of both peptides. Immunoreactive somatostatin varied from less than 1 ng/mg of protein to almost 500 ng/mg of protein. The range of immunoreactive calcitonin was 0.3 to 30 μg/mg of protein. Although somatostatin-like immunoreactivity was always less than that of calcitonin, the levels in certain series were as high as those found in neural or endocrine tissues used for in vitro studies of somatostatin elaboration. No significant correlation was found between tissue levels of these two peptides. Two tumor lines were generated by initiation of tumor growth with cells from primary monolayer cultures. Levels of both immunoreactive calcitonin and somatostatin significantly differed from those of the parent lines, which were maintained by serial passage of tissue fragments only. Plasma somatostatin-like immunoreactivity assessed in two tumor series with high (149 ng/mg of protein) and low (1.5 ng/mg of protein) tissue levels was 3100 and 50 pg/ml, respectively. Gel filtration chromatography of tissue and blood extracts showed a predominant peak (>90%) of immunoreactive somatostatin eluting at the position of the native hormone. Three other peaks were resolved in the tissue extract with estimated molecular weights of 14,000, 8,700, and 5,000. The high level of somatostatin-like immunoreactivity and the presence of multiple large forms suggest that certain tumor lines will prove valuable for studies of somatostatin biosynthesis and secretion.

INTRODUCTION

We have recently reported the establishment of several tumor series of transplantable rat MTC3 (22). Each series has been characterized and distinguished on the basis of tumor growth rate and calcitonin content. Light microscopic and ultrastructural studies indicated that they closely resembled human MTC (15).

The rat tumors have provided a convenient, abundant source of tissue for studies of calcitonin secretion and biosynthesis. This has facilitated the isolation and characterization of calcitonin mRNA. When translated in vitro, this mRNA directed the synthesis of a putative M.W. 17,500 preprocalcitonin (2).

MATERIALS AND METHODS

Propagation of MTC Tumor Series. The origin, transplantation protocol, and care of the tumor-bearing rats have been reported previously (22). Tumors were serially propagated by implanting 10 to 25 mg of tumor tissue fragments beneath the kidney capsule of 3- to 5-week-old WAG/Rij rats (6, 7). The tissue was obtained from anesthetized donor animals near the end of their predicted life spans (22). During alternate passages of tumor cells, rats were given injections under the kidney capsule of 1 to 5 x 10^6 cells from primary monolayer cultures prepared from transplanted tumors (18). Tumors derived from cell cultures were serially transplanted with intermittent passage into monolayer culture and subsequent return to in vivo growth. Alternate passages of type 2-2-7 tumor cells also markedly improved the plating efficiency of tumor cell suspensions from <10% to >60%.

Tissue Extraction. Tissue fragments from the same portion of the tumor used for transplantation were lyophilized and later extracted in 1 ml of 0.1 M HCl using a Brinkmann Polytron. The extracts were centrifuged in a Beckman microfuge, and the supernatant was assayed for immunoreactive somatostatin and calcitonin. Protein concentrations were estimated by the method of Lowry et al. (14) using bovine serum albumin as standard. Recovery of somatostatin-like immunoreactivity was assessed by adding synthetic somatostatin (Peninsula Laboratories, San Carlos, Calif.) to the acid before extraction of liver fragments. Of 640 pg of somatostatin added, the recovery was 506 ± 102 (S.E.) pg (n = 4).

For gel filtration chromatography, 4 g of tissue were extracted in 20 ml of 0.1 M HCl containing 8 M urea, 5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.), and Trasylol (Sigma Chemical Co.), 130 KIU/ml (12).
The extract was clarified by centrifugation.

**Plasma Extraction.** In order to estimate the level of somatostatin-like immunoreactivity in plasma, blood samples were taken from the jugular vein with a syringe containing EDTA and Trasylol to provide approximate final concentrations of 8.7 mM and 500 KIU/ml, respectively (13). The blood was centrifuged, and 0.2-ml aliquots of plasma were extracted with acetone by the method of Arimura et al. (3). For chromatography, whole blood collected in EDTA:Trasylol from the renal vein draining the tumor-bearing kidney was extracted with acetic acid and acetone (8). To assess recovery, normal rat blood was collected with only EDTA present and then allowed to sit for 1 hr at room temperature before addition of Trasylol and synthetic somatostatin. Of 256 pg of somatostatin added to each plasma sample, the recovery was 240 ± 40 (S.E.) pg (n = 4).

**Gel Filtration.** Both tissue and blood extracts were chromatographed on a 1.5- x 93-cm column of Sephadex G-50 equilibrated with 0.1 M HCOOH containing 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride and 130 KIU Trasylol per ml (12). The residue from the blood extract was dissolved in 3 ml of the same HCl:urea solution used to extract the tissue. Fractions of 3.3 ml were collected. The column was calibrated with radioiodinated Tyr1-somatostatin (13) and Trasylol as well as with unlabeled cytochrome c (Boehringer Mannheim, Inc., Indianapolis, Ind.). Fractions from gel filtration of the tissue extract were assayed directly; those from the blood extract were lyophilized, and the residue was suspended in assay diluent. In the latter case, urea eluting at the salt volume of the column interfered with tracer-antibody binding and gave rise to an apparent small peak of immunoreactive material.

**Radioimmunoassays.** The somatostatin radioimmunoassay was performed with antisera R141E (a gift of Dr. R. Eide, University of Minnesota School of Medicine, Minneapolis, Minn.) and 125I-labeled Tyr1-somatostatin. R141E cross-reacts with the midportion of somatostatin, and the validity of rat somatostatin measurements with this antisera has been well documented (3). The assay diluent was 0.02 M sodium phosphate (pH 7.5), containing 1 mM EDTA, 0.005% Merthiolate, 0.1% gelatin, and 0.03% Brij-35 (Sigma Chemical Co.). The antisera was used at a final dilution of 1:60,000. After an overnight incubation at 4°C, phase separation was accomplished with the addition of 1 μl of normal rabbit serum and 20 μl of goat anti-rabbit γ-globulin. Under these assay conditions, 1 to 2 pg of somatostatin were detectable, and 50% displacement of tracer occurred with 15 to 20 pg.

We had noted previously that radioimmunoassay measurements of calcitonin in certain types of lung cancer reflected degradation of tracer rather than of immunoreactive calcitonin (21). Accordingly, some radioimmunoassays were modified so that prior to goat anti-rabbit γ-globulin addition, 1 μl of antisera R141E was added instead of normal rabbit serum. This was enough to immunoprecipitate both labeled and unlabeled immunoreactive somatostatin. For tissue and plasma extracts, the amount of tracer immunoprecipitated was not significantly different from that precipitated in assay tubes that contained different known amounts of synthetic somatostatin.

The calcitonin radioimmunoassay was performed with a 1:20,000 final dilution of antisera RB3/5A, radioiodinated synthetic human calcitonin, and synthetic rat calcitonin standards (19, 20). The assay diluent and method of phase separation were as described for the somatostatin assay. Antisera RB3/5A recognizes amino acid residues 22 to 32 of calcitonin.

Under the assay conditions used here, the minimum detectable level of rat calcitonin was approximately 100 pg/tube; 50% displacement of tracer occurred at 600 to 700 pg.

**Statistical Methods.** Tests of significance were performed using Student's t test. A possible significant correlation between tissue immunoreactive somatostatin and calcitonin was tested by linear regression analysis (4).

**RESULTS**

Both immunoreactive somatostatin and calcitonin were detected in all 16 tumor series (Table 1). However, wide concentration ranges for both peptides were observed. Tissue somatostatin-like immunoreactivity varied from approximately 1 to 500 ng/mg of protein (0.6 to 300 pmol/mg of protein). In several tumor series, the levels were as high as were those reported for any nonneoplastic neural or endocrine tissue (11, 28). Nonetheless, the immunoreactive somatostatin content of each tumor series was lower than that of immunoreactive calcitonin, which ranged from 0.30 to 30 μg/mg of protein (85 to 8600 pmol/mg of protein).

There was no significant correlation between the levels of immunoreactive somatostatin and calcitonin. The 1-2-9 and 2-2-6 tumor series had nearly identical high levels of immunoreactive calcitonin, but they differed 5-fold in their immunoreactive somatostatin content. The 1-1-2 tumor series had one-tenth the amount of immunoreactive calcitonin found in 2-2-6 tumors but 2.5-fold more somatostatin-like immunoreactivity. Finally, the 2-4-4 tumor series had relatively low levels of both peptides.

Analyses of tumors accumulated from as many as 10 transplant generations indicated that the tumor series maintained predictable levels of immunoreactive calcitonin (22). Since somatostatin-like immunoreactivity has been monitored for only 1 or 2 transplant generations, the constancy of tumor immunoreactive somatostatin levels during serial in vivo propagation cannot yet be assessed.

Alternate passage of 2 stable tumor series significantly altered the levels of both peptides (Table 2). The WE tumor series, generated by alternate passage of type 1-1-4 tumors, had one-fourth the level of immunoreactive calcitonin and one-

<table>
<thead>
<tr>
<th>Tumor series</th>
<th>Calcitonin content (pg/mg of protein)</th>
<th>Somatostatin content (ng/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1-2</td>
<td>2.10 ± 0.45 (11)</td>
<td>64.6 ± 12.8 (7)</td>
</tr>
<tr>
<td>1-1-4</td>
<td>4.65 ± 1.34 (8)</td>
<td>87.7 ± 25.1 (8)</td>
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<tr>
<td>1-1-5</td>
<td>2.98 ± 1.41 (9)</td>
<td>69.1 ± 19.7 (8)</td>
</tr>
<tr>
<td>1-2-4</td>
<td>16.0 ± 2.5 (15)</td>
<td>24.2 ± 10.0 (17)</td>
</tr>
<tr>
<td>1-2-5</td>
<td>15.1 ± 2.8 (21)</td>
<td>9.64 ± 4.3 (18)</td>
</tr>
<tr>
<td>1-2-6</td>
<td>13.5 (1)</td>
<td>98.2 (1)</td>
</tr>
<tr>
<td>1-2-8</td>
<td>24.1 ± 3.5 (8)</td>
<td>146 ± 46 (7)</td>
</tr>
<tr>
<td>2-2-1</td>
<td>21.9 ± 19.7 (2)</td>
<td>227 ± 6 (2)</td>
</tr>
<tr>
<td>2-2-2</td>
<td>24.6 ± 4.5 (4)</td>
<td>18.9 ± 5.8 (4)</td>
</tr>
<tr>
<td>2-2-7</td>
<td>7.92 ± 1.32 (29)</td>
<td>2.67 ± 1.98 (27)</td>
</tr>
<tr>
<td>2-2-10</td>
<td>22.6 ± 3.7 (10)</td>
<td>1.71 ± 0.76 (8)</td>
</tr>
<tr>
<td>2-3-3</td>
<td>6.62 ± 2.53 (9)</td>
<td>110 ± 94 (9)</td>
</tr>
<tr>
<td>2-4-3</td>
<td>0.29 ± 0.05 (2)</td>
<td>0.77 ± 0.23 (2)</td>
</tr>
<tr>
<td>2-4-4</td>
<td>0.28 ± 0.87 (9)</td>
<td>4.74 ± 2.06 (9)</td>
</tr>
<tr>
<td>2-4-5</td>
<td>0.64 ± 0.21 (4)</td>
<td>131 ± 120 (3)</td>
</tr>
<tr>
<td>2-4-6</td>
<td>3.78 ± 0.35 (2)</td>
<td>60.3 ± 12.4 (2)</td>
</tr>
</tbody>
</table>

*Mean ± S.E.*

Numbers in parentheses, number of observations.
sixtieth the level of immunoreactive somatostatin of the parent series. The CA tumor series also had significantly lower tissue immunoreactive calcitonin than did the parent (type 2-2-7) tumors, but somatostatin-like immunoreactivity was almost 50-fold greater.

Rats bearing CA tumors had higher plasma immunoreactive somatostatin levels than did rats bearing tumors of the parent (2-2-7) tumor series (Chart 1). Plasma samples were collected after 3.5 months of tumor growth. Based upon predicted growth rates, the tumors in both series were approximately the same size. Rats bearing type 2-2-7 tumors had mean plasma levels of somatostatin-like immunoreactivity of 50 pg/ml (range, 10 to 141 pg/ml). In contrast, the CA tumor-bearing rats had a mean plasma level of 3,100 pg/ml (range, 517 to 10,500 pg/ml).

Gel filtration chromatography of tissue and blood extracts was performed to compare the size of tumor and plasma species with the size of synthetic somatostatin. For these comparisons, a previously characterized chromatography system was used that resolved somatostatin from several high-molecular-weight immunoreactive somatostatin species (12). More than 90% of the tumor (Chart 2) and blood (Chart 3) immunoreactive somatostatin eluted in the position of the synthetic peptide. In the tumor extract, at least 3 early eluting species, comprising 3% of the total immunoreactive somatostatin, were also detected. Their estimated molecular weights were 5,000, 8,700, and 14,000. Radioimmunoassay of serial

![Chart 2](chart2.png)

**Chart 2.** Gel filtration chromatography of an acid-urea extract of a type 2-2-1 tumor. Somatostatin-like immunoreactive material (15.9 µg) was applied to the column. The elution positions of Blue Dextran (V₀), cytochrome c (C), Trasylol (T), somatostatin (SRIF), and urea (V₆) are also indicated.

![Chart 3](chart3.png)

**Chart 3.** Gel filtration chromatography of an acid:acetone extract of renal vein blood from a rat bearing a type 2-2-1 tumor. The residue from the extraction was dissolved in the acid:urea buffer used to extract the tumor tissue. Somatostatin-like immunoreactive material (9.8 ng) was applied to the column. For explanation of symbols, see Chart 2.
dilutions of each of the 4 tissue species generated antibody-tracer displacement curves with slopes not significantly different from that observed for synthetic somatostatin (data not shown).

DISCUSSION

Immunoreactive somatostatin and calcitonin were detected in each of our 16 established rat tumor series and in the 2 series generated by alternate passage of tissue and cells derived from primary monolayer cultures. High plasma somatostatin-like immunoreactivity was associated with high tissue levels.

There was no obvious relationship between the tissue levels of immunoreactive somatostatin and calcitonin. This argues against a common gene for the 2 peptides. Each of the tumor lines may also be comprised of different proportions of multiple cell types, each of which may elaborate one or more than one peptide. There are at least 2 types of parafollicular cells in normal rat thyroid, one containing calcitonin and the other containing both calcitonin and somatostatin (25). Neoplastic forms of each cell type may be present in the rat tumors.

The predominant form of immunoreactive somatostatin resolved by gel chromatography was most likely the M.W. 1,600 native peptide. Proof that this species is somatostatin, however, will require further biochemical characterization. Three distinct higher-molecular-weight forms, comprising about 3% of the immunoreactive material, were also observed in the tissue extract. The apparent molecular weights of these species were 5,000, 8,700, and 14,000. Although several laboratories have reported forms of somatostatin-like immunoreactivity with molecular weights ranging from 3,000 to 15,000, the variety of chromatography and sample preparation methods makes any comparison of results difficult (8, 12, 17, 23, 28). With this in mind, we selected a method reported to resolve species with molecular weights of 6,000, 10,000, and 15,000 in mouse hypothalamus (12).

The rat tumor series may prove of great importance for studies of calcitonin and somatostatin biosynthesis and secretion. Taking advantage of the large amounts of tissue available, we have already begun to exploit these tumors to characterize high molecular weight forms of calcitonin. A 1,050-nucleotide mRNA has been isolated and translated to yield a precursor of calcitonin with a molecular weight of 17,500 (1, 2). We have also been able to demonstrate, by lectin affinity chromatography of tumor extracts, glycoprotein forms of calcitonin (5). Radiolabeling of monolayer cultures has identified high-molecular-weight calcitonin precursors which appear identical to the glycoprotein species (5).

Similar investigations are now possible for somatostatin. The immunoreactive somatostatin content of certain rat MTC series is equivalent to that of murine hypothalamus and pancreatic islets, tissues relied upon previously for biosynthesis studies (9, 16, 27). Preliminary experiments indicate that somatostatin mRNA can be isolated from these tumors and translated in vitro. Indeed, the larger species of immunoreactive somatostatin present in the tumors are most probably biosynthetic precursors.

ACKNOWLEDGMENTS

We thank Dr. Robert Elde for his gift of somatostatin antisera and his assistance in the initial stages of this study. We also thank Jane O'Neill, Stephen Sabo, Michael Yoon, Candice Disch, Geraldine Galloway, and Jula Taylor for their assistance.

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