Pro-opiomelanocortin gene expression in silent corticotroph-cell adenoma and Cushing’s disease

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The silent corticotroph-cell adenoma (SCCA) is characterized by the presence of immunoreactive adrenocorticotropic hormone (ACTH) in the tumor tissue in patients without symptoms of Cushing’s disease. To elucidate the pathophysiology of SCCA, the expression of pro-opiomelanocortin (ACTH precursor) genes was studied in a patient with SCCA and in three patients with Cushing’s disease. Pro-opiomelanocortin messenger ribonucleic acid (mRNA) was found in the SCCA tissue to a greater degree than in the adenomas of the patients with Cushing’s disease. Northern blot analysis revealed that the size of pro-opiomelanocortin mRNA present in the SCCA tissue was indistinguishable from that in the adenomas associated with Cushing’s disease. A ribonuclease mapping study indicated that there were no point mutations in the coding sequence of pro-opiomelanocortin mRNA present in the SCCA tissue. Because of the presence of pro-opiomelanocortin mRNA and immunoreactive ACTH in the adenoma tissue, it is proposed that translation of the mRNA and subsequent accumulation of ACTH precursor occurred in the SCCA. Thus, the absence of Cushing’s disease symptoms in this SCCA could not be caused by abnormality in the coding sequence of the pro-opiomelanocortin gene or in ribonucleic acid processing. The occurrence of abnormality at or after the translational steps was strongly suggested.

KEY WORDS • silent corticotroph-cell adenoma • pituitary tumor • adenoma • pro-opiomelanocortin • Cushing’s disease • ribonucleic acid

PITUITARY adenomas can be classified into two groups, functioning and nonfunctioning adenomas. Since nonfunctioning pituitary adenomas do not secrete excessive amounts of pituitary hormones, clinical symptoms due to such hypersecretion may be absent; however, clinical symptoms may be associated with neural tissue compression by a tumor mass or hyposecretion of pituitary hormones. Several recent investigations have demonstrated that some nonfunctioning tumors can synthesize and secrete pituitary hormones such as subunits of glycoprotein hormones. The silent corticotroph-cell adenoma (SCCA) is one such nonfunctioning adenoma. Despite the presence of immunoreactive adrenocorticotropic hormone (ACTH) in the tumor, it is not associated with the symptoms of Cushing’s disease. Clinical symptoms in SCCA’s are similar to those observed in other nonfunctioning pituitary tumors. Plasma ACTH and cortisol levels in most cases of SCCA are normal, although an occasional case may show high plasma ACTH levels without hypercortisolism.

The pathophysiology of SCCA is still unknown, although the production of defective hormones lacking bioactivity or the destruction of hormones in the tumor cell have been proposed. We and others have reported cases of SCCA in which gel chromatography revealed that the molecular size of immunoreactive ACTH was larger than that of authentic ACTH. The larger molecular form was demonstrated to be less bioactive or to show no bioactivity. Since ACTH is known to be derived from the ACTH precursor pro-opiomelanocortin by peptide processing, the larger form of ACTH might be caused by abnormal peptide processing. One such example can be found in a case of proinsulinemia. Point mutation of the coding sequence of the insulin gene resulted in an amino acid substitution at the site of peptide processing (lysine-arginine to lysine-histidine); thus, the larger, unprocessed, biologically inactive proinsulin was formed and secreted.

To elucidate the pathophysiology of SCCA, pro-opiomelanocortin gene expression was analyzed in de-
Pro-opiomelanocortin gene expression
tail in a case of SCCA and compared with three cases of Cushing’s disease; however, the molecular size and biological activity of immunoreactive ACTH could not be investigated.

Materials and Methods
Patients, Pituitary Samples, and Immunohistochemistry

Fifteen patients with nonfunctioning pituitary adenomas were operated on in the Department of Neurosurgery, Nagoya University School of Medicine, in 1985 and 1986. Adenoma tissue was collected from these cases during surgery. A portion of each specimen was fixed with 10% formaldehyde and embedded in paraffin for immunohistochemical studies. The remaining tissues were kept frozen at –70°C for analysis of ribonucleic acid (RNA).

Indirect immunoperoxidase staining for ACTH was performed as described by Nakane and Pierce using rabbit antibody against human 1-24 ACTH, and peroxidase-labeled anti-rabbit immunoglobulin G goat antibody as the second antibody. Sections of tissue 5 μm thick were treated with 0.3% H₂O₂ before immunoreaction to block endogenous peroxidase activity. After reaction with the primary and secondary antibodies, an antigen-antibodies complex was visualized by incubation with diaminobenzidine solution. The slides were counterstained with hematoxylin. Adenoma tissue from three patients with Cushing’s disease was used as positive controls and, in place of antiserum, normal rabbit antibody as the second antibody. Sections of tissue 5 μm thick were treated with 0.3% H₂O₂ before immunoreaction to block endogenous peroxidase activity. After reaction with the primary and secondary antibodies, an antigen-antibodies complex was visualized by incubation with diaminobenzidine solution. The slides were counterstained with hematoxylin. Adenoma tissue from three patients with Cushing’s disease was used as positive controls and, in place of antiserum, normal rabbit serum was used as a negative control.

Among the 15 nonfunctioning adenomas studied, ACTH immunoreactivity was demonstrated in the tissue of only one tumor. The patient was a 37-year-old man whose chief complaints were visual disturbance and easy fatigability. Computerized tomography demonstrated a suprasellar enhancing mass (Hardy grade IIIb). There was a small low-density area in the center of the mass which was found at surgery to be old hematoma. The plasma ACTH level was 12 pg/ml and the plasma cortisol level was 6.8 μg/dl in the early morning after overnight fasting. Other anterior pituitary hormone levels were also within normal limits. Thus, this patient was considered to have SCCA because there was no hypercortisolism despite ACTH immunoreactivity in the tumor tissue. In contrast, the three patients with Cushing’s disease (the same cases as were used in the immunohistochemical studies) showed microadenoma and typical symptoms of hypercortisolism. The preoperative clinical data for the patient with SCCA and the three patients with Cushing’s disease are summarized in Table 1.

**Total RNA Extraction from Tumor Tissue**

Total RNA was extracted by the guanidinium/cesium chloride method described by Glisin, et al. In brief, tumor tissue (10 to 50 mg wet weight) was homogenized in 2.5 ml of 4 M guanidinium thiocyanate/5 mM sodium citrate (pH 7.0)/0.1 M β-mercaptoethanol/0.5% Sarkosyl. The homogenate was layered on a 2-ml cushion of 5.7 M cesium chloride/0.1 M ethylenediaminetetra-acetic acid (EDTA) (pH 7.5) in a centrifuge tube and centrifuged at 35,000 rpm for 15 hours at 20°C. The RNA pellet was suspended in 2 M lithium chloride. After lithium precipitation, the RNA was dissolved in water and precipitated with 0.1 vol of 3 M sodium acetate and 2.5 vol of 100% ethanol. The RNA was recovered by centrifugation and the amount of RNA was estimated by spectrophotometry.* The recovery of total RNA per mg of wet tissue was about 2 μg in all cases.

**RNA Dot Hybridization**

Hybridization of RNA was performed as described by Thomas. The total RNA from the SCCA was dissolved to 1.4 μg/μl, whereas the total RNA from the adenomas associated with Cushing’s disease was dissolved to 0.14 μg/μl. From each RNA sample, 1 μl was added to 19 μl of 50% denionized formamide/6% formaldehyde and denatured for 1 hour at 50°C. Each treated RNA (20 μl) was serially diluted with 15 x standard saline citrate (0.15 M NaCl, 0.05 M trisodium citrate) in a 96-well microtiter plate and the final volume was adjusted to 100 μl. These diluted samples were applied on a nitrocellulose sheet using a 96-hole Minifold apparatus. The nitrocellulose sheet was baked for 90 minutes at 80°C to immobilize the RNA and was stored in a desiccator until hybridization.

In order to detect pro-opiomelanocortin messenger RNA (mRNA), the third exon of pro-opiomelanocortin deoxyribonucleic acid (DNA) was cloned into the Sma I site of the plasmid pSP 65. The Sma I fragment of pro-opiomelanocortin exon 3 includes all cod-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs), Sex</th>
<th>Symptoms &amp; Signs</th>
<th>Plasma ACTH (pg/ml)</th>
<th>Plasma Cortisol (μg/dl)</th>
<th>Tumor Size†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCA</td>
<td>37, M</td>
<td>visual disturbance</td>
<td>12</td>
<td>6.8</td>
<td>IIIb</td>
</tr>
<tr>
<td>C1</td>
<td>18, F</td>
<td>obesity</td>
<td>105</td>
<td>17.3</td>
<td>I</td>
</tr>
<tr>
<td>C2</td>
<td>18, F</td>
<td>obesity, hypertension</td>
<td>83</td>
<td>45.5</td>
<td>I</td>
</tr>
<tr>
<td>C3</td>
<td>37, F</td>
<td>obesity, hypertension, diabetes mellitus</td>
<td>55</td>
<td>21.6</td>
<td>II</td>
</tr>
<tr>
<td>normal range</td>
<td>10-50</td>
<td></td>
<td>6.5-13.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SCCA = silent corticotroph-cell adenoma; Cases C1 to C3 suffered from Cushing’s disease. ACTH = adrenocorticotropic hormone. Plasma samples were obtained in the early morning after overnight fasting.

† Tumor size assessed according to Hardy.10

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* Spectrophotometry system, Model H65-BX, manufactured by Hitachi, Tokyo, Japan.
† Nitrocellulose sheet, type BA85, and Minifold apparatus manufactured by Schleicher & Schuell, Keene, New Hampshire.
ing regions of ACTH and β-lipotropin (Fig. 1 shaded area).26

A single-stranded RNA probe was prepared by the SP6 RNA synthesis method.19 For this, pSP 65 containing the pro-opiomelanocortin exon 3 Sma I fragment was linealized by Pst I digestion. It was then transcribed by SP6 RNA polymerase in the presence of 32P-labeled cytidine triphosphate. Template DNA was removed by deoxyribonuclease treatment. The nitrocellulose sheets were prehybridized for 4 hours at 60°C in solution H, composed of 50% deionized formamide/50 mM sodium phosphate (pH 6.0)/0.8 M NaCl/1 mM EDTA/0.05% bovine serum albumin/0.05% Ficoll/0.05% polyvinylpyrrolidone/0.01% denatured salmon sperm DNA. They were then hybridized overnight at 60°C with 32P-labeled RNA probe in 10 ml of solution H. The sheets were washed five times for 20 minutes at 65°C in 50 mM NaCl/20 mM sodium phosphate (pH 6.5)/1 mM EDTA/0.1% sodium dodecyl sulfate (SDS); they were then dried on 3-M paper and autoradiographed overnight using Kodak X-Omat AR film in a cassette at -70°C. The amount of pro-opiomelanocortin mRNA was evaluated by densitometric examination of the autoradiogram of RNA dot hybridization (Table 2).

**Northern Blot Analysis**

Northern blot analysis was conducted by the method of Lehrach, et al.18 In the case of the SCCA 2 μg of total RNA was used, and in the Cushing’s disease cases 0.5 μg was used. As a negative control, 16 μg of total RNA of Hep G2 (a human hepatoma-derived cell line) was also used. Each RNA sample was denatured with 1 M glyoxal/50% dimethylsulfoxide for 1 hour at 50°C; a 0.25 vol of loading buffer (0.25% bromophenol blue/0.25% xylene cyanole FF/15% Ficoll type 400) was added, and the mixture was electrophoresed on a 0.8% agarose gel in 10 mM sodium phosphate buffer (pH 7.0). The gel was blotted onto a nitrocellulose sheet, which was baked and stored in the same manner as described above. Hybridization, washing, and autoradiography were performed as before.

**Ribonuclease Mapping**

Ribonuclease (RNase) mapping19,31 was utilized to detect abnormalities in the mRNA sequences. Single-stranded 32P-labeled RNA probe was synthesized after Pst I treatment of the plasmid as described above. In theory, the labeled RNA probe (1141 bases) should hybridize the coding sequence of pro-opiomelanocortin mRNA, and the resultant hybrid (778 bases) should be protected from RNase digestion (Fig. 1). However, if there is any sequence that does not form a hybrid, the portion will be digested.

Samples of RNA (5 μg for the SCCA and 0.5 μg for the Cushing’s disease adenomas) were dissolved in 30 μl of hybridization buffer (80% deionized formamide/40 mM piperazine-N,N'-bis-2-ethane sulfonic acid (pH 6.4)/400 mM NaCl/1 mM EDTA) containing 32P-labeled RNA probe (3 × 10^5 cpn); they were then heated at 85°C for 5 minutes and incubated at 45°C overnight. Three hundred microliters of RNase digestion buffer (10 mM Tris-HCl (pH 7.5)/5 mM EDTA/300 mM NaCl) containing 10 μg/ml of RNase A and 2 μg/ml of RNase T1 was added, and the mixture was incubated at 37°C for 60 minutes. The reaction was terminated by the addition of 20 μl of 10% SDS and 50 μg of proteinase K, followed by incubation at 37°C for 15 minutes, phenol extraction, and ethanol precipitation in the presence of yeast transfer RNA as a carrier. The samples were denatured, fractionated on 8 M urea-denaturing acrylamide gel, and autoradiographed.

**TABLE 2**

**Correlation of the tumor size and total amount of POMC mRNA***

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Tumor Size† (mm)</th>
<th>Tumor Volume (cu cm)</th>
<th>POMC mRNA/total RNA</th>
<th>Total POMC mRNA/Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCA</td>
<td>42 × 30 × 27</td>
<td>17.80</td>
<td>1.0</td>
<td>1.00</td>
</tr>
<tr>
<td>C1</td>
<td>8‡</td>
<td>0.27</td>
<td>8.4</td>
<td>0.13</td>
</tr>
<tr>
<td>C2</td>
<td>8‡</td>
<td>0.27</td>
<td>23.2</td>
<td>0.35</td>
</tr>
<tr>
<td>C3</td>
<td>12‡</td>
<td>0.90</td>
<td>12.5</td>
<td>0.62</td>
</tr>
</tbody>
</table>

* POMC = pro-opiomelanocortin; mRNA = messenger ribonucleic acid.
† Tumor size was established by the radiological or operative findings. By assigning the POMC mRNA level in the silent corticotomy-cell adenoma (SCCA) as 1.0, the levels in the Cushing’s disease adenomas (Cases C1 to C3) could be calculated.
‡ Greatest diameter of the tumor.

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**FIG. 1.** Schematic drawings of SP6 ribonucleic acid (RNA) synthesis method and ribonuclease (RNase) mapping. Plasmid pSP 65 containing the Sma I fragment of pro-opiomelanocortin (POMC) exon 3 (template DNA) was linealized by digestion with Pst I. The RNA probe was transcribed by SP6 RNA polymerase in the presence of 32P-labeled cytidine triphosphate. The labeled RNA probe (1141 bases) should hybridize the coding region for adrenocorticotropic hormone and β-lipotropin (shaded area) in POMC messenger RNA. The resultant hybrid (778 bases) should be protected from RNase digestion.
Pro-opiomelanocortin gene expression

Results

Immunohistochemical Findings

The samples of SCCA and from the three Cushing’s disease adenomas showed cells staining positively for ACTH (Fig. 2). The ACTH-positive cells were scattered in the SCCA sample, while most of the cells showed positive staining in the cases of Cushing’s disease.

RNA Dot Hybridization

Figure 3 shows the results of RNA dot hybridization in the SCCA and the three Cushing’s disease adenomas. Considering the amount of mRNA in the SCCA as 1 unit, the amount in the adenoma tissues of Cushing’s disease ranged between 0.84 and 2.32. Since the amount of applied total RNA from the SCCA patient was 10 times more than in the other patients, the amount of pro-opiomelanocortin mRNA per equivalent RNA input in the cases of Cushing’s disease was 8.4- to 23.2-fold greater than in SCCA (Table 2).

On the other hand, the tumor mass of the SCCA was much larger than that of the Cushing’s disease adenomas when the volume of the tumor mass was estimated by radiological or operative findings. Assuming that the pro-opiomelanocortin-positive cells were distributed evenly in the tumor, the total amount of pro-opiomelanocortin mRNA in each tumor can be estimated because RNA recovery from the tissue was similar in all cases. When the total amount of mRNA in the SCCA was assigned as 1.0, those in the Cushing’s disease tumors were 0.13 to 0.62. Thus, the total amount of pro-opiomelanocortin mRNA in the tissue of the SCCA was more than that of the Cushing’s disease adenomas.

Northern Blot Analysis

Results of the Northern blot analysis are shown in Fig. 4. A single band was observed in the lanes of the SCCA and Cushing’s disease adenoma tests. No band was observed in the lane of the Hep G2 cell line. The size of the mRNA was estimated to be approximately 1200 bases, as reported previously.

Results of Ribonuclease Mapping

In the RNA probe (1141 bases) transcribed from plasmid containing an Sma I fragment of pro-opiomelanocortin exon 3, 778 bases will be protected from RNase A and T1 digestion by hybridization with normal pro-opiomelanocortin mRNA (Fig. 1). A band with 778 base pairs was demonstrated in the case of the SCCA and in the three cases of Cushing’s disease (Fig. 5). Although the size of the RNA probe should be only 1141 bases, there were a few smaller ones (Fig. 5, probe lane). These RNA’s were believed to be produced by incomplete extension by SP6 RNA polymerase. When pro-opiomelanocortin mRNA is hybridized with these smaller probes, the shorter ones would be protected. Indeed, the hybrid was smaller in all of the cases, but there were no differences in those bands between the SCCA and the Cushing’s disease adenomas. This suggested that there were no point mutations in the coding sequence of pro-opiomelanocortin in the SCCA.

Discussion

Silent corticotroph-cell adenomas, which possess ACTH immunoreactivity in the adenoma tissue without symptoms of Cushing’s disease, are rare. In our university, two SCCA’s (6%) were found among 31 nonfunctioning adenomas. This incidence is in agreement with the findings of Black, et al., who reported three SCCA’s (8.1%) among 37 nonfunctioning adenomas. However, SCCA’s are of clinical importance because the recurrence rate and incidence of pituitary
The total RNA from the silent corticotroph-cell adenoma (SCCA) tissues (lane S) and from three cases of Cushing’s disease were serially diluted and blotted on a nitrocellulose membrane (lanes C₁ to C₃). The amount of RNA from the SCCA was 1.4 μg in the uppermost dot and that from the Cushing’s disease adenomas was 0.14 μg. After hybridization with pro-opiomelanocortin probe, the amount of pro-opiomelanocortin messenger RNA (mRNA) was estimated by densitometry. When the amount of mRNA in the SCCA was expressed as 1 unit, the amounts in the adenoma tissue of the patients with Cushing’s disease ranged between 0.84 and 2.32. Since the amount of total RNA from the SCCA was 10 times more than from the Cushing’s disease adenomas, the amount of pro-opiomelanocortin mRNA per equivalent total RNA was calculated (Table 2).

Northern blot analysis revealed that the size of pro-opiomelanocortin mRNA in this SCCA was indistinguishable from that in the tissues of the three Cushing’s disease adenomas. Moreover, the RNase mapping study suggested that no point mutations were present in the coding sequence of pro-opiomelanocortin mRNA in the SCCA. The possibility of frame shift of the coding sequence is unlikely because of the presence of ACTH immunoreactivity in the adenoma tissue. Thus, translation of the mRNA and the accumulation of the product were most probably present in the tissue of this SCCA.

The RNA dot hybridization study indicated that the total amount of pro-opiomelanocortin mRNA present in the SCCA tissue was greater than in the Cushing’s disease adenomas. If so, why did this SCCA not present the symptoms of hypercortisolism? Since no abnormality in the size or coding sequence of the pro-opiomelanocortin mRNA was demonstrated, translational

apoplexy are high. It has also been reported that SCCA’s could develop the symptoms of hypercortisolism after several years. This is the first report to demonstrate the analysis of the pro-opiomelanocortin gene expression in SCCA’s, although the molecular size and biological activity of the ACTH molecule from our SCCA patient could not be investigated. Northern blot analysis revealed that the size of pro-opiomelanocortin mRNA in this SCCA was indistinguishable from that in the tissues of the three Cushing’s disease adenomas. Moreover, the RNase mapping study suggested that no point mutations were present in the coding sequence of pro-opiomelanocortin mRNA in the SCCA. The possibility of frame shift of the coding sequence is unlikely because of the presence of ACTH immunoreactivity in the adenoma tissue. Thus, translation of the mRNA and the accumulation of the product were most probably present in the tissue of this SCCA.

The RNA dot hybridization study indicated that the total amount of pro-opiomelanocortin mRNA present in the SCCA tissue was greater than in the Cushing’s disease adenomas. If so, why did this SCCA not present the symptoms of hypercortisolism? Since no abnormality in the size or coding sequence of the pro-opiomelanocortin mRNA was demonstrated, translational
efficiency in the SCCA could be less than that in the tissue of the Cushing's disease adenomas. Rapid degradation of ACTH produced in the tumor tissue is another possibility. Abnormal peptide processing leading to the formation of a biologically inactive or non-secretable form of ACTH might be also considered.

Acknowledgments

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References


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