Comparison of ACTH Secretion in Cushing’s Adenoma and Clinically Silent Corticotroph Adenoma by Cell Immunoblot Assay

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Abstract. Immunocytochemical staining and cell immunoblot assay (CIBA) were performed in adenoma tissue from five patients with Cushing’s disease and three patients with clinically silent corticotroph adenomas. All five patients with Cushing’s disease showed hypersecretion of ACTH (130, 190, 331, 120, and 130 pg/ml), high levels of serum cortisol (26.6–44.0 μg/dl), and symptoms of Cushing’s disease. All three patients with silent corticotroph adenoma showed hypersecretion of ACTH (110, 140, and 160 pg/ml) and normal levels of serum cortisol (11.4–26.8 μg/dl). The size of the pituitary adenoma on magnetic resonance imaging was smaller in patients with Cushing’s disease (mean 8.2 mm) than in patients with silent corticotroph adenoma (mean 26.7 mm) (p = 0.001). Transsphenoidal surgery was performed to totally resect the adenoma tissue. Immunostaining for ACTH showed diffuse ACTH-immunopositive cells in all eight adenomas. CIBA technique showed a good correlation between percentage of ACTH-immunopositive cells and level of plasma ACTH in patients with Cushing’s disease but no correlation between the two parameters in patients with silent corticotroph adenoma. The percentage of ACTH-secreting cells and the amount of hormone secreted by a single cell are too low in silent corticotroph adenomas to cause an increase in plasma ACTH level corresponding to the large tumor size.

Key words: Cushing’s disease, Silent corticotroph adenoma, Cell immunoblot assay


SILENT corticotroph adenoma is a term used to describe a subset of nonfunctioning adenoma containing immunoreactive ACTH but without clinical signs of Cushing’s disease [1]. Such adenomas may show high biological activity, large size on magnetic resonance (MR) imaging, and low levels of serum cortisol [2–6]. They are clinically characterized by the absence of symptoms of hypercortisolism, no laboratory data consistent with hypercortisolism, and tumoral ACTH immunoreactivity [7]. There are no known differences in immunocytochemistry between Cushing’s disease and silent corticotroph adenoma [1, 8].

We treated three patients with silent corticotroph adenoma identified preoperatively because of high levels of plasma ACTH in the absence of signs of Cushing’s disease. The present study investigated the immunocytochemistry and percentage of ACTH-secreting cells in these three cases of clinically silent corticotroph adenoma and five cases of Cushing’s disease. Cell immunoblot assay (CIBA) of cultured cells obtained from the pituitary adenomas was used to investigate the hormone-secreting cells.

CIBA showed a good correlation between the plasma ACTH level and the percentage of ACTH-immunopositive cells in Cushing’s disease but no correlation in silent corticotroph adenomas. We discuss the characteristics of silent corticotroph adenomas compared with Cushing’s disease, including plasma ACTH and cortisol level, MR imaging findings, immunocytochemistry, and CIBA.
Patients and Methods

Patients with Cushing’s disease and silent corticotroph adenoma

There were five patients with Cushing’s disease and three patients with silent corticotroph adenoma.

Table 1 shows the individual characteristics and Table 2 compares the clinical and endocrinological findings. The diagnoses were based on the high levels of plasma ACTH, MR imaging findings, and positive immunocytochemical staining for ACTH. ACTH RIA was performed using Allegro ACTH kit.

Table 1. Clinical, endocrinological, radiological, immunohistochemical, and cell immunoblot assay (CIBA) findings of individual patients with Cushing’s disease and silent corticotroph adenoma

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Sex</th>
<th>Clinical findings</th>
<th>Plasma ACTH (pg/ml)</th>
<th>Serum cortisol (µg/dl)</th>
<th>Urine 17-OHCS (mg/day)</th>
<th>Dexamethasone suppression test</th>
<th>Rate of increase of plasma ACTH CRH</th>
<th>Immunostaining</th>
<th>Tumor size (mm)</th>
<th>CIBA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>m</td>
<td>hypertension, central obesity, edema, skin pigmentation</td>
<td>130</td>
<td>28.0</td>
<td>44.3</td>
<td>no change</td>
<td>decreased</td>
<td>not tested</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>f</td>
<td>hypertension, central obesity, edema</td>
<td>190</td>
<td>26.6</td>
<td>not tested</td>
<td>no change</td>
<td>decreased</td>
<td>189%</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>m</td>
<td>hypertension, central obesity, edema, skin pigmentation</td>
<td>331</td>
<td>34.4</td>
<td>9.7</td>
<td>no change</td>
<td>decreased</td>
<td>163%</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>f</td>
<td>hypertension, central obesity, edema</td>
<td>120</td>
<td>30.4</td>
<td>24.0</td>
<td>no change</td>
<td>decreased</td>
<td>not tested</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>f</td>
<td>hypertension, central obesity, edema</td>
<td>130</td>
<td>44.0</td>
<td>31.3</td>
<td>no change</td>
<td>decreased</td>
<td>249%</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>m</td>
<td>visual disturbance</td>
<td>110</td>
<td>11.4</td>
<td>not tested</td>
<td>not tested</td>
<td>not tested</td>
<td>3</td>
<td>30</td>
<td>0.9</td>
</tr>
<tr>
<td>7</td>
<td>38</td>
<td>m</td>
<td>visual disturbance</td>
<td>160</td>
<td>17.8</td>
<td>not tested</td>
<td>not tested</td>
<td>not tested</td>
<td>106%</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>54</td>
<td>f</td>
<td>visual disturbance</td>
<td>140</td>
<td>26.8</td>
<td>not tested</td>
<td>not tested</td>
<td>not tested</td>
<td>214%</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

a) Cases 1–5: patients with Cushing’s disease, Cases 6–8: patients with silent corticotroph adenoma.  
b) Normal ranges: ACTH 10–60 pg/ml, cortisol 5.6–12.5 µg/dl, urine 17-OHCS 3.18–11.5 mg/day.  
c) Tumor size = maximum diameter of the tumor on magnetic resonance imaging.

m: male, f: female.

Table 2. Clinical and endocrinological comparison of patients with Cushing’s disease and silent corticotroph adenoma

<table>
<thead>
<tr>
<th></th>
<th>Cushing’s adenoma (n = 5)</th>
<th>Silent corticotroph adenoma (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>15–36 yr mean: 26.6 yr</td>
<td>31–54 yr mean: 41 yr</td>
</tr>
<tr>
<td>M/F</td>
<td>2 to 3</td>
<td>2 to 1</td>
</tr>
<tr>
<td>Symptoms</td>
<td>Cushing’s syndrome</td>
<td>Mass effects (visual symptoms)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>No</td>
<td>One case (33%) recurred after 27 months</td>
</tr>
<tr>
<td>Invasion</td>
<td>No</td>
<td>One case (33%)</td>
</tr>
<tr>
<td>Apoplexy</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Plasma ACTH</td>
<td>180 ± 39.7 pg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>136.7 ± 14.5 pg/ml</td>
</tr>
<tr>
<td>Serum cortisol</td>
<td>32.7 ± 3.1 µg/dl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.7 ± 4.5 µg/dl</td>
</tr>
<tr>
<td>Tumor size</td>
<td>8.2 ± 1.7 mm&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.7 ± 3.3 mm</td>
</tr>
<tr>
<td>Immunocytochemical staining</td>
<td>3 + : four cases; 2 + : one case</td>
<td>3 + : two cases; 2 + : one case</td>
</tr>
<tr>
<td>Cell immunoblot assay</td>
<td>11.2 ± 5.4%&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.1 ± 2.7%</td>
</tr>
</tbody>
</table>

T test: a) p = 0.451, b) p = 0.038, c) p = 0.001, d) p = 0.385
SILENT CORTICOTROPH ADENOMA

(Nihon Medi-physics Co. Ltd. Hyogo, Japan) by SRL Inc (Tachikawa Japan). Cushing’s disease was distinguished from silent corticotroph adenoma by the presence or absence of clinical symptoms.

Immunocytochemical staining

Surgically resected tumor tissues were stored in 20% formalin, embedded in paraffin, and sliced into sections 4–6 μm thick for immunocytochemical studies. Immunocytochemical staining for ACTH, GH, PRL, TSH, LH, and FSH were performed by an indirect peroxidase technique, using immunohistology kits purchased from DAKO Corporation (Carpinteria, CA, USA). Antiserum against the following hormones were used: ACTH (L1810), GH (L1814), PRL (L1837), TSH (L1842), LH (L1827), and FSH (L1810) (all from DAKO Corporation). Immunoreactivity was visualized with 3,3′-diaminobenzidine and sections were counterstained with Carazzi’s hematoxylin. We examined at least 300 cells in 5 different areas of the specimen to identify immunopositive cells for ACTH. Degree of immunoreactivity was classified as 3+ for more than 75% of cells stained, 2+ for 25% to 74% of cells stained, 1+ for 1% to 24% of cells stained, and 0 for no cells stained.

Pituitary cell suspension

Surgical specimens (100–300 mg) were used to prepare dispersions of pituitary cells as previously described [9–11]. After cell counting, the pituitary cells were suspended at a density of 1 × 10⁶ or 2 × 10⁶ cells/ml in Earle’s balanced salt solution supplemented with 20 mM HEPES, and then immediately used for CIBA.

CIBA

CIBA allows incubation of dispersed cells in an incubation chamber with a very low and constant height. This chamber method can evaluate the percentage of hormone secretion in total cell populations in an 8 mm² area within the incubation chamber [9, 10]. Dispersed cell suspensions (100 μl) were infused into the incubation chamber by capillary action. The suspension was then incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 60 min. After incubation, the transfer membranes were incubated at room temperature with 10% bovine serum albumin in 20 mM Tris-buffered saline containing 0.05% NaN₃, pH 7.6 for 120 min to block unoccupied binding sites.

After the blocking procedure, the transfer membrane was immunostained for ACTH. Primary antiserum was rabbit anti-human ACTH diluted at 1 : 100 (L1801, DAKO Corporation). Secondary antiserum was biotinylated donkey anti-rabbit immunoglobulin (Amersham, Arlington Heights, IL, USA) at 1 : 500 dilution. The transfer membrane was incubated for 40 min with the secondary antiserum with streptavidin-alkalinephosphatase (Amersham) at 1 : 3,000 dilution. The blots were visualized with 5-bromo-4-chloro-3-indolyl phosphate disodium and nitroblue tetrazolium chloride (Amersham) applied for 3 min. The transfer membrane was then rinsed with water and dried. Immunopositive blots for ACTH in the 8 mm² area were counted under a microscope. Then 100 μl of cell suspension diluted with Turk solution was infused into the incubation chamber and the total cells in the same area were counted under a microscope. The percentage of hormone-secreting cells in the total cells in the 8 mm² area was calculated from the number of immunopositive blots/the total number of cells [11].

Results

Hormonal assays

Plasma ACTH (p = 0.451) and serum cortisol (p = 0.038) levels were greater in patients with Cushing’s disease than in those with silent corticotroph adenoma (Table 2). Urine 17-OHCS level was high in three out of 4 Cushing patients (Table 1).

Immunocytochemical staining

Immunoreactivity for ACTH was 3+ in four cases and 2+ in one case of Cushing’s disease, and 3+ in one case and 2+ in two cases of silent corticotroph adenomas. All other pituitary hormones were detected at 1+ in scattered cells. LH was detected in Case 1, LH, FSH, and TSH were detected in Case 5, and GH was detected in Case 6.
CIBA

Percentages of ACTH cells were 0.6–32.0% (mean 11.2 ± 5.4%) in cases of Cushing’s disease and 0.9–9.6% (mean 4.1 ± 2.7%) in cases of silent corticotroph adenomas (Table 1). There was no difference between the percentage of Cushing’s disease and silent corticotroph adenomas (p = 0.385). The blot became darker in proportion to the amount of hormone secreted by a single cell [10].
The gray values of cell blots were scanned and changed into binary images by personal computer using ScanJX V4.21 and Adobe Photoshop 4.01. The threshold value for drawing binary images was decided by the disappearance of small blots. We counted blots greater than the threshold value of 90 degrees among all immunopositive cells for ACTH in the 8 mm² area. Blots more than 90 degrees in cases of silent corticotroph adenomas (0%, 3.6%, 49.0%) were less than in those of Cushing’s disease (13.5%, 0%, 28.6%, 30.8%, 23.1%) (binary images not shown) (Fig. 1).

Fig. 1 Photographs showing blots immunoreactive for ACTH in patients with Cushing’s disease and patients with silent corticotroph adenoma. The intensity of staining was weaker in Cases 6–8 than in Cases 1–5.
A: Case 1, B: Case 2, C: Case 3, D: Case 4, E: Case 5, F: Case 6, G: Case 7, H: Case 8.
CIBA and plasma ACTH level

There was a good correlation ($r^2 = 0.93$, $p = 0.008$) between the percentage of hormone-secreting cells and plasma ACTH level in patients with Cushing's disease (Fig. 2), whereas there was no correlation ($r^2 = 0.048$) between these two parameters in patients with silent corticotroph adenoma (Fig. 3).

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**Fig. 2.** Graph showing a good correlation between the percentage of hormone-secreting cells and the plasma ACTH level in five patients with Cushing's disease. Tumor size of four patients was almost the same except in Case 2.

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**Fig. 3.** Graph showing no correlation between the percentage of hormone-secreting cells and the plasma ACTH level in three patients with clinically silent corticotroph adenoma. Tumor size was almost the same.
Discussion

Clinically silent corticotroph adenomas have been described recently [1–5, 7, 8, 12–15]: 17 (5.7%) silent corticotroph adenomas were detected among 300 surgically removed pituitary adenomas [1]; 3 of 37 non-functioning adenomas showed immunostaining for ACTH [16]; and a large series of 23 patients with clinically silent corticotroph tumors of the pituitary gland treated at the Mayo Clinic over a 22-year period with a mean follow-up of 4.9 years was retrospectively analyzed [5]. The characteristic clinical and biological features of these silent corticotroph adenomas were as follows: Absence of clinical symptoms of Cushing’s disease, no or only minor increases in serum ACTH levels, normal serum cortisol concentrations, high frequency of symptoms attributable to mass effects, large tumor size, frequent hemorrhagic infarctions with subsequent cystic changes, sphenoid or cavernous sinus invasion, and frequent need for postoperative radiation therapy and/or reoperation to treat residual tumor or major tumor regrowth.

Silent corticotroph adenoma can be divided into three subtypes based on morphological, ultrastructural, and immunohistochemical findings [15]. Subtype 1 adenomas are morphologically and ultrastructurally similar to the adenomas associated with Cushing’s disease, but ACTH or cortisol excess does not occur in the patients. Subtype 2 tumors have some features of functional corticotropic adenomas, except for the smaller ultrastructural size of the secretory granules and the absence of type I microfilaments. Subtype 3 silent corticotroph adenomas are more varied and may secrete other pituitary hormones, in addition to ACTH, but the ultrastructural features differ from corticotroph adenomas. Therefore, subtype 3 tumors were thought to be unrelated to silent corticotroph adenomas. Recently, the differences in subtype 1 and subtype 2 were investigated by hematoxylin and eosin staining, immunoreactivity for ACTH, and periodic acid-Schiff staining [5]. Hematoxylin and eosin staining showed basophilic in subtype 1 and chromophobic in subtype 2 [5]. Immunoreactivity for ACTH was moderate to strong in subtype 1 and scattered in subtype 2 [5]. We did not perform ultrastructural studies in our seven pituitary adenomas and so we could not assign the adenomas according to Horvath’s classification [1]. However, it would seem that our three silent corticotroph adenomas belonged to subtype 1 based on basophilic staining and moderate to strong immunoreactivity for ACTH.

Cushing’s disease and silent corticotroph adenoma show differences in sex-related incidence, frequency of preoperative hyperprolactinemia, frequency of radiographic/gross invasion, frequency of intratumoral apoplexy, and serum cortisol levels [5]. Silent corticotroph adenomas differ markedly from the adenomas associated with Cushing’s disease, especially in large tumor size, absence of clinical symptoms, minor increase in plasma ACTH level, normal plasma cortisol concentration, and major tumor regrowth [2–4, 6, 13]. Our three patients with silent corticotroph adenoma showed minor increases in plasma ACTH, normal cortisol concentration, and large tumor size.

Cushing’s disease and silent corticotroph adenoma type I show no pathological differences [5]. Similarly, we found no differences in immunocytochemical staining. However, we found obvious differences between patients with Cushing’s disease and those with silent corticotroph adenomas in both clinical/endocrinological characteristics and the activity of hormone secretion (Table 2). Minor differences were found in plasma ACTH and serum cortisol levels between Cushing’s disease and silent corticotroph adenoma. Silent corticotroph adenomas show no clinical symptoms of Cushing’s disease despite the slightly increased plasma ACTH levels. ACTH secreted from silent corticotroph adenomas cannot cause an increase in serum cortisol level. The mechanism responsible for the endocrine inactivity of silent corticotroph adenomas is unknown. However, three mechanisms have been proposed: The hormonal secretion is too low to produce serum and clinical changes [1, 2]; the hormonal product is catalyzed in situ by lysosomes [14]; and ACTH secreted by the tumor could be biologically inactive (so-called big ACTH) [1, 3, 6–8, 13, 15]. The clinical silence might be caused by a minor variant of ACTH leading to lower biological activity of ACTH. Our study indicated that both the amount of hormone secreted by a single cell and the percentage of hormone-secreting cells were smaller in silent corticotroph adenoma than in Cushing’s adenoma. We previously examined the amount of hormone secreted by a single pituitary cell by CIBA [9–11]. This investigation
demonstrated a low percentage of hormone-secreting cells and low amount of hormone secreted by a single cell in silent corticotroph adenomas. This finding suggested that the reason why plasma ACTH levels were not correlated with CIBA in silent corticotroph adenomas is that the secretory activity is low in spite of the same immunopositivity in immunocytochemical staining as Cushing’s disease.

The present finding of a good correlation between the percentage of hormone-secreting cells and the plasma ACTH level in Cushing’s disease suggests that the plasma ACTH level is determined by the percentage of hormone-secreting cells. The plasma ACTH levels are low in spite of the large tumor size in silent corticotroph adenomas. Therefore, plasma ACTH level may be related to the percentage of hormone-secreting cells in microadenoma of Cushing’s disease. Plasma ACTH level increases with the size of the tumor in Cushing’s disease [17, 18]. Therefore, there was no correlation (p = 0.291) between the plasma ACTH level and the tumor size in our patients with Cushing’s disease. The only factor which correlated to plasma ACTH level was the percentage of immunopositive cells for ACTH. Silent corticotroph adenomas have increased plasma ACTH level but not increased serum cortisol level. While the slightly elevated plasma ACTH level may be induced by stress [5], our study demonstrated ACTH secretion from tumor cells. It is possible that Cushing’s syndrome is absent in patients with silent corticotroph adenomas because biologically inactive ACTH is being secreted from a small number of ACTH-secreting cells.

References


