Identification of Somatotrophs, Mammosomatotrophs, and Mammotrophs by Sandwich Cell Immunoblot Assay in GH-Secreting Adenomas and Prolactinomas: Correlations between the Proportions of Hormone Secreting Cells and Tumor Size

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Abstract. Sandwich cell immunoblot assay (sandwich CIBA) was used to identify somatotrophs (GH cells), mammosomatotrophs, and mammotrophs (PRL cells) in pituitary tumors obtained from patients with GH-secreting adenomas and prolactinomas. The mean serum GH level was 177.6 ng/ml in 19 patients with GH-secreting adenomas and the mean PRL level was 2,129 ng/ml in 9 patients with prolactinomas. GH-secreting adenomas could be divided into 3 groups according to the proportions of the cell types. The GH cell dominant type had more than 70% GH cells. The mammosomatotroph cell dominant type had more than 80% mammosomatotrophs. The nondominant type had no dominant cell type. There was a good correlation ($r^2=0.804$) between serum GH levels and tumor size in patients with the GH cell dominant type ($n=10$). The nondominant type ($n=8$) had a low serum level of GH except for one tumor. The mammosomatotroph cell dominant type ($n=1$) showed high serum levels of both GH and PRL. All prolactinomas had a predominance of PRL cells. Sandwich CIBA is a simple method for detecting GH cells, mammosomatotrophs, and PRL cells, and useful for classification for GH-secreting adenomas.

Key words: Pituitary adenoma, GH-secreting adenoma, Prolactinoma, Sandwich cell immunoblot assay
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GH-SECRETING adenomas can be divided into acidophil stem cell adenomas, mammosomatotroph adenomas, and somatotroph adenomas according to the proportions of the cell types, whereas prolactinomas contain only a single cell type. To determine whether other techniques of classification of the subtypes in GH-secreting adenomas and prolactinomas are possible, in this study we classified GH-secreting adenomas and prolactinomas based on the proportions of their GH cells, mammosomatotrophs, and

PRL cells, and investigated the relationships between the subtypes and the clinical manifestations.

Many techniques have been developed to identify GH cells, mammosomatotrophs, and PRL cells. Colloidal gold immunoelectron microscopy has shown that both acidophil stem cells and mammosomatotroph cells secrete both GH and PRL [1, 2], and that the mammosomatotroph cell may be considered to be a mature type of the acidophil stem cell [3]. Previously, PRL and GH were reported to be mainly synthesized and secreted by mammotrophs and somatotrophs, respectively [4–9], but the existence of mammosomatotroph cells has been confirmed [1, 3, 9–23].

Reverse hemolytic plaque assay (RHPA) [19, 24] can detect mammosomatotrophs in cell cultures and

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the double immunocytochemical labeling technique using gold particles of different sizes can show cells containing both GH granules and PRL granules. The double immunostaining method [20, 25] and the immunocytochemistry-mirror section method can also show the coexistence of mammosomatotroph cells [22], but these methods are difficult to use compared with the RHPA and colloidal gold techniques. Chamber cell immunoblotting assays can quantify hormone secretion from individual pituitary cells [26], and has been used to detect GH cells and PRL cells, but not mammosomatotrophs. However, the sandwich cell immunoblot assay (sandwich CIBA) [27, 28] can detect mammosomatotrophs as well as GH cells and PRL cells. Therefore, sandwich CIBA is a simple method to detect the hormones secreted by individual cells and also reveals the cell proportions of GH cells, PRL cells, and mammosomatotrophs in cell cultures.

We investigated in the present study the proportions of GH cells, PRL cells, and mammosomatotrophs in specimens of GH-secreting adenoma and prolactinoma to evaluate the correlation between the cell types and the clinical manifestations of tumor size and serum hormone levels.

Materials and Methods

Patients and endocrinological examination

In this study, we examined pituitary specimens obtained via the transsphenoidal approach from 19 patients, 6 males and 13 females aged 15 to 59 years (mean 42.4 years), with GH-secreting adenomas manifesting as elevation of serum GH level (more than 5 ng/ml), and nine patients, 6 males and 3 females aged 34 to 66 years (mean 51.0 years), with prolactinomas manifesting as hyperprolactinemia (more than 30 ng/ml). All tumors were demonstrated by magnetic resonance (MR) imaging. Tissue was obtained from the center of the pituitary adenoma to avoid contamination by normal pituitary tissue. Immunostaining of formalin-fixed, paraffin-embedded sections excluded the possibility of contamination by normal pituitary tissue which shows immunoreactivity to GH, PRL, LH, FSH, TSH, and ACTH.

Endocrinological studies were performed to meas-

ure the basal levels of serum GH, PRL, LH, FSH, TSH, and ACTH. Serum hormone levels were also evaluated with such stimuli as insulin induced hypoglycemia (insulin 0.1 U/kg, iv), TRH (500 mg, iv), LHRH (100 mg, iv), and bromocriptine (2.5 mg, po). Patients with GH-secreting adenoma were considered to have a positive response to insulin when the serum GH level increased to more than 150% of the basal GH value, and a positive response to bromocriptine when the serum GH level decreased to less than 50% of the basal GH value. All three stimuli were given at the same time and consequently we could not evaluate the elevation of serum GH level by only TRH. Patients with prolactinomas were considered to have a positive response to TRH when the serum PRL level increased to more than 150% of the basal PRL value, and a positive response to bromocriptine when the serum PRL level decreased to less than 50% of the basal PRL value.

Morphological studies

All patients underwent MR imaging and computed tomography with contrast enhancement. The maximum diameter of the enhanced lesion on MR images was measured.

Immunohistochemical staining

Surgical specimens were fixed for more than 6 hours at room temperature in 20% buffered formalin, and embedded in paraffin. Sections (4 mm thick) were then prepared for the indirect immunoperoxidase-staining using such antisera as anti-human GH (L1814), PRL (L1837), LH (L1827), FSH (L1810), TSH (L1842) and ACTH (L1801) (all from DAKO, Carpinteria, CA). The staining was visualized with 3,3'-diaminobenzidine. 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 [27–30]. After cell counting, the pituitary cells were suspended at a density of 2×10^5 cells/ml

in Earle's balanced salt solution supplemented with 20 mM HEPES (ESH), and then immediately used for sandwich CIBA.

Sandwich CIBA

In this study, we used the novel sandwich CIBA technique [27, 28] which can detect two different hormones secreted from the same cell. Pieces of polyvinylidene difluoride transfer membrane (Immobilon, Millipore, Bedford, MA), 40×28 mm for the lower layer and 30×18 mm for the upper layer, were prewetted in 100% methanol for 20 sec, rinsed in distilled water for 5 min, and equilibrated with ESH for 60 min. The larger piece of transfer membrane was placed on a glass microscope slide with the water-repellent side up to serve as the lower layer of the sandwich. Cell suspension (30 ml) was applied to the center of the transfer membrane. The smaller piece of the transfer membrane was then placed on the cell suspension droplet with the water-repellent side down to act as the upper layer of the sandwich. The transfer membrane sandwich was then incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 60 min.

After incubation, the transfer membrane sandwich was penetrated at 22 sites with a pair of thin needles held 0.68 mm apart (Fig. 1). The pairs of transfer membranes were immersed in ESH and carefully separated with forceps. The transfer membranes were incubated at room temperature with 10% bovine serum albumin in 20 mM Tris-buffered saline containing 0.05% NaN_3 , pH 7.6 for 120 min to block unoccupied binding sites. After the blocking procedure, the transfer membrane was immunostained for either GH or PRL. Primary antisera were rabbit anti-human GH diluted at 1:15,000 (A570, DAKO), and rabbit anti-human PRL diluted at 1:15,000 (A569, DAKO). Secondary antiserum was biotinylated donkey anti-rabbit immunoglobulin (Amersham, Arlington Heights, IL) used diluted at 1:500. The transfer membrane was incubated for 40 min with the secondary antiserum with streptavidin-alkaline phosphatase (Amersham) diluted at 1:3,000. The blots were visualized with 5-bromo-4-chloro-3-indolyl phosphate disodium and nitroblue tetrazolium chloride (Amersham) for 3 min. The transfer membrane was then rinsed with water and air dried.

Cell blots detected on the transfer membranes were

photographed by a microscope so that each pair of the reference points was in the center of the photograph. Cell blots as well as reference points in each print were copied onto tracing paper. At least 300 blots were analyzed to detect the composition of cell types.

Concerning cross reactivity, the immunoreactivity of anti-GH serum (A570, DAKO) was considered to be 100% for human GH and 1% for PRL and the immunoreactivity of anti-PRL serum (A569, DAKO) was 100% for PRL by radioimmunoassay (manufacturer's data). Therefore, we ignored the cross reactivity between the two antibodies as few cells were

SANDWICH CELL IMMUNOBLOT ASSAY

Fig. 1. Flow diagram of the methods used to perform sandwich CIBA. A drop of cell suspension is placed on a transfer membrane (A) and then covered with another transfer membrane (B). After incubation at 37°C , the two transfer membranes were stuck together at 22 sites with a pair of thin sewing needles to determine the position of the cell blots and then separated. The upper transfer membrane is immunostained with GH antiserum, the lower transfer membrane with PRL antiserum. If a single cell is a mammosomatotroph, cell blots are found at the same positions on the different transfer membranes in a mirror-image position and found at the same positions on the photographs of the transfer membranes.

Table 1. Hormonal data, immunohistochemical staining of formalin-fixed, paraffin-embedded sections, and results of sandwich CIBA and chamber CIBA in 28 patients with pituitary adenomas.

	Case	Age	Sex	Serum GH (ng/ml)		Serum PRL (ng/ml)	
				Insulin ^{a)}	bromocriptine ^{b)}	TRH ^{c)}	bromocriptine ^{b)}
GH adenoma (GH-dominant)	1	47	M	308/1650	334/458	3/12.1	3/0.6
	2	43	F	100/260	560/140	NP	NP
	3	47	F	550/1400	490/310	21/59.8	13.7/5.1
	4	50	M	122/802	86/39.8	4.5/16.4	4.4/0.5
	5	15	F	140/140	100/74	1.2/3.8	1.1/1
	6	52	F	110/320	67/4.9	2.6/13.8	NP
	7	40	M	572/380	426/400	8.3/21.3	10.1/1.2
	8	50	F	6.8/10.3	9.0/7.8	4.1/35.3	4/1.0
	9	59	F	24/154	31/28	39/41	NP
	10	54	F	NP	133/63	6.7	NP
GH adenoma (Non-dominant)	11	49	F	20/30	13/27	NP	2.7/4.3
	12	39	M	39/21	24.1/12	3.3	NP
	13	21	F	56	15/15	NP	30/20
	14	44	F	26/35	26/9.8	26/35	NP
	15	45	M	97/130	79/5.6	130/250	74/16
	16	55	F	92/111	110/121	4.4	NP
	17	28	F	390/400	390/220	20/49	20/1.1
	18	47	M	21/428	NP	40.4	NP
Mammotroph cell adenoma	19	21	F	70/500	70/3.1	18/187	18/1.1
Prolactinoma	20	57	M	0.2/0.22	0.2/0.14	1559/1917	1559/73
	21	48	M	0.52/2.1	NP	2557/3881	3721/617
	22	40	M	1.64	NP	NP	3631/790
	23	34	M	0.08/12	NP	198/286	283/35.6
	24	49	F	0.12/3.79	NP	746/820	NP
	25	41	M	1.03/2.7	NP	413/521	556/134
	26	62	F	0.76/1.44	NP	458/625	NP
	27	62	M	0.16/3.7	NP	1950/2194	1534/230
	28	66	F	1.03/4.6	1.84/0.53	NP	6258/1085

M: male, F: female, NP: not performed.

^{a)} Insulin: basal value/maximum value after intravenous injection of insulin 0.1 U/kg.

^{b)} Bromocriptine: basal value/minimum value after oral administration of bromocriptine 2.5 mg.

^{c)} TRH: basal value/maximum value after intravenous injection of TRH 500 µg.

^{d)} Immunostaining: 3=immunoreactive cells more than 75%, 2=25–74%, 1=1–24%, 0=not stained.

immunoreactive for PRL in GH-secreting adenoma (Cases 1, 9) and no cells were immunoreactive for GH in prolactinoma (Case 28) by sandwich cell immunoblot assay (Table 1). Also, to decrease the cross reactivity, we diluted the antiserum as much as possible for minimal detection of immunopositive blots.

Chamber CIBA

Chamber CIBA [27] allows incubation of dispersed cells in an incubation chamber with a very low and constant height. This chamber method enabled us to evaluate the percentage of hormone secretion in total cell populations in an 8 mm square area within the incubation chamber.

Immunostaining ^{d)} (grade)		Sandwich CIBA ^{e)} (%)			Chamber CIBA ^{f)} (%)	
anti-GH	anti-PRL	GH cell	PRL cell	mammosomatotroph cell	GH cell	PRL cell
3	3	97.8	1.3	0.9	90.7	3.9
3	2	84.5	4.1	11.4	11.5	0.6
3	0	96.0	2.3	1.7	53.3	NP
3	0	85.1	2.7	12.2	53.7	3.3
3	3	76.1	7.0	16.9	75.5	2.9
3	3	84.4	7.5	8.1	63.4	4.4
2	0	88.1	4.8	7.1	24.8	0.4
NP	NP	80.4	9.8	9.8	40.4	2.1
NP	NP	98.0	2.0	0.0	87.5	0.1
3	3	84.0	4.0	12.0	67.8	2.1
3	2	50.0	33.3	16.7	85.8	35.2
1	0	50.0	31.2	18.8	12.3	2.1
3	0	36.1	24.1	39.8	21.6	4.2
3	3	55.6	28.5	15.9	30.3	13.5
2	2	48.0	36.0	16.0	35.0	18.0
2	0	43.1	21.6	35.3	41.5	4.3
3	3	47.5	3.7	48.8	35.0	4.8
3	3	32.9	45.8	21.3	85.2	70.6
3	3	8.9	1.3	89.8	40.4	39.7
1	3	1.9	95.6	2.5	3.4	41.5
0	3	5.6	86.9	7.5	2.6	25.8
0	3	2.8	95.8	1.4	1.4	32.4
0	3	12.3	72.3	15.4	6.1	33.9
0	3	16.2	76.6	7.4	0.06	0.2
0	2	6.5	87.0	6.5	2.0	63.9
0	3	0.9	96.8	0.9	0.01	14.0
0	3	18.3	76.3	5.4	0.9	67.9
NP	NP	0.0	100.0	0.0	0.01	14.0

^{e)} Sandwich CIBA: GH cell=immunopositive cells for only GH, PRL cell=immunopositive cells for only PRL, mammosomatotroph cell=immunopositive cells for both GH and PRL.

^{f)} Chamber CIBA: GH cell=immunopositive cells for only GH/total count of pituitary cells in 8 mm square, PRL cell=immunopositive cells for only PRL/total count of pituitary cells in 8 mm square.

Dispersed cell suspensions (100 μ l) 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 immunostained in the same way as described for sandwich CIBA. Then 100 μ l of cell suspension diluted with Turk solution was infused into the incubation chamber and total cells in the same area were counted under a microscope. The percentage

of hormone-secreting cells among the total cells in an 8 mm square area was calculated as the number of immunopositive blots/the total number of cells.

Results

Endocrinological examination

Positive responses of serum GH to insulin-induced

hypoglycemia were recognized in 8 of 17 patients, and positive responses to bromocriptine in 8 of 18 patients with GH-secreting adenoma. Positive responses of serum PRL to TRH were recognized in 10 of 12 patients and positive responses to bromocriptine were recognized in 8 of 11 patients with GH-secreting adenoma.

Serum GH levels were within the normal limit in the patients with prolactinomas. No response of serum PRL to TRH was recognized in five patients (Cases 20, 22, 23–27), but responses to bromocriptine were recognized in all seven patients (Cases 20–23, 25, 27, 28) who underwent the bromocriptine test (Table 1).

Immunohistochemical staining

Immunohistochemical staining was performed in pituitary specimens obtained from 17 of 19 patients with GH-secreting adenomas. All 17 specimens showed immunoreactivity for GH and 11 of 17 specimens showed immunoreactivity for PRL.

Immunohistochemical staining was performed in pituitary specimens from 8 of 9 patients with prolactinomas. One specimen showed immunoreactivity for both GH and PRL. The other seven specimens showed immunoreactivity for only PRL (Table 1).

Among the 19 GH-secreting adenomas and 9 prolactinomas, two specimens (Cases 6, 14) showed immunoreactivity for LH and FSH and one specimen (Case 18) for TSH. There were no specimens with immunoreactivity for ACTH.

Sandwich CIBA

GH-secreting adenomas could be divided into three groups according to the composition of GH, PRL, and mammosomatotroph cells. The GH cell dominant type was characterized by a GH cell composition of more than 70% (Fig. 2). The non-dominant cell type showed no preponderance of GH, PRL or mammosomatotroph cells (Fig. 3). The mammosomatotroph cell dominant type was characterized by a mammosomatotroph cell composition of more than 80% (Fig. 4). The GH cell dominant type (Cases 1–10) had a composition of $87.4 \pm 2.4\%$ (mean $\pm$ SEM) GH cells, $4.6 \pm 0.9\%$ PRL cells, and $8.0 \pm 1.8\%$ mammosomatotrophs. The non-dominant cell type (Cases 11–18) had a composition of $45.4 \pm 2.7\%$ GH cells, $28.0 \pm 4.4\%$ PRL cells, and $26.6 \pm 4.5\%$ mammosomatotrophs. The mammosomatotroph cell dominant type (mammosomatotroph cell adenoma [31], Case 19) had a composition of 8.9% (mean) GH cells, 1.3% PRL cells, and

Fig. 2. Photographs of sandwich CIBA of GH cell dominant type adenoma. The upper transfer membrane (a) was immunostained with GH antiserum and the lower transfer membrane (b) with PRL antiserum. Note that many GH-immunopositive blots (upper) but few PRL-immunopositive blots (lower) are found at the same positions with reference to two pinhole marks in the center of each photograph. Almost all cells are somatotroph cells. The only blot on the lower transfer membrane is a mammotroph. The diameter of the hole is 45 μ m. Primary antisera used were as follows: anti-human GH (A570; DAKO, Carpinteria, CA., USA) at a dilution of 1 : 15,000; anti-human PRL (A569; DAKO, Carpinteria, CA., USA) at a dilution of 1 : 15,000.

Fig. 3. Photographs of sandwich CIBA of nondominant cell type adenoma. The upper transfer membrane (a) was immunostained with GH antiserum and the lower transfer membrane (b) with PRL antiserum. Note that there are more blots for anti-PRL than for the GH dominant cell adenoma in Fig. 2. The adenoma cells consist of somatotrophs, mammotrophs, and mammosomatotroph cells.

Fig. 4. Photographs of sandwich CIBA of mammosomatotroph cell dominant type adenoma (Case 19). The upper transfer membrane (a) was immunostained with GH antiserum and the lower transfer membrane (b) with PRL antiserum. Note that almost all blots showed immunopositivity for both GH and PRL. This adenoma is probably a mammosomatotroph adenoma based on the population of mammosomatotroph cells and endocrinological data.

89.8% mammosomatotrophs (Table 1).

Eight of the nine prolactinomas secreted both GH and PRL. However, the percentage of immunopositive cells for GH was lower than 10% in six of these nine adenomas (Fig. 5). Prolactinomas had a composition of $7.2 \pm 2.3\%$ (mean $\pm$ SEM) GH cells, $87.5 \pm 3.4\%$ PRL cells, and $5.2 \pm 1.6\%$ mammosomatotrophs (Table 1).

Chamber CIBA

The percentage of hormone-secreting cells in total cell populations is shown in Table 1. The percentages of GH cells and PRL cells in GH cell dominant type adenomas were $56.9 \pm 8.1\%$ and $2.2 \pm 0.5\%$, respectively. The percentages of GH cells and PRL cells in nondominant cell type adenomas were $43.3 \pm 9.9\%$ and $19.1 \pm 8.3\%$, respectively. The per-

Fig. 5. Photographs of sandwich CIBA of prolactinoma. The upper transfer membrane (a) was immunostained with GH antiserum and the lower transfer membrane (b) with PRL antiserum. Note that almost all blots showed immunopositivity for only PRL (lower).

centages of GH cells and PRL cells in prolactinomas were $1.8 \pm 0.7\%$ and $32.6 \pm 7.5\%$, respectively. Case 28 indicated a difference in the percentage of GH cells between chamber CIBA and sandwich CIBA because one immunopositive cell for GH was found in the 8 mm square area.

Tumor size and serum hormone level

The mean tumor diameter was 18.6 mm for the GH cell dominant type, 24.4 mm for the nondominant cell type, and 13 mm for the mammosomatotroph cell dominant type. Patients with the GH cell dominant type showed a good correlation ($r^2 = 0.804$) between serum GH level and tumor size (Fig. 6 upper). Patients with the nondominant cell type had a low serum GH level even with large tumors except for Case 17 (Fig. 6 lower). Patients with the mammosomatotroph cell dominant type had a higher serum PRL level (18 ng/ml) than those with the GH cell dominant type (10.2 ± 4.1 ng/ml).

The mean diameter of tumors was 24.6 ± 4.0 mm for prolactinomas. There was no good correlation between the tumor size and serum PRL level ($r^2 = 0.421$).

Clinical features and cell type

Acromegaly was found in 8 patients and diabetes mellitus in 5 of the 10 patients with the GH cell dominant type. In contrast, acromegaly was found

in three patients and diabetes mellitus in one of the 8 patients with the nondominant cell type.

Discussion

Mammosomatotrophs can be detected in specimens of pituitary gland or adenoma using several methodologies, including the double immunostaining method [20, 25], the reverse hemolytic plaque assay [19], and the colloidal gold modification [32, 33]. Colloidal gold immunoelectron microscopy enables the identification of mammosomatotrophs by employing gold particles of differing sizes that have been conjugated to separate antibodies. The colloidal gold modification deals with fixed cells. In contrast, the reverse hemolytic plaque assay enables microscopic visualization of hormone release from individual living cells in culture, providing direct evidence for the existence of cells that release both GH and PRL. To improve the sensitivity of these techniques to detect two hormones, we used sandwich CIBA to detect mammosomatotrophs in human pituitary adenomas.

Two morphological patterns were found in GH-secreting adenomas by double immunocytochemical labeling [32], adenomas that contain only GH-positive cells and adenomas that contain varying proportions (10–98%) of PRL-positive cells. The great majority of cells positive for PRL were also positive for GH. A positive correlation was found between

Fig. 6. Correlation between serum GH and the maximum diameter of the tumor in GH cell dominant type adenomas (upper) and nondominant cell type adenomas (lower). There was a correlation between the two parameters for the GH cell dominant type. However, there was no correlation between the two parameters for the nondominant cell type.

the serum PRL levels and the percentage of mammosomatotrophs. We used sandwich CIBA, which is more sensitive and technically simpler than other methods, to classify GH-secreting adenomas and prolactinomas by the composition of GH cells, PRL cells, and mammosomatotroph cells. In particular, this sandwich CIBA method was effective for the detection of mammosomatotrophs.

Advanced methods of immunocytochemical analysis have allowed the presence of mammosomatotrophs in pituitary adenomas to be confirmed by other investigators. The percentage of mammosomatotrophs in the tumors of patients with acromegaly has been reported as 0–53% [9], 4–20% [19], and 0–80% [1, 32]. In our study, the per-

tages of mammosomatotrophs in the 19 GH-secreting adenomas and nine prolactinomas were 0.9–89.8% (mean 20.1%) and 0–15.4% (mean 5.2%), respectively. Bassetti *et al.* investigated 22 GH secreting adenomas by double immunocytochemical labeling technique and found eleven adenomas consisted of cells positive only for GH and other adenomas consisted of cells (10–98%) positive for PRL besides cells positive only for GH [32]. Zurschmiede *et al.* reported the proportion of mammosomatotrophs by means of double immunocytochemical labeling technique were 0.0–53.0% in 15 pituitary adenomas causing acromegaly and 1.6–34.3% in 11 prolactinomas [9]. Our data are consistent with these data previously reported [1, 9, 32].

We found in the present study immunoreactivity for both GH and PRL in 11 (64.7%) of 17 GH-secreting adenomas and 1 (12.5%) of 8 prolactinomas. Immunohistochemistry of GH-secreting adenomas has demonstrated immunoreactivity for PRL, even though the serum PRL levels were not substantially elevated. Eight (Cases 1, 5, 6, 10, 11, 14, 17, 19) of 14 GH-secreting adenomas with normoprolactinemia (PRL level <30 ng/ml) showed positive immunostaining for PRL.

Mammotrophs may contribute more to PRL than GH levels [34]. However, we did not find a good correlation between PRL levels and the percentage of mammotrophs. Therefore, we speculate that an adenoma with a higher content of mammotrophs will respond more to bromocriptine. However, serum GH responses

to bromocriptine in our patients with GH-secreting adenomas were not strictly related to the presence of mammotrophs in both the GH dominant type (Fig. 7 upper) and the nondominant type (Fig. 7 lower).

Table 1 shows that the immunohistochemical data were not consistent with the results of sandwich CIBA in many cases. For example, sandwich CIBA showed only a few PRL cells in Cases 1 and 17, which showed grade 3 immunoreactivity for PRL in formalin-fixed, paraffin-embedded sections. Since a similar discrepancy was found in many cases, we supposed that most immunoreactive cells in these cases stored but did not secrete hormone.

We did not find a good correlation between PRL levels and the percentage of mammotrophs as previously reported [34], but we supposed that this

Fig. 7. Correlation between the percentage of mammotroph cells and the change of serum GH after bromocriptine for GH cell dominant type adenomas (upper) and nondominant cell type adenomas (lower). There was no correlation between the two parameters. MS cells: mammotroph cells.

discrepancy can be accounted for by the fact that mammosomatotrophs do not secrete as much PRL as the PRL cells in PRL-secreting adenomas.

Chamber CIBA

We previously reported that the percentage of PRL-secreting cells in prolactinomas determined by chamber CIBA was correlated to the percentage of PRL-secreting cells in prolactinomas determined by reverse hemolytic plaque assay [35, 36]. Therefore, we considered chamber CIBA to be a good method to measure the proportion of hormone-secreting cells. However, we cannot divide GH-secreting adenomas into subtypes using only chamber CIBA because there was no difference in the proportion of GH cells and PRL cells between the GH-dominant type and non-dominant type. Therefore, we used sandwich CIBA to detect mammosomatotrophs as well as GH cells and PRL cells. Unlike chamber CIBA, we can measure the proportions of GH, PRL, and mammosomatotroph cells in all immunopositive blots. Of course, the sum of the percentages of GH, PRL, and mammosomatotroph cells is 100%.

Sandwich CIBA

GH-secreting adenomas can be divided into three types according to pathophysiology: mixed GH-PRL cell adenomas, acidophil stem cell adenomas which contain both GH and PRL, and mammosomatotroph cell adenomas which are considered to be a mature type of acidophil stem cell adenoma [37, 38]. Our study showed that GH-secreting adenomas can be divided into three groups according to the composition of GH, PRL, and mammosomatotroph cells. The clinical characteristics were well correlated with this classification. Acromegaly and diabetes mellitus were more frequently recognized in patients with the GH cell dominant type than those with the nondominant cell type. Patients with the GH cell dominant type had a level of serum GH proportional to the size of the adenoma. In contrast, patients with the nondominant cell type had a low level of serum GH even with a large tumor. There were many differences in hormonal tests, tumor size, and clinical features between the GH cell dominant type and nondominant cell type. An atypical GH-secreting adenoma showed a slightly high level of serum

GH despite the large tumor size. This atypical GH-secreting adenoma was classified as the nondominant type. We also found that in some atypical GH-secreting adenomas, there were GH-immunopositive cells scattered in the formalin-fixed, paraffin-embedded sections. Serum GH was not lowered to normal levels after surgery in patients with a slightly high level of serum GH even when the tumor resection was subtotal. We consider that the reason was the presence of such scattered GH cells in the tumor.

Case 19 was previously described [31] as a patient with mammosomatotroph cell adenoma because electron microscopy showed no oncocyctic changes such as poorly developed rough-surfaced endoplasmic reticulum membranes, inconspicuous Golgi complexes or giant mitochondria [37]. A pituitary adenoma which secretes both GH and PRL in the same cell is thought to be an acidophil stem cell adenoma or a mammosomatotroph cell adenoma. We cannot distinguish a mammosomatotroph cell adenoma from an acidophil stem cell adenoma on the basis of clinical data, immunohistochemical findings, and sandwich CIBA. Acidophil stem cell adenoma may be an adenoma composed of immature cells representing the common progenitor of GH and PRL cells [37]. However we can distinguish mammosomatotroph cell adenoma from acidophil stem cell adenoma by the presence of oncocyctic changes under the electron microscope. Mammosomatotroph cell dominant type adenomas corresponded to mammosomatotroph cell adenoma based on the clinical findings, endocrinological data, and electron microscopy findings. We propose that mammosomatotroph cell adenoma can be defined as a tumor with a composition of more than 80% mammosomatotrophs.

The present sandwich CIBA study indicated that there are three types of GH-secreting adenomas with different characteristics relating to hormone level and tumor size. In contrast, prolactinomas have a consistent cell composition. This method may give more information on mammosomatotroph cell adenoma.

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References

1. Bassetti M, Spada A, Arosio M, Vallar L, Brina M, Giannattasio G (1986) Morphological studies on mixed growth hormone (GH)- and prolactin (PRL)-secreting human pituitary adenomas. Coexistence of GH and PRL in the same secretory granule. *J Clin Endocrinol Metab* 62: 1093–1100.
2. Goluboff IG, Ezrin C (1969) Effect of pregnancy on the somatotroph and the prolactin cell of the human adenohypophysis. *J Clin Endocrinol Metab* 29: 1535–1538.
3. Horvath E, Kovacs K, Killinger DW, Smyth H, Weiss MH, Ezrin C (1983) Mammosomatotroph cell adenoma of the human pituitary: A morphologic entity. *Virchows Arch A Pathol Anat Histopathol* 398: 277–289.
4. Corenblum B, Ssiek AMT, Horvath E, Kovacs K, Ezrin C (1976) Human mixed somatotrophic and lactotrophic pituitary adenomas. *J Clin Endocrinol Metab* 42: 857–863.
5. Farquhar MG, Dellman HD, Johnson JA, Klachko DM (1977) Secretion and crinophagy in prolactin cells. In: *Comparative Endocrinology of Prolactin*. Plenum Press, New York, pp. 37–94.
6. Guyda H, Robert F, Colle E, Hardy J (1973) Histologic, ultrastructural and hormonal characterization of a pituitary tumor secreting both hGH and prolactin. *J Clin Endocrinol Metab* 36: 531–547.
7. Martin JB (1973) Neural regulation of growth hormone secretion. *N Engl J Med* 288: 1384–1393.
8. Mashiter K, Adams E, Beard M, Holley A (1977) Bromocriptine inhibits prolactin and growth hormone release by human pituitary tumors in culture. *Lancet* 2: 197–198.
9. Zurschmiede C, Landolt AM (1987) Distribution of growth hormone and prolactin human adenohypophysis. *Virchows Arch B Cell Pathol Incl Mol Pathol* 53: 308–315.
10. Felix IA, Horvath E, Kovacs K, Smyth HS, Killinger DW, Vale J (1986) Mammosomatotroph adenoma of the pituitary associated with gigantism and hyperprolactinemia: A morphological study including immunoelectron microscopy. *Acta Neuropathol (Berl)* 71: 76–82.
11. Halmi NS (1982) Occurrence of both growth hormone- and prolactin-immunoreactive material in the cells of human somatotrophic pituitary adenomas containing mammatrophic elements. *Virchows Arch A Pathol Anat Histopathol* 398: 19–31.
12. Heitz PU, Landolt AM, Zenklusen HR, Kasper M, Reubi JC, Oberholzer M, Roth J (1987) Immunocytochemistry of pituitary tumors. *J Histochem Cytochem* 35: 1005–1011.
13. Ishibashi T, Shiino M (1989) Co-localization pattern of growth hormone (GH) and prolactin (PRL) within the anterior pituitary cells in the female rat and female musk shrew. *Anat Rec* 223: 185–193.
14. Ishikawa H, Nogami H, Kamio M, Suzuki T (1983) Single secretory granules contain both GH and prolactin in pituitary mixed type adenoma. *Virchows Arch A Pathol Anat Histopathol* 399: 221–226.
15. Kameya T, Tsumuraya M, Adachi I, Abe K, Ichikizaki K, Toya S, Demura R (1980) Ultrastructure, immunohistochemistry and hormone release of pituitary adenomas in relation to prolactin production. *Virchows Arch [Pathol Anat]* 387: 31–46.
16. Kanie N, Kageyama N, Kuwayama A, Nakane T, Watanabe M, Kawaoi A (1983) Pituitary adenomas in acromegalic patients: An immunohistochemical and endocrinological study with special reference to prolactin-secreting adenoma. *J Clin Endocrinol Metab* 57: 1093–1101.
17. Landolt AM, Heitz PHU (1986) Alpha-subunit-producing pituitary adenomas: Immunocytochemical and ultrastructural studies. *Virchows Arch A Pathol Anat Histopathol* 409: 417–431.
18. Landolt AM, Rothenbuhler V (1978) Extracellular growth hormone deposits in pituitary adenoma. *Virchows Arch A Pathol Anat Histopathol* 378: 55–65.
19. Lloyd RV, Anagnostou D, Cano M, Barken AL, Chandler WF (1988) Analysis of mammosomatotropic cells in normal and neoplastic human pituitary tissues by the reverse hemolytic plaque assay and immunocytochemistry. *J Clin Endocrinol Metab* 66: 1103–1110.
20. Nakane PK (1968) Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat. *J Histochem Cytochem* 16: 557–560.
21. Nikitovich-Winer BM, Atkin J, Maley BE (1987) Colocalization of prolactin and growth hormone within specific adenohypophyseal cells in male, female and lactating female. *Endocrinology* 121: 625–630.
22. Teramoto A (1980) Immunocytochemical studies on the functioning pituitary adenomas. *No to Shinkei* 32: 1163–1174 (In Japanese).
23. Trouillas J, Girod C, Lheritier M, Claustat B, Dubois MD (1980) Morphological and biochemical relationships in 31 human pituitary adenomas with acromegaly. *Virchows Arch [Pathol Anat]* 389: 127–142.
24. Leong DA, Lau SK, Sinha YN, Kasiser DL, Thorner MO (1985) Enumeration of lactotropes and somato-

- tropes among male and female pituitary cells in culture: evidence in favor of a mammosomatotrope subpopulation in the rat. *Endocrinology* 116: 1371–1378.
25. Nakane PK (1970) Classifications of anterior pituitary cell types with immunoenzyme histochemistry. *J Histochem Cytochem* 18: 9–20.
 26. Kendall ME, Hymer WC (1987) Cell blotting: a new approach to quantify hormone secretion from individual rat pituitary cells. *Endocrinology* 121: 2260–2262.
 27. Arita J (1993) Analysis of the secretion from single anterior pituitary cells by cell immunoblot assay. *Endocr J* 40: 1–15.
 28. Arita J, Kojima Y, Yamamoto I, Kimura F (1994) Somatotropes and thyrotropes in the rat anterior pituitary gland cosecrete substance P: Analysis by the sandwich cell immunoblot assay. *Neuroendocrinology* 60: 567–574.
 29. Arita J, Kojima Y, Kimura F (1991) Identification by the sequential cell immunoblot assay of a subpopulation of rat dopamine-unresponsive lactotrophs. *Endocrinology* 128: 1887–1894.
 30. Arita J, Kojima Y, Kimura F (1992) Lactotrophs secreting small amounts of prolactin reveal great responsiveness to thyrotropin-releasing hormone: Analysis by the sequential cell immunoblot assay. *Endocrinology* 130: 3167–3174.
 31. Kojima Y, Arita J, Kuwana N (1995) Mammosomatotroph adenoma cells secrete both growth hormone and prolactin. *Neurol Med Chir (Tokyo)* 35: 791–796.
 32. Bassetti M, Arosio M, Spada A, Brina M, Bazzoni N, Faglia G, Giannattasio G (1988) Growth hormone and prolactin secretion in acromegaly: Correlations between hormonal dynamics and immunocytochemical findings. *J Clin Endocrinol Metab* 67: 1195–1204.
 33. Kineman RD, Faught WJ, Frawley LS (1991) Mammosomatotropes are abundant in bovine pituitaries: influence of gonadal status. *Endocrinology* 128: 2229–2233.
 34. Frawley LS, Bookfor FR (1991) Mammosomatotropes: presence and functions in normal and neoplastic pituitary tissue. *Endocr Rev* 12: 337–355.
 35. Bookfor FR, Hoeffler JP, Frawley LS (1986) Analysis by plaque assays of GH and prolactin release from individual cells in cultures of male pituitaries. *Neuroendocrinology* 42: 64–70.
 36. Kojima Y, Arita J, Kuwana N, Kimura F (1995) Dopamine responsiveness of human prolactinoma cells as determined by the reverse hemolytic plaque assay. *Endocr J* 42: 355–360.
 37. Melmed S, Braunstein GD, Horvath E, Ezrin C, Kovacs K (1983) Pathophysiology of acromegaly. *Endocr Rev* 4: 271–290.
 38. Porter TE, Wiles CD, Frawley LS (1991) Evidence for bidirectional interconversion of mammotropes and somatotropes: Rapid reversion of acidophilic cell types to pregestational proportions after weaning. *Endocrinology* 129: 1215–1220.