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CONFOCAL LASER SCANNING MICROSCOPY IN IMMUNOHISTOCHEMICAL STUDY OF PITUITARY ADENOMAS

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We retrospectively studied 30 pituitary adenomas to determine the intracellular distributions of prolactin (PRL) and growth hormone (GH) on the same section by double immunofluorescence staining and subsequent observation by confocal laser scanning microscopy (CLSM). The clinical presentations of these tumors were hyperprolactinemia (10 cases), acromegaly (10 cases), and nonfunctioning (10 cases). Of the 10 cases of hyperprolactinemia, 8 were pure PRL- secreting adenomas and 2 were mixed somatotroph-lactotroph adenomas (MSLA). Of the 10 cases of acromegaly, 3 were MSLA, 4 were mammosomatotroph adenomas (MSA), and 3 were pure GH-secreting adenomas. Among the 10 nonfunctioning tumors, there were 2 pure GH-secreting adenomas, one pure PRL-secreting adenoma, one MSA, one MSLA, and 5 negative cases for both PRL and GH.

We conclude that pituitary adenomas with clinical hyperprolactinemia are mostly pure PRL-secreting adenomas, whereas those with acromegaly are mostly mixed PRL-GH-secreting adenoma. In addition, pituitary adenomas without clinical endocrinopathy have diverse hormonal expressions. The method we used in this study by CLSM is technically easy and thus can be performed on a routine basis for more definite classifications of pituitary adenomas.

Introduction

Pituitary adenomas were conventionally classified into acidophilic, basophilic and chromophobe adenomas based on their hematoxylin and eosin (HE) staining characteristics. After the introduction of immunohistochemical staining into the field of neuropathology, these classification were replaced by those based on the immunohistochemical hormonal expression. The most common pituitary adenomas based on such classifications are prolactin (PRL)-secreting adenoma, growth hormone (GH)-secreting adenoma, ACTH-secreting adenoma, TSH-secreting adenoma, gonadotropin-secreting adenoma and null cell adenoma (adenomas negative for all hormonal staining). However, the current classification of human pituitary adenomas recognizes several tumor types, which elaborate more than one hormone[1)]. The most common of these is the mixed PRL-GH adenomas. These include the bimorphous mixed somatotroph-lactotroph adenoma (MSLA) as well as the monomorphous mammosomatotroph adenoma (MSA)[2)3)] and acidophil stem cell adenoma (ASCA)[4)5)]. The MSLA is composed of two distinct cell populations of somatotrophs and lactotrophs[6)]. The ASCA along with the MSA refers to tumors with PRL and GH expressions in single cells.

The MSAs are tumors with acromegaly, while the ASCAs are tumors with hyperprolactinemia.

Ultrastructural examinations[3)~6)] and the localizations of PRL and GH within tumor cells using electron microscopic immunocytochemistry[2)7)8)] have enabled the above subclassifications. These methods are technically demanding and time-consuming and thus cannot be employed in routine diagnostic workups. Confocal laser scanning microscopy (CLSM) has the advantages of low background fluorescence, high resolution, and the provision of reconstructions to three-dimensional (3D) images. With the aid of a computer system, CLSM can be used to visualize different fluorescence intensities in the same slide, both separately and simultaneously.

We conducted a retrospective study of thirty cases of histologically confirmed pituitary adenomas, using double immunofluorescence staining of PRL and GH with subsequent observation by CLSM. The goals of this study were ① to establish a standard technique and procedure of double immunostaining and observation by CLSM, ② to determine whether, with the use of CLSM, the above classification of pituitary tumors to be performed on a routine basis, and ③ to establish a general understanding of the hormonal expressions of various pituitary adenomas.

Materials and Methods

Pituitary adenoma

Thirty cases of histologically confirmed pituitary adenomas were chosen for this study. According to the clinical presentation, there were 10 cases with hyperprolactinemias, 10 cases with acromegalies, and 10 cases of nonfunctioning tumors. The clinical presentation of hyperprolactinemia was defined as symptoms of galactorrhea-amenorrhea syndrome and/or a serum PRL level over 200 ng/ml. The clinical presentation of acromegaly was defined as the clinical feature of acromegaly and/or a serum level of GH over 5 ng/ml. A nonfunctioning tumor was defined as the lack of both the clinical presentation of hormone-related symptoms and an elevated serum level of pituitary hormones. The clinical data were summarized in Table 1.

Immunofluorescence staining

Buffered formalin-fixed paraffin-embedded tissue blocks were obtained from the Neuropathology Laboratory of Tokyo Women's Medical College. Six-μm-thick sections were cut, dewaxed in xylene, and rehydrated through regressively graded ethanols to water. The sections were incubated within normal goat serum to block the nonspecific binding of the primary antibody. The primary antibody was a mixed solution of 1 : 50 polyclonal antiserum raised in rabbits to human PRL (DAKO, Denmark) and a mouse monoclonal antibody to human growth hormone (DAKO). The goat serum-treated sections were incubated in the primary antibody for 1 hour. The sections were then washed in 0.1 M Tris buffer (pH 7.6) and incubated with the secondary antibody for 1 hour. The secondary antibody was a mixed solution of 1 : 50 fluorescein-isothiocyanate (FITC)-labeled anti-rabbit IgG (Vector Labora-

Table 1 Clinical summary of 30 cases of pituitary adenomas

Clinical presentation	No. of cases	Sex	Age y.o. (average)	Microadenoma	Macroadenoma
Hyperprolactinemia	10	Male 3 Female 7	24~62(31.5)	4	6
Acromegaly	10	Male 3 Female 7	32~59(45.8)	4	6
Nonfunctioning	10	Male 3 Female 7	22~76(49.3)	2	8

tories, Burlingame, CA) and Texas red-labeled anti-mouse IgG (Vector). After washing, slides were mounted with coverslips. Controls were incubations with the omission of the primary antibody. Autofluorescence was assessed on sections not treated with either antibody.

These sections were then observed under a confocal laser scanning microscope (LSM-GB200, Olympus, Tokyo, Japan). For the assessment of the FITC fluorescence, a 488 nm wavelength argon laser beam was used; for the Texas red fluorescence, a 543.5 nm wavelength helium-neon laser beam was used. The emission was monitored using a 535 nm wavelength band pass filter for the FITC and a 610 nm wavelength long-pass filter for the Texas red. The design of the CLSM allows us to visualize different fluorescence values in the same microscopic field both separately and concurrently. Thus, we can observe not only the double staining pattern , but also the single staining pattern of each hormone.

Analysis

Before investigating the staining characteristics of these cases, we evaluated the background fluorescence with the omission of the primary antibody, because the background fluorescence cannot be completely eliminated even with the use of CLSM. All diagnoses were made after both separate and simultaneous investigations of the PRL and GH staining patterns of each case. A positive staining of either hormone was established mainly by the separate investigations of them. In contrast with the darkish color of the background fluorescence, the positive staining appeared brighter.

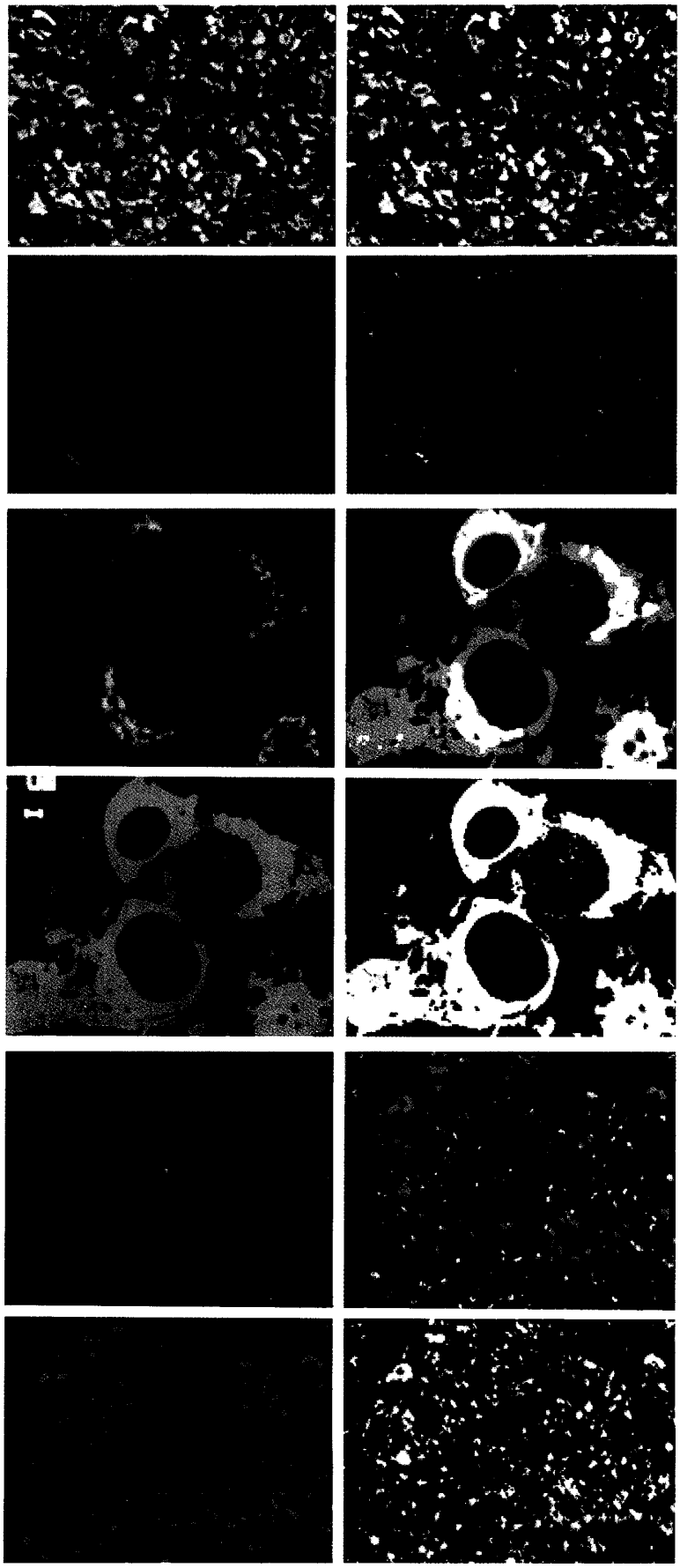
The staining intensity of each hormone was categorized into ++, +, ±, and − according to the number and distribution of the positive cells. If over 50% of the tumor cells were positive, it was categorized as ++; if 25~50% were positive, it was termed +; if a significant number of positive cells existed in almost every low-power field, but the total number was less than 25%, it was termed ±; finally, if no positive cells or only a few positive cells were found in limited areas, it was categorized as −. A tumor was considered positive for one hormone if its staining pattern of that hormone fell into the category of ++, +, or ±. If the staining pattern was −, the tumor was considered negative for that hormone.

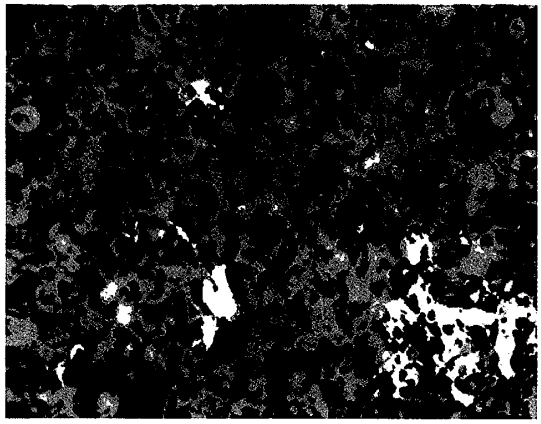
The double staining pattern was examined mainly to determine whether different hormones existed in the same cells. In a mixed PRL-GH-secreting tumor, if positive cells for both hormones were found, the tumor was called a MSA. In contrast, if no cells were positive for either hormone, the tumor was called a MSLA. Because of the influence of the background fluorescence of the other hormone, some cells positive for only one hormone might occasionally appear somewhat yellowish in color. In such circumstances, instead of drawing the conclusion that the tumor was a MSA, we re-examined the separate findings of each hormone to determine whether such yellowish cells were actually positive for both hormones. If cells positive for both hormones were then identified, the tumor was classified as a MSA.

Results

Photomicrographs were taken with the use of a confocal laser scanning microscope (CLSM) after the double immunofluorescence staining of prolactin (FITC: green) and growth hormone (Texas red: red) of various pituitary adenomas. These pictures were selected to show the advantage of our methodology. Because the background fluorescence could not be completely eliminated even with the use of CLSM, the color of the fluorescence may appear somewhat yellowish when the positive staining of one hormone is mixed with the background fluorescence of the other hormone. The diagnosis of MSA and MSLA was ascertained by separate investigations of the staining patterns of PRL and GH.

Based on the aforementioned observation criteria, in the 10 cases of clinical hyperprolactinemia, we found 8 pure PRL-secreting adenomas (Fig. 1) and 2 MSLAs. None of the





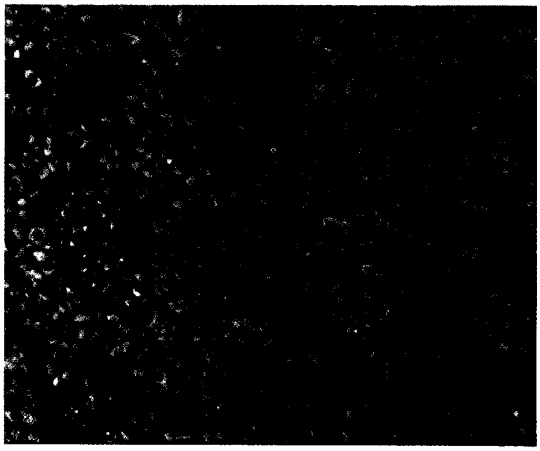


Fig.1	Fig.2
Fig.3	Fig.5
Fig.4	

cases with hyperprolactinemia was an MSA. Of the 10 cases of clinical acromegaly, 3 were MSLAs (Fig. 2), 4 were MSAs (Fig. 3), and only 3 were pure GH-secreting adenomas (Fig. 4). Of the 10 cases of clinical nonfunctioning tumors, there were 2 pure GH-secreting adenomas, 1 pure PRL-secreting adenoma, 1 MSA, 1 MSLA and 5 negative cases of both hormones (Fig. 5). These results are summarized in Table 2.

Discussion

Pituitary adenomas are classified into several subtypes based on immunohistochemical staining. The most common pituitary adenomas are PRL-secreting adenoma and GH-secreting adenoma and sometimes we found tumors with both hormonal expression. Tumors with both GH and PRL secreting abilities (mixed PRL-GH-secreting adenoma) have been morphologically classified as monomorphous bihormonal acidophil stem cell adenomas (ASCAs) and mammosomatotroph adenomas (MSAs) as well as bimorphous mixed somatotroph-lactotroph adenomas (MSLA). Since pituitary adenomas with different hormonal secreting abilities have somewhat different clinical courses and respond to different pharmacological agents, such classification may be of clinical significance in the future. This subclassification for pituitary tumors was based mainly on double immunogold technique of electron microscopy[9]. Although some authors have attempted the double immunostaining method[10], it has its inherent deficiencies in that, ① it is time-consuming, since the staining procedure has to be repeated for different antigens;

Fig. 1 A pure PRL-secreting adenoma
Upper left: FITC fluorescence (green) showed that over 50% of cells were positive for PRL. **Lower left:** Texas red fluorescence (red) showed no cells positive for GH. The darkish red spots in this picture represent the background fluorescence. **Upper right:** Double staining picture of the FITC and Texas red. This is the combined picture of the above upper and lower left photographs. The green color of FITC turned somewhat yellowish because of the influence of the background fluorescence of Texas red. **Lower right:** Monochrome picture.

Fig. 2 Photomicrograph of a GH-secreting adenoma
There are many GH positive cells and some PRL-positive cells. Since PRL and GH exist in different cells, this is an MSLA. Because of the strong Texas red background, the green color of FITC looked somewhat yellowish.

Fig. 3 A high-power magnification picture of an MSA demonstrating the utility of CLSM for studying the subcellular distribution of secretory granules
Upper left: FITC fluorescence showed PRL-secreting granules accumulated eccentrically in the cytoplasm. **Lower left:** Texas red fluorescence showed GH-secreting granules distributed evenly throughout the cytoplasm. **Upper right:** This double staining picture clearly depicts the different distributions of the PRL- and GH-secreting granules in the same cell. **Lower right:** Monochrome picture.

Fig. 4 A pure GH-secreting adenoma
Upper left: FITC fluorescence showed no cells positive for PRL. However, some background fluorescence could not be completely eliminated. **Lower left:** Texas red fluorescence showed many cells positive for GH. The positive staining is bright red in color. The darkish red spots represent background fluorescence. **Upper right:** Double staining picture. The red color of GH appears somewhat yellowish because of the mixing with the background of the green FITC stain. Therefore, investigating the two fluorescence both separately and together is crucial for a definite diagnosis. **Lower right:** Monochrome picture.

Fig. 5 Double staining of a nonfunctioning adenoma showing negative staining for PRL and GH
The fluorescence of a positive staining is of bright color. The darkish red and darkish green colors in this picture are the background fluorescence.

Table 2 Classification of 30 cases of pituitary adenomas

Case No.	Clinical presentation	PRL*	GH*	Mammosomatotroph**	Tumor type
1	Acromegaly	+	++	−	MSLA
2	Acromegaly	+	+	+	MSA
3	Acromegaly	−	+	−	Pure GH adenoma
4	Acromegaly	−	+	−	Pure GH adenoma
5	Acromegaly	−	+	−	Pure GH adenoma
6	Acromegaly	+	++	−	MSLA
7	Acromegaly	+	++	+	MSA
8	Acromegaly	++	+	+	MSA
9	Acromegaly	+	+	−	MSLA
10	Acromegaly	+	+	+	MSA
11	Hyperprolactinemia	+	−	−	Pure PRL adenoma
12	Hyperprolactinemia	+	±	−	MSLA
13	Hyperprolactinemia	+	−	−	Pure PRL adenoma
14	Hyperprolactinemia	++	−	−	Pure PRL adenoma
15	Hyperprolactinemia	++	−	−	Pure PRL adenoma
16	Hyperprolactinemia	+	−	−	Pure PRL adenoma
17	Hyperprolactinemia	++	+	−	MSLA
18	Hyperprolactinemia	+	−	−	Pure PRL adenoma
19	Hyperprolactinemia	++	−	−	Pure PRL adenoma
20	Hyperprolactinemia	++	−	−	Pure PRL adenoma
21	Nonfunctioning	+	+	+	MSA
22	Nonfunctioning	−	−	−	
23	Nonfunctioning	−	−	−	
24	Nonfunctioning	±	+	−	MSLA
25	Nonfunctioning	−	−	−	
26	Nonfunctioning	−	+	−	Pure GH adenoma
27	Nonfunctioning	−	−	−	
28	Nonfunctioning	−	+	−	Pure GH adenoma
29	Nonfunctioning	−	−	−	
30	Nonfunctioning	+	−	−	Pure PRL adenoma

PRL : prolactin, GH : growth hormone, MSLA : mixed somatotroph-lactotroph adenoma, MSA : mammosomatotroph adenoma.

*For PRL and GH : ++ ; positive cells>50%, + ; positive cells>25%, ± ; a few positive cells found in virtually every low-power field, − ; no positive cells or only a few positive cells were found in some low-power fields.

**For Mammosomatotroph : + ; cells positive for both hormones were found, − ; no cells positive for either hormone.

② the results are difficult to interpret since if the concentration of one hormone exceeds that of the other, the double staining yields 1 color, suggesting that only 1 hormone is present in the cell; and ③ the subcellular distribution of secretory granules cannot be determined.

In this study, we determined GH and PRL expressions of 30 pituitary adenomas by double immunofluorescence staining with subsequent observation by confocal laser scanning microscopy. Such methodology has been shown to be useful for the study of mammosomatotroph cells[11]. However, it has never been applied to a wide range of pituitary adenomas for the study of hormonal expressions in various pituitary adenomas. Double immunofluorescence staining can be performed with routine formalin-fixed paraffin-embedded sections and can be completed in one day. Thus we believe it can be performed as a routine procedure.

CLSM is now established as a valuable tool for obtaining high-resolution images and 3D

reconstruction of a variety of biological specimens. With the aid of a CLSM computer system, different fluorescence values in the same microscopic field can be visualized both individually and as a whole. Because the double staining pattern can sometimes be misleading, the individual investigation of each hormone is of critical importance for the interpretation of the results. In addition, the high resolution of CLSM along with its high magnification provided us a useful tool for studying the subcellular distribution of secretory granules of pituitary adenomas. Such a study could previously be accomplished only by electron microscopy.

In their immunohistochemical and electron microscopic study, Kovacs and Horvath[1] classified 67 human pituitary adenomas associated with hyperprolactinemia into 5 types. Nine adenomas containing GH cells were included in their investigation; they grouped these adenomas into those in which GH and PRL were present in 2 different cell types and those in which these hormones were present in the same cell. The former type was termed mixed somatotroph-lactotroph stem cell adenoma, and the latter type was termed acidophil stem cell adenoma. The findings reported by Kovacs and Horvath correspond in part with ours. Among the 10 cases of hyperprolactinemia of the present series, 8 were pure PRL-secreting adenomas. However, we found no so-called acidophil stem cell adenomas in our cases. The only two cases of hyperprolactinemia containing positive GH cells in our series were both MSLA.

Kanie et al[10] studied surgical specimens from 55 acromegalic patients and found that GH was present in all adenomas and PRL was found in 25 of them (45.5 %). With the cases with positive PRL staining, they performed double immunostaining which revealed that 15 of them had cells containing both GH and PRL. However, Kanie et al suggested that the incidence of such tumors may be higher than estimated by their study because of the difficulty of interpretation of the double immunostaining. Among the 10 cases of acromegaly in our series, only 3 were pure GH-secreting adenomas. Four were MSA and 3 were MSLA. Our results are thus in keeping with the above report.

Clinically, a nonfunctioning pituitary adenoma is defined as a pituitary tumor without endocrinological symptoms and with normal serum levels of hormones. Howng et al reported 14 patients with nonfunctioning pituitary adenomas; all were negative for both PRL and GH[12]. In our series, however, only half of the 10 cases of nonfunctioning pituitary adenoma were negative for both PRL and GH. Of the other 5 cases, 2 were pure GH-secreting adenomas, 1 was a pure PRL-secreting adenoma, 1 was a MSA, and 1 was a MSLA. It has been reported that MSA and ASCA are common type of mixed PRL-GH-secreting adenomas[1)13)]. However, among the 11 present cases of mixed PRL-GH-secreting adenomas, 6 were MSLA and 5 were MSA. We had no so-called ASCA in this series. It might be difficult to detect weak GH expression of ASCA by immunofluorescence staining. Further investigation must be needed.

In conclusion, most of the present tumors with clinical hyperprolactinemia were pure PRL-secreting adenomas. In contrast, most of the tumors with acromegaly were mixed PRL-GH-secreting adenomas (mostly MSA). We believe that the further classification of mixed PRL-GH-secreting adenomas into different entities based on their hormonal expressions may not only be of pathological interest, but may also be of clinical significance in the future. Our methodology comprised of the double immunofluorescence staining of PRL and GH with subsequent observation by confocal laser scanning microscopy has proved to be reliable and easy for interpretation. Furthermore, it is simpler, faster, and more cost-effective compared with all of the other methodologies reported in the literature. We therefore believe that it can be performed as a routine pathological procedure.

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共焦点レーザー顕微鏡（CLSM）を用いた下垂体腺腫の免疫組織化学的検討

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下垂体腺腫のうち成長ホルモンとプロラクチン産生腫瘍は一般的にはpure growth hormone secreting adenoma（PGA），pure prolactin secreting adenoma（PPA）とmixed growth hormone-prolactin secreting adenoma（MGPA）に分類されている．更に詳細に分類するとMGPAはacidophil stem cell adenoma（ASCA），mammosomatotroph adenoma（MSA），およびmixed somatotroph-lactotroph adenoma（MSLA）に分けられている。ASCAとMSAは両ホルモンを持っている細胞からなる腫瘍で，MSLAは両ホルモンをそれぞれ別々の細胞が産生している腫瘍といわれている．今回30例の下垂体腺腫の手術例についてこのような詳細な検討をするため，両ホルモンの細胞内分布について二重標識蛍光抗体法を行い共焦点レーザー顕微鏡（CLSM）で観察し検討した．プロラクチン産生腫瘍とされた10例ではPPAが8例，MSLAが2例であった．成長ホルモン産生腫瘍の10例ではMSLAは3例，MSAは4例，PGAが3例であった．非機能性腫瘍の10例では両ホルモンとも認められぬもの5例，PGAが2例，PPA，MSA，MSLAが1例ずつであった．これらの結果よりプロラクチン産生腫瘍ではPPAが多いが，成長ホルモン産生腫瘍ではMGPAが多く，そのうちでもMSAが大部分を占めていることがわかった．CLSMによる下垂体腺腫の観察の報告は今までなく，分泌顆粒の細胞内分布などもよく見え，二重染色による観察も簡便であり，下垂体腺腫の詳細な分類に役立つことがわかった．