COMPARATIVE STUDIES ON TISSUE CORTICOSTEROID CONTENT AND MORPHOLOGICAL FEATURE IN FUNCTIONING ADRENOCORTICAL ADENOMA AND HYPERPLASIA*

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For the purpose of clarifying the functional significance of clear- and compact-type cells, correlative hormonal and morphological studies were performed on adrenocortical tumors associated with primary aldosteronism, Cushing's syndrome or non-dyshormonal symptoms. The content of aldosterone, corticosterone and cortisol in adrenal tissue was estimated by radioimmunoassay, and, on the surface of a quantitative sample, the ratio of its constituent cell-type was examined in sections with oil red O stain and/or H-E stain.

The content of aldosterone and corticosterone was significantly higher in primary aldosteronism ($P < 0.001$) than in Cushing's syndrome and in non-functioning tumor, with a mean value of 1.22 ± 0.15 and 7.52 ± 1.05 ng/mg tissue, respectively. In Cushing's syndrome, cortisol content showed a high value, 9.27 ± 1.60 ng/mg tissue. The steroid content was different in each case, and varied with parts of the quantitative sample even in the same case.

Though the correlation of aldosterone content and compact-type cell population in primary aldosteronism showed a negative trend and that of cortisol content and compact-type cell in Cushing's syndrome had a positive, neither correlation was statistically significant.

Morphologically, the adrenocortical adenomas are mainly composed of clear-type cells and compact-type cells, although the ratio of their constituent cell-type varies with the kind of hypercorticalism. According to Symington and his co-workers (20, 21, 22), the clear cell of the zona fasciculata represents a storage zone of steroid precursors and the compact cell of the zona reticularis is an active zone in the biosynthesis of steroid hormones. In terms of this conception, and from histochemical and ultrastructural findings, it has been suggested that the clear-type cell plays a part in the storage of steroid precursors, and that the compact-type cell is the source of steroid secretion (25, 26). However, another investigator speculated that aldosterone in primary aldosteronism was just released as a clear cell (18).

While many quantitative investigations on steroid content in human adrenocortical tumors have been performed (1, 4, 5, 8, 9, 10, 11, 13, 14, 19), there has only been one study on the relationship between hormone content and mor-

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phological findings (18).

In the present study, an attempt was made to clarify the functional significance of clear- and compact-type cells in functioning adrenocortical adenoma and hyperplasia.

MATERIALS AND METHODS

Adrenal tissues surgically resected from 12 patients with primary aldosteronism and 2 patients with Cushing's syndrome were examined. For comparative purposes, 6 cases of non-functioning adenoma, adenomatous hyperplasia and micronodular hyperplasia were obtained at autopsy within two hr after death. The above cases are shown in Table 1. All adrenal tissues were either extracted or frozen within one hr after removal according to the following procedure:

I. Preparation of histological section and quantitative sample

For histological examination, the adrenal tissue was cut into 10 μ m-thick-sections in a cryostat. Then, quantitative samples of each case were punched out by a hole puncher (Fig. 1) and weighed. Samples for assay were each preserved in a vial with cold acetone (about 3 ml) at -20°C until hormonal analysis could be done. Continuously, as illustrated in Fig. 2, parallel 10 μ m-thick-sections were made again. The frozen sections were stained with oil red O and/or hematoxylin and eosin. A different part of the tumor was fixed in 10% formalin and embedded in paraffin. Paraffin sections were prepared with hematoxylin-eosin, Mallory-Azan or Gridley silver stains.

II. Measurement of tissue corticosteroid content

Based on the method of Fukuchi *et al.* (6, 7, 8), the content of aldosterone, corticosterone and cortisol in adrenal tissue were measured by radioimmunoassay. Anti-aldosterone, -corticosterone and -cortisol-3-BSA antiserum (1 : 100) were obtained from Japan Bulcon Inc. and stored at -40°C . Before use, they were diluted with pH 8.0, 0.05 M borate buffer, containing 0.1% gamma-venin (Hoechst Japan Co.) and methanol for optimum sensitivity to 1 : 50,000, 1 : 20,000 and 1 : 50,000, respectively.

Extraction and Purification: Acetone in a sample tube stocked at -20°C was dried using warm air below 40°C , and 1 ml redistilled methanol was added to the dried sample tube. After mixing, the methanol was transferred to two tubes with volumes of 0.5 ml and 0.25 ml. Approximately 10,000 cpm ^3H -aldosterone and

Fig. 1. Hole puncher for collecting quantitative sample. The upper is an inner cylinder and the lower, an outer one. $\times 0.9$

FIG. 2. Procedure for preparation of histological section and quantitative sample.

cortisol (New England Nuclear Co.) were added to the tube containing 0.5 ml methanol, and in the same manner, labeled corticosterone (New England Nuclear Co.) was added to the 0.25 ml methanol tube. Each sample was dried and applied to paper chromatography. The dried extract was spotted with methanol: dichloromethane (1 : 1) on the paper strip. This step was repeated. Chromatography was carried out in the system, hexane : benzene : methanol : water (1 : 9 : 5 : 2.5). After equilibration in a chromatography tank for 5 hr at 30°C, the mobile phase was added and the strips for aldosterone and cortisol developed for 12–16 hr. The sample for corticosterone ran in the same system for 3–5 hr. A paper chromatogram scanner (Aloka, model JPC-213) was used to locate the ^3H -aldosterone, cortisol and corticosterone. The paper strip was cut out and eluted with 5 ml of methanol. Two 1 ml and 0.1 ml (or 0.5 ml) aliquots were transferred into assay tubes in duplicate and dried. The 0.5 ml eluates for recovery determination were transferred to scintillation counting vials and dried under air.

Immunoassay: For the standard curve, 0, 10, 20, 50, 100, 200, 500 and 1,000 pg of each steroid (Sigma Co.) were prepared. Approximately 2,000 cpm labeled steroids were added to each standard tube. All of the samples and standard tubes were dried simultaneously under air below 40°C. Dried samples and standards were dissolved in 0.25 ml of the appropriate diluted antiserum using a Vortex mixer in a cold room at 4°C. All tubes were placed in a water bath at 25°C for 30 min and stored in the cold room for 16 hr. Then cold saturated ammonium sulfate, 0.25 ml, was added to every tube, and the tubes were again mixed on the Vortex for 15 sec. After 30 min in the cold room, the tubes were centrifuged at 3,000 rpm for 15 min at 4°C and then 0.3 ml of supernatant was transferred to a scintillation vial. To those samples dried for recovery, 0.3 ml of half saturated ammonium sulfate was added. Bray's scintillation fluid (PPO 8 g, POPOP 0.4 g, naphthalene 120 g,

FIG. 3. The histologic appearance on the surface of quantitative samples. Left: primary aldosteronism. Right: Cushing's syndrome. Oil red O stain. $\times 60$

dissolved in 40 ml of ethyleneglycol, 200 ml of methanol and 1,760 ml of dioxane) was added to all vials. All samples were counted by Packard tri-carb liquid scintillation spectrometer for 10 min. Steroid concentration in every sample was read off the standard curve and calculated.

III. Determination of the ratio of constituent cell-types on the surface of quantitative sample.

The punched-out section (No. 2 in Fig. 2) was put on the whole one (No. 1 in Fig. 2) and together prepared for microscopic examination. Then, every region taken off the quantitative sample was taken a color film slide (Fig. 3). The slide was projected on the paper and traced. The traced paper was cut according to constituent cell-types. After weighing every cut paper, the percentage of clear- and compact-type cells was calculated.

RESULTS

I. Morphological findings.

On microscopic examination, all but one tumor were adenoma (Case 3: adenomatous hyperplasia, Table 1). As a rule, clear-type cells with vacuolated lipid-rich cytoplasm were prominent in the adenoma with primary aldosteronism. Most of the small groups of compact-type cells with granularly eosinophilic, lipid-poor cytoplasm scattered around the delicate, fibrovascular septa. Nuclear and cellular pleomorphism was mild to moderate in degree. Occasionally, transitional type (25, 26) and glomerulosa-type cells (15, 23) were seen. The former had a eosinophilic cytoplasm with a moderate amount of lipid and the latter could usually be

TABLE 1. *Cases of primary aldosteronism, Cushing's syndrome and control*

Primary aldosteronism						
Case No.	Age	Sex	Size (cm) Site	Serum K (mEq/L)	Plasma Aldosterone (pg/ml)	
1	41	F	1.3×0.9×1.5 left	2.4-4.6	443.9	
2	28	F	1.6×1.5×1.1 left	2.7-3.7	987.8	
3	24	M	1.4×0.7×0.8 right	3.1	313.7	
4	42	F	2.2×2.3×1.8 left	2.0-4.3	690	
5	28	F	1.5×1.4×0.9 right	1.9-2.2	761.5	
6	45	F	2.5×1.5×1.2 left	2.0		
7	44	M	1.3×1.2×0.9 right	1.7-4.0	380	
8	51	F	1.7×2.6×1.4 left	1.6-2.0	564.5	
9	36	F	2.5×2.4×1.5 left	1.4-3.7	730	
10	39	M	1.0×1.3 left	4.1	300	
11	39	F	1.8×1.5×1.2 right	1.9-4.7	348	
12	37	F	2.0×1.7×1.7 left	2.6-3.6	430	
Cushing's syndrome						
Case No.	Age	Sex	Size (cm) Site	Plasma Cortisol (μg/dl)	Urine	
					17 OHCS	17 KS (mg/day)
13	45	F	3.3×2.7×1.9 right	29-41	9.8-19.8	3.4-6.0
14	19	F	multiple nodules right	13.4-16.2	11.8-15.7	5.4-10.8
Control						
Case No.	Age	Sex	Size (cm), Weight Site	Pathological finding in adrenal gland	Major diagnosis at autopsy	
15	71	M	1.3×1.3 right	Adenoma	Car. lung	
16	76	M	10 g left	Micronodular hyperplasia	Diabetes mellitus	
17	63	M	0.8×0.5×0.9 left	Adenomatous hyperplasia	Aortic aneurysm	
18	66	M	2.5×2.0×1.5 left	Adenoma	Car. pancreas	
19	70	F	0.8×0.7×0.9 right	Adenomatous hyperplasia	Car. urinary bladder	
20	60	M	9 g right	Micronodular hyperplasia	Car. Stomach	

found under the capsule. In general, the width of the zona glomerulosa in the adrenal cortex adjacent to adenoma with primary aldosteronism was increased.

Of the two cases of Cushing's syndrome, one (Case 13) was adenoma and the other (Case 14) was adenomatous hyperplasia. Case 13 adenoma was composed of the clear- and the compact-type cells. Their proportions were almost equal. The areas which appeared yellow macroscopically consisted on clear-type cells and the brown-coloured areas revealed the compact-type cells. The resected right adrenal gland of Case 14 showed multiple nodules of various sizes. They were composed predominantly of the compact-type cells and compressed the related cortex. With oil red O stain, a moderate amount of lipid was found in some areas.

The control group is presented in Table 1. None of them had hormonal symptoms and they were found accidentally at autopsy. Microscopically, there were two each of adenoma, adenomatous hyperplasia and micronodular hyperplasia, none of which showed malignancy.

II. Tissue corticosteroid content

The hormone contents in adrenal tissues of cases of primary aldosteronism, Cushing's syndrome and control are shown in Table 2. In primary aldosteronism, the mean aldosterone content was 1.22 ng/mg tissue, varying between 0.11 (Case 11) and 3.94 (Case 7). The corticosterone content varied from 0.69 (Case 2) to 33.71 (Case 4) with a mean of 7.52 ng/mg tissue. Compared with Cushing's syndrome and control cases, these aldosterone and corticosterone contents were statistically significant ($P < 0.001$). The cortisol content was similar to that of the control cases. There was no difference in corticosteroid content between adenoma and adenomatous hyperplasia. Although the data is not included in Table 2, the steroid content of adrenal tissue adjacent to the adenomas of four patients with primary aldosteronism (Case 2, 3, 8, 9) was also measured. The contents were almost the same as the control.

In Cushing's syndrome, the mean of cortisol content was 9.27 ng/mg tissue with

TABLE 2. *Corticosteroid contents in adrenal tissue*

Case	Corticosteroid		
	Aldosterone	Corticosterone	Cortisol
Primary aldosteronism 12 cases	$1.22 \pm 0.15^*$ (50) $P_1 < 0.001$ $P_2 < 0.001$	$7.52 \pm 1.05^*$ (57) $P_1 < 0.001$ $P_2 < 0.001$	4.61 ± 0.45 (50)
Cushing's syndrome 2 cases	$0.11 \pm 0.01^*$ (23) $P_3 < 0.02$	2.41 ± 0.74 (22)	$9.27 \pm 1.60^*$ (23) $P_1 < 0.02$ $P_3 < 0.05$
Control 6 cases	0.07 ± 0.01 (22)	2.37 ± 0.44 (23)	5.18 ± 0.83 (22)

Mean $\pm$ SE (ng/mg tissue)

*: Significant by t-test

P_1 : Primary aldosteronism vs. Cushing's syndrome

P_2 : Primary aldosteronism vs. Control

P_3 : Cushing's syndrome vs. Control

( ): Number of samples

a range of 0.27 (Case 13) to 33.80 (Case 14).

These contents were higher than those of primary aldosteronism and the control group. The cortisol of Case 13 showed a low level compared to Case 14, and the aldosterone content was slightly high.

Figs. 4, 5 and 6 indicate the individual values for aldosterone, corticosterone and cortisol. Wide variations were found within each group, particularly corticosteroids in primary aldosteronism and cortisol in Cushing's syndrome. Namely, the content appeared to vary in each case and between different parts of the same case. Moreover, in a few samples of the control group, the cortisol content showed a high value (Fig. 6).

Among the primary aldosteronism and Cushing's syndrome group, there was no correlation between tissue corticosteroid content and tumor size, plasma hormone level and other laboratory data.

III. Comparison between corticosteroid content and morphological feature

About half of the samples of primary aldosteronism were deleted from this study because frozen sections were not available.

Although the glomerulosa-type cells were seen on paraffin section, they were

FIG. 4.

FIG. 5.

FIG. 4. Aldosterone contents (ng/mg tissue) in adrenal tissues from patients with primary aldosteronism, Cushing's syndrome and control. Horizontal bars indicate mean value.

FIG. 5. Corticosterone contents (ng/mg tissue) in adrenal tissues from patients with primary aldosteronism, Cushing's syndrome and control. Horizontal bars indicate mean value.

FIG. 6.

FIG. 7.

FIG. 6. Cortisol contents (ng/mg tissue) in adrenal tissues from patients with primary aldosteronism, Cushing's syndrome and control. Horizontal bars indicate mean value.

FIG. 7. Relationship between corticosteroid content (●: aldosterone, ▲: corticosterone, □: cortisol) and clear-type cell in primary aldosteronism ($P = n.s.$).

not included in the quantitative samples. While the transitional type cells were occasionally found in some samples, these cells were included in clear-type cells because it was difficult to determine them on frozen sections with oil red O stain.

For an analysis of the functional significance of clear- and compact-type cells, the relationship between tissue corticosteroid content and the percentage of constituent cell-type on the surface of a quantitative sample is shown in Figs. 7, 8 and 9. For the sake of convenience, the abscissa indicates the number of clear-type cells in percent. Though the correlation of steroid content in adrenal tissue of primary aldosteronism and clear-type cell percent revealed a positive trend, the correlation coefficient (r) was not statistically significant. There were no direct correlations between tissue steroid content and clear-type cell in Cushing's syndrome and control.

DISCUSSION

The histological and lipid histochemical features of primary aldosteronism and Cushing's syndrome were similar to those previously reported (3, 15, 16, 23, 25, 26). That is to say, the clear-type cell with vacuolated lipid-rich cytoplasm is more prominent in primary aldosteronism, and Cushing's syndrome is composed

FIG. 8.

FIG. 9.

FIG. 8. Relationship between corticosteroid content (●: aldosterone, ▲: corticosterone, □: cortisol) and clear-type cell in Cushing's syndrome ($P=n.s.$).

FIG. 9. Relationship between corticosteroid content (●: aldosterone, ▲: corticosterone, □: cortisol) and clear-type cell in control ($P=n.s.$).

predominantly of the compact-type cell with granularly eosinophilic, lipid-poor cytoplasm. The terms of clear- and compact-type cells in this report are in accordance with Tsuchiyama (25, 26). They are not considered the cell origin but express morphologic peculiarity.

From this hormonal study with radioimmunoassay, the mean content of aldosterone and corticosterone in primary aldosteronism was found to be significantly higher than in Cushing's syndrome and control. Although conflicting reports are made about corticosterone contents, high aldosterone and corticosterone are characteristic of primary aldosteronism (1, 5, 8, 9, 10, 11, 13, 14, 17). Furthermore, cortisol was contained almost the same as in the control. It is a well-known fact that cortisol, as well as aldosterone, is produced in incubation study of primary aldosteronism (2). A high content of cortisol is a consistent feature of Cushing's syndrome (4, 5, 11, 19). Contrary to expectations, Cushing's syndrome showed high aldosterone content. According to a report of Louis and Conn (11), a significant amount of aldosterone was presented. However, further study will be necessary to estimate the content of aldosterone, because only a few cases of Cushing's syndrome were studied.

Hormone contents in the control group generally revealed low values, except in

a few samples. Thus the quantitative study may be useful for distinguishing functioning and non-functioning tumors. As previously mentioned, various results have been reported with regard to hormone content. Most of those reports were determined by one sample assay about one case. The corticosteroid content in the author's study also varied with the region of quantitative sample even in the same case. Therefore, it is probably desirable that the measurement of corticosteroid content is performed from many parts of adrenal tissue in one case.

On histological examination of a nontumorous portion of adrenal tissue in primary aldosteronism, the broadening of the zona glomerulosa was seen. This finding has been pointed out by others (2, 23, 24, 25). The steroid content of these portions was within normal range in this study. It may not suggest a hyperfunctional state, but the exact nature is obscure.

Up to date, many studies have been performed using various methods in order to define the morphological features and the functional meaning of hyperadrenocorticism. However, the details of the functional significance of its constituent cell-types have not been fully established. Tsuchiyama suggested that the clear-type cells of the adenoma with primary aldosteronism played a role in the storage of steroid precursors but not in production or secretion of the hormone (25, 26). This is based upon histochemical and ultrastructural observations that those cells showed the following characteristics: heavy concentration of cholesterol; sparse distribution of phospholipid and acid phosphatase; little mitochondria and other organelles. Moreover, he thought that compact-type cells were the source of secretory activity, quite contrary findings to the clear-type cells. Concerning the relation between corticosteroid content and morphological constituent cell-type, one observation has been made by Sasano (18). He compared the histological feature on a maximum cut surface of adenoma in primary aldosteronism with hormone content in another part of it. According to his study, the aldosterone content showed a low value in cases of high clear-type cell proportion, and conversely, compact-type cells had a conspicuously high content. From these results, he indicated that those two type cells were not directly concerned with storage and release of aldosterone, but that "clear cell" as defined by Neville *et al.* (15) had a functional significance about superfluous production and secretion of aldosterone. In other words, the release of aldosterone in primary aldosteronism was performed by the clear cell.

In general, active synthesis of hormone will be nearly always accompanied by release into the blood. Therefore, if the clear-type cell represents a storage state of steroid precursors and if the compact-type cell is a secretory condition, the following observations can be made in this study. In primary aldosteronism, the correlation between aldosterone content and compact-type cell population has a negative trend, and that between corticosterone content and clear-type cell shows a positive. Moreover, a negative correlation is supposed between cortisol content and compact-type cell in Cushing's syndrome. Although there were no direct correlations between corticosteroid contents in adrenal tissue and constituent cell-types, some tendencies were recognized in this study. Namely, the same trends as mentioned above were shown in primary aldosteronism. These do not conflict with the foregoing assumption that the secretion of aldosterone is not performed by the clear-type cell. Even if it does, the quantity of aldosterone secretion may be very small. Furthermore, it may be possible that the clear-type cell stores not only the steroid precursors but also

the final products, as aldosterone and cortisol, in the process of steroid synthesis. On the other hand, there was a positive trend between cortisol content and compact-type cell population in Cushing's syndrome. In regard to this point, some possibilities should be taken into consideration: first, cortisol synthesis in compact-type cell is more active than its release. Second, release of cortisol is carried out as a clear-type cell. Third, independent storage, synthesis and secretion have no relationship to constituent cell-type. Making conclusions about these points is not simple. With regard to aldosterone and cortisol in the control group, the same possibilities must be considered.

It has been suggested that the amount of mitochondria, smooth endoplasmic reticulum and lipid may be closely related to steroid synthesis (12). There are some ultrastructural differences in each constituent cell of primary aldosteronism and Cushing's syndrome, although they appear to be identical in light microscopy. Those dissimilarities of relationship between hormone content and constituent cell-type may be relevant to the morphological difference and a kind of corticosteroid. About corticosteroid synthesis, release and storage in functioning adrenocortical adenoma and hyperplasia, various kinds of complicated mechanisms can be supposed. The functional significance of the two cell-types was not fully established from this correlative study, and studies using various methods should be done in future.

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