Adrenocortical Zonation in Humans under Normal and Pathological Conditions

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Context: Aldosterone synthase (CYP11B2) and steroid 11β-hydroxylase (CYP11B1) catalyze the terminal steps for aldosterone and cortisol syntheses, respectively, thereby determining the functional differentiation of human adrenocortical cells. Little is known, however, about how the cells expressing the enzymes are actually distributed in the adrenals under normal and pathological conditions.

Objective: The objective of the study was to determine the localization of CYP11B2 and -B1 in human adrenal specimens by using developed antibodies capable of distinguishing the two enzymes from each other.

Results: Under normal conditions, CYP11B2 was sporadically detected in the zona glomerulosa, whereas CYP11B1 was entirely detected in the zonae fasciculata-reticularis. Adrenocortical cells lacking both enzymes were observed in the outer cortical regions. In addition to conventional zonation, we found a variegated zonation consisting of a subcapsular cell cluster expressing CYP11B2, which we termed aldosterone-producing cell cluster, and a CYP11B1-expressing area. Aldosterone-producing adenomas differed in cell populations expressing CYP11B2 from one another, whereas CYP11B1-expressing and double-negative cells were also intermingled. Adenomas from patients with Cushing's syndrome expressed CYP11B1 entirely but not CYP11B2, resulting in atrophic nontumor glands. The nontumor portions of both types of adenomas bore frequently one or more aldosterone-producing cell clusters, which sustained CYP11B2 expression markedly under the conditions of the suppressed renin-angiotensin system.

Conclusion: Immunohistochemistry of the human normal adrenal cortex for CYP11B2 and CYP11B1 revealed a variegated zonation with cell clusters constitutively expressing CYP11B2. This technique may provide a pathological confirmatory diagnosis of adrenocortical adenomas. **(*J Clin Endocrinol Metab* 95: 2296–2305, 2010)**

The adrenal cortex of mammals consists of three major cell zones, which differ from each other in morphology and steroidogenesis. The most potent mineralocorticoid, aldosterone, is synthesized in the outermost zone, the zona glomerulosa (zG). Cortisol and/or corticosterone are formed in the midzone, the zona fasciculata (zF). In some species including humans, adrenal androgens are produced in the innermost zone, the zona reticularis (zR) (1). The classical view

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Abbreviations: APA, Aldosterone-producing adenoma; APCC, aldosterone-producing cell cluster; CPA, cortisol-producing adenoma; CS, Cushing's syndrome; CYP11B1, steroid 11β-hydroxylase cytochrome P450; CYP11B2, aldosterone synthase cytochrome P450; CYP17, steroid 17α-hydroxylase and C17-20 lyase cytochrome P450; 3βHSD, 3β-hydroxysteroid dehydrogenase; PA, primary aldosteronism; RCC, renal cell carcinoma; TF, Trigger factor; UTUC, upper urinary tract urothelial carcinoma; zF, zona fasciculata; zFR, zonae fasciculata-reticularis; zG, zona glomerulosa; zR, zona reticularis.

of the functional zonation of the adrenal cortex was established based on histological examinations and biochemical studies on steroids produced from isolated tissue fragments (2–4).

The molecular basis of the functional zonation has been derived mainly from studies using experimental animals including rodents. The differential production of the steroid hormones in rodents, especially for either aldosterone or corticosterone, is attributed to the spatially restricted expression of the two enzymes responsible for the terminal steps of their syntheses. These two enzymes are aldosterone synthase cytochrome P450 or P450aldo (CYP11B2) and steroid 11β-hydroxylase cytochrome P450 or P450$_{11\beta}$ (CYP11B1). These enzymes catalyze the syntheses of aldosterone and corticosterone, respectively, from the common substrate 11-deoxycorticosterone (5–8), which is synthesized from cholesterol by successive actions of CYP11A, 3β-hydroxysteroid dehydrogenase (3βHSD), and CYP21. These latter enzymes are expressed in both the zG and zF. The localization of the two CYP11B enzymes by means of immunohistochemical examination confirmed the classical view and further revealed the histological details of the functional zonation. Under normal dietary sodium conditions, only a small cell population in the zG expresses CYP11B2, whereas the zF cells express entirely CYP11B1 (9). Cells devoid of both CYP11B enzymes, namely functionally undifferentiated cells, exist in the outer cortical region including the zG (10). In adult rat adrenal cortex, the undifferentiated cells are comprised of the zG cells without CYP11B2, which is inducible when the renin-angiotensin system is activated (9, 11), and possible stem or progenitor cells (12–17).

In humans, CYP11B2 and -B1 also catalyze the terminal steps of aldosterone and cortisol syntheses, respectively (18–20). The production of cortisol, but not corticosterone, as the major glucocorticoid is due to 17α-hydroxylation catalyzed by CYP17 in humans. Thus, expression of either CYP11B2 without CYP17 or CYP11B1 with CYP17 determines the corticosteroids synthesized in human adrenocortical cells. Based on immunohistochemistry, the localization of CYP17 has been specified to the zonae fasciculata-reticularis (zFR) (21). However, little is known about the localization of the two CYP11B proteins because immunohistochemical techniques for the two enzymes have not been established for human specimens prepared for routine histopathological examinations. Although *in situ* hybridization studies on mRNAs for the two enzymes have been performed by different research groups, consistent localization of the mRNAs has not been unambiguously defined for normal adrenal cortex as well as pathological glands in humans (22–24).

The present study reveals the functional differentiation of adrenocortical cells in humans under normal and pathological conditions by demonstrating immunohistochemical localization of the two CYP11B enzymes. The successful application of the immunological detection of the two enzymes to formalin-fixed paraffin-embedded specimens of human samples provides a useful tool for a definitive diagnosis of adrenocortical tumors. The immunohistochemical localization leads to the novel finding of cell clusters markedly expressing CYP11B2 in the normal adrenal cortex and also in nontumor portions of adrenocortical adenomas, even under the suppressed renin-angiotensin system. This observation suggests that aldosterone is produced constitutively in such a cell cluster and inducibly in the zG of the conventional zonation under normal conditions.

Materials and Methods

Adrenal specimens

Adrenal glands from eight patients with renal cell carcinoma (RCC), a patient with upper urinary tract urothelial carcinoma (UTUC), 24 patients with primary aldosteronism (PA), and six patients with adrenal Cushing's syndrome (CS) were examined. The patients with RCC had no clinical manifestation of hypertension, and the patient with UTUC had mild hypertension that was controlled by nicardipine. Diagnosis of PA and CS was confirmed on the basis of The Endocrine Society clinical practice guidelines of PA (25) and CS (26), respectively (see supplemental data, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The present study was approved by the Medical Ethics Committee of the School of Medicine, Keio University.

Antibodies

Antibodies to human CYP11B2 or -B1 were raised in rabbits and affinity-purified as we described previously (19). Because the two enzymes have 93% identical residues in their amino acid sequences (18), the respective peptides corresponding to amino acid residues 80-90 (RYNLGGPRMVC for CYP11B2 and RYDLGGAGMVC for CYP11B1) were used for immunization. The sulfhydryl group of the carboxy terminal cysteine residue was used for conjugation with equine myoglobin (19) and with column media for affinity preparation of the antibodies (9). Anti-3βHSD antiserum was raised in rabbits against purified bovine 3βHSD (Yamazaki, T., unpublished result). Rabbit anti-guinea pig CYP17 antiserum was described previously (27).

Immunoblotting

An inner and outer layer of a normal adrenal (case 9), a nontumor and tumor portion of an aldosterone-producing adenoma (APA; case 27), and *Escherichia coli* cells expressing recombinant CYP11B2 or -B1 proteins were examined using anti-CYP11B2 and anti-CYP11B1 antibodies (1:40,000 dilution) with an ECL Plus reagent pack (GE Healthcare, Buckinghamshire, UK). Blocking peptides, which had the same amino acid sequences as those used for immunization, were

TABLE 1. Functional characterization of normal adrenal cortex and adenomas from primary aldosteronism and CS

		Adrenal cortex		Tumor			
Case no.	Clinical diagnosis	CYP11B2	CYP11B1	Size (mm)	CYP11B2	CYP11B1	Figure
1	RCC	zG	zFR(+++)				2, A–G
2	RCC	zG, APCC	zFR(+++)				
3	RCC	zG, APCC	zFR(+++)				2, H, I
4	RCC	zG, APCC	zFR(+++)				
5	RCC	zG, APCC	zFR(+++)				
6	RCC	APCC	zFR(+++)				2, J–O
7	RCC	APCC	zFR(+++)				
8	RCC	APCC	zFR(+++)				
9	UTUC	APCC	zFR(+++)				1A
10	PA	zG, APCC	zFR(++)	2, 8[a]	IV, III	II, II	Suppl A–D
11	PA	zG	zFR(+++)	5	IV	I	
12	PA	zG, APCC	zFR(+++)	6	IV	I	
13	PA	zG, APCC	zFR(++)	7	II	II	
14	PA	—	zFR(++)	7	IV	—	
15	PA	APCC	zFR(+)	7	III	I	3, O–R
16	PA	zG, APCC	zFR(++)	8	III	II	
17	PA	zG	zFR(+++)	9	II	II	
18	PA	—	zFR(++)	9	IV	I	
19	PA	zG, APCC	zFR(+++)	9	II	II	3, F–N
20	PA	—	zFR(++)	9	IV	I	3, A–E
21	PA	—	zFR(+)	11	II	I	
22	PA	—	zFR(+++)	12	III	II	
23	PA	—	zFR(++)	12	IV	I	
24	PA	APCC	zFR(++)	14	III	II	
25	PA	—	zFR(+++)	14	III	I	
26	PA	zG	zFR(++)	14	III	II	
27	PA	APCC	zFR(++)	14	I	II	1A
28	PA	—	zFR(+)	15	II	II	
29	PA	APCC	zFR(++)	15	II	II	
30	PA	APCC	zFR(++)	15	III	II	
31	PA	—	zFR(+)	16	III	II	Suppl D
32	PA	—	zFR(+++)	20	III	II	Suppl D
33	PA	—	Atrophic (±)[b]	20	I	III	
34	CS	APCC	Atrophic (±)[b]	15	—	IV	4, D–O
35	CS	—	Atrophic (±)[b]	25	—	IV	
36	CS	APCC	Atrophic (±)[b]	25	—	IV	4, A–C
37	CS	APCC	Atrophic (±)[b]	25	—	IV	
38	CS	APCC	Atrophic (±)[b]	30	—	IV[c]	
39	CS	—	Atrophic (±)[b]	32	—	IV[c]	

Intensities of CYP11B1 staining in normal and nontumor glands: ±, 0–10%; +, 11–50%; ++, 51–90%; +++, 91–100% of the average intensity in zFR of normal adrenals. Cell population positive for CYP11Bs in tumors: —, not detected; I, up to 10%; II, 11–50%; III, 51–90%; IV, 91–100%.

[a] The smaller tumor in case 10 was subjected to laser capture microdissection for the experiments in supplemental data.

[b] zFR of nontumor glands were atrophic.

[c] Twenty and 60% of the tumor cells in cases 38 and 39 were weakly positive for CYP11B1, respectively.

used at 0.25 μM for verification of the specificities. The experimental procedures are described in the supplemental data.

Immunohistochemistry

Serial sections (4 μm) from formalin-fixed paraffin-embedded specimens were deparaffinized and treated with Target retrieval solutions (Dako, Glostrup, Denmark) at pH 9 for staining of CYP11B2 and -B1 and at pH 6 for staining of 3βHSD and CYP17. Immunohistochemical staining was performed using a Dako autostainer universal system according to the manufacture's protocol. Anti-CYP11B2 and anti-CYP11B1 antibodies were used at 1:10,000 and 1:5,000 dilutions, respectively. Antisera specific to 3βHSD and CYP17 were used at 1:10,000 and 1:4,000 dilutions, respectively. Secondary antibodies used were an Envision reagent coupled with horseradish peroxidase (Dako) or a simple stain reagent coupled with alkaline phosphatase (Nichirei, Tokyo, Japan). Peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Dako). Alkaline phosphatase activity was visualized with 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (Dako). To show CYP11B2 and -B1 with different colors on a single specimen, the sections were first immunostained for CYP11B2 with alkaline phosphatase reaction. Subsequently the sections were treated again with antigen retrieval reagents to remove the bound antibodies and were stained for CYP11B1 with peroxidase reaction.

Results

Antibodies specific to human CYP11B2 and -B1

Immunoblotting was performed to characterize the antibodies raised against human CYP11B2 and -B1 using a normal adrenal gland (case 9, Table 1) and an APA sample (case 27). An outer and inner layer from the normal adrenal and a nontumor and tumor portion from the APA were examined. The antibody raised against CYP11B2 gave a weak signal with a molecular mass of 48.5 kDa from the outer layer (Fig. 1A, lane1) but no detectable signal from the inner layer or from the nontumor portion (lanes 2 and 3). The tumor portion of the APA showed a well-defined band (lane 4). The antibody raised against CYP11B1 gave an easily detectable signal with a molecular mass of 50 kDa from both layers from the normal gland (lanes 5 and 6); the signal intensity of the inner layer was higher than that of the outer layer. A strong signal was observed from the nontumor portion of the APA sample (lane 7). The tumor portion also showed a considerable signal, although an APA (lane 8). These results agreed well with our previous observations (19).

To confirm there was no cross-reactivity of the two antibodies between CYP11B2 and -B1, bacterially produced recombinant fusion proteins (TF-hB2 and TF-hB1) were tested by immunoblot analysis. The results showed that anti-CYP11B2 antibody recognized only TF-hB2 (Fig. 1B, lanes 1 and 2), whereas anti-CYP11B1 antibody recognized only TF-hB1 (lanes 3 and 4).

In the experiments mentioned above, antigenic peptides were used to examine whether they could block the immunoreactivity of the two antibodies. The antibodies were preincubated with the free peptides and then used for immunoblotting of the tissue homogenates and the recombinant proteins. The signals obtained with either antibody were blocked specifically by addition of its own peptide but not by addition of the other (Fig. 1A, lanes 9–24, and B, lanes 5–12). Thus, these results indicated that the two antibody preparations reacted specifically with either CYP11B2 or -B1.

Additionally, the expression levels of mRNAs for CYP11B2 and -B1 in tissue specimens including a nontumor portion of an APA and tumor portions of APAs were examined using laser capture microdissection and quantitative RT-PCR analysis. The mRNAs results were consistent with the results from the immunoblotting (Fig. 1) and the immunohistochemistry described below (Figs. 2–4) (see supplemental data).

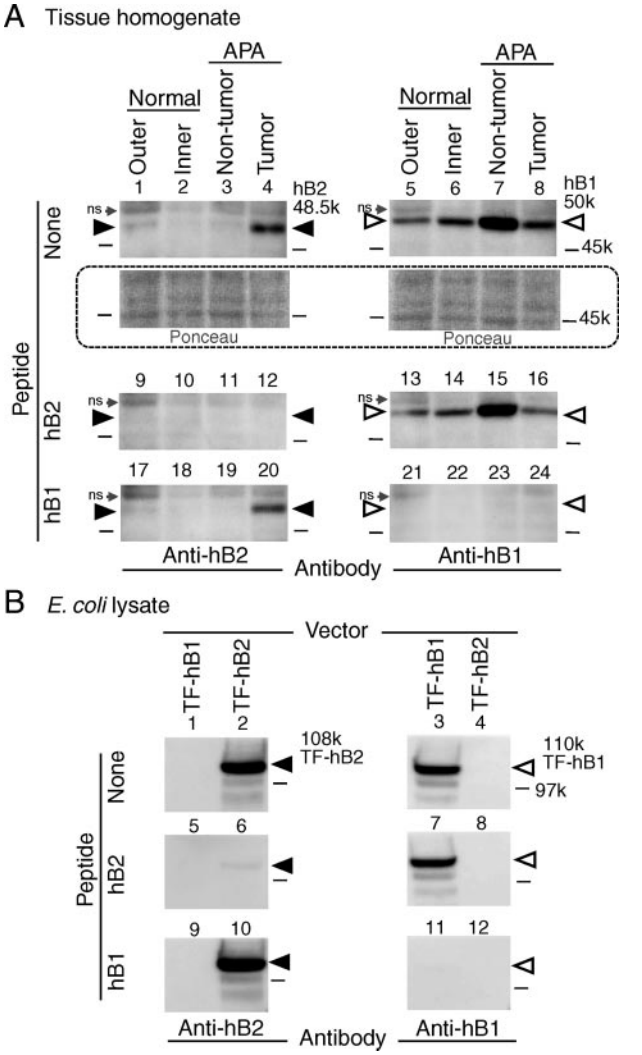

FIG. 1. Antibodies specific to human CYP11B2 and -B1. Immunoblotting using anti-human CYP11B2 or -B1 antibodies was performed to detect the two enzymes in tissue homogenates and their recombinant proteins. A, Tissue homogenates (20 μg of protein) prepared from an outer (lanes 1, 5, 9, 13, 17, and 21) and an inner layer (lanes 2, 6, 10, 14, 18, and 22) of a normal adrenal sample (case 9) and a nontumor (lanes 3, 7, 11, 15, 19, and 23) and a tumor portion (lanes 4, 8, 12, 16, 20, and 24) of an APA (case 27) were analyzed. Ponceau S staining of the blots (lanes 1–4 and 5–8) after electroblotting shows that almost equal amounts of protein were loaded. B, Lysates of *Escherichia coli* cells expressing human recombinant CYP11B2 or -B1 protein (200 ng) fused to Trigger factor (TF) were analyzed. Antigenic peptide hB2 (lanes 9–16 of A and lanes 5–8 of B) or hB1 (lanes 17–24 of A and lanes 9–12 of B) were added at 0.25 μM to the primary antibody solutions to verify the antibody specificities. CYP11B2 and -B1 extracted from the tissues are indicated by *arrowheads* with molecular sizes of 48.5 and 50 kDa, respectively. Recombinant fusion proteins TF-hB2 and -hB1 produced by *E. coli* cells are indicated by *arrowheads* with molecular sizes of 108 and 110 kDa, respectively. A nonspecific band is indicated with ns. Positions of 45 and 97 kDa molecular size markers are indicated with a *dash*.

Normal adrenal cortices in humans

Immunohistochemical examination using the two antibodies in a successive manner enabled us to detect CYP11B2 and -B1 simultaneously in a single adrenal section. We found that normal adrenocortical tissue exhibited two types of distribution of the two enzymes: the conventional distribution (Fig. 2, A–G) and the variegated distribution (Fig. 2, H–O), as discussed below.

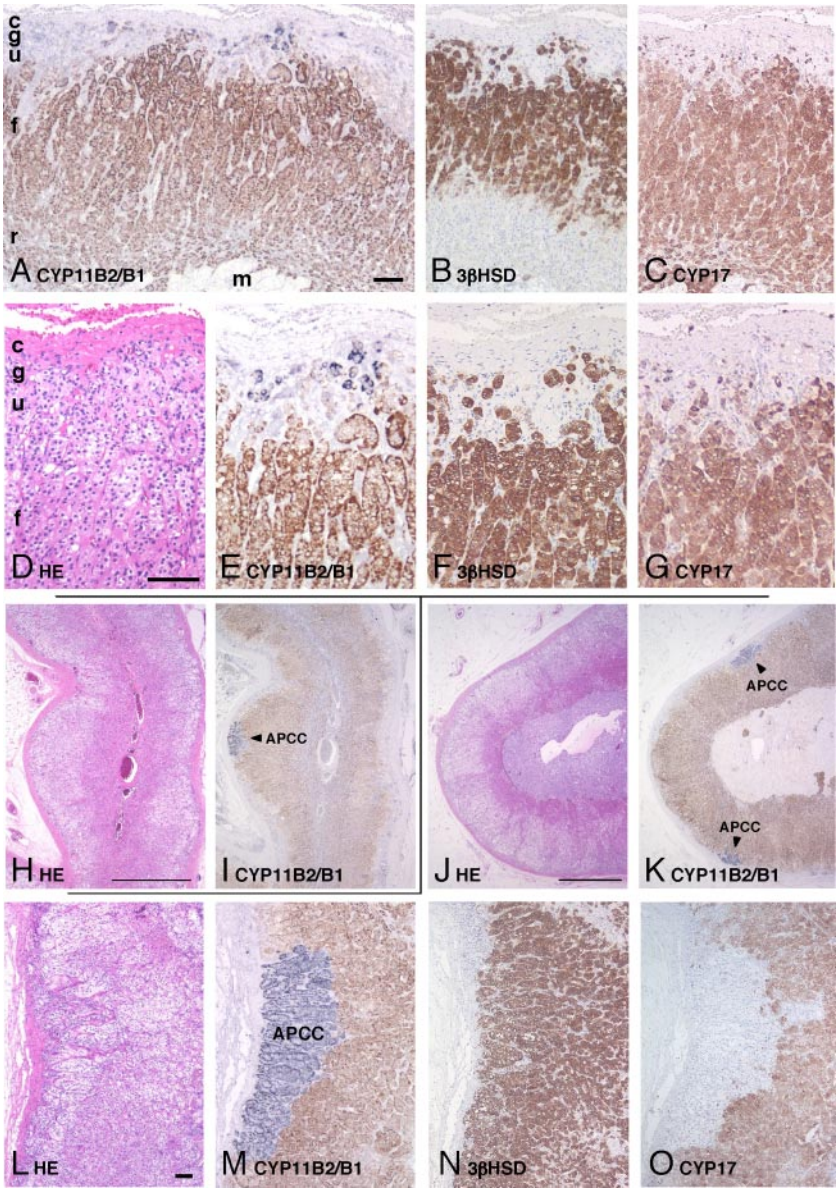

FIG. 2. Conventional and variegated zonation of normal adrenal cortex in humans. Serial sections prepared from formalin-fixed, paraffin-embedded specimens of normal adrenal glands from patients with RCC (A–G, case 1; H and I, case 3; J–O, case 6) were examined histologically and immunohistochemically. D, H, J, and L, Hematoxylin-eosin staining. A, E, I, K, and M, Double immunostaining for CYP11B2 (*blue*) and -B1 (*brown*). B, F, and N, Immunostaining for 3βHSD (*brown*). C, G, and O, Immunostaining for CYP17 (*brown*). Nuclei were counterstained with hematoxylin after immunostaining for 3βHSD and CYP17. A–G, Conventional adrenocortical zonation with sporadic expression of CYP11B2 in the zG and overall expression of CYP11B1 in the zF and zR. Note that double staining for CYP11B2 and -B1 indicates the presence of adrenocortical cells devoid of both enzymes in the outer area. H–O, Variegated zonation consisting of subcapsular APCCs expressing CYP11B2 and the remaining areas expressing CYP11B1. The medulla is absent in the innermost areas of H and I. c, g, u, f, r, and m are capsule, zG, undifferentiated zone, zF, zR, and medulla, respectively. *Bars*, 100 μm (A, D, and L); 1000 μm (H and J).

The normal adrenocortical tissues from the nine patients with RCC or UTUC exhibited conventional histology consisting of the zG, zF, and zR (Fig. 2D for case 1, Fig. 2H for case 3, Fig. 2J for case 6; Table 1). Double immunostaining of sections from cases 1–5 for CYP11B2 and -B1 showed that CYP11B2 (*blue*) was sporadically detected in the zG, whereas CYP11B1 (*brown*) was entirely detected in the zF and zR (Fig. 2, A and E, for case 1). It was noted that cells devoid of both CYP11B2 and -B1 were observed in the outer areas including the zG. Negative controls (normal rabbit IgG or secondary antibody only) gave no signals, and preincubation of the antibodies with appropriate amounts of each of their antigenic peptides blocked the signals (not shown), suggesting that the immunostainings were specific.

Localization of 3βHSD and CYP17 was examined for comparison with the localizations of CYP11B2 and -B1. Cells expressing 3βHSD evidently corresponded to the zG cells expressing CYP11B2 and the zF cells expressing CYP11B1 (Fig. 2, B and F). 3βHSD was hardly detected in the zR. Cells expressing CYP17 corresponded to those expressing CYP11B1 (Fig. 1, C and G). Thus, the spatially restricted expression of CYP11B2 and -B1 agreed well with the conventional zonation of the adrenal cortex.

Interestingly, eight of the nine normal adrenals (cases 2–9) exhibited a variegated pattern consisting of cell clusters markedly expressing CYP11B2 with the remaining areas expressing CYP11B1 (Fig. 2, H–O, for cases 3 and 6). On the basis of morphological criteria, these normal adrenal cortices exhibited the conventional zonation (Fig. 2, H and J; note that the medulla is not seen in panel H). However, double staining for CYP11B2 and -B1 revealed cell clusters showing signals of CYP11B2 with a width of 200-1300 μm and a depth of 100–500 μm beneath the capsule (Fig. 2, I, K, and M). Such cell clusters, termed aldosterone-producing cell clusters (APCCs) in the present study, were composed of morphological zG cells in contact with the capsule and inner columnar zF-like cells forming cords along sinusoids (Fig. 2, L and M). The cells in the remaining subcapsular areas other than APCCs were devoid of both CYP11B enzymes (Fig. 2I) or expressed only CYP11B1 (Fig. 2K). Expression of 3βHSD but not CYP17 in APCCs was consistent with their ability to synthesize aldosterone (Fig. 2, N and O).

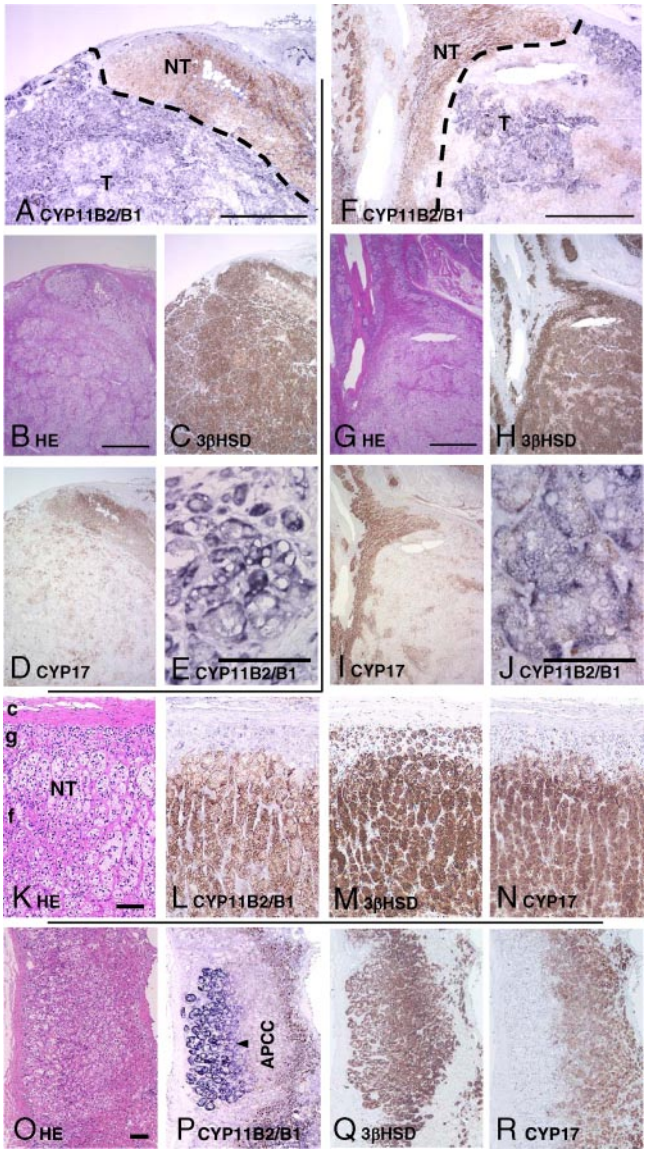

FIG. 3. Functional characterization of APAs and their nontumor portions. Sections of adrenals from patients with primary aldosteronism (A–E, case 20; F–N, case 19; O–R, case 15) were examined histologically and immunohistochemically. B, G, K, and O, Hematoxylin-eosin staining. A, E, F, J, L, and P, Double immunostaining for CYP11B2 (*blue*) and -B1 (*brown*). C, H, M, and Q, Immunostaining for 3βHSD (*brown*). D, I, N and R, Immunostaining for CYP17 (*brown*). Nuclei were counterstained with hematoxylin after immunostaining for 3βHSD and CYP17. A and E, Tumor consists of greater than 95% cell populations positive for CYP11B2 and a few percent populations positive for CYP11B1. F and J, Tumor consists of 40% cell populations positive for CYP11B2, 20% positive for CYP11B1, and 40% of double-negative cells. K–N, Nontumor portion exhibits the conventional zonation consisting of the zG with only sporadic expression of CYP11B2 and the zFR with expression of CYP11B1. O–R, Nontumor portion contains an APCC and that the regions outside the APCC are negative for CYP11B2 and negative or weakly positive for CYP11B1. T, Tumor; NT, nontumor. The boundary between tumor and nontumor regions is indicated by *dashed lines*. Bars, 100 μm (E, J, K, and O); 1000 μm (A, B, F, and G).

Based on the two patterns of the distribution of CYP11B2-expressing cells as described above, the functional zonation in humans was classified into the conventional zonation with sporadic expression of CYP11B2 in the zG and the novel variegated zonation with an APCC.

APAs

Twenty-four APAs were immunohistochemically examined for CYP11B2 and -B1. They were found to contain varied cell populations expressing CYP11B2 (Table 1). Figure 3 shows two cases: one was almost entirely positive for CYP11B2 (Fig. 3, A and E, for case 20); the other was approximately 40% positive for it (Fig. 3, F and J, for case 19). Similarly, cell populations expressing CYP11B1 were different from one another; a few percent in the former (Fig. 3, A and E) and 20% in the latter (Fig. 3, F and J). Taken together, the APAs essentially consisted of at least three cell types: CYP11B2-positive and -B1-negative cells; CYP11B2-negative and -B1-positive cells; and double-negative cells. Double-positive cells were not detectable under the experimental conditions. The cell populations expressing CYP11B2 appeared to be inversely correlated with the tumor sizes and with the cell populations expressing CYP11B1 (Table 1). Expression of 3βHSD was detected throughout the tumors, irrespective of tumor sizes and the cell populations expressing CYP11B2 or -B1 (Fig. 3, C and H). The localization of CYP17-expressing cells in the tumors almost corresponded to that of CYP11B1-expressing cells (Fig. 3, D and I). Thus, these results suggested that APAs produced both aldosterone and cortisol.

Nontumor portions of APAs

Two examples of nontumor portions from the 24 APAs are shown in Fig. 3; one exhibited the conventional zonation histologically and immunohistochemically (Fig. 3, K-N, for case 19); the other contained an APCC and exhibited no or weak expression of CYP11B1 in the non-APCC area (Fig. 3, O and P, for case 15). Ten of the 24 cases contained APCCs (Table 1), even under the suppressed renin-angiotensin system (<9.8 pg renin per milliliter plasma). Similar to the expression profile in the APCCs of normal adrenal cortex, the APCCs in the nontumor portions expressed CYP11B2 and 3βHSD but not CYP11B1 and CYP17.

Cortisol-producing adenomas (CPAs) and their nontumor portions

Two of six cases of CPAs are shown in Fig. 4 (Fig. 4, A–C, for case 36 and Fig. 4, D-O, for case 34). CYP11B1 was detected throughout the tumors, whereas CYP11B2 was not (Fig. 4, A and E). 3βHSD and CYP17 were also detected throughout the tumors (Fig. 4, C, F, and G). These results suggested that the CPAs produced cortisol but not aldosterone.

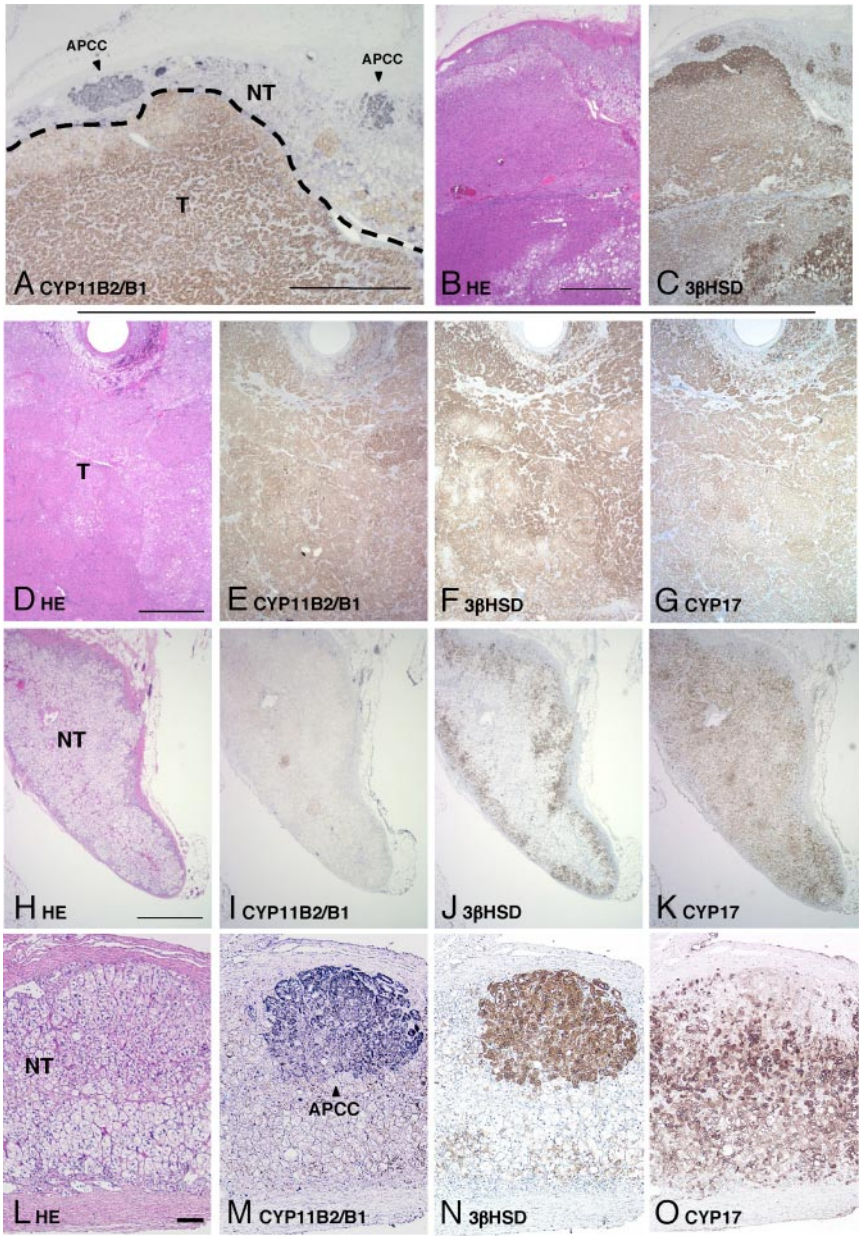

FIG. 4. Functional characterization of CPAs and their nontumor portions. Sections of adrenals from patients with CS (A–C, case 36; D–O, case 34) were examined histologically and immunohistochemically. B, D, H, and L, Hematoxylin-eosin staining. A, E, I, and M, Double immunostaining for CYP11B2 (*blue*) and -B1 (*brown*). C, F, J, and N, Immunostaining for 3βHSD (*brown*). G, K, and O, Immunostaining for CYP17 (*brown*). Nuclei were counterstained with hematoxylin after immunostaining for 3βHSD and CYP17. A–C, Tumor portion is negative for CYP11B2 and 95% positive for CYP11B1 and the remaining nontumor portion is atrophic but contains two APCCs. D–G, Tumor is negative for CYP11B2 and greater than 95% positive for CYP11B1. H–K, Nontumor portion is atrophic and negative for CYP11B2 and hardly detectable for CYP11B1. L–O, An APCC in the nontumor portion; the APCC is an example contains cells positive for CYP17. The medulla is absent in the innermost areas of H–K and L–O. T, Tumor; NT, nontumor. The boundary between tumor and nontumor regions is indicated by *dashed lines*. *Bars*, 100 μm (L); 1000 μm (A, B, D, and H).

The nontumor portions of the CPAs were atrophic (Fig. 4, B, H, and L) and faintly immunostained for CYP11B1 (Fig. 4, A, I, and M; Table 1). CYP11B2 was hardly detectable (Fig. 4I), whereas 3βHSD and CYP17 were expressed weakly (Fig. 4, J and K). APCCs were found frequently in four of six cases (Fig. 4, A and M; Table 1). It was again noted that the APCCs evidently expressed CYP11B2 under the suppressed renin-angiotensin system in the patients (<9.8 pg renin per milliliter plasma) due to excess mineralocorticoid activity caused by overproduction of cortisol. 3βHSD was expressed in the APCCs (Fig. 4, C and N). Although CYP17 was not expressed in most APCCs in the atrophic nontumor portions of CPAs, the APCC shown in Fig. 4O contained CYP17-expesssing cells. Thus, the nontumor portions of CPAs were inactive for production of corticosteroids except for APCCs.

Discussion

In the present study, immunohistochemical detection of human CYP11B2 and -B1 clearly visualized functional differentiation of adrenocortical cells. This was enabled by development of a pair of specific antibodies that distinguished between the two enzymes and by the application of the antibodies to standard formalin-fixed paraffin-embedded specimens. The establishment of the immunohistochemical technique led us to first discover the variegated zonation of the gland and confirm the conventional zonation under normal and pathological conditions (Fig. 5).

The variegated zonation in the human adrenal cortices under normal conditions is a novel finding obtained in the present study. The finding raises a question about physiological and developmental roles of an APCC. The functional and morphological features of APCCs may provide important clues to these roles. First, the fact that APCCs frequently occur in normal adrenal cortices and nontumor portions of adenomas suggests that the human adrenal cortex consists of the variegated zonation and the conventional zonation. Second, the feature that cells in an APCC form columnar cell arrangements along sinusoids similar to those of the zF supports the view that an APCC retains the normal histology, at least in part. Third, compared with the conventional zG cells that ex-

Cells	Expression of steroidogenic enzymess				Synthesis of aldosterone or cortisol	Zonation
	CYP11B2	CYP11B1	3βHSD2	CYP17		
Normal						
zG, induced	+	-	+	-	Aldosterone	Conventional
zG, uninduced	-	-	-	-	-	Variegated
zU						
zF	-	+	+	+	Cortisol	
zR	-	+	-	+	-[a]	
APCC	+	-	+	-[b]	Aldosterone	
Adenoma						
APA cell type 1	+	-	+	-	Aldosterone	
cell type 2	-	+	+	+	Cortisol	
cell type 3	-	-	+	-	-	
CPA	-	+	+	+	Cortisol	

FIG. 5. Expression profiles of steroidogenic enzymes in normal adrenocortical and adenoma cells and proposed adrenocortical zonation in humans. A small population of the zG cells is induced by endocrine systems such as the renin-angiotensin system to express CYP11B2 for production of aldosterone. Uninduced cells in the zG and putative progenitor cells in undifferentiated cell zone (zU) are devoid of both CYP11Bs and 3βHSD and CYP17. The zF cells that express CYP11B1 together with 3βHSD and CYP17 produce cortisol. The zR cells synthesize neither aldosterone nor cortisol but produce adrenal androgen such as dehydroepiandrosterone (indicated with a) due to expression of CYP17. APCCs express autonomously CYP11B2 to produce aldosterone under the normal or the pathological conditions of the suppressed renin-angiotensin system. APCCs do not express CYP17 except for some of them in nontumor atrophic portion of CPAs (indicated with b; Fig. 4O). APAs consist of at least three cell types, as indicated, in different populations from one another. The three types of cells each produce aldosterone, cortisol, or none of them. CPAs produce cortisol but not aldosterone.

press CYP11B2 only sporadically under control of the renin-angiotensin system (reviewed in Refs. 1 and 28), APCCs express CYP11B2 remarkably, even under the pathological conditions of PA and CS in which the endocrine system is suppressed. On the basis of these observations, we propose that aldosterone production in the human adrenal cortex is constitutive in APCCs of the variegated zonation and inducible in the zG of the conventional zonation.

An APCC is morphologically isolated with a defined boundary from the neighboring areas with CYP11B1 expression, albeit without an apparent fibrous capsule. This feature contrasts with the conventional zonation in which steroidogenic functions of cells change radially inward with cell arrangements from the subcapsular zG to the inner zF. Previous studies using chimeric and transgenic animals (29, 30) suggested that adrenocortical cells arranging centripetally are clonal cells. Therefore, the conventional zonation is based on two principles of cell arrangements, namely the centripetal arrangements of clonal cells and the concentric arrangements of functionally differentiated cells (10, 31). In this context, an APCC could not occur on the basis of the two principles for the conventional zonation. Thus, development of an APCC in the human adrenal cortex may involve a cellular mechanism different from those for the conventional zonation. Moreover, because the autonomous expression of CYP11B2 in APCCs is shared with APAs, it is possible that an APCC develops into an APA.

Besides the finding of the variegated zonation, the primary observation of the present study is that CYP11B2 is expressed in the zG, whereas CYP11B1 is expressed in the zFR of the adrenal cortex in human (Fig. 5). The distribution of the two enzymes is consistent with the functional differentiation of the conventional zonation. The presence of the functionally undifferentiated cells devoid of the two enzymes in the outer regions further indicates that the histology of the conventional zonation in humans is essentially the same as that in rodents (10). By analogy with rats (12–17), it is possible in humans that the cell populations devoid of the two enzymes consist of uninduced zG cells and stem-like or progenitor cells (Fig. 5). Direct evidence for the presence of stem-like or progenitor cells would provide more insights into development and maintenance of the adrenal cortex.

Another key steroidogenic enzyme responsible for the functional differentiation of human adrenocortical cells is CYP17, which carries out two separate reactions: 17α-hydroxylation and C17-20 cleavage. Adrenocortical expression of CYP17 in human and some other mammals, but not in rodents, results in the production of cortisol as the major glucocorticoid and also androgen such as dehydroepiandrosterone. The present study, which showed the localization of CYP17 in the zFR, verified the production of cortisol in the zFR of humans. Furthermore, our data demonstrating the colocalization of CYP17 with CYP11B1 in APAs, as well as in CPAs, histochemically proved that the tumors were able to synthesize cortisol.

Previously a few research groups performed *in situ* hybridization examinations on mRNAs for the two CYP11B enzymes with human specimens (22–24). Their results that the zG of the nontumor portions expresses CYP11B2 mRNA agreed well with our present immunohistochemical result for CYP11B2 in the conventional zonation. As for CYP11B1 mRNA, Enberg *et al.* (23) reported expression of CYP11B1 mRNA in the zF, consistent with our results, whereas the other groups reported conflicting observations that CYP11B1 mRNA was coexpressed with CYP11B2 in the zG (22, 24). On the other hand, our results obtained using highly specific antibodies indicated that the two enzymes are expressed in a mutually exclusive manner. Thus, the present immunohistochemical technique provided to give more conclusive and clearer im-

ages than those collected from the *in situ* hybridization techniques.

In situ hybridization examinations also showed that nonpathological portions of APAs had cell clusters expressing CYP11B2 mRNA (23, 24). By comparison of their results for mRNA expression profile with our results for the enzyme proteins, such cell clusters seem to be very similar to APCCs. As shown in the present study, however, the normal adrenal cortices frequently (eight of nine) contained APCCs, indicating that an APCC occurs normally in the human gland.

It is unknown about how adrenocortical adenomas develop. Besides the notion that an APA originates from an APCC, the undifferentiated cells with the putative pluripotency might be another candidate because APAs contain double-negative cells in addition to cells expressing CYP11B2 or -B1 (Fig. 5). On the other hand, because CPAs consist almost of cells expressing CYP11B1 but not those expressing CYP11B2 (Fig. 5), the zF cells might be an origin of a CPA. These putative candidates might undergo neoplastic changes to develop adenomas in combination with altered regulation of the steroidogenic gene expression.

Highly sensitive immunohistochemical detection of CYP11B2 as well as -B1 in surgically excised specimens is clinically of great significance in understanding and diagnosing pathologic conditions of adrenal disorders based on the following considerations. First, because CYP11B2 is expressed in APAs but not CPAs whereas CYP11B1 is expressed in both, detection of CYP11B2 is of importance in diagnosing whether a tumor is an APA or a CPA. Second, patients with an APA with a low level of CYP11B2 mRNA expression tend to have a poor prognosis of blood pressure (32). The tendency supports the importance of highly sensitive detection of CYP11B2. Third, because much less is known about pathophysiological conditions of nontumor PAs, such as idiopathic hyperaldosteronism and unilateral adrenal hyperplasia, the immunohistochemical detection of CYP11B2 and -B1 would provide new insights into their pathophysiologies. Finally, compared with previous *in situ* hybridization techniques, which are generally complicated and result in low-resolution imaging, the present immunohistochemical technique is more effective and suitable for a pathological confirmatory diagnosis of adrenocortical adenomas. Our technique can be further used for other rare clinical conditions such as glucocorticoid-remediable aldosteronism, familial aldosteronisms type II, and adrenocortical carcinomas.

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