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Review

roidogepic Enzymes in Adrenal Cortex Localization of Ste and Its Disorders

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I. Introduction

As is well known, adrenal cortex produces and secrete steroids, which originate from circulating or stored cholesterol. All corticosteroids are syn-thesized through a series of complicated enzymatic steps from cholesterol (Fig. 1). Corticosteroids are classified into three groups, glucocorticoids, min-eralocorticoids and adrenal androgens based on their biological actions. In human adrenal cortex associated with abnormal corticosteroidogenesis, the lesions are always associated with complicated morphological features, whether hyperplasia or neoplasms. Therefore, determining which types of cortical cells produce which classes of steroid hor-mones is essential to studying steroid metabolism in human and experimental animals. A majority of the studies of steroidogenesis dealing with adrenal have been performed by biochemical methods. However, biochemical studies alone are by no means sufficient to provide significant data on adrenal steroid metabolism. Therefore, it is important to know the lo-calization o f each specific steroidogenic enzyme when studying adrenal steroid production and metabolism.

II. Immunohistochemistry and in situ hybridization of steroidogenic enzymes

Recent advances in biochemical and molecular biology techniques have made it possible to purify

all the types of the enzymes involved in specific steroid hormones production [1, 2]. Subsequently, specific antibodies have been generated from these

purified products and corresponding DNA Se-quences.

Demonstration of the presence of the enzyme(s) involved in specific steroidogenesis strongly indi-cates that the steroid hormones catalyzed by the enzyme(s) are produced in cells where the enzyme is located. Based on this principle, we first suc-ceeded in determining the distribution of steroido-genesis in the adrenal gland and its disorders by an immunohistochemical method employing anti-bodies and by in situ hybridization employing cDNA or oligonucleotides against steroidogenic enzymes. These include P450scc (side chain-cleav-age) [3], 3j3HSD (hydroxysteroid dehydrogenase) [4], P450c21(21-hydroxylase) [5, 6], P450~17 (17a-hy-droxylase) [7-9] and P450c11 (11/3-hydroxylase) [10].
It is very important to choose the antibodies and DNA probes as well as the modes of processing the specimen. Antibodies employed in the immunolocalization of steroidogenesis should not only recognize the specific immunoreactivity on the tissue sections but also have the capacity to in-hibit in vitro enzymatic reaction. When only surgi-cal materials were available, the antibodies should recognize denaturation-resistant epitopes of the enzymes. Details of the characteristics of the anti-bodies employed for immunostain of surgical pa-thology materials of human adrenal and its disor-ders are described in detail in the references above.

Prompt fixation for brief duration is desired as in immunohistochemistry of other antigens. Other-wise, false negative results are expected. Once it is

properly processed, immunoreactivity of ste-roidogenic enzymes can be retained in 10% forma-lin fixed and paraffin-embedded materials in the

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surgical pathology files, as in other antigens. In general, immunohistochemistry o f steroidogenic en-zymes can be performed in 10% formalin-fixed and par-affin-embedded materials, i f the materials are properly processed. Immunostaining can be performed by avidin-biotin peroxidase complex or peroxidase-antiperoxidase methods.
For in situ hybridization of steroidogenic en-zymes, we employed 4 or 8% paraformaldehyde, adjusted to pH 7.4, for fixation, immediately fixed for 18 to 24 h at 4°C, and embedded in paraffin. It is better to store these blocks at 4°C, if possible. Denhardt-coated clean glass slides may be used for placing the specimens. We used oligonucleotides or cDNA labelled with 35S-dATP and 35S or 3H-

dCTP, respectively. It is very important to select appropriate DNA sequences when designing oli-gonucleotide probes, including G/C content of the probes of steroidogenic enzymes, properly de-signed oligonucleotide probes can yield much better results of mRNA hybridization of steroidogenic enzymes in tissue sections than cDNA probes, be-cause redundant or conserved nucleotide se-

quences can be avoided during probe design and synthesis, and a higher specific activity probe can be much more easily obtained [9]. It is also pos-sible to perform simultaneous immunohistochem-

istry and in situ hybridization of steroidogenic en-zymes on the tissue section. This method made it

possible to detect expression of steroidogenic en-zymes in the same cells at both protein and mRNA levels, especially with combination of colorimetric reaction for immunohistochemistry and silver dots on autoradiography which represent mRNA hy-
bridization of steroidogenic enzymes. Technical
details of in situ hybridization of steroidogenic en-zymes including simultaneous immunohistochem-istry and in situ hybridization were previously re-

ported by the authors [9].
In the following chapters, the new findings on localization of steroidogenesis in the adrenal and its disorders which could be revealed only by these approaches and could contribute to the better un-derstanding of adrenal steroid metabolism will be summarized.

III. Normal adrenal cortex
- -functional zonation-

Adrenal cortex is composed of three different
zones, the zona glomerulosa, fasciculata and
reticularis. It has been postulated by various bio-
chemical and conventional morphological methods

Fig. 1. Pathway of
modifications

corticosteroidogenesis in human from reference 17 with some

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that the zona glomerulosa is involved in the bio-synthesis of mineralocorticoid while the zonae fasciculata-reticularis produce and secrete gluco-corticoid and adrenal androgens. This functional compartmentalization is well-known but has not been directly confirmed. Immunolocalization and in situ hybridization revealed that cytochrome P450c17, a cytochrome P450 variant which is re-quired in glucocorticoid and adrenal androgen bio-synthesis but not in mineralocorticoid biosynthe-sis, was absent from the zona glomerulosa (Fig. 2), while cytochrome P450aldo, which is present at least in rat and human adrenals and is considered

to be engaged exclusively in aldosterone biosyn-thesis was demonstrated only in the zona

glomerulosa (Fig. 3). Therefore, the sites o f specific steroid production in the normal adrenal glands, i.e. what zones o f the cortex produce what kinds o f steroids, were first revealed by immunohistochemistry and in situ hybridization o f steroidogenic enzymes.

IV. Incidentalomas
-hormonally active or inactive?-

With the rapid and remarkable development of radiologic techniques, adrenal masses as small as

0.5 cm can now be detected [11]. Thus, increased numbers of adrenocortical lesions have been de-

tected in patients without clinical evidence of adrenocortical hormonal abnormalities. A major-ity of these lesions are of adrenocortical origin, i.e., adrenocortical nodules and neoplasms. This find-ing may merely reflect the fact that nodules or neo-

plasms have been detected frequently at autopsies

[12-14]. These lesions have been termed as "incidentaloma" or "non-functioning adrenocorti-
cal tumors" or "hormonally inactive adrenocortical tumors". Morphologically, these lesions cannot be differentiated from adrenocortical adenoma associ-

ated with Cushing's syndrome or primary aldosteronism [11, 13, 15, 16]. Therefore, it is im-

portant to determine whether there are any differ-ences of the patterns of the expression of ste-roidogenic enzymes between hormonally active and inactive adrenocortical neoplasms in order to obtain a better understanding of steroid metabo-lism in these incidentaloma.

Immunohistochemical analysis of steroidogenic enzymes in these hormonally inactive adrenocorti-cal tumors also demonstrated immunoreactivity of all the enzymes involved in corticosteroidogenesis in the tumor cells [17]. There are no significant differences of the patterns of expression of ste-

Fig. 2. Immunohistochemistry of cytochrome P450c17 in normal dog adrenal gland. Immunoreactivity was observed in the zona fasciculata (F) but not in the zona glomerulosa (G) (X 200).

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roidogenic enzymes between hormonally active and inactive tumors. These findings indicate that a majority o f incidentally detected adrenocortical lesions can synthesize cortisol and, possibly biologically active corticosteroids including cortisol but in insufficient amounts to cause hypercortisolism [11, 13, 15, 16, 17]. This is especially true in the cases with cortical at-rophy in attached non-neoplastic adrenals, autono-mous neoplastic production and secretion of corti-sol may be insufficient to cause clinical and routine laboratory abnormalities but sufficient to subtly al-ter the hypothalamic-pituitary-adrenal axis by sup-pressing ACTH and/or CRF production [17]. The question then arises as to whether there are any adrenocortical neoplasms which do not have the capacity to produce any corticosteroids or not, i.e., the presence or absence of the true "non-func-tioning" adrenocortical neoplasms. We recently experienced relatively large adrenal neoplasms which are composed of compact cells with abun-dant lipid-sparse eosinophilic cytoplasm (Fig. 4) [18]. The patients did not have any adrenocortical hormonal abnormalities [18]. Election microscopic examination revealed that eosinophilic appearance of the cytoplasm of tumor cells was due to abun-dant mitochondria with occasional presence of intramitochondria crystal [18]. We designated

these tumors, which were not reported in the lit-erature, as "adrenocortical oncocytoma" because morphological characteristics including ultrastruc-tural findings were very similar to oncocytoma ob-served in the other tissues. Interestingly, these tu-mor cells were immunohistochemically negative for any steroidogenic enzymes except for scattered immunoreactivity of P450scc [18]. Therefore, this adrenocortical oncocytoma was considered as "true non-f unctioning adrenocortical tumor". Increasing num-bers of adrenocortical oncocytoma have been re-

ported in the literature. It may have been very difficult to characterize the hormonal features of

this rare but unique adrenocortical neoplasms without application of immunohistochemistry of steroidogenic enzymes.

V. Primary aldosteronism
-hyperplasia versus adenoma-

Etiology of hyperaldosteronism with suppressed

plasma renin activity of primary aldosteronism is adrenocortical hyperplasia or adenoma. Adreno-cortical adenoma comprises the great majority of the patients with primary aldosteronism but it is sometimes difficult to differentiate aldosteronoma

Fig. 3. Immunolocalization of cytochrome P450aldo in normal rat adrenal gland. Immunoreactivity was observed in the zona glomerulosa (G) but not in the zona fasciculata (F) (X 300).

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from hyperplasia or idiopathic hyperaldosteronism (IHA) based on hormonal, and/or radiographical findings, in the patients with primary aldosteronoma. This is especially so in the cases of bilateral aldosteronoma, IHA with nodular hyperplasia and aldosteronoma with secondary adrenocortical nodules in the attached non-neo-

plastic adrenal.
In aldosteronoma, the zona glomerulosa of the attached non-neoplastic adrenal is expected to demonstrate atrophy due to suppressed renin-an-

giotensin system. The zona glomerulosa demon-strated normal or hyperplasia in the adrenals with
aldosteronoma, which has been termed as "para-
doxical hyperplasia" of the zona glomerulosa. Therefore, this paradoxical hyperplasia makes it difficult to differentiate IHA, especially the cases associated with nodules from aldosteronoma, in the surgically resected adrenals associated with primary aldosteronism. Histopathological differ-entiation between IHA and aldosteronoma in the

resected adrenal is very important because unilat-eral adrenalectomy generally relieves hypertension in the patients with aldosteronoma but not neces-sarily so in those with IHA. Etiology of paradoxi-cal hyperplasia is unknown but the morphologi-

cally hyperplastic zona glomerulosa is not consid-ered to be involved in complete aldosterone bio-synthesis because of relatively low tissue content of aldosterone and the absence of well-developed smooth endoplasmic reticulum and mitochondria in the zona glomerulosa cells [19]. Immuno-localization of steroidogenic enzymes demon-strated that the zona glomerulosa cells in the adrenals with IHA exhibited marked immunoreac-

tivity of all the enzymes except for P450c17 (Fig. 5) while those in the non-neoplastic adrenals with aldosteronoma did not have increased expression of the enzymes except for P450c21 (Fig. 6). This observation indicates that excessive production of aldosterone does not occur in the zona

glomerulosa with paradoxical hyperplasia and this finding is considered to be of great value in evalu-ating surgical pathology specimens of the adrenals associated with primary aldosteronism. It is con-sidered that immunolocalization o f steroidogenic en-zymes is considered as the only reliable diagnostic method o f differentiating IHA from aldosteronoma in some patients with primary aldosteronism, especially in the cases o f bilateral adenoma and/or marked paradoxi-cal hyperplasia o f the zona glomerulosa.

Fig. 4. Histopathological feature of adrenocortical oncocytoma. Tumor was composed almost entirely of the cells with abundant eosinophilic cytoplasm. Tumor cells
were negative for all steroidogenic enzymes with an exception of scattered immunoreactivity of P450scc (X 250).

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VI. Cushing's syndrome
- primary pigmented nodular adrenocortical
disease (PPNAD) and ACTH-independent
adrenocortical macronodular hyperplasia
(AIMAH)-

A great majority of Cushing's syndrome is caused by excessive ACTH production from pitu-itary gland and adrenocortical tumors with au-tonomous cortisol production. PPNAD and AIMAH are rare but established and important cause of Cushing's syndrome at this juncture [20]. Therefore, it is necessary to evaluate the cortico-steroid metabolism associated with these unique adrenocortical disorders. It is especially important to determine the localization of steroidogenesis,

particularly what types of the cells produce corti-sol, because these adrenocortical disorders were associated with complicated morphological fea-

tures.

PPNAD is characterized by an ACTH-indepen-dent adrenocortical hyperfunction without demon-strable autonomously functioning neoplasms and is associated with highly characteristic morpho-logical features that involve both adrenal glands exhibiting multiple peculiar nodules with black

pigmentation at the cut surface [21-23]. Immu-noreactivity (Fig. 7) and in situ hybridization sig-nals (Fig. 8) of the steroidogenic enzymes was in-tense in almost all of the cells in the adrenocortical nodules, in contrast to the adrenocortical nodules in the adrenals with ACTH dependent bilateral adrenocortical hyperplasia and Cushing's

Fig. 5. Immunohistochemistry of 3I3HSD in the adrenal with IHA. Immunoreactivity was observed in both hyperplastic zona glomerulosa (G) and fasciculata
(F) but immunoreactivity in the zona glomerulosa was stronger than that in the zona fasciculata (X 175).

Fig. 6. Immunohistochemistry of 3I3HSD in the non-neoplastic attached adrenal of aldosteronoma. Immunoreactivity was scattered and weak in the morphologically hyperplastic zona glomerulosa
(G). Immunoreactivity in the zona fasciculata (F)
was prominent (X 175).

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adenoma which demonstrated heterogeneity of ex-

pression of steroidogenic enzymes [24]. This result indicates that almost all o f the cells in the nodules pro-duce cortisol and are associated with an increased pro-duction of the enzyme protein, which can also explain

the presence of hypercortisolism despite small sizes of the adrenals with PPNAD. The internodular cortex

of the adrenals with PPNAD was negative for the enzymes except for sporadic immunoreactivity of 3 J3HSD, which is also consistent with ACTH-inde-

Fig. 7. Immunohistochemistry of P450c17 in the adrenal with PPNAD. Immunoreactivity
is only observed in the nodules (N) but not in internodular cortex (C) (X 200) [30].

Fig. 8. In situ hybridization study of P450c17 in the adrenal with PPNAD. Hybridization signals of 3H-labeled probe; which are observed as black dots, i.e., silver grains on autoradiography are predominantly present over the nodules (N). C: internodular
cortex (X 200).

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pendent hypercortisolism. Immunoreactivity and
in situ hybridization signals of steroidogenic en-zymes is observed in a small cluster of cortical cells with abundant eosinophilic cytoplasm located at the zona reticularis but not in adjacent non-nodular cortex, which may support an abnormal develop-ment of the zona reticularis as a possible pathogen-esis of the disorders. Thus, immunolocalization and mRNA in situ hybridization analysis o f steroidogenic enzymes not only revealed pathophysiology including intracortical steroid metabolism but also provided new insights into the etiology o f PPNAD.
In addition to PPNAD, cases in which bilateral adrenocortical macronodules are observed in the

presence of suppressed ACTH levels have recently been reported in the literature [25-27]. These le-sions have been assigned various terms, and ACTH-independent bilateral macronodular adrenocortical hyperplasia (AIMAH) is the most frequently used descriptive term [28]. Steroid me-tabolism and endocrine-pathologic correlation of AIMAH has not been well-studied. Adrenal

glands of AIMAH are composed of two different characteristic cell types, clear and compact cells. Generally, clusters of compact cells are dispersed in the clear cortical cells. Immunoreactivity to P450scc, P450c21 and P450c11 was observed in

both clear and compact cortical cells with compact cells displaying more intense staining as reported in Cushing's adenoma and ACTH-dependent bilat-eral adrenocortical hyperplasia. However, Immu-noreactivity and mRNA hybridization signals of P450c17 was observed predominantly in small compact cells, while those of 3J3HSD occurred ex-clusively in clear cortical cells [29] (Figs. 9, 10). This differential expression o f 3/3HSD and P450c17 in clear and compact cortical cells has been observed only in AIMAH among the adrenocortical disorders associ-ated with Cushing's syndrome. Further investiga-tions are required to obtain the correlation of this differential expression of 3/3HSD and P450c17 to cortisol production in the adrenals with AIMAH but distribution of the enzymes is considered to represent ineffective corticosteroidogenesis, i.e., progesterone produced as a result of 313-hydroxysteroid dehydrogenation in the large clear cortical cells may not be effectively converted to cortisol because P450c17 is present only in compact cortical cells. It may be premature but interesting to postulate that this ineffective corticosteroidogenesis may contribute to the relatively low production of corti-sol per tissue in AIMAH. As described above, immuno-histochemistry and in situ hybridization o f ste-roidogenic enzymes provided novel findings in these

Fig. 9. Immunohistochemistry of P450c17 in the adrenal with AIMAH. Immunoreactivity is predominantly observed in clusters of compact cortical cells (X 200) [15].

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adrenocortical disorders associated with hyper-cortisolism. It was demonstrated that these unique dis-orders are different from other adrenocortical disorders

associated with hypercortisolism, not only in morphol-ogy but also in the distribution of expression of ste-roidogenic enzymes. Therefore, PPNAD and AIMAH were considered as an independent entity of hyper-cortisolism.

VII. Adrenocortical carcinoma

Difficulty with discerning malignancy in adreno-cortical neoplasms has been well-known, even by histopathologic examination of the resected speci-mens [30-33]. Numerous studies have focused on the possible biochemical differentiation between benign and malignant adrenocortical neoplasms. However, biochemical differentiation by hormonal studies has not provided satisfactory results. No specific patterns of steroidogenesis are associated with adrenocortical malignancy in adults, with the possible exception of neoplastic adrenocortical feminization [15].
No independent markers of adrenocortical ste-roidogenesis have not been reported as described above but steroid metabolism in human adreno-

cortical carcinoma exhibits a relatively characteris-tic feature compared to that of both adrenocortical adenoma and the normal adrenal cortex. This fea-

ture has been summarized as low efficiency of ste-roidogenesis, including overproduction of minor

products of adrenocortical steroidogenesis or pre-cursor steroids [15, 34-37]. In our recent study of localization of steroidogenic enzymes in nine cases of human adrenocortical malignancy [38], a num-ber of carcinoma cells did not express all the en-zymes required for the synthesis of biologically ac-tive corticosteroids (Figs. 11, 12), which may ex-

plain an increased level of precursor steroid secre-tion. In addition, a discrepancy between mRNA and protein expression, which is not usually ob-served in the normal adrenal cortex and adreno-

cortical adenoma, was occasionally observed at least in P450c17. Therefore, immunolocalization and in situ hybridization of steroidogenic enzymes revealed that ineffective corticosteroidogenesis ob-served in adrenocortical malignancy was considered to be due to disturbance of the expression of steroidogenic enzymes in individual carcinoma cells both at mRNA and protein levels, which may occur through the process o f malignant transformation.

Fig. 10. Immunohistochemistry of 33HSD in the adrenal with AIMAH, the adjacent section of Fig. 9. Immunoreactivity was present only in the clear cortical cells and compact cortical cells were negative for the enzymes (X 200) [30].

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Fig. 11. Immunohistochemistry of 3fHSD in adrenocortical carcinoma. Immunoreactivity was observed in most of carcinoma cells in three areas designated by *, ** and *** in
this field. Immunoreactivity of the enzyme was not detected either in the septum
(S) or the capsule (C) (X200) [38].

Fig. 12. Immunohistochemistry of P450c21 in adrenocortical carcinoma. The section is a serial section of the specimen shown in Fig. 11. Immunoreactivity of the enzyme
was observed in carcinoma cells in two areas (*, **) but a majority of the cells were negative in one area (***) in this field. Immunoreactivity was not observed in the septum (S) or the capsule (C) (X200) [38].

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VIII. Summary

Immunolocalization and in situ hybridization analysis of steroidogenic enzymes demonstrated the localization of steroidogenesis in the adrenal cortex and its disorders. The findings obtained

provided new insights into adrenocortical hor-monal metabolism, especially through establishing endocrine-pathological correlation.

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