Localization of Steroidogenic Enzymes in Adrenal Cortex and Its Disorders

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I. Introduction

As is well known, adrenal cortex produces and secretes steroids, which originate from circulating or stored cholesterol. All corticosteroids are synthesized through a series of complicated enzymatic steps from cholesterol (Fig. 1). Corticosteroids are classified into three groups, glucocorticoids, mineralocorticoids 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 hormones 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 localization of 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 sequences.

Demonstration of the presence of the enzyme(s) involved in specific steroidogenesis strongly indicates that the steroid hormones catalyzed by the enzyme(s) are produced in cells where the enzyme is located. Based on this principle, we first succeeded in determining the distribution of steroidogenesis in the adrenal gland and its disorders by an immunohistochemical method employing antibodies and by in situ hybridization employing cDNA or oligonucleotides against steroidogenic enzymes. These include P450scc (side chain-cleavage) [3], 3βHSD (hydroxysteroid dehydrogenase) [4], P450c21 (21-hydroxylase) [5, 6], P450c17 (17α-hydroxylase) [7-9] and P450c11 (11β-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 inhibit in vitro enzymatic reaction. When only surgical materials were available, the antibodies should recognize denaturation-resistant epitopes of the enzymes. Details of the characteristics of the antibodies employed for immunostain of surgical pathology materials of human adrenal and its disorders are described in detail in the references above. Prompt fixation for brief duration is desired as in immunohistochemistry of other antigens. Otherwise, false negative results are expected. Once it is properly processed, immunoreactivity of steroidogenic enzymes can be retained in 10% formalin fixed and paraffin-embedded materials in the

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In general, immunohistochemistry of steroidogenic enzymes can be performed in 10% formalin-fixed and paraffin-embedded materials, if the materials are properly processed. Immunostaining can be performed by avidin-biotin peroxidase complex or peroxidase-antiperoxidase methods.

For in situ hybridization of steroidogenic enzymes, 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 oligonucleotide probes, including G/C content of the probes of steroidogenic enzymes, properly designed oligonucleotide probes can yield much better results of mRNA hybridization of steroidogenic enzymes in tissue sections than cDNA probes, because redundant or conserved nucleotide sequences can be avoided during probe design and synthesis, and a higher specific activity probe can be much more easily obtained [9]. It is also possible to perform simultaneous immunohistochemistry and in situ hybridization of steroidogenic enzymes on the tissue section. This method made it possible to detect expression of steroidogenic enzymes 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 hybridization of steroidogenic enzymes. Technical details of in situ hybridization of steroidogenic enzymes including simultaneous immunohistochemistry and in situ hybridization were previously reported 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 understanding 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 biochemical and conventional morphological methods
that the zona glomerulosa is involved in the biosynthesis of mineralocorticoid while the zonae fasciculata-reticularis produce and secrete glucocorticoid 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 required in glucocorticoid and adrenal androgen biosynthesis but not in mineralocorticoid biosynthesis, 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 biosynthesis was demonstrated only in the zona glomerulosa (Fig. 3). Therefore, the sites of specific steroid production in the normal adrenal glands, i.e. what zones of the cortex produce what kinds of steroids, were first revealed by immunohistochemistry and in situ hybridization of 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 detected in patients without clinical evidence of adrenocortical hormonal abnormalities. A majority of these lesions are of adrenocortical origin, i.e., adrenocortical nodules and neoplasms. This finding may merely reflect the fact that nodules or neoplasms have been detected frequently at autopsies [12–14]. These lesions have been termed as “incidentaloma” or “non-functioning adrenocortical tumors” or “hormonally inactive adrenocortical tumors”. Morphologically, these lesions cannot be differentiated from adrenocortical adenoma associated with Cushing’s syndrome or primary aldosteronism [11, 13, 15, 16]. Therefore, it is important to determine whether there are any differences of the patterns of expression of steroidogenic enzymes between hormonally active and inactive adrenocortical neoplasms in order to obtain a better understanding of steroid metabolism in these incidentaloma.

Immunohistochemical analysis of steroidogenic enzymes in these hormonally inactive adrenocortical 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-
roidogenic enzymes between hormonally active and inactive tumors. These findings indicate that a majority of incidentally detected adrenocortical lesions can synthesize cortisol and, possibly biologically active corticosteroids including cortisol but in insufficient amounts to cause hypercortisolism [11, 13, 16, 17]. This is especially true in the cases with cortical atrophy in attached non-neoplastic adrenals, autonomous neoplastic production and secretion of cortisol may be insufficient to cause clinical and routine laboratory abnormalities but sufficient to subtly alter the hypothalamic-pituitary-adrenal axis by suppressing 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-functioning" adrenocortical neoplasms. We recently experienced relatively large adrenal neoplasms which are composed of compact cells with abundant lipid-sparse eosinophilic cytoplasm (Fig. 4) [18]. The patients did not have any adrenocortical hormonal abnormalities [18]. Electron microscopic examination revealed that eosinophilic appearance of the cytoplasm of tumor cells was due to abundant mitochondria with occasional presence of intramitochondria crystal [18]. We designated these tumors, which were not reported in the literature, as "adrenocortical oncocytoma" because morphological characteristics including ultrastructural findings were very similar to oncocytoma observed in the other tissues. Interestingly, these tumor cells were immunohistochemically negative for any steroidogenic enzymes except for scattered immunoreactivity of P450scc [18]. Therefore, this adrenocortical oncocytoma was considered as "true non-functioning adrenocortical tumor". Increasing numbers of adrenocortical oncocytoma have been reported 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. Adrenocortical adenoma comprises the great majority of the patients with primary aldosteronism but it is sometimes difficult to differentiate aldosteronoma
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-neoplastic adrenal. In aldosteronoma, the zona glomerulosa of the attached non-neoplastic adrenal is expected to demonstrate atrophy due to suppressed renin-angiotensin system. The zona glomerulosa demonstrated normal or hyperplasia in the adrenals with aldosteronoma, which has been termed as “paradoxical 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 differentiation between IHA and aldosteronoma in the resected adrenal is very important because unilateral adrenalectomy generally relieves hypertension in the patients with aldosteronoma but not necessarily so in those with IHA. Etiology of paradoxical hyperplasia is unknown but the morphologically hyperplastic zona glomerulosa is not considered to be involved in complete aldosterone biosynthesis 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]. Immunolocalization of steroidogenic enzymes demonstrated that the zona glomerulosa cells in the adrenals with IHA exhibited marked immunoreactivity 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 evaluating surgical pathology specimens of the adrenals associated with primary aldosteronism. It is considered that immunolocalization of steroidogenic enzymes is considered as the only reliable diagnostic method of differentiating IHA from aldosteronoma in some patients with primary aldosteronism, especially in the cases of bilateral adenoma and/or marked paradoxical hyperplasia of 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 (X250).
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-
titary 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,
adenoma which demonstrated heterogeneity of expression of steroidogenic enzymes [24]. This result indicates that almost all of the cells in the nodules produce cortisol and are associated with an increased production 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 3βHSD, which is also consistent with ACTH-inde-
pended hypercortisolism. Immunoreactivity and in situ hybridization signals of steroidogenic enzymes 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 development of the zona reticularis as a possible pathogenesis of the disorders. Thus, immunolocalization and mRNA in situ hybridization analysis of steroidogenic enzymes not only revealed pathophysiology including intracortical steroid metabolism but also provided new insights into the etiology of 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 lesions have been assigned various terms, and ACTH-independent bilateral macronodular adrenocortical hyperplasia (AIMAH) is the most frequently used descriptive term [28]. Steroid metabolism 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 P450sc, 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 bilateral adrenocortical hyperplasia. However, immunoreactivity and mRNA hybridization signals of P450c17 was observed predominantly in small compact cells, while those of 3βHSD occurred exclusively in clear cortical cells [29] (Figs. 9, 10). This differential expression of 3βHSD and P450c17 in clear and compact cortical cells has been observed only in AIMAH among the adrenocortical disorders associated with Cushing’s syndrome. Further investigations are required to obtain the correlation of this differential expression of 3βHSD 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 3β-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 cortisol per tissue in AIMAH. As described above, immunohistochemistry and in situ hybridization of steroidogenic 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 (×200) [15].](image-url)
adrenocortical disorders associated with hypercortisolism. It was demonstrated that these unique disorders are different from other adrenocortical disorders associated with hypercortisolism, not only in morphology but also in the distribution of expression of steroidogenic enzymes. Therefore, PPNAD and AIMAH were considered as an independent entity of hypercortisolism.

VII. Adrenocortical carcinoma

Difficulty with discerning malignancy in adrenocortical neoplasms has been well-known, even by histopathologic examination of the resected specimens [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 steroidogenesis have not been reported as described above but steroid metabolism in human adrenocortical carcinoma exhibits a relatively characteristic feature compared to that of both adrenocortical adenoma and the normal adrenal cortex. This feature has been summarized as low efficiency of steroidogenesis, including overproduction of minor products of adrenocortical steroidogenesis or precursor steroids [15, 34–37]. In our recent study of localization of steroidogenic enzymes in nine cases of human adrenocortical malignancy [38], a number of carcinoma cells did not express all the enzymes required for the synthesis of biologically active corticosteroids (Figs. 11, 12), which may explain an increased level of precursor steroid secretion. In addition, a discrepancy between mRNA and protein expression, which is not usually observed in the normal adrenal cortex and adrenocortical adenoma, was occasionally observed at least in P450c17. Therefore, immunolocalization and in situ hybridization of steroidogenic enzymes revealed that ineffective corticosteroidogenesis observed 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 of malignant transformation.

Fig. 10. Immunohistochemistry of 3βHSD 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 (×200) [30].
Fig. 11. Immunohistochemistry of 3βHSD 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) (×200) [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) (×200) [38].
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 hormonal metabolism, especially through establishing endocrine-pathological correlation.

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References


