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IMMUNOHISTOCHEMISTRY OF THE ADRENAL IN PRIMARY ALDOSTERONISM

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Abstract

Purpose of the review—Primary aldosteronism (PA) is a major cause of hypertension; aldosterone-producing adenomas (APA) cause about half of PA; idiopathic hyperplasia of adrenal glomerulosa cells are responsible for the rest. A surprising variety of mutations have recently been identified in ion channels and pumps in a significant number of APA. This review addresses histological and molecular aspects of APA and the surrounding adrenal.

Recent findings—Specific antibodies against the CYP11B2 and CYP11B1 enzymes, the last enzyme in aldosterone and cortisol synthesis, respectively, allow for the first time study of the steroidogenic capabilities of cells within the APA and adjacent adrenal. Cells expressing CYP11B2 may be scattered and/or in clusters throughout the normal adrenal zona glomerulosa. APA differ widely in the number of cells expressing CYP11B2; some did not express it at all, but were surrounded by cells, some in clusters or micronodules, that expressed CYP11B2. Some APAs also comprised cells expressing both CYP11B1 and CYP17A1. In some samples analysis of the tissue adjacent to APA detected ion channel and pump mutations heretofore associated only with APA.

Summary—APAs have a complex structure and expression of steroidogenic enzymes.

Keywords

Primary aldosteronism; Aldosterone-producing adenomas; Aldosterone-producing cell clusters; CYP11B2 immunohistochemistry

Introduction

The adrenal gland was first described by Bartolomeo Eustachios in 1563, but its definitive linkage to a clinical condition was established by Thomas Addison in 1849 demonstrating by autopsy that destruction of the adrenal cortex produced the clinical symptoms of adrenal insufficiency (1). Early investigations of adrenal extracts initially focused on their effects on

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carbohydrate metabolism leading to the isolation of a variety of hormones recognized as steroidal in nature and finally the identification of cortisol as the mayor glucocorticoid produced by the adrenal (2). Early techniques involved isolation of steroids by crystallization from fractions of adrenal extracts, leaving a fraction that resisted crystallization dubbed the amorphous fraction, that when found to have significant effect on sodium and potassium metabolism, was then called electrocortin. Through the efforts of Simpson and Tait in London and Reichstein in Basel in collaboration with then Ciba Pharmaceutical Company electrocortin was isolated. Elucidation of its structure led to its definitive name, aldosterone (3). In 1955, Jerome Conn described the syndrome of primary aldosteronism (PA) caused by an aldosterone-producing adrenal tumor (4). It was subsequently recognized that a single adrenal adenoma was not the only source of excessive aldosterone production. It is now known that the two mayor causes are a unilateral aldosterone-producing adenoma (APA) and bilateral zona glomerulosa hyperplasia (idiopathic hyperaldosteronism, IHA); less frequent causes include unilateral micro or macro nodular hyperplasia, aldosterone-producing carcinoma and three familial causes of PA.

The normal adrenal cortex

The adrenal cortex is composed of an outer area, zona glomerulosa, ZG, corresponding to ~5% of the cortex, comprising compact cells with sparse lipid droplets forming rounded nests or clusters. The ZG is the site of aldosterone synthesis. Immediately below is the zona fasciculata, ZF, corresponding to ~75% of the cortex, with larger lipid droplets laden cells that appear microscopically as clear cells forming columns. The ZF is the site of cortisol synthesis. The inner cortex corresponds to the zona reticularis with compact cells of intermediate size between the ZG and ZF (5) where adrenal androgens are synthesized.

Enzymes that define the different areas of the adrenal cortex

Biosynthesis of adrenal steroids involves multiple enzymes common to all the zonas plus unique enzymes for the last steps of specific steroid synthesis that define each zona. The regulation of aldosterone and cortisol synthesis is exerted at two rate limiting steps in the pathway. The first and common step is transport of cholesterol into the mitochondria by StAR protein, where cholesterol undergoes side-chain cleavage to produce pregnenolone which diffuses from the mitochondria, followed, in the ZG, by conversion to progesterone by the 3β -hydroxysteroid dehydrogenase (HSD3B) in the endoplasmic reticulum. Progesterone is then 21-hydroxylated to deoxycorticosterone and it is transported back into the mitochondria where the second rate limiting step occurs, the sequential hydroxylation by the CYP11B2 leading to the synthesis of aldosterone. CYP11B2 is normally only expressed in the ZG. In the ZF, pregnenolone is 17-hydroxylated by the CYP17A1 hydroxylase and then acted by the 3β -hydroxysteroid dehydrogenase to 17α -hydroxyprogesterone which will be 21-hydroxylated to 11-deoxycortisol and then in the mitochondria acted by the CYP11B1 to form cortisol. The CYP11B2 enzyme is transcriptionally regulated for the synthesis of aldosterone. The CYP11B enzymes are 93% homologous at the amino acid level making it difficult to generate specific antibodies (6). It has been recently proposed that HSD3B is a second rate limiting enzyme (7). There are two isoforms of HSD3B in the human; HSD3B1

is expressed in the ZG, HSD3B2 in the ZF (7). Intriguingly, aldosterone producing adenomas were found to be immunoreactive for HSD3B2, the ZF isoform (7).

The development of polyclonal (8) and monoclonal antibodies (6) has allowed a more clear differentiation between the ZG and ZF. Not all cells in the normal human ZG express significant amounts of CYP11B2, perhaps a reflection of the high sodium intake of most people. There are two normal patterns of CYP11B2-expressing ZG cells, one of sporadic and scattered cells underneath the adrenal capsule, the other of clusters of cells named aldosterone-producing cell clusters (APCC) which are present even under conditions of a suppressed renin-angiotensin system (6, 8) (Figure 1A, 1B). The APCCs appear to be active in the production of aldosterone as the transcriptome of the APCC are similar to the other ZG cells, but have higher levels of mRNA for the CYP11B2 (9). Infant adrenals have more CYP11B2 immunoreactive cells and the cluster pattern seems to predominate (6); older individuals have fewer clusters (10). Newborn infants have higher plasma aldosterone and a physiological resistance to aldosterone due to a low expression of the mineralocorticoid receptor (11). The CYP11B1 is expressed in a more uniform fashion throughout the ZF. In the normal human adrenal there are also a few CYP11B1-expressing cells touching the adrenal capsule and mingled with some CYP11B2 expressing cells in human adrenal glands (6) (Figure 1). In contrast, rodent adrenals show a clear delineation between the CYP11B1 expressing cells in the ZF and the CYP11B2 expressing cells in the ZG. The rat adrenal ZG comprises a 4–10 cell deep layer underneath the capsule. In between the rat ZG and ZF there is an area of cells that express neither CYP11B1 or CYP11B2 that has been called the undifferentiated zone or stem cell area (12, 13). In the human, there are also cells that express neither enzyme mingling with the CYP11B2 and CYP11B1 cells close to the adrenal capsule that do express the HSD3B (6, 8, 14) and are probably either stem cells or steroidogenic latent cells that when stimulated during sodium depletion acquire a ZG phenotype as occurs in rodents when placed on a sodium deficient diet (12, 13). This cannot be tested in the human.

Aldosterone-producing adenomas and idiopathic hyperaldosteronism

Histopathology using H & E staining has shown that APA and the surrounding cells are highly heterogeneous. Frequently the adenoma presents with hyperplasia of the ZG with microscopic or macroscopic nodules in addition to the APA (5). Most APA have pleiotropic cellular characteristics with variable mixtures of larger clear fasciculata-type cells, small lipid-laden cells of glomerulosa phenotype, and compact reticularis-type cells (15). The majority of APA have a predominance of fasciculata-type cells with fewer small lipid-laden cells of glomerulosa phenotype and compact reticularis-type cells. In other APAs the glomerulosa type cells predominate (Azizan, 2013 #13165). Most adenomas are monoclonal, but some are described as polyclonal (16). The differentiation between an adenoma and a nodule is not possible at the microscopic level as there is seldom a capsule to delineate an adenoma, nor clonality is determined.

Aldosterone-producing adenomas and somatic mutations

The most significant advance in the understanding of APAs came from the discovery of several somatic mutations in the selectivity filter of the potassium inward rectifying channel, KIRK4 coded by the *KCNJ5* gene (17). Approximately 30% of APA of the original study had a *KCNJ5* gene mutation that altered the selectivity for potassium (17). Subsequent studies of APA genes have shown an incidence of *KCNJ5* mutations between 36–75%. They are most common in Asian populations (18–25). Among those of European origin who have a *KCNJ5* mutation, in women are more often affected (20–26). Other somatic mutations associated with APA include those in the *ATP1A1* gene coding for the sodium-potassium ATPase alpha subunit (15–27), the *ATP2B3* (27) gene coding for the calcium ATPase, the calcium channel *CACNA1D* (15–28), and the *CACNA1H* (29). *CACNA1H* mutations have been found in young patients (29).

Using polyclonal antibodies Nishimoto *et al* (8) described high heterogeneity in CYP11B2 immunoreactivity in APA. While some tumors were almost entirely stained, others had as few as 40% CYP11B2 positive cells. Fewer cells in the APAs were immunoreactive for CYP11B1 and a variable proportion of double negative stained cells were present, but it was not technically possible to demonstrate double positive cells in this study (8). In some adrenals the APA was surrounded by conventional zonation with no staining in the ZG for either the CYP11B1 or the CYP11B2 enzymes. However, a significant proportion, in addition to the APA there were subcapsular APCCs stained with the CYP11B2 antibody (8). In other studies with these antibodies, in a few adrenals the adenomas were negative for CYP11B2, but there were distinctly positive APCC (30–31). Unfortunately the polyclonal antibodies appear to provide poor resolution and the manuscripts published to date only show figures at low magnification. The development of a mouse monoclonal antibody for the human CYP11B2 and a rat monoclonal antibody for the human CYP11B1 have facilitated studies and advanced the field (6–32–33).

The few adrenals available from patients with IHA show strong diffuse immunoreactivity for the CYP11B2 enzyme (Figure 1C) throughout the ZG that is distinct from cells expressing the CYP11B1 and CYP17A1 enzymes, however the level of CYP11B2 expression as assessed by semi-quantitative H-scoring system did not differ from normal adrenals (33). The expression of the HSD3B in the ZG of IHA patients appears to be significantly greater than in normal adrenals in two separate studies (33–34). These results suggest that the level of the CYP11B2 might not be solely responsible for the hyperaldosteronism in IHA. A form of salt-sensitive hypertension with hyperaldosteronism found in mice null for the Cryptochrome-1 and -2 genes was found to be associated with a significant increase in the HSD3B6 isoform expressed in the ZG of these mice and postulated to be a rate limiting enzyme in the synthesis of aldosterone in addition to the CYP11B2 (35). The HSD3B6 of the mouse corresponds to the HSD3B1 of the human. HSD3B1 is also reported in the human ZG with higher levels found in the ZG of patients with IHA (7). In patients with APA, HSD3B2 is more abundant than HSD3B1, but HSD3B1 correlates better with the expression of CYP11B2 and plasma aldosterone secretion (36). The expression of HSD3B1 was also found to be higher in patients whose adenomas had a *KCNJ5* mutation (36). Thus, in

addition to a dysregulation of CYP11B2 expression, excessive HSD3B1 activity may exacerbate PA by providing more substrate.

In one study of APAs using the monoclonal antibodies the tumors comprised a majority of CYP11B2 immunoreactive cells (Figure 1D), however the proportion was highly variable with some cells expressing neither CYP11B2 or CYP11B1, and a few scattered cell expressing CYP11B1. In addition, there were a few cells expressing both CYP11B1 and CYP17A1, CYP11B2 and CYP11B1, and CYP11B2 with CYP17A1 (33). Expression of CYP11B2 in the surrounding ZG was less than that in the normal or IHA adrenal ZG, but APCCs were not uncommon. In a different study using polyclonal antibodies the APAs were highly heterogeneous and immunohistochemical evaluation using the H-scoring system was initially suggested to correlate with aldosterone synthesis (30), but this was not confirmed and the relative immunointensity and percentage of CYP11B2 immunoreactivity did not appear to reflect overproduction of aldosterone (33).

In a study by Volpe (31), 24 patients with unilateral production of aldosterone that were treated by adrenalectomy were divided into 3 groups according to APA CYP11B1 and CYP11B2 expression patterns. Group A, 17 of 24 patients, had immunoreactivity for the CYP11B2 in the adenoma; 11 of the 17 also had variable number of CYP11B1 immunoreactive cells. Group B, 3 of the 24 patients, had an adrenocortical tumor that was CYP11B1 positive, but devoid of CYP11B2-positive cells, and several cortical nodules of variable size with strong immunoreactivity for CYP11B2. In group C, 4 of 24 patients, no tumor was found in the specimen, however they had a hyperplastic ZG with nodules of variable size positive for CYP11B2 staining. Groups B and C probably correspond to unilateral adrenocortical aldosterone-producing hyperplasia. Although the aldosterone production appeared be lateralized before the adrenalectomy, these patients were not cured on follow up (31). Similar findings were reported by Nanba *et al* (30). These results support our earlier hypothesis that patients with bilateral but asymmetrical adrenal hyperplasia which on adrenal vein sampling appears unilateral, improve, but are not cured of the hyperaldosteronism as the bulk of aldosterone production is removed by the unilateral adrenalectomy (37). Assessment of residual adrenal autonomy by the fludrocortisone suppression test of patients that were properly diagnosed as having unilateral production of aldosterone and underwent unilateral adrenalectomy revealed that only 62% were biochemically cured. The rest were improved; their aldosterone levels were lower, but remained abnormal; plasma renin activity increased, and post fludrocortisone aldosterone decreased, but not to the same degree as patients who were cured (38). Patients who were biochemically cured had a higher possibility of their hypertension also being cured.

Intratumor heterogeneity of steroidogenesis

Double immunofluorescence in APAs from 27 patients with CYP11B2 and CYP11B1 antibodies and triple immunofluorescence in APAs from 21 patients with CYP11B2, CYP11B1 and CYP17A1 antibodies was reported. In addition to CYP11B2 positive cells, three types of intratumoral hybrid cells were found using the double immunofluorescence: type A, CYP11B1 & CYP11B2 co-expression, type B, CYP11B2 & CYP17A1 co-expression and type C, CYP11B1 & CYP17A1 co-expression (32). Double negative cells

were the predominant cell type; in most tumors most cells were hormonally non-functional and only a small number of tumor cells synthesized the bulk of the steroids (32). On average type A cells comprised 2.1% of the APAs; type B and C cells made up a mean of 0.6% each (32). Triple immunofluorescence revealed very few triple-positive cells (mean of 0.14% of the APA cells). The hybrid cell type A were smaller than the triple negative cells and might represent steroidogenically active cells with lower lipid droplets previously described as ZG-like cells in APA (32). The small numbers of the hybrid type C cells and the fact that the ZF is regulated normally would explain why cortisol synthesis is not generally increased in patients with APA. Type B cells expressing CYP11B2 and CYP17A1 may be responsible for the elevation of 18-oxocortisol which is frequently reported in patients with APA (39). The ratio of area and cell number of hybrid cell types A/B/C and triple positive cells do not appear to correlate with the status of somatic mutations of ion channels (KCNJ5 and ATPase mutations) (32). These studies show that during the formation of adenomas, the normal pattern of expression of enzymes that are specific for each zona of the adrenal becomes deranged in a few cells

What are aldosterone-producing cell clusters and what role they play in adenoma formation?

APCCs are clusters of cells expressing the CYP11B2 below the capsule or protrude into the ZF. They are frequently seen in adrenals with an APA where one would expect that the high aldosterone would suppress aldosterone production in the ZG surrounding the adenoma and might represent cells with autonomous production of aldosterone. APCC scores were higher in normal adrenals from women (9), however women, particularly those who are premenopausal, produce more aldosterone (40–41). In a recent study, it was hypothesized that the APCCs arise from the ZG as a result of somatic mutations that result in autonomous aldosterone production (9). Microarray data from a comparison of APCC, ZG, ZF and ZR RNA obtained by Laser Capture Microdissection of normal adrenals showed very significant differences between the transcriptome of ZG vs. ZF and ZR; the APCC transcriptome was very similar to the ZG. Expression of the CYP11B2 was significantly higher in the APCC than the ZG and there were a few other differentially expressed genes shared between them (9). To assess whether APCCs harbored somatic mutations similar to those found in APAs, new generation sequence using a custom target multiplexed amplicons for the entire coding sequences of genes associated with somatic mutations identified in APA was performed and compared to paired normal adrenal tissue. From 23 informative APCC samples from formaldehyde fixed paraffin embedded samples 8 (35%) harbored somatic mutations that have been shown to drive aldosterone secretion in APA. Mutations were found in the calcium channel, voltage-dependent, L-type α_1 D-subunit (*CACNA1D* gene; 6 of 23 APCCs) and ATPase, sodium/potassium α_1 polypeptide (*ATP1A1* gene; 2 of 23 APCCs) which were not present in the adjacent normal ZG. Interestingly no mutations of the *KCNJ5* gene were found. These findings suggest that APCCs might be a precursor of APAs (9).

Does adenoma formation start with an APCC?

A recent study of two resected adrenals with an APA and multiple micronodules, some of nodules that stained positive for CYP11B2 were located within an APCC. The subcapsular portions of these micronodules had a ZG-like morphology and were positive only for the CYP11B2 enzyme. The inner portion of the nodules had a ZF-like morphology and was heterogeneous, comprising CYP11B1 and CYP11B2 positive cells. These micronodules appeared to be a micro-APAs (42). The subcapsular and inner portions did not appear to have a border between them. The inner micro-APA had a mutation of the KCNJ5 (Gly151Arg) whereas the APCC did not. Using macrodissection from FFPE sections and sequencing from several large APCC-like and micro-APAs showed that two of them had different mutations of the same gene, KCNJ5, suggesting a different origin (42). Because only the micro-APA had the mutation it might have played a role in the transition from APCC-like histology to micro-APA-like histology. Another APCC-like and micro-APA-like pair in this adrenal had a novel *ATP1A1* deletion (p.Ile322_Ile325del) and a substitution (p.Ile327Ser). The second adrenal in the study had fewer micronodules some of which appeared to have similar APCCs and micro-APAs. Sequencing demonstrated a novel mutation of the *ATP1A1* gene (L337M) in the micro-APA (42). These results suggest that cells within an APCC were in transition to form an APA.

Conclusion

the advent of new immunohistochemistry, microscopic tissue sampling and sequencing capabilities has demonstrated the astonishing plasticity of the adrenal cortex and the complexity of the molecular basis of Primary Aldosteronism.

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Bullet points

- Aldosterone-producing adenomas are frequently due to somatic mutations of potassium and calcium channels or sodium/potassium and calcium ATPases.
- Monoclonal antibodies against CYP11B2 demonstrate highly heterogeneous patterns of expression in aldosterone-producing adenomas and adjoining zona glomerulosa.
- Aldosterone-producing adenomas frequently have cells that co-express the CYP11B2 with the CYP11B1 and/or the CYP17A1 enzymes.

Figure 1.

A: Double staining of the CYP11B1 in pink and the CYP11B2 in green. CYP11B1 cells extend in some areas touching the capsule and some are mingling with CYP11B2 cells. Some of the CYP11B2 staining show an Aldosterone-Producing cell cluster type pattern.

B: Shows scattered pattern of expression of the CYP11B2 next to the capsule.

C: Expression of the CYP11B2 cells in a case of idiopathic hyperaldosteronism.

D: Adrenal gland with an adenoma stained in brown for the CYP11B2 and the rest of the adrenal stained for the CYP11B1 in green.