Virchows Archiv A Pathol Anat (1988) 413:313–318

Virchows Archiv A
Pathological Anatomy and Histopathology

Immunohistochemical study of cytochrome P-450$_{11\beta}$-hydroxylase in human adrenal cortex with mineralo- and glucocorticoid excess*

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Summary. Cytochrome P-450 specific for steroid 11β-hydroxylation (P-450$_{11\beta}$) was immunohistochemically demonstrated in the adrenal glands of human, pig and bovine and of mineralo- and glucocorticoid excess using a specific monoclonal antibody against P-450$_{11\beta}$ of bovine adrenocortical mitochondria. P-450$_{11\beta}$ was present in all three cortical zones of the histologically normal adrenal glands of bovine, pig and human, particularly in the zona fasciculata (ZF) and reticularis (ZR). The P-450$_{11\beta}$ immunoreactivity was intensive in cortical micronodules and inner ZF and ZR in Cushing's disease, and relatively intensive in the zona glomerulosa (ZG) and outer ZF in idiopathic hyperaldosteronism (IHA), corresponding to the sites of active steroidogenesis. In adenomas with Cushing's syndrome and primary aldosteronism, compact cells were generally stained well. In the adrenal glands attached to the adenomas, immunoreactivity was observed only focally in ZG cells but not in ZF and ZR cells.

Key words: Adrenal cortex – Hypercorticism – Adrenocortical adenoma – Cytochrome P-450 – Steroid 11β-hydroxylase

Introduction

In order to understand the control mechanism involved in steroidogenesis of both normal and pathological adrenal glands, it is essential to know the intracortical and intrazonal distribution of steroidogenic enzymes. Histochemical analysis and immunohistochemical studies of corticosteroids themselves have many limitations and there are difficulties in their interpretation, but immunohistochemical analysis using specific antibodies against the enzyme protein have made it possible to observe the distribution of immunoreactivity of the enzyme in the adrenal cortex. We have previously demonstrated the immunohistochemical localization of 21-hydroxylase (P-450$_{C21}$) in normal bovine and normal and disordered human adrenal cortex using specific rabbit antiserum against P-450$_{C21}$ (Sasano et al. 1988a, 1988b).

Cytochrome P-450$_{11\beta}$ is a protohaeme-containing monooxygenase system in adrenocortical mitochondria, known to catalyze 11β- and 19-hydroxylation primarily, and the two-step conversion of corticosterone to aldosterone in adrenocortical steroidogenesis (Okamoto et al. 1982; Kim et al. 1983; Wada et al. 1984, 1985). P-450$_{11\beta}$ has been purified from bovine adrenocortical mitochondria and its enzymatic and physical properties have been extensively studied (Okamoto et al. 1982; Kim et al. 1983; Momoi et al. 1983; Wada et al. 1984, 1985; Yanagibashi et al. 1986). However, there are still controversies in the identity of the enzyme involved in the two final steps of aldosterone biosynthesis (Watanuki et al. 1978; Lauber et al. 1987). Recently Okamoto et al. (Sugano et al. 1985) isolated the monoclonal antibodies against bovine adrenocortical cytochrome P-450$_{11\beta}$ and one of them (MAb 258) was found to react with P-450$_{11\beta}$ in adrenocortical mitochondria of bovine, rat, human and other animal species.

In this study, the intracortical distribution of P-450$_{11\beta}$ was examined in adrenocortical hyperplasia with Cushing's disease, idiopathic hyperaldosteronism (IHA), in adrenocortical adenomas with Cushing's syndrome and in primary aldosteronism, using a streptoavidin biotin method and the mono-

* This study was in part supported by a Grant from the Ministry of Health and Welfare "Disorders of adrenal hormone" Research Committee, Japan

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clonal antibody, MAb 258. For a control study bovine and swine adrenal glands and surgically obtained histologically normal human adrenal glands were examined in the same way.

Materials and methods

Specimens from bovine and porcine adrenal glands were obtained immediately after sacrifice at a local slaughter house. After the removal of surrounding connective and adipose tissues, the specimens were cut into small pieces and fixed immediately. All the human materials employed in this study were surgical specimens in the files of the Department of Pathology, Tohoku University School of Medicine. Histologically normal adrenal glands were obtained from 5 patients undergoing bilateral adrenalectomy for advanced breast cancer and from 2 patients subjected to radical nephrectomy for renal cell carcinoma. No endocrine abnormalities were observed in any case. Surgical material from bilateral adrenocortical hyperplasia were obtained from six cases of Cushing's disease and five cases of IHA. The adrenals in Cushing's disease were obtained prior to trans-sphenoidal surgery of the pituitary gland and those of IHA were obtained when medical management of the patients was difficult. A well-circumscribed tumour was resected from twelve patients with primary aldosteronism (aldosteronoma) and ten cases of Cushing's syndrome (Cushing's adenoma). Clinical and endocrine findings of primary aldosteronism or Cushing's syndrome were evident prior to operation in all the cases.

From seven monoclonal antibodies against purified bovine adrenocortical cytochrome P-450$_{11\beta}$, only one antibody (MAb 258) reacted with a protein in the adrenocortical mitochondria of human and other animal species. Characterization of this MAb 258 has been described in detail by Okamoto et al. (Sugano et al. 1985; Lauber et al. 1987).

Tissues from the bovine and swine adrenal glands were fixed in 4% paraformaldehyde buffered at pH 7.4 or PLP (periodate-lysine paraformaldehyde) fixative buffered at pH 7.4 for 24 to 48 h at 4° C, or 100% methanol or 10% neutral formalin fixative for two to three days at room temperature. The fixed specimens were mainly cut in paraffin sections 2.5 μm thick and mounted on regular glass slides. Part of the fixed specimen was frozen and cut in a cryostat for 6 μm thick sections which were mounted on albumin-coated glass slides. No significant differences in the distribution of immunoreactivity were observed between the various fixatives employed in this study, and between specimens of paraffin and frozen section.

Human surgical materials fixed in 10% neutral formalin solution were processed to paraffin sections 2.5 μm thick and mounted on glass slides.

For immunocytochemistry, deparaffinized paraffin sections were put into methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Frozen sections were put into 0.0 M PBS pH 7.2 containing 0.3% hydrogen peroxide for 10 min. They were washed in three changes of 0.01 M PBS, pH 7.2 for five min each and treated with 1% normal goat serum for 30 min.

The immunohistochemical method employed was Biotin-Streptavidin (B-SA) amplified method using StrAviGen B-SA immunostaining system (BioGenex Laboratories, Dublin, CA). After washing with PBS, sections were treated sequentially with anticytochrome P-450$_{11\beta}$ (MAb 258) (1/100 to 1/500 dilution), for 18 h at 4° C in a moist chamber, and with biotinylated goat antimouse-immunglobulin in PBS and peroxidase conjugated streptavidin in PBS, each for 30 min at room temperature in a moist chamber. The sections were washed with cold PBS between incubations. A final wash was followed by immersion of the reacted sections for 5 to 10 min in a solution containing 0.05% Tris-HCl pH 7.6, 0.66 mM 3.3′-diaminobenzidine and 2 mM hydrogen peroxide. Specific staining was identified by the presence of brown reaction products. The reacted sections were finally counterstained with 1% methyl green and mounted with a glycerol-gelatin water-soluble medium. Control sections were incubated with normal mouse serum and 0.01 M PBS instead of primary antibody.

Results

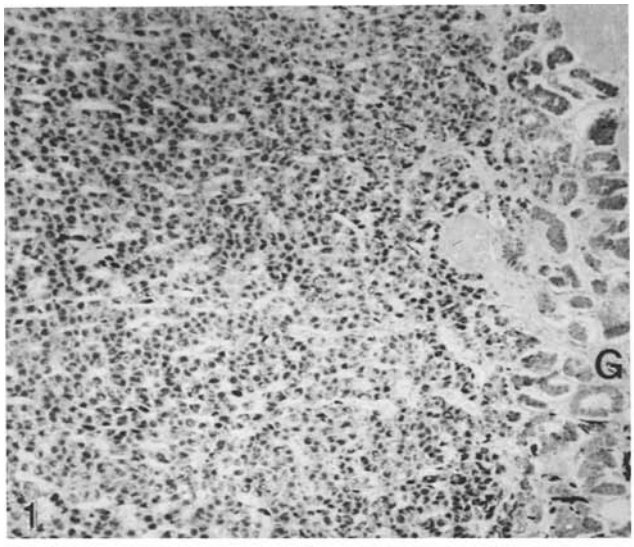
In bovine and porcine adrenal glands the P-450$_{11\beta}$ immunoreacivity was observed in the parenchymal cells of all three zones of the adrenal cortex. The capsule, medulla and sinusoidal cells of the adrenal glands were unstained. The zona fasciculata and reticularis were intensely stained for P-450$_{11\beta}$ but the immunoreactivity in the zona glomerulosa was faint (Fig. 1). In the controls, immunohistochemical staining was completely absent. No significant differences were observed in immunohistochemical findings between the bovine and porcine adrenals.

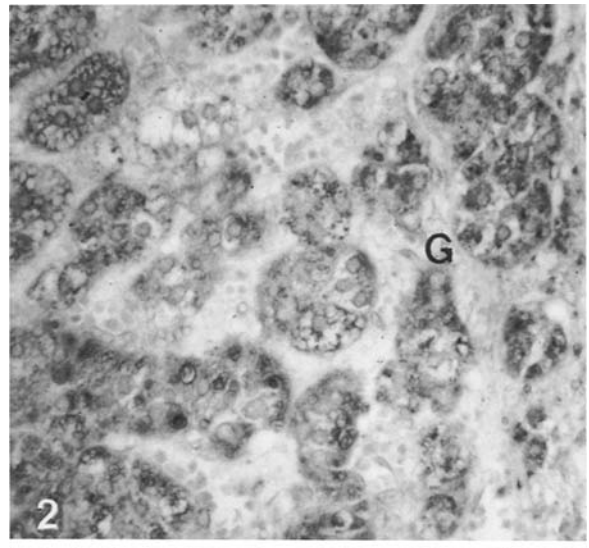
In normal human adrenal glands, the intraadrenal distribution of immunoreactivity was the same as that of bovine and porcine adrenal glands. The P-450$_{11\beta}$ immunoreactivity was evident in adrenocortical cells in the zona fasciculata and reticularis. The staining appeared as compact and fine granules at high magnification. Immunoreactivity was faint in the zona glomerulosa but less so when compared with the bovine and porcine adrenal cortex.

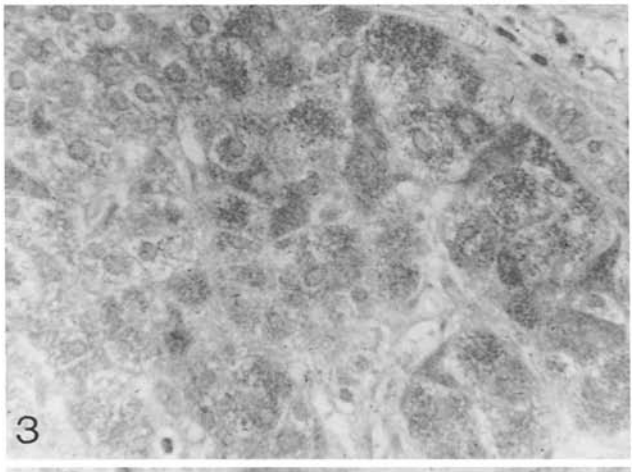
In idiopathic hyperaldosteronism the histopathological findings have been described in detail (Sasano et al. 1988c). The P-450$_{11\beta}$ immunoreactivity was more prominent in cells of hyperplasia of the zona glomerulosa and outer fasciculata (Fig. 2). The intensity of the staining in the zona glomerulosa was the same as that of the zona reticularis in three cases.

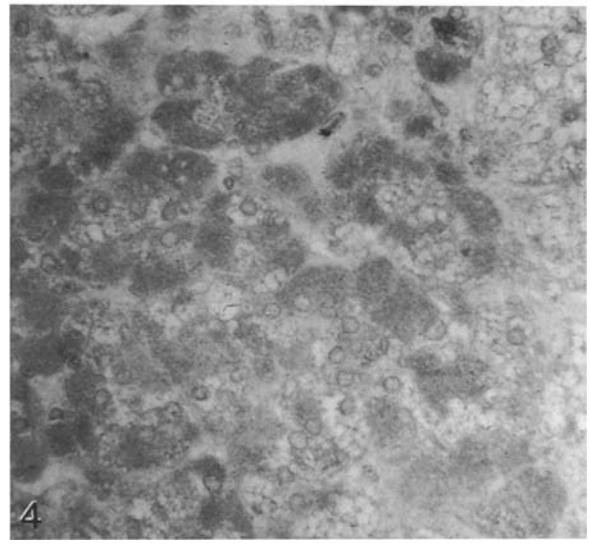
In Cushing's disease all the adrenal glands showed cortical hyperplasia. The staining was intensive in the inner fasciculata and reticularis, particularly in the cells of a cortical micronodule (Fig. 3). The immunoreactivity in zona glomerulosa cells was faint.

In aldosteronoma tumour cells with relatively intensive immunoreactivity to P-450$_{11\beta}$ were generally small and compact (Fig. 4) or posessed of large nuclei. Large clear tumour cells were also positive for P-450$_{11\beta}$ but the immunoreactivity was less intensive than in the tumour cells. In the attached adrenal glands of aldosteronoma, P-450$_{11\beta}$ immu-









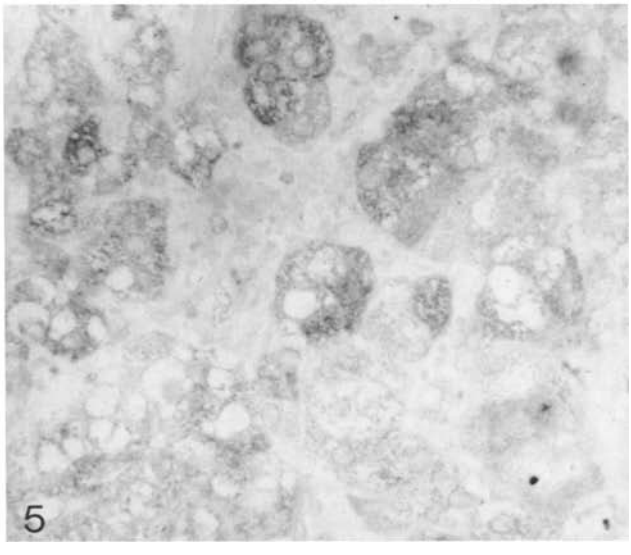


Fig. 1. Immunoreactivity to P-450$_{11\beta}$ in the zona glomerulosa (*G*) and fasciculata of bovine adrenal gland fixed in 100% methanol. × 200

Fig. 2. Marked immunoreactivity to P-450$_{11\beta}$ in the zona glomerulosa (*G*) of the adrenal gland from a patient with IHA. × 400

Fig. 3. Intensive immunoreactivity to P-450$_{11\beta}$ in a cortical micronodule of the adrenal gland with Cushing's disease. × 400

Fig. 4. Immunoreactivity to P-450$_{11\beta}$ in an aldosteronoma. Compact tumour cells show more intensive immunoreactivity. × 400

Fig. 5. Immunoreactivity to P-450$_{11\beta}$ in an adrenocortical adenoma with Cushing's syndrome. × 400

noreactivity was not observed in the zona fasciculata and reticularis but some cells in the zona glomerulosa were weakly positive for P-450$_{11\beta}$.

In adenomas with Cushing's syndrome, compact tumour cells arranged in small cords or alveoli showed relatively intensive immunoreactivity for P-450$_{11\beta}$ (Fig. 5), clear lipid-laden tumour cells were more weakly stained. In the attached adrenal glands, no immunoreactivity was observed in the atrophic zona fasciculata and reticularis but some cells in the zona glomerulosa were faintly stained.

Discussion

It is known that an adrenocortical mitochondrial cytochrome P-450$_{11\beta}$ is an indispensable enzyme in the biosynthesis of both cortisol and aldosterone (Marusic et al. 1973; Momoi et al. 1983; Yanagibashi et al. 1986) but the enzymatic properties of this P-450$_{11\beta}$ have only recently been identified (Wada et al. 1984, 1985). Intraadrenal localization of P-450$_{11\beta}$ has been analyzed in the bovine (Mitani et al. 1982; Sugano et al. 1985; Ishimura et al. 1985) and porcine (Geuze et al. 1987) adrenal cortex by employing specific monoclonal (Sugano et al. 1985; Ishimura et al. 1985) and polyclonal (Mitani et al. 1982; Geuze et al. 1987) antibodies against P-450$_{11\beta}$. These findings and our own, using monoclonal antibodies against a common epitope of P-450$_{11\beta}$ in various animals species have all shown that immunoreactivity was intensive in the zona fasciculata and reticularis and was faint in the zona glomerulosa in bovine, porcine and human adrenal glands. This intracortical distribution of immunoreactivity is consistent with the biochemical distribution of P-450$_{11\beta}$ in the bovine adrenal gland (Yagi et al. 1983), and intracortical distribution of corticosteroids in the human adrenal gland (Dickerman et al. 1984).

In adrenocortical lesions with gluco- and mineralocorticoid excess, cells with strong immunoreactivity correspond to those which are hyperfunctional. In IHA, zona glomerulosa cells, which had faint reactions in the normal adrenal glands, showed prominent immunoreactivity to P-450$_{11\beta}$. This immunohistochemical finding is consistent with clinical and morphological findings in IHA (Neville and O'Hare 1982; Sasano et al. 1988c) and the role of P-450$_{11\beta}$ in aldosterone biosynthesis (Marusic et al. 1973; Momoi et al. 1983; Yanagibashi et al. 1986). A primary site of ACTH effect is considered to be the conversion of cholesterol to pregnenolone (cholesterol side chain cleavage, Stone and Hechter 1954) but Kramer et al. (1983) demonstrated that ACTH had long term effects in promoting the synthesis and activity of 11β-hydroxylase. The intensive immunoreactivity in cells of cortical micronodules and inner zona fasciculata and reticularis cells in the adrenal with Cushing's disease, confirm directly the ACTH stimulation of P-450$_{11\beta}$ in Man. In functioning adrenocortical adenomas, compact cells had more intensive immunoreactivity than clear lipid-ladened cells, suggesting active corticosteroidogenesis in those cells. This finding is consistent with our previous investigations of the distribution of immunoreactivity to P-450$_{C21}$ (Sasano et al. 1988b) and of in vitro steroid production by adrenocortical adenoma cells (Sasano et al. 1980).

Analysis of the attached adrenal glands with functioning adrenocortical adenomas may be of interest in understanding the pathophysiology of those disorders. The adrenal glands attached to aldosteronoma have, in most cases, been known to show hyperplasia of the zona glomerulosa where aldosterone is synthesized (Neville and O'Hare 1982). This finding is inconsistent with the low plasma renin levels observed in patients with aldosteronoma and is considered pathophysiologically as a paradoxical hyperplasia. Sasano (1975) showed a decreased histochemical activity of 18-hydroxycorticosterone dehydrogenase, low level of tissue aldosterone concentration and a decrease in number of mitochondria in zona glomerulosa and outer fasciculata cells of the attached adrenal glands of aldosteronoma. However, the immunoreactivity to P-450$_{C21}$ was present exclusively in the zona glomerulosa of those adrenal glands (Sasano et al. 1988b). Our present investigation showed only some glomerulosa cells showed positive immunoreactivity for P-450$_{11\beta}$ with no immunoreactivity in the zona fasciculata and reticularis. The findings showed that most of microscopically hyperplastic zona glomerulosa cells in these adrenal glands do not have complete aldosterone biosynthesis but possibly can metabolise up to 21-hydroxylation, which suggests an incomplete aldosterone biosynthesis. In the attached adrenal glands of adrenocortical adenoma with Cushing's syndrome, the intracortical distribution of P-450$_{11\beta}$ is consistent with atrophy of the zona fasciculata and reticularis and suppressed ACTH level due to excess glucocorticoids produced by adenoma and persistence of the zona glomerulosa and its mineralocorticoid synthesis.

P-450$_{11\beta}$ was recently demonstrated to catalyze not only 11β-hydroxylation but also conversions of corticosterone to 18-hydroxycorticosterone and aldosterone in aldosterone biosynthesis (Wada et al. 1984, 1985; Yanagibashi et al. 1986). How-

ever, there are still some controversies whether those reactions are catalyzed by a single protein with regulators stimulating or suppressing the final two steps of aldosterone biosynthesis without affecting the 11β-hydroxylation in cortisol synthesis, or by two distinct proteins (Watanuki et al. 1978; Ohnishi et al. 1986; Yanagibashi et al. 1986; Lauber et al. 1987). Lauber et al. (1987) recently demonstrated that this monoclonal antibody (MAb 258) reacted with a 49K protein in the mitochondria of the zona glomerulosa from rats with stimulated aldosterone biosynthesis, but with a 51K protein in the mitochondria of the zona glomerulosa from rats with suppressed aldosterone biosynthesis, and that of the zona fasciculata from both groups of the rats. In this study, the intensive immunoreactivity to P-450$_{11\beta}$ was observed in cells with both excessive gluco- and mineralocorticoid synthesis in the human adrenal glands.

These results suggest that this monoclonal antibody recognized not only the enzyme exclusively involved in 11β-hydroxylation in mineralo- and glucocorticoid biosynthesis, but also that involved in the conversion of corticosterone to 18-hydroxycorticosterone and to aldosterone in the human adrenal gland. This has been observed in the rat adrenal gland. It is currently unknown whether biosynthesis from deoxycorticosterone to aldosterone is catalyzed by one enzyme or two enzymes in Man. Relevant to this is the observation that in addition to 11β-hydroxylase deficiency, isolated deficiency of corticosterone methyl oxidase II deficiency has been reported in Man (Rösler 1984) and human cDNA clone encoding P-450$_{11\beta}$ has recently been isolated and the structural gene for P-450$_{11\beta}$ was demonstrated on the long arm of chromosome 8 (Chua et al. 1987). However, further investigations are necessary to clarify the role of P-450$_{11\beta}$ in both gluco- and mineralocorticoid biosynthesis in the human adrenal cortex.

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Accepted May 30, 1988