Immunohistochemical Study of Cytochrome b5 in Human Adrenal Gland and in Adrenocortical Adenomas from Patients with Cushing’s Syndrome

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Abstract. Cytochrome b5, a component of the electron transfer system increases the relative activity of 17α,20-lyase to 17α-hydroxylase of P450c17 in vitro. In the present study, immunohistochemical analysis of cytochrome b5 was performed in the human adrenal gland and in adrenocortical adenomas from patients with Cushing’s syndrome. In the human adrenal gland, cytocrome b5 was stained in all three adrenocortical layers but the staining was most remarkable in the zona reticularis. All of the adenomas were composed mainly of compact cells, which exhibited immunoreactive staining for cytochrome b5 as well as for P450c17 and 3β-hydroxysteroid dehydrogenase (3β-HSD). The distribution of b5 in the adenomas was correlated with that of P450c17 rather than with that of 3β-HSD. The immunoreactive staining for cytochrome b5 appeared to be more prominent in the two adenomas that produced relatively high concentrations of adrenal androgens than in adenomas that produced low concentrations of adrenal androgens. These results immunohistochemically support the functional association of b5 with androgen production through interaction with P450c17 and the previous finding that higher concentrations of cytochrome b5 are associated with greater production of adrenal androgens in adrenocortical adenomas from patients with Cushing’s syndrome.

Key words: Cytochrome b5, Immunohistochemistry, Adrenocortical adenomas, Cushing’s syndrome, Adrenal androgens

THE steroidogenic microsomal enzyme cytochrome P450c17 catalyzes both the 17α-hydroxylation required for the production of cortisol and the 17,20-lyase reaction leading to the production of adrenal androgen synthesis [1, 2]. The gene CYP17 encoding human P450c17 is a single copy gene [3] and has been mapped to chromosome 10 [4]. Both the 17α-hydroxylase and 17,20-lyase activities of this enzyme are simultaneously co-induced by ACTH [5] and impaired by congenital defects in P450c17 caused by various mutations in the CYP17 gene [6]. Nevertheless, the secretion of cortisol and adrenal androgens is dissociated in many clinical situations, for example, in adrenarche, puberty, aging, and recovery, from adrenal suppression [7]. The steroidogenic abnormalities in patients with Cushing’s syndrome secondary to adrenocortical adenoma also show a dissociation between cortisol and adrenal androgens. The sera from these patients contain a low concentration of adrenal androgens, but a high concentration of cortisol. This indicates that a factor other than P450c17 regulates the ratio of the activity of 17α-hydroxylase
to that of 17,20-lyase. The proteins of the electron transfer system are good candidates for such regulation, because the relative activity of 17,20-lyase to 17α-hydroxylase is increased several times by an increase in the ratio of either cytochrome b5 (b5) [8, 9] or NADPH-cytochrome P450 reductase (Red) [10] to P450c17 in vitro. More decisively, cytochrome b5 has recently been shown to cause more than a 10 fold increase in 17,20-lyase activity of E. coli cells engineered to express a large amount of human P450c17 [11].

This regulation by electron transfer proteins may also occur in vivo. Two adrenocortical adenomas from patients with Cushing’s syndrome that produced exceptionally high concentrations of androgens contained more b5 and greater 17,20-lyase activity than other adenomas that produced low concentrations of androgens [12]. In addition, the increased 17,20-lyase activity in the two adenomas was partially but significantly antagonized by an antibody to b5, suggesting a functional association of b5 with 17,20-lyase activity in those adenomas [13]. We have extended this finding by examining the immunohistochemical staining of b5 in these adenomas in the present study.

Materials and Methods

Experimental materials and enzymatic activities

Under the informed consent, normal adrenal gland was obtained from a 55-y.o male patient with renal carcinoma who underwent hemilateral nephrectomy at operation.

Four patients with a clinical diagnosis of Cushing’s syndrome secondary to benign adrenocortical adenoma were also studied. The age, sex, and laboratory findings of these patients were summarized in a previous report [12]. Cases 1, 2, 3 and 4 in the present study were cases 1, 8, 3 and 5, respectively in the previous study [12]. Briefly, these patients were classified into two groups on the basis of their serum and/or urine concentrations of adrenal androgens. Cases 1 and 2 had high adrenal androgen concentrations and cases 3 and 4 had low adrenal androgen concentrations. Values for the enzymatic activities of 17α-hydroxylase, 17,20-lyase and 3β-hydroxysteroid dehydrogenase (3β-HSD) and the cytochrome b5 concentration in microsomes from the adenomas of the five patients were available from previous studies [12-14] and are summarized in Table 1. Based on 95% confidence limits, 17,20-lyase activities in cases 1 and 2 were significantly higher than those in cases 3 and 4. Activities of 17α-hydroxylase and 3β-HSD in cases 1 and 2 were not significantly different from those in cases 3 and 4, respectively. Consequently, the ratio of 17,20-lyase to 17α-hydroxylase activity in cases 1 and 2 were about four fold greater than in those other cases. Moreover, a functional association between b5 and 17,20-lyase activity in these adenomas was suggested, because the microsomal concentration and the degree of the expression of b5 in cases 1 and 2 were significantly higher than those in other cases with lower 17, 20-lyase activity [12, 13] and

<table>
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<th>Patient</th>
<th>17-KS (µmol/day)</th>
<th>Lyase (pmol/mg/min)</th>
<th>17-OHase (nmol/mg/min)</th>
<th>Lyase/17-OHase (x 10^-3)</th>
<th>3β-HSD (nmol/mg/min)</th>
<th>Cyt b5 (nmol/mg)</th>
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<td>3.33</td>
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<td>0.66</td>
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<tr>
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<tr>
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<tr>
<td>4</td>
<td>15.9</td>
<td>11</td>
<td>2.58</td>
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Levels of urine 17-ketosteroids (17-KS) and enzymatic activities are from previous studies [12-14]. Designation of mg in each unit indicates mg protein. –, not determined; 17-OHase, 17α-hydroxylase; Lyase, 17,20-lyase; Cyt b5, cytochrome b5. Although cytochrome b5 content in case 3 was not measured, relative lower expression of b5 in case 3 compared with those in cases 1 and 2 has been proven by Northern and Western blot analysis [12].
because the increased 17,20-lyase activity in the two adenomas was partially but significantly antagonized by an antibody to b5 [13]. No significant difference in NADPH-cytochrome P450 reductase activity was observed among all adenomas [13], suggesting that P450 reductase is not responsible for the difference in 17,20-lyase activity in these adenomas.

Histology and immunohistochemistry

Adrenal tissues were fixed in 10% formalin solution and 2.5 mm thick paraffin sections were cut for immunohistochemical studies. After deparaffinization, the sections were put into methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. They were washed in three changes of 0.01 M phosphate-buffered saline, pH 7.2 for 5 min each and treated with 1% normal swine serum for 30 min. After a washing with phosphate-buffered saline, sections were treated sequentially with antibodies to P450c17 (kindly donated by Dr. MR Waterman, Vanderbilt University, USA), 3β-HSD [15] or cytochrome b5 (donated by Dr. O Yubisui, Kochi University, Japan) overnight at 4 °C in a moist chamber, then incubated with swine anti-rabbit IgG for 30 min at room temperature in a moist chamber (1:40 dilution), and then with peroxidase-antiperoxidase complexes for 30 min at room temperature in a moist chamber (1:80 dilution). The sections were washed with cold phosphate-buffered saline between incubations. A final wash was followed by immersion of the reacted sections for 5 to 20 min in a solution containing 0.05 M Tris-HCl buffer, pH 7.6, 0.55 mM 3,3’-diaminobenzene, and 2 mM hydrogen peroxide. Specific staining was identified by the presence of the brown reaction product. The sections were finally counterstained for nuclei with 1% methyl green and mounted with a glycerol-gelatin water-soluble medium. To establish the specificities of the immunohistochemical staining with each antibody, control sections were incubated with respective matched serum obtained from the same rabbit prior to immunization.

Results

In human adrenal gland, cytocrome b5 was stained in all three adrenocortical layers. Most intense staining of cytochrome b5 was observed in the area of zona reticularis (Fig. 1).

All four adrenocortical adenomas from patients with Cushing’s syndrome were composed mainly of compact cells, with a relatively eosinophilic cytoplasm. Clusters of clear tumor cells were also observed in cases 1, 3, and 4. In the tumor, immunoreactivity to P450c17 and 3β-HSD was seen in all the adenomas. Immunoreactive b5 was observed predominantly in the compact tumor cells. The distribution of the immunoreactive b5 in adrenal adenomas was correlated with that of

Fig. 1. Immunohistochemical staining of cytochrome b5 in normal human adrenal cortex (x 50). ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis.
P450c17 but not with that of 3β-HSD in all adenomas. Figure 2 is such an example observed in the adenoma from case 1. The number of tumor cells positive for b5 and the relative immunoreactivity of b5 in the tumor cells appeared to be much greater in the two cases of adenoma (cases 1 and 2) that produced relatively higher concentrations of adrenal androgens than in the other cases of adenoma (cases 3 and 4) (Fig. 3).

In attached non-neoplastic adrenal tissue, 3β-HSD was sporadically present, but immunoreactive P450c17 was not observed in this atrophied cortex. Immunoreactivity to b5 was weakly and sporadically observed in the inner fasciculata and reticularis of the attached non-neoplastic adrenal. Immunoreactivity as described above was not observed in the negative controls.

**Discussion**

Cytochrome b5 is a heme protein that serves as an electron donor in a variety of reactions including the desaturation of fatty acids in animal liver [16], reduction of methemoglobin in erythrocytes [17], and the reduction of microsomal cytochrome P450 [18]. In addition, cytochrome b5 has been reported to potentiate the monooxygenase reactions of rat hepatic microsomal cytochrome P450 [18–20] and P4502B1 [21]. Cytochrome b5 also increases the ratio of the activity of 17,20-lyase to 17α-hydroxylase catalyzed by a microsomal P450, P450c17 in vitro [8, 9, 11]. Thus, the abundance of available cytochrome b5, by determining this activity ratio, may regulate the amount of adrenal androgen produced by steroidogenic tissues.

In the present study, cytochrome b5 was present in all three adrenocortical layers in adrenal gland
by immunoreactive staining. The immunoreactivity was seen most intensely in zona reticularis. Since P450c17 is reported to be present only in zona fasciculata and reticularis, leading to the production of cortisol and adrenal androgens, respectively [22], these results support the functional association of b5 with production of adrenal androgens especially in zona reticularis. Although it is clear that b5 in zona glomerulosa is not associated with androgen production, the functional significance of b5 in zona glomerulosa is not yet known.

On the other hand, the immunoreactivity of cytochrome b5 in the adenomas was observed in compact tumor cells, the usual main cell type in Cushing’s adenoma [22]. Because of the correlation between b5 immunoreactivity and 17,20-lyase activity in these tumors [12, 13], it is likely that b5 immunoreactivity occurs in the same areas of the tumor as immunoreactivity for P450c17. Actually, in all four cases that we examined, distribution of b5 within the tumor was correlated with that of P450c17 rather than with that of other microsomal enzymes, 3β-HSD. These results also support the functional association of b5 with androgen production through interaction with P450c17 in the adrenocortical adenomas.

Although it is difficult to compare the amounts of immunoreactivity for b5 in different tissue sections, the relative immunoreactivity in tumor cells positive for b5 and the ratio of b5 positive tumor cells appeared to be greater in the two adenomas (cases 1 and 2) with high 17,20-lyase activity than in the other adenomas (cases 3 and 4) with low 17,20-lyase activity. These data are consistent with our previous finding that relatively more b5 was produced, as evidenced by Northern and Western blot analysis [12] and by biochemical assay [13], in two adenomas with high 17,20-lyase

Fig. 3. Immunohistochemical staining of cytochrome b5 in Cushing’s adrenocortical adenomas from cases 1 (upper left), 2 (upper middle), 3 (upper right), and 4 (lower right) (x 50). No staining was observed with preimmune serum for cytochrome b5 in an adenoma from case 4 (lower left). Staining procedure for nuclei with 1% methyl green was omitted only in the adenoma from case 2.
activity, than in five other adenomas with low 17,20-lyase activity. The mechanism for the different expression of b5 in these various cortical adenomas is not clear. ACTH does not seem to cause the effect, because b5 is not induced by ACTH in cultured adrenocortical cells (unpublished observation). Anyway, the different expression of b5 in the adenomas is an interesting observation because it suggests the biochemical heterogeneity of Cushing's adrenocortical adenomas.

Thus, by applying immunohistochemical techniques, b5 can be localized to steroidogenic tissues. In addition, immunoreactive staining for b5 occurs abundantly in the zona reticularis in the normal human adrenal cortex and in the compact cells of adrenocortical adenomas from patients with Cushing's syndrome. The relative b5 immunostaining and the proportion of tumor cells positive for b5 were much greater in adrenocortical adenomas with higher 17,20-lyase activity, supporting the view that cytochrome b5 may regulate the 17,20-lyase activity as reported in in vitro studies [12, 13].

Acknowledgment

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References

16. Strittmatter P, Spatz L, Corcoran D, Rogers MJ,