Synaptophysin immunoreactivity in adrenocortical adenomas: a correlation between synaptophysin and CYP17A1 expression

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Abstract

Design and methods: The adrenal cortex is not considered to be an intrinsic part of the diffuse neuroendocrine system, but adrenocortical neoplasms possess neuroendocrine properties. In this study, we examined synaptophysin (SYP) and neural cell adhesion molecule (NCAM) expression in adrenocortical adenomas in relation to adrenal function.

Results: Immunohistochemical analysis showed that 50.7 and 98.6% of the cortical adenomas showed SYP and NCAM immunoreactivities respectively. There was no apparent difference in NCAM immunoreactivity among the adenomas. However, the immunostaining for SYP was significantly stronger in cortisol-producing adenomas (CP A) than in aldosterone-producing adenomas (APA), nonfunctioning adenomas (NFA), showing no clinical or endocrinological abnormality, or adenomas associated with preclinical Cushing’s syndrome (preCS). Western blotting and real-time PCR demonstrated that the expression level of SYP protein and mRNA was significantly higher in CP A than in APA or NFA. Additionally, the SYP mRNA level showed a positive correlation with CYP17A1 mRNA. In addition to the plasma membrane, mitochondria, and smooth endoplasmic reticulum, SYP immunoreactivity was detected in the Golgi area, which is known to be involved in the regulation of mitochondrial cholesterol and the transport of steroid intermediates. It was unexpected that the ratio of positive cells for SYP in preCS was less than that in APA and NFA. However, further examination is required, because the number of preCS cases we investigated was very small.

Conclusions: We propose that SYP expression in adrenocortical cells may be involved in some aspect of adrenal function such as transport or secretion of glucocorticoids.

Introduction

Hyperfunction of the adrenal cortex is associated with either hyperplasia, adenoma, or carcinoma. There are principally three forms of adrenocortical hyperfunction, namely, primary aldosteronism (PA), Cushing’s syndrome (CS), and congenital adrenal hyperplasia, although extremely rare cases such as androgen- or estrogen-secreting adrenocortical tumors have been reported (1, 2). Immunohistochemistry (3–5) and in situ hybridization (6, 7) for steroidogenic enzymes have been attempted, as it is difficult to determine the adrenal function from the histological findings alone. However, the mechanism involved in the release of steroids from the cortical cells remains unknown, due to the absence of obvious secretory granules in the cells.

Although the adrenal cortex is not an intrinsic part of the diffuse neuroendocrine system (DNS) (8), neuroendocrine differentiation, as evidenced by immunohistochemical detection of neuroendocrine markers, is encountered in some adrenocortical tumors (9–13). An integral membrane protein, synaptophysin (SYP; molecular weight, 38-kDa) is involved in synaptic vesicle formation (14) and is well accepted as a neuroendocrine marker (15). On the other hand, the expression of the SYP gene family is not restricted to neuronal and neuroendocrine differentiation in rats or humans (16, 17). Neural cell adhesion molecule (NCAM) is a cell surface glycoprotein involved in cell–cell interactions and is thought to play a role in axonal growth and cell migration (18, 19). NCAM expression is noted not only in neuronal and neuroendocrine tissues, but also in endocrine tissues (20). In the adrenal cortex, staining for NCAM is restricted to the definitive zone (DZ) in the fetus and the zona glomerulosa (ZG) in adults, both of which express aldosterone synthase (12, 21). Although two large NCAM protein isoforms, with apparent molecular masses of 180- and 140-kDa, have been characterized, the 180-kDa isoform is more common in neuronal tissues and the 140-kDa isoform has been
noted in endocrine tissues (20). Hence, it is possible that NCAM may play a different role in neuronal and endocrine tissues.

These observations led us to the suggestion that both SYP and NCAM may be involved in some aspect of adrenal function. In this study, we examined the expression of SYP and NCAM in association with endocrine function in adrenocortical adenomas by using immunohistochemistry, western blotting, and real-time PCR.

Materials and methods

Tissue samples

Of the human adrenal tissues listed in Nagasaki University Graduate School of Biomedical Sciences between 2001 and 2008, 29 cases of aldosterone-producing adenomas (APA), 20 cases of cortisol-producing adenomas (CPA), two cases of APA with CS, two cases of APA with preclinical CS (preCS), two cases of adenomas associated with preCS, and 16 cases of clinically silent nonfunctioning adenomas (NFA) were identified (see Supplementary Table 1, which can be viewed online at http://www.eje-online.org/supplemental/). The plasma aldosterone concentration (PAC)/plasma renin activity (PRA) ratio (≥25) was used in screening for PA. Subsequently, PA was diagnosed on the basis of an elevated PAC and suppressed PRA. The diagnosis of CS was based on specific clinical symptoms such as obesity, moon face, buffalo hump, abdominal striae, etc. elevated plasma cortisol concentration with lack of diurnal rhythm; and a suppressed plasma ACTH level. A diagnosis of preCS was made on the basis of: a) the presence of adrenal incidentaloma with lack of specific clinical symptoms of CS, b) abnormal circadian rhythm (normal plasma cortisol levels at early morning and elevated plasma cortisol levels at late evening), c) demonstration of autonomous secretion of cortisol by the overnight mg dexamethasone suppression test, d) inhibition of ACTH secretion, e) postoperative adrenal insufficiency or atrophy of the attached adrenal cortex, etc. Clinically silent adrenal incidentalomas showing no signs of PA, CS, or preCS were classified as NFA. Imaging of the adrenal glands with computerized tomography and scintigraphy was done for detection of adrenal masses. Furthermore, all patients were examined by adrenal venous sampling to determine the aldosterone-to-cortisol ratio on the right and left sides. Adrenocortical adenomas were diagnosed according to the histopathologic criteria proposed by Weiss et al. (22, 23). As controls, we used adrenal glands obtained from patients undergoing adrenalectomy together with pancreaticectomy or nephrectomy for pancreatic or renal cancer, who did not reveal any endocrine abnormalities.

Immunohistochemistry

Paraflin-embedded tissues were cut on a microtome to a thickness of 4 μm. After deparaffinization, heat-induced epitope retrieval with Dako target retrieval solution (pH 6.0; DakoCytomation, Kyoto, Japan) was performed. The tissue sections were allowed to react overnight at 4 °C with anti-SYP (SY38 clone, dilution 1/20; Dako), anti-NCAM (123C3 clone, dilution 1/20; Zymed Corp., South San Francisco, CA, USA), or anti-Golgi apparatus protein 1 precursor (GLG1; dilution 1/75; Sigma–Aldrich) antibodies. Double staining with SYP and GLG1 was also performed using the HISTOSTAIN-DS KIT (Zymed). For determination of antibody specificity, immunostaining was prevented by preincubation of anti-SYP antibody with an excess of recombinant human SYP (Thermo Fisher Scientific, Fremont, CA, USA), and nonimmune mouse or rabbit sera were substituted for anti-NCAM or GLG1 antibodies respectively. The data obtained were expressed as scores ranging from 1 (<10% positive cells) to 2 (10–30% positive cells), to 3 (30–50% positive cells), and to 4 (50–100% positive cells). In cases with no reaction, a score of 0 was recorded.

Western blotting

Five cases of APA and seven cases of CPA were used for the detection of SYP and NCAM proteins by western blotting. Frozen adenoma tissues were homogenized on ice for 1 min in lysis buffer containing protease inhibitor cocktail (Nakarai, Kyoto, Japan). To obtain detailed information on the subcellular distribution of SYP immunoreactivity, homogenates of adrenal tissues were also fractionated into mitochondria, microsome derived from fragmented smooth endoplasmic reticulum (sER), and plasma membrane, as described previously (24). The protein extracts (40 μg each) were subjected to SDS-12% PAGE. After the proteins were transferred onto an Immobilon-P membrane (Nihon Millipore Ltd, Tokyo, Japan), the membrane was reacted with anti-SYP (dilution 1/50; Dako) or anti-NCAM (dilution 1/50; Zymed) for 1 h at room temperature. The optical densities were measured by NIH image and were standardized by β-actin.

Real-time PCR

To examine the expression of SYP, 140-kDa NCAM, CYP11B2, and CYP17A1 mRNAs, we performed real-time PCR amplification in seven cases of APA, 13 cases of CPA, and four cases of NFA. Total RNA was collected from frozen adenoma tissues using GenElute Mammalian Total RNA kit (Sigma–Aldrich). After the reverse transcriptase reaction, LightCycler Quick System 330 (Roche Diagnostics K K) was used for the real-time PCR (7). Sequence-specific primers were designed and assigned the following GenBank accession
numbers: SYP (X06389: 225–249 and 357–333), 140-kDa NCAM (M17410: 919–937 and 993–972), CYP11B2 (X54741: 2637–2658 and 2724–2699), and CYP17A1 (M14564: 1203–1226 and 1339–1315). Expression levels were standardized by 18S rRNA (M10098: 124–148 and 256–232). All products were checked by electrophoresis using 3% agarose gels and ethidium bromide staining with u.v. visualization to ensure the specificity of the PCR products and the absence of nonspecific bands. Relative quantitation of gene expression was performed using the relative standard curve method.

**Ethics**

All patients signed a form of informed consent prepared in accordance with the rules outlined by the Nagasaki University Ethics Committee.

**Statistical analysis**

Differences were analyzed with Spearman’s correlation coefficient by rank test, Student’s t-test, Pearson’s correlation coefficient test, and χ² for independent test. Results were expressed as the mean ± S.E.M.

**Results**

In the control adrenal cortices, immunoreactivity for SYP was present in the nerve fibers, appearing as small dots, while NCAM immunostaining was present in the ZG and outer zona fasciculata (ZF) (data not shown). In the adenomas, SYP immunoreactivity was detected along the plasma and lipid membranes (Fig. 1a), and cytoplasm. Immunoreactivity manifesting as large dots was also observed in the perinuclear Golgi area, which well accorded with the localization of GLG1 staining (Fig. 1b). The immunoreaction for NCAM was limited to the plasma membrane (Fig. 1c). There was no evidence of positive immunostaining in the negative control sections (Fig. 1d).

The results of staining for SYP and NCAM are summarized in Tables 1 and 2. Of the 71 cases of adrenocortical adenomas, 36 (50.7%) and 70 (98.6%) showed SYP and NCAM immunoreactivities respectively, although the component cells of adenomas were not all positive for SYP or NCAM. With the exception of one case where a staining score 3 was recorded, the APA showed only a faint (staining score 1) or no SYP immunoreactivity. All cases of preCS (containing APA with preCS) and 15 out of 16 cases of NFA also showed no SYP immunoreactivity. On the other hand, intense staining of SYP was observed in all cases of CPA (staining score 3–4). The intensity of staining for SYP was significantly stronger in the adrenocortical adenomas associated with overt CS than in the adenomas not associated with overt CS (Spearman’s correlation coefficient by rank test, P < 0.01). Unexpectedly, the intensity of SYP immunoreactivity in preCS was less than that in APA and NFA. A significant relationship between the intensity of staining for NCAM and clinical symptoms was not observed, although there was a tendency to intensely stain among the APA.

Western blotting demonstrated a 38-kDa SYP band in the control adrenal gland (containing adrenal medulla) and all cases of CPA, but not in the APA cases (Fig. 2a). Immunoreactivity was present in all the subcellular

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Staining score: 1+, 10%; 2+, 10–30%; 3+, 30–50%; 4+, >50%. Intensity of staining: APA > PA + CS > APA = NFA > PA + preCS = preCS (P < 0.01).

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<th>Table 2</th>
<th>Immunoreactivity for neural cell adhesion molecule in adrenocortical adenomas.</th>
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Staining score: 1+, <10%; 2+, 10–30%; 3+, 30–50%; 4+, >50%. Intensity of staining: no significant difference was observed.
fractions of mitochondria, microsome, and plasma membrane (Fig. 2b). Meanwhile, all cases examined showed mainly a 140-kDa band reacting with MAB against NCAM (Fig. 2a), and the optical density of the band was slightly higher in AP A ($n = 5; 1.06 \pm 0.03$) than in CPA ($n = 7; 0.76 \pm 0.11$) (Student’s $t$-test, $P < 0.05$; Fig. 2c).

Real-time PCR (Fig. 3a) demonstrated that the expression level of SYP mRNA was about tenfold higher in CPA ($n = 13; 1.20 \pm 0.12 \times 10^{-4}$) than in APA ($n = 7; 0.10 \pm 0.03 \times 10^{-4}$) and NFA ($n = 4; 0.11 \pm 0.05 \times 10^{-4}$) (Student’s $t$-test, $P < 0.001$). On the other hand, 140-kDa NCAM mRNA was more highly expressed in APA ($n = 7; 0.97 \pm 0.05 \times 10^{-6}$) and NFA ($n = 4; 1.05 \pm 0.08 \times 10^{-6}$) than in CPA ($n = 13; 0.80 \pm 0.03 \times 10^{-6}$, $P < 0.05$). To determine whether or not the expression of SYP or NCAM mRNA correlated with steroidogenesis, we also examined the expression of CYP11B2 and CYP17A1 mRNAs, encoding aldosterone synthase, the enzyme for the final step of aldosterone synthesis, and 17α-hydroxylase (17α-OH), the limiting enzyme in cortisol synthesis respectively. As expected, high levels of CYP11B2 and CYP17A1 mRNAs were observed in APA and CPA respectively (data not shown). High expression of CYP17A1 mRNA in CPA was also confirmed by in situ hybridization (see Supplementary Table 2, which can be viewed online at http://www.eje-online.org/supplemental/).

The expression level of SYP mRNA correlated positively with that of CYP17A1 mRNA (Pearson’s correlation coefficient test, $n = 24; r = 0.8532, P < 0.001$; Fig. 3b). No significant correlation was found between NCAM mRNA and endocrine function.

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**Figure 2** Western blot analysis. A 38-kDa SYP band is detected in the control adrenal and all cases of CPA, but not in APA (a, upper panel; c, left panel). SYP immunoreactivity was present in the fractions of mitochondria (Mit), microsome (Mic), and plasma membrane (Plas) (b). All cases demonstrate 140-kDa NCAM band (a, middle panel), but the optical density of the band standardized by β-actin (a, lower panel) is slightly higher in APA than in CPA (c, right panel). Dotted lines show the mean value in APA and CPA tissues respectively. $^* P < 0.01$, $^* P < 0.05$. Cn, control adrenal (containing the medulla).

**Figure 3** Analysis of SYP, 140-kDa NCAM and CYP17A1 mRNA expression by real-time PCR. (a) The results are shown as the ratio of SYP (left panel) and 140-kDa NCAM (right panel) to 18S rRNA. Dotted lines show the mean value in APA, CPA, and NFA tissues respectively. $^* P < 0.01$, $^* P < 0.05$. (b) A correlation between SYP and CYP17A1 mRNA expression. The expression level of SYP mRNA shows a positive correlation with CYP17A1 mRNA ($P < 0.01$).
Discussion

Previous studies (9–13) reported that SYP and NCAM were ubiquitously expressed in adrenocortical tumors, but detailed examination of the differences in the expression among adrenal disorders has not been extensively performed. In this study, we examined SYP and NCAM expression in adrenocortical tumors using immunohistochemistry, in relation to adrenal function. The specificity of the immunoreactivities was confirmed by a preabsorption test or by substitution of nonimmune mouse sera in place of the primary antibodies. Western blotting, using the same antibodies as in the immunohistochemistry, also demonstrated a specific band: 38-kDa in SYP (12, 14) and 140-kDa in NCAM (12). Hence, the immunostaining in this study was specific with no false positives.

The implication of the finding that adrenocortical adenomas exhibit neuroendocrine properties such as SYP and NCAM, despite the fact that the adrenal cortex is not an intrinsic part of the DNS, is as yet little understood (8). Haak et al. (10) suggested the possibility that the staining cells might be derived from remnants of the fetal adrenal cortex and that silent genes might emerge under physiological conditions, as a result of dedifferentiation of adrenocortical cells. The DZ cells in the human fetal cortex are thought to be a progenitor (stem) population, capable of migrating into other zones (21). Aldosterone synthase and other steroidogenic enzymes are expressed at late gestation by DZ cells, like ZG cells in the adult adrenal (10, 25). Additionally, as NCAM protein and mRNA are expressed in DZ cells as well as ZG cells (12, 21), this hypothesis could explain why NCAM expression was present in most of the adenomas, especially APA. However, the hypothesis fails to account for the significant difference in SYP expression among the adenomas. In this study, CPA exhibited a high ratio of positive cells for SYP, whereas APA, the adenomas associated with preCS, and NFA showed few positive cells. Our result differed from the report of Li et al. that there seemed to be no correlation between SYP expression and endocrine clinical syndrome (13). However, our findings were supported by the results of western blotting and real-time PCR, demonstrating that the expression level of SYP protein and mRNA was significantly higher in CPA than in APA or NFA. In general, a nonspecific- or over-staining sometimes appears, depending on the antibody (especially polyclonal antibody), in the kidney and adrenal gland. Li et al. used a SYP polyclonal antibody in addition to monoclonal (13), whereas we used only monoclonal to avoid nonspecific binding as much as possible. Therefore, the type of antibody used may account for the differing results.

SYP is one of the major polypeptide components of the small electron-translucent vesicles of neurons and DNS cells (14, 16), and its presence in endocrine tumors correlates with the presence of cytoplasmic dense core granules (26). Furthermore, SYP is thought to contribute fundamentally to controls of the exocytotic process in non-DNS cells, such as thymic epithelial cells (16, 17). In adrenal glands, the biosynthetic steps involved in steroidogenesis are well elucidated (4, 27), but the secretory mechanism involved in the release of steroids from the adrenocortical cells is little understood, because the intracellular localization of the steroids is obscure. In addition to the plasma membrane, SYP immunostaining was present in the cytoplasm. Western blotting using subcellular fractions indicated that the immunoreactivity was localized in fractions containing mitochondria and microsome deriving from fragmented sER expressing the P450 enzymes necessary for steroid hormone production (27). Additionally, we demonstrated in this study that the SYP mRNA level showed a positive correlation with the mRNA level of CYP17A1 encoding 17z-OH, but not CYP11B2 mRNA encoding aldosterone synthase. Furthermore, of particular interest is the observation that the staining for SYP was detected in the Golgi area, as proved by GLG1 staining. The Golgi apparatus in the adrenal cortex has been thought to be involved in the regulation of mitochondrial cholesterol, the transport of steroid intermediates, and the regulation of plasma and lysosomal proteins (28, 29). Bassett & Pollard (30) reported that Golgi-related coated vesicles in rat ZF cells might be related to the transport of corticosterone (corresponding to cortisol in human) to the plasma membrane, based on the correlation between the vesicle numbers and plasma corticosterone concentration. Similar vesicle-like structures have been reported to be present also in human adrenocortical tumors and in primary pigmented nodular adrenocortical disease (9, 31, 32). The data thus far collectively imply that SYP expression may be involved in adrenal function, such as in the transport or secretion of glucocorticoids.

The expression level of SYP may be dependent upon the amount of steroid production in each cell. This would also explain why SYP was undetectable in adenomas associated with preCS. Additionally, it was no surprise to find heterogeneity of SYP staining in the same adrenal tumor, because the component cells of tumors do not all consistently synthesize or secrete steroid hormones at the same level. However, it was unexpected that the intensity for SYP staining in preCS was weaker than that in APA or NFA. Further examination is required to confirm whether there is actually any difference among APA, NFA, and preCS, because the number of preCS cases we investigated was very small. On the other hand, the real-time PCR showed that APA and NFA expressed SYP mRNA at the same, albeit very low, level, which is consistent with the fact that both APA and NFA have the potential for cortisol synthesis within the normal range (33).

Although we found no evidence of any relationship between NCAM and adrenal function, the results of immunohistochemistry, western blotting, and real-time
PCR led us to the suggestion that SYP expression in adrenocortical adenomas may be associated with functions such as the transport or secretion of at least glucocorticoids. However, not all cases conformed to our postulation, because there was one case each among the APA and NFA cases in which there was an intense SYP immunoreactivity (staining scores 3 and 2), but no oversecretion of cortisol. Adrenocortical tumors have also been known to express some neuropeptides (34, 35), and, hence, the possibility that SYP expression may be related to the secretion of neuropeptides cannot be completely ruled out. Further investigation into SYP expression in adrenocortical lesions, especially in vivo experiments, is required.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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