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Somatostatin receptors in human adrenal gland tumors - immunohistochemical study

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Abstract: Somatostatin receptors subtypes (SSTR 1-5) were demonstrated in surgically obtained adrenal gland tumors by means of immunohistochemistry (IHC). Results of the present study demonstrate that somatostatin receptors are expressed in adrenal tumors in a varied manner which is specific in each case. It provides different diagnostic and therapeutic possibilities.

Key words: Somatostatin receptor subtypes - Adrenal gland - Immunohistochemistry

Introduction

Somatostatin (SST) is a well known neurohormone peptide that occurs in two molecular forms with 14 or 28 aminopeptide residues. It is widely distributed in the central and peripheral nervous system, the pituitary gland and other tissues such as the pancreas, adrenals, intestine, kidneys, prostate, placenta and the immune system cells. SST exerts a number of different biological effects, predominantly of inhibitory nature (for review see: [1]). One of the most encouraging aspects of somatostatin effects and, particularly in the context of tumor diseases therapy, is its antiproliferative action [2]. SST as well as its synthetic analogs acts via specific receptors which are present on the surface of the target cells. Five subtypes of the SST receptor have been identified so far, i.e. SSTR 1-5 with two variants (2A and 2B) of the type 2 receptor [3,4]. These belong to a group of 7 transmembrane domains linked with the G protein [5] and are encoded by 5 genes which are present on separate chromosomes [6,7]. In the cell the SST receptors can be found on the cell membrane or in the cytoplasm region. Particular SSTR subtypes can occur alone or be grouped together in the same cell. SSTR are present both in normal and in tumor tissues which enables their response to applied SST analogs (for review see: [8]). If the investigated tumor shows the expression of more than one subtype of the SST receptor it cannot be predicted via which of these the SST analog action will be exerted. Expression of the receptors in a given tumor enables to apply a ligand that would react specifically with the only detected receptor or a SST multiligand which would react specifically with several receptors. The SST analogs - octreotide and lanreotide, which are widely applied at present, act mainly via 2 and 5 receptor subtypes and thus, are not always effective in the therapy of tumors expressing other receptor subtypes . The use of the SST multiligands that express the affinity to majority of SSTR subtypes and would therefore act more effectively in a given tumor has been postulated. Different techniques are being currently applied to detect SST receptors in human tumors. Octreoscan, receptor scintigraphy using indium labeled SST analogs - 111 In-pentetreotide (111 In-DTPA) octreotide [9] or technetium labeled (99mTc-EDDA/HYNIC-Tate) octreotate [10] have been proved most useful *in vivo*. These methods allow to determine *in vivo* the tumor localization and size with good precision, however their effectiveness depends on the presence of subtype 2 SSTR in the tumor. Moreover in the detection of adrenal tumors, receptor scintigraphy is difficult because of the strong signals from kidneys. Nevertheless, the immunohistochemical (IHC) technique performed on

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paraffin embedded tissue specimens obtained from surgically removed tumors seems the best ex vivo *in vitro* method in detecting subcellular distribution of SST receptors. The observations of negative results obtained with octreoscan in pheochromocytoma or neuroendocrine tumors (NET) where the presence of SST receptors in the investigated tumors was demonstrated using the immunohistochemical method solely have been published [11,12]. As a consequence, patients with negative results of receptor scintigraphy and with positive immunohistochemical reaction for SST receptors lost their chance of treatment with SST analogs. On the other hand, in approximately 15% of patients with positive octreoscan there were no SST receptors found in the tumor, which excludes any successful treatment with SST analogs. Currently applied methods of molecular biology (PCR, RT-PCR, Western-blotting) are useful exclusively in detecting receptor mRNA, while giving no insight into receptor localization. Apart from that, these methods are often burdened with false positive results.

Despite the fact that the adrenal gland has long been known as the target organ for SST and SST receptors have been found there in physiological conditions [13,14] data concerning the occurrence of SSTR in human adrenal gland tumors are still scarce and often conflicting [15-18]. Somatostatin analogs are not routinely used in treatment of adrenal tumors so far. The aim of our study was to assess systematically the presence of all the 5 subtypes of SSTR (including 2A and 2B SSTR isoforms) in surgically treated human adrenal tumors.

Material and methods

In this study 21 surgically treated adrenal gland tumors were assessed. The material was obtained from 15 women and 6 men aged between 42 and 67 years (mean age 55 years). Histological examination of the operated tumors revealed adrenal adenoma in 11 cases (9 clinically nonfunctioning tumors, 1 case of Conn's disease and 1 case of Cushing's syndrome), adrenal cortex hyperplasia associated with ectopic release of ACTH in 1 case, adrenal cortex cancer in 2 cases and pheochromocytoma in 7 cases.

Bouin's fixed, dehydrated and paraffin embedded 8-μm sections were immunostained using commercially available rabbit polyclonal antisera raised against carboxyl-terminal fragments of specific human somatostatin receptor subtypes (GRAMSCH Laboratories, Schwabhausen, Germany): SSTR 1 (named SS-840 antibody, corresponding to amino acid sequence 377-391 of the receptor's peptide chain), SSTR 2A (SS-800, specific for 355-369 sequence), SSTR 2B (SS-860, specific for 342-356 sequence), SSTR 3 (SS-850, specific for 381-395 sequence) SSTR 4 (SS-880, specific for 374-388 sequence) and SSTR 5 (SS-890, specific for 350-364 sequence). The immunohistochemical procedures were performed as previously described [19]. The working dilution of antibodies was 1:1000 (diluted in 0.05 M TRIS buffer, pH=7.6 containing 2% goat serum). Following overnight incubation in 4°C in humidified chamber with primary antibodies, the cells were treated with anti-rabbit IgG biotinylated goat antibody (1:800, DAKO, Denmark) and streptavidin complex (Strept ABC/HRP, DAKO, Denmark). The immunoreaction was visualized with 3,3'-diaminobenzidine (DAB, DAKO, Denmark) solution. For negative controls the primary antibody was replaced by normal goat serum. The immunoreactive intensity for specific receptor proteins was assessed semiquantitatively using a descriptive method as follows: strong staining (+++), moderate staining (++), weak staining (+) and pale staining (+/-). Subcellular distribution pattern of SSTR subtypes - membranous or cytoplasmic was also determined.

Results

Adrenal adenomas

The samples obtained from 11 cases of adrenal adenoma were characterized by markedly differentiated results. Multiple SSTR subtypes were found to coexist in each of them. Most of the specimens showed mixed distribution pattern of the receptors - cytoplasmic and membranous with one of them being predominant in the particular cases (Table 1).

Strong and moderate, both cytoplasmic and mambranous immunostatining of SSTR 1 was demostrated in 7 (63,6%) cases (Fig.1). Strong staining was detected in the cell membranes in 2 specimens and moderate staining in the cytoplasm was found in another 2 specimens. In case of SSTR 2A strong or moderate immunostaining with exclusively cytoplasmic distribution was shown in 3 (27.3%) slides, whereas strong or moderate staining with mixed, cytoplasmic and membranous distribution was found in 4 slides (Fig. 2) and moderate or weak membranous staining - in 3 cases. Mixed weak staining was found in 1 case. Staining for SSTR 2B was restricted to the cytoplasm and was characterized by strong or moderate intensity occurred in 4 (36.4%) specimens; mixed distribution - cytoplasmic and membranous occurred in 3 cases and exclusively membranous localization was seen in 2 cases. In the 2 remaining samples weak cytoplasmic or weak mixed cytoplasmic and membranous staining was found.

Staining for SSTR 3 was also characterized by mixed distribution and various intensity. Strong and moderate staining which was distributed both in the cytoplasm and the membranes was shown in 6 specimens. Strong or moderate staining in the cell cytoplasm was detected in 2 cases, strong or moderate staining in the cell membrane - in 2 cases and weak staining with mixed localization - in 1 case.

Moderate immunostaining for subtype 4 receptor was found in 2 (18.2%) cases of adrenal adenoma the intensity was weak or pale in 4 (36.4%) cases, nevertheless in each of the 6 slides SSTR 4 showed cytoplasmic localization. No presence of SSTR 4 was found in the remaining 5 (45.5%) cases. In 1 slide we found quite a strong staining in the cells of the adrenal medulla which were present in the specimen.

Similarly to SSTR 1, 2A, 2B and 3, SSTR 5 was characterized by mixed distribution - cytoplasmic and membranous. Strong staining in the cytoplasm was present in 4 (36.4%) cases, whereas in 7 (63.6%)

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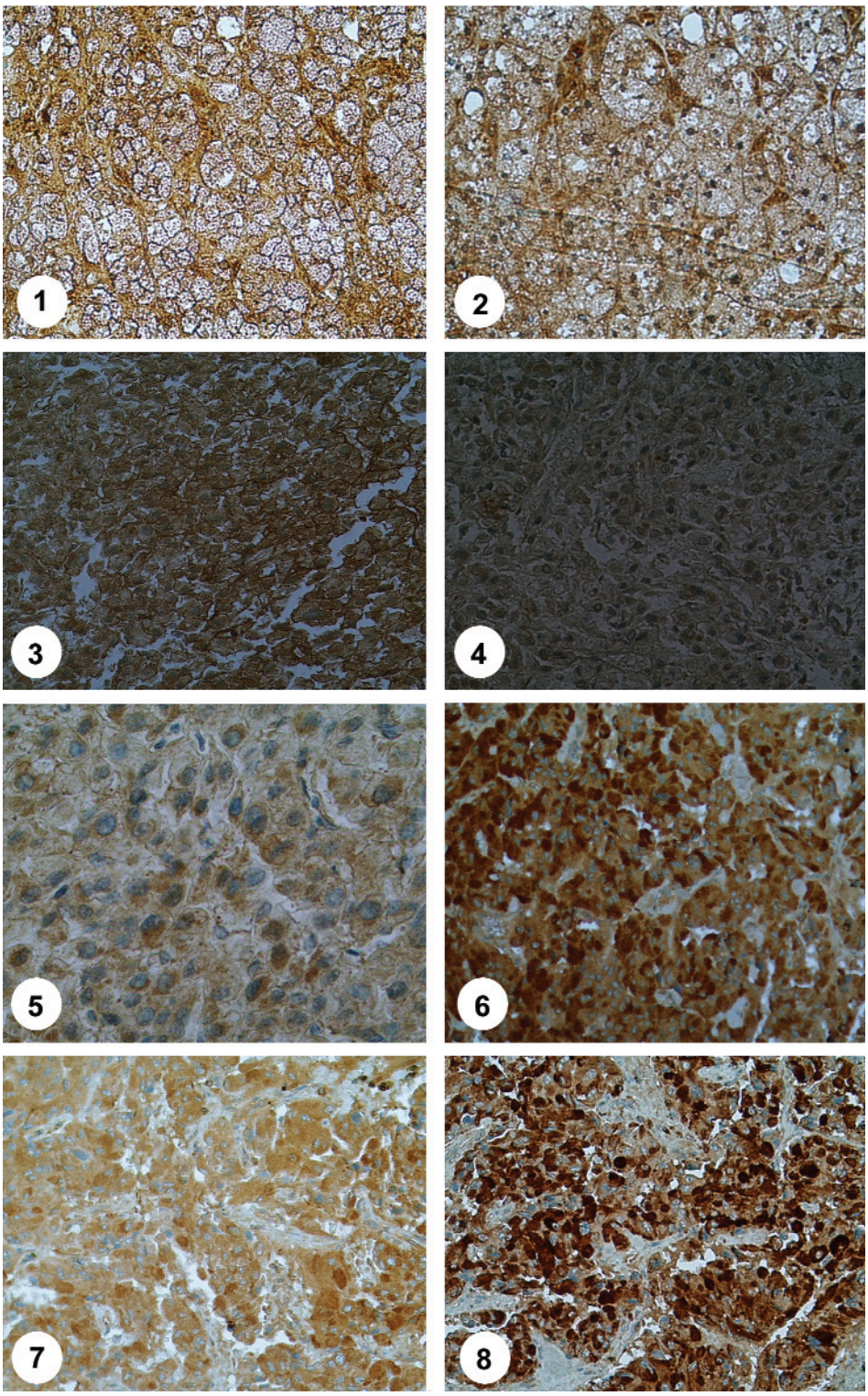

Figs. 1-2. Adrenal cortex adenoma. **Fig. 1**. Nr 2444, SSTR 1 (magnification ×200). **Fig. 2**. Nr 2444, SSTR 2A (magnification ×200). **Figs. 3-5.** Adrenal cortex carcinoma. **Fig. 3.** Nr 1846, SSTR 2A (magnification ×200). **Fig. 4.** Nr 1846, SSTR 2B (magnification ×200). **Fig. 5.** Nr 1846, SSTR 3 (magnification ×400). **Figs. 6-8.** Pheochromocytoma. **Fig. 6.** Nr 1926, SSTR 2A (magnification ×200). **Fig. 7**. Nr 1926, SSTR 2B, (magnification ×200). **Fig. 8.** Nr 1926, SSTR 3, (magnification ×200).

specimens it was distributed in both the cytoplasm and the membranes.

Adrenal cortex carcinomas

Except for SSTR 4, all subtypes of SSTR were found to coexist in 2 cases of adrenal cortex carcinoma (Table 2). SSTR 1, 2A, 2B, 3, 5 were present in 1 case and were distributed both in the cytoplasm and the cell membrane with predominantly moderate and weak intensity of staining (Fig. 3,4,5). In the latter case immunostaining was only seen in the cytoplasm.

Receptor subtypes SSTR 1-5, excluding SSTR 4 were detected in the specimen obtained from a patient

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Table 1. Somatostatin receptor subtypes in adrenal cortex adenomas.

No.	No. of patient	Histopatological determination	SSTR 1	SSTR 2A	SSTR 2B	SSTR 3	SSTR 4	SSTR 5
1.	1847	Adrenal cortex adenoma (Cushing's syndrome)	++/+++ mem/cytopl	++/+++ mem/cytopl	++/+++ mem/cytopl	++/+++ mem/cytopl	negative (++/+++ in medulla cells)	+++ mem/cytopl
2.	1931	Adrenal cortex adenoma	+++ mem	++/+++ mem	++ mem	+++ mem	negative	+++ mem/cytopl
3.	2270	Adrenal cortex adenoma	++ mem/cytopl	++ mem (+++ cytopl of single cells)	+-/+ mem/cytopl (+++ cytopl of single cells)	++ mem/cytopl (++/+++ cytopl of single cells)	negative	+++ mem/cytopl
4.	2444	Adrenal cortex adenoma	++/+++ cytopl/mem	++/+++ cytopl/mem	++ mem/cytopl	+++/++ cytopl/mem	negative	+++/+++ mem/cytopl
5.	328360/02	Adrenal cortex adenoma	+++/++ cytopl/mem	+++ cytopl	+++ cytopl	++ cytopl	+ cytopl	+++ cytopl
6.	328759/03	Adrenal cortex adenoma	++/+++ cytopl/mem	+/+ cytopl/mem	+ cytopl	+/+ cytopl/mem	+/- cytopl	++/++ cytopl/mem
7.	332298/02	Adrenal cortex adenoma	++ cytopl	++ cytopl	+/++ cytopl	++/++ cytopl/mem	+ cytopl	+++ cytopl
8.	333348/02	Adrenal cortex adenoma	+++/++ cytopl/mem	++/++ cytopl/mem	+++ cytopl	++/++ cytopl/mem	++ cytopl	+++ cytopl
9.	333809/03	Adrenal cortex adenoma	+++/+++ cytopl/mem	++/+++ cytopl/mem	++/+ cytopl/mem	++/+ cytopl/mem	+ cytopl	+++/+++ cytopl/mem
10.	332935/02	Adrenal cortex adenoma	++ cytopl	+++ cytopl	+++ cytopl	+++ cytopl	++ cytopl	+++ cytopl
11.	2271	Adrenal cortex adenoma (Conn's)	+++ mem	+ mem	++ mem	++ mem	negative	+++ mem/cytopl

Abbreviations: mem - membranous localization; cytopl - cytoplasmatic localization; strong staining (+++), moderate staining (++), weak staining (+) and pale staining (+/-).

with adrenal cortex hyperplasia due to ectopic release of ACTH by thymoma (nr 2350) and were distributed both in the cell membrane and the cytoplasm (Table 3). Strong staining was found for SSTR 1 and moderate - for SSTR 5.

Pheochromocytomas

In 6 (85%) out of 7 examined specimens SSTR subtypes 1, 2A, 2B, 3 and 5 coexisted and the staining was defined as strong or moderate (Table 4). Strong immunostaining for SSTR 1 was detected in 3 (42.8%) cases. Strong staining for SSTR 2A was found in 4 (57.14%) cases (Fig. 6), for SSTR 2B - in 1 (14.28%) case, SSTR 3 - in 3 (42.8%) cases (Fig. 8) and for SSTR 5 - in 4 (57.14%) specimens. No presence of SSTR 4 was found in 6 (85.71%) cases. In 1 case this subtype of receptor was demonstrated in moderate staining (14.28%). Weak or pale staining for receptors SSTR 1, 2A, 2B, 3 and 5 was shown in only 1 specimen (nr 2132). In all the examined specimens SST receptors were localized in the area of the cell cytoplasm which showed a positive staining (Fig. 7). Local strong or moderate staining in the endothelium was demonstrated in one specimen.

Discussion

Results of the present study demonstrate that adrenal tumors which derive both from the cortex and the medulla of the organ show strong expression of somatostatin receptors, which is consistent with earlier reports. All the examined adrenal cortex adenomas were characterized by positive staining for SSTR 1, SSTR 2A, SSTR 2B, SSTR 3 and SSTR 5 of different intensity. Staining for SSTR 4 which was found negative in 45% or pale (36.4%) was an exception. Due to the paucity of the material it is impossible to answer

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Table 2. Somatostatin receptor subtypes in adrenal cortex carcinomas.

No.	No. of patient	Histopatological determination	SSTR 1	SSTR 2A	SSTR 2B	SSTR 3	SSTR 4	SSTR 5
1.	1846	Adrenal cortex carcinoma	+++ cytopl/mem	++/+ cytop/mem	+/+ cytopl/mem	+/+++ cytopl/mem	negative	++ /++ cytopl/mem
2.	2304	Adrenal cortex carcinoma	+ cytopl	+/++ cytopl	+/++ cytopl	+ cytopl	negative	++/+++ cytopl

Abbreviations: mem - membranous localization; cytopl - cytoplasmatic localization; strong staining (+++), moderate staining (++), weak staining (+) and pale staining (+/-).

Table 3. Somatostatin receptor subtypes in adrenal cortex hyperplasia.

No.	No. of patient	Histopatological determination	SSTR 1	SSTR 2A	SSTR 2B	SSTR 3	SSTR 4	SSTR 5
1.	2350	Adrenal cortex hyperplasia (ectopic release of ACTH)	+++ cytopl	+ cytopl	+ mem	+/+ mem/cytopl	negative	++/+ cytopl/mem

Abbreviations: mem - membranous localization; cytopl - cytoplasmatic localization; strong staining (+++), moderate staining (++), weak staining (+) and pale staining (+/-).

Table 4. Somatostatin receptor subtypes in pheochromocytomas.

No.	No. of patient	Histopatological determination	SSTR 1	SSTR 2A	SSTR 2B	SSTR 3	SSTR 4	SSTR 5
1.	1829	Pheochromocytoma	++/+++ cytopl (++/+++ endo)	++ mem/cytopl (0/++ endo)	++ cytopl (0/+++ endo)	++ cytopl (++/+++ endo)	negative	+++ cytopl (+++ endo)
2.	1830	Pheochromocytoma	++ cytopl	+ cytopl	+ cytopl	++ cytopl	negative	++/+++ cytopl
3.	1926	Pheochromocytoma	++ cytopl	++/+++ cytopl	+ cytopl	++/+++ cytopl	negative	+/++ cytopl
4.	2132	Pheochromocytoma	0/+ cytopl	+_/+ cytopl	+_/+ cytopl	+_/+ cytopl (+++ single cells)	negative	+_/++ cytopl
5.	330357/04	Pheochromocytoma	+++ cytopl	+++ cytopl	++ cytopl	+++ cytopl	negative	+++ cytopl
6.	332974/03	Pheochromocytoma	++ cytopl	+++ cytopl	++ cytopl	++ cytopl	negative	++ cytopl
7.	333153/10	Pheochromocytoma	+++ cytopl	+++ cytopl	+++ cytopl	+++ cytopl	++ cytopl	+++ cytopl

Abbreviations: mem - membranous localization; cytopl - cytoplasmatic localization; strong staining (+++), moderate staining (++), weak staining (+) and pale staining (+/-).

the question whether there is any difference in SSTR expression between hormonally active and nonfunctioning adenomas. Unger *et al.* [17] examined 7 nonfunctioning adenomas and found positive immunostaining for SSTR 1, 2A and 3 in 2/3 of cases. Almost all nonfunctioning adenomas showed the expression of subtype SSTR 5 with positive staining in 30-60% of cells. Almost all cases of Cushing's syndrome and Conn's syndrome that were studied by these authors were positive for SSTR subtypes 1-5 with positive staining in 30% of cells. All the SSTR subtypes, except for SSTR 4 were also found in both cases of adrenal carcinoma in the present study. In the experience of Unger *et al.* 50% of adrenal carcinomas (4 cases)

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showed the expression of SSTR 2A and SSTR 3 with positive immunostaining in 30-100% of cells. Interestingly, SSTR1 subtype did not occur in any of them. Similarly, we demonstrated positive staining for all SSTR subtypes but SSTR 4, which occurred in moderate intensity in only 1 (14.3%) case. Receptor 2A and 5 subtypes were expressed in 57.14% of cases and SSTR 1 and 3 in equal amounts - 42.8%. In the study by Pasquali *et al.* [20] expression of SSTR 3 was detected in 90% of studied pheochromocytomas while that of SSTR 2A in only 25%; the remaining SST receptors were expressed in still smaller percentage of cases. Using immunohistochemical methods, Mundschenk *et al.* [13] found the presence of SSTR 3 in 90%, SSTR 2A - in 25%, SSTR 4 - in 10%, SSTR 5 - in 15% and SSTR 1 - in 8% of studied pheochromocytomas. Unger *et al.* [17] demonstrated the expression of SSTR 1, 2A and 5 in 1/3 of the studied pheochromocytomas with 30% positive staining cells whereas SSTR 3 occurred in all of the cases and the percentage of cells with positive staining was 60%.

Diversity of reported results has also been reflected in the studies on the expression of SST receptors that have been conducted using molecular biology techniques, particularly the PCR. The mRNA for mainly SSTR 5 was found in a series of human corticotropic adenomas [21]. No presence of SSTR 5 in any of 10 studied pheochromocytomas was shown by this method and SSTR 1 was detected in almost all cases [18]. Expression of all the 5 subtypes was equally distributed in aldosterone producing adenomas (Conn's syndrome). Using autoradiography, Epelbaum *et al.* [15] earlier found the presence of mRNA of all the five subtypes of SSTR in pheochromocytomas and they concluded that SSTR 2 and SSTR 4 mRNA occurred most frequently in these tumors, whereas SSTR 5 was identified in only 6% of cases.

Although majority of immunopositive cells were characterized by homogenous distribution in the area of engaged tissue, marked concentration of intensely immunoreactive cells was found in 2 cases of adrenal adenomas (1847, 2270) and 1 case of pheochromocytoma (2132) that have been analyzed in the present study, nevertheless general staining for a given receptor remained weak like, for example in the cells of the adrenal medulla or in the cytoplasm region. This observation indicates differences in the receptor density in individual cases. Cellular localization of investigated receptors should also be taken into consideration. SSTR subtypes that have been assessed in this study were located both in the cell cytoplasm and in the cell membrane. Other authors [16,22,23] also report cytoplasmic localization of SSTR 1, 3, 4, 5, the membranous distribution of SSTR 2A and expression of SSTR 3 both in the cell membrane and in the cytoplasm. Hofland *et al.* (24) observed expression of SSTR 2 in the cell membrane and Reubi *et al.* [25] using another specific antibody (R2-88) showed both cytoplsmic and membranous expression of SSTR 2A in pheochromocytomas. This phenomenon could be explained by the so called receptor internalization by a specific ligand, which is essential in the *in vivo* ligand-receptor interaction. Thus, localization of the receptor results from its subcellular "processing" due to synthetic activity, mechanisms of binding receptor to a ligand or receptor desensitization. Reubi *et al.* [25] consider subcellular distribution of SSTR 2A as the effect of autocrine activity of somatostatin that is produced by the tumor (e.g. pheochromocytoma), which can result in spontaneous internalization of the receptor and in avoiding its binding to the ligand. However, it has not been elucidated thus far, how subcellular localization of receptors which are detected by immunohistochemistry affects the expected biological effects. Results of the present study confirm high efficacy of immunohistochemical investigation of SSTR expression using antibodies specific for a given receptor subtype - a method considered supplementary or even equally effective as methods of molecular biology or diagnostic imaging. Usefulness of this method can even be considered superior to those due to lower costs of reagents (molecular biology), shorter time of providing results and lack of using radioactive reagents (receptor scintigraphy). Besides, receptor mRNA detected by molecular biology techniques (PCR, RT-PCR) does not seem a reliable proof of its presence. In fact, it is genetic material detected rather than the receptor protein being a real target for somatostatin and its analogs. Despite and partly due to their marked sensitivity, methods of molecular biology often provide results that are falsely positive and are associated with expression of somatostatin receptors in structures such as vascular endothelium or tumor infiltrating immunocytes.

Conclusion

Results of the present study demonstrate that somatostatin receptors are expressed in adrenal tumors in a varied manner which is specific in each case. It provides different diagnostic and therapeutic possibilities. Our data provide the rationale to therapeutic trials of somatostatin analogs in adrenal tumors. The diversity and coexistence of several somatostatin receptor subtypes in one tumor unables unequivocal prediction which receptor subtype would react with somatostatin analog. Therefore, apart from applying octreotide and lanreotide, newly synthesized multiligand analogs such as SOM 230 or KE 108 should be considered also as therapeutic agents in adrenal tumors. The decision as to therapeutic strategy choice should be taken individually in each case of adrenal tumor.

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