Ectopic expression of serotonin\textsuperscript{7} receptors in an adrenocortical carcinoma co-secreting renin and cortisol

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Abstract

Abnormal expression of membrane receptors has been previously described in benign adrenocortical neoplasms causing Cushing’s syndrome. In particular, we have observed that, in some adrenocorticotropic hormone (ACTH)-independent macronodular adrenal hyperplasia tissues, cortisol secretion is controlled by ectopic serotonin\textsuperscript{7} (5-HT\textsubscript{7}) receptors. The objective of the present study was to investigate \textit{in vitro} the effect of serotonin (5-hydroxy tryptamine; 5-HT) on cortisol and renin production by a left adrenocortical carcinoma removed from a 48-year-old female patient with severe Cushing’s syndrome and elevated plasma renin levels. Tumor explants were obtained at surgery and processed for immunohistochemistry, \textit{in situ} hybridization and cell culture studies. 5-HT-like immunoreactivity was observed in mast cells and steroidogenic cells disseminated in the tissue. 5-HT stimulated cortisol release by cultured cells. The stimulatory effect of 5-HT on cortisol secretion was suppressed by the 5-HT\textsubscript{7} receptor antagonist SB269970. In addition, immunohistochemistry showed the occurrence of 5-HT\textsubscript{7} receptor-like immunoreactivity in carcinoma cells. mRNAs encoding renin as well as renin-like immunoreactivity were detected in endothelial and tumor cells. Cell incubation studies revealed that the adrenocortical tissue also released renin. Renin production was inhibited by 5-HT but was not influenced by ACTH and angiotensin II (Ang II). In conclusion, the present report provides the first demonstration of ectopic serotonin receptors, i.e. 5-HT\textsubscript{7} receptors, in an adrenocortical carcinoma. Our results also indicate that 5-HT can influence the secretory activity of malignant adrenocortical tumors in an autocrine/paracrine manner. The effects of 5-HT on adrenocortical tumor cells included a paradoxical inhibitory action on renin production and a stimulatory action on cortisol secretion involving 5-HT\textsubscript{7} receptors.

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Introduction

We have previously shown that, in the normal human adrenal gland, serotonin (5-hydroxy tryptamine; 5-HT), released by perivascular mast cells, stimulates aldosterone and cortisol production through a paracrine mechanism involving 5-HT\textsubscript{4} receptors positively coupled to adenylyl cyclase and calcium influx (Contesse \textit{et al.} 2000). We have also observed that 5-HT is able to stimulate cortisol secretion in adreno corticotrophic hormone (ACTH)-independent macronodular adrenocortical hyperplasias (AIMAHs) and adenomas responsible for Cushing’s syndrome (Bertherat \textit{et al.} 2005, Contesse \textit{et al.} 2005). In these lesions, 5-HT is abnormally detected in clusters of
steroidogenic cells that harbor therefore a mixed, i.e. both steroidogenic and neuroendocrine, phenotype (Bertherat et al. 2005, Contesse et al. 2005). The receptors that mediate the action of 5-HT in cortisol-producing AIMAHs and adenomas both include eutopic 5-HT₄ and ectopic 5-HT₇ receptors (Cartier et al. 2003, Contesse et al. 2005, Louiset et al. 2006). Conversely, the effect of 5-HT on the secretory activity and expression of 5-HT₄ and 5-HT₇ receptors have never been studied in adrenocortical carcinoma tissues.

Renin and the other components of the renin angiotensin system, i.e. angiotensinogen, angiotensin converting enzyme and angiotensin I and II, have been detected in the rat and human adrenal cortex (Racz et al. 1992, Mulrow 1998). Prorenin mRNA has also been identified in adrenocortical cells indicating that renin is locally synthesized (Racz et al. 1992, Mulrow 1998). However, the physiological and physiopathological roles of the adrenocortical renin–angiotensin system are not clearly established. Several observations suggest that the local renin–angiotensin system may be involved in adrenocortical carcinogenesis: 1) angiotensin II (Ang II) has been shown to stimulate in vitro the mitogenic activity of bovine adrenocortical cells (Gill et al. 1977); 2) renin angiotensin systems are now considered as important actors of carcinogenesis in many tissues (Yoshiji et al. 2004); 3) expression of renin, angiotensinogen, angiotensin-converting enzyme, and their messengers has been described in adrenocortical neoplasms including carcinomas (Racz et al. 1992). In addition, two cases of adrenocortical tumors associated with elevated plasma renin concentrations have also been reported, indicating that, after its local synthesis, renin can be released in the plasma by tumor cells (Iimura et al. 1986, Yamanaka et al. 2000).

In the present study, we report a case of adrenocortical carcinoma co-secreting cortisol and renin. After surgery, immunohistochemistry, in situ hybridization, and cell culture studies were conducted to investigate the role of 5-HT in the regulation of cortisol and renin release by the tumor tissue. In addition, the possible occurrence of ectopic 5-HT₇ receptors was investigated by pharmacological and immunohistochemical approaches.

**Table 1 Biological data during preoperative and postoperative periods**

<table>
<thead>
<tr>
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<th>Preoperative</th>
<th>One month postoperative</th>
<th>Five month postoperative</th>
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<tr>
<td>Kalemia</td>
<td>2.6 mmol/l</td>
<td>3.5 mmol/l</td>
<td>3.2 mmol/l</td>
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<tr>
<td>Plasma renin concentration (pmol/l; 0.16 &lt; N &lt; 0.45)</td>
<td>2.09 pmol/l</td>
<td>0.26 pmol/l</td>
<td>2.13 pmol/l</td>
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<tr>
<td>Urinary free cortisol (nmol/day; 55 &lt; N &lt; 250)</td>
<td>3650 nmol/day</td>
<td>48 nmol/day</td>
<td>2034 nmol/day</td>
</tr>
<tr>
<td>Plasma aldosterone concentration (pmol/l; 90 &lt; N &lt; 760)</td>
<td>325 pmol/l</td>
<td>236 pmol/l</td>
<td>369 pmol/l</td>
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**Subject and methods**

**Case report**

A 48-year-old female patient was referred to our Department of Endocrinology for severe Cushing’s syndrome revealed by hypertension (195/100 mmHg) and hypokalemia (kalemia: 2.6 mmol/l). Hormonal assays performed without any treatment showed ACTH-independent (ACTH < 1.1 pmol/l) hypercortisolism (urinary free cortisol: 3650 nmol/day, normal: 50–220 nmol/day; Table 1). They also revealed an increase in recumbent plasma renin level, i.e. 2.09 pmol/l (normal: 0.16–0.45 pmol/l; Table 1), as well as elevated plasma androgen concentrations reaching 14.5 pmol/l (normal: 0.95–11.7 pmol/l) for dehydroepiandrosterone sulfate (DHEAS), 25.5 nmol/l (normal: 1.4–8.4 nmol/l) for androstenedione, and 4.65 nmol/l (normal: 0.70–2.10 nmol/l) for testosterone. Plasma aldosterone levels were within normal range: 325 pmol/l (normal: 90–760 pmol/l). Thoracoabdominal computerized tomography (CT)-scan revealed the presence of a large heterogeneous left adrenal tumor measuring 12 cm in diameter highly suggestive of adrenocortical carcinoma. In addition, CT-scan showed that the tumor came into contact with the renal hilum and compressed renal artery. There was no evidence for extra-adrenal metastasis. The patient was then placed under mitotane (6 g/day orally; Lysodren, Laboratoire Hra Pharma, Paris, France) and spironolactone (150 mg/day orally; Aldactone, Laboratoire Pfizer, Paris, France) therapy in order to inhibit corticosteroid synthesis and cortisol binding to the mineralocorticoid receptor respectively as a preparation for adrenalectomy. However, three weeks later, aggravation of clinical and biological signs of hypercortisolism in spite of the above-mentioned treatments led to immediate surgical removal of the adrenal mass. Part of the tumor tissue was obtained for in vitro studies after informed consent of the patient. Pathological examination of the tumor confirmed the diagnosis of adrenocortical carcinoma (Weiss score: 9). Immediately after surgery, the patient developed cardiovascular collapse that rapidly resolved under treatment with vasopressor drugs and volume loading.
Toxic cytolytic hepatitis and pancytopenia led to the interruption of mitotane treatment. One month postoperative biological assays showed normalization of plasma renin (0.26 pmol/l) and urinary free cortisol (48 nmol/day; Table 1) levels as well as kalemia (3.5 mmol/l) in the absence of any antiglucocorticoid or antialdosterone treatment. A moderate reduction in plasma aldosterone levels was also noticed (236 pmol/l). Plasma cortisol level was normal at 0800 h (516 nmol/l, normal: 250–850 nmol/l) but was incompletely suppressed during a 1 mg dexamethasone suppression test (123 nmol/l, normal: <50 nmol/l). CT-scan did not show any residual adrenal tumor tissue but revealed the presence of small lung lesions suggestive of metastases. Mitotane could not be reintroduced because of persisting liver function abnormalities. Five months after surgery, hormonal assays revealed a reincrease in plasma renin (2.13 pmol/l), aldosterone (369 pmol/l), and urinary free cortisol concentration (2034 nmol/day) associated with the reappearance of hypokalemia (kalemia: 3.2 mmol/l; Table 1), and elevated DHEA-S (12.7 pmol/l), androstenedione (13.3 nmol/l) and testosterone (2.78 nmol/l) levels. CT-scan showed local recurrence of the tumor reaching 3.5 cm in diameter associated with multiple retroperitoneal lymphadenopathies, and liver, pulmonary, bone, and brain metastases. Cytotoxic chemotherapy was considered but the patient died a short time after cerebral hemorrhage probably favored by brain metastasis.

Histology and immunohistochemistry

Deparaffinized and rehydrated sections were stained with hematoxylin–eosin–saffron or kit RAL 555 (CML, Nemours, France). Sections were also incubated with antibodies directed against 17α-hydroxylase (1:100; provided by Drs V Luu The and G Pelletier, Laval University Medical Center, Québec, Canada), renin (2D12, 1/500; a gift of Dr P Corvol, INSERM U833, Collège de France, Paris, France), neuron-specific enolase (NSE, 1:200; Dako, Trappes, France), protein S100 (1:400; Dako), chromogranin A (1:300; Dako), synaptophysin (1:100; Dako), human mast cell tryptase (1/1000, ABD Serotec, Cergy Pontoise, France), 5-HT (1:4000; Sigma), and 5-HT7 receptor (1:100; Sigma). The sections were then incubated with streptavidin–biotin–peroxidase or alkaline phosphatase complex and the enzymatic activity was revealed with diaminobenzidine or permanent red, respectively (Envision System, Dako). Some tissue sections were counterstained with hematoxylin. Semiquantitative evaluation of immunohistochemical reactions was performed using a modified version of the score previously published by Zhang et al. (2003). This histological score integrates both the number of positive cells and staining intensity.

In situ hybridization

In situ hybridization for renin was performed as previously described (Sibony et al. 1995, Schutz et al. 1996). Briefly, paraffin sections were deparaffinized, rehydrated, heated in a microwave oven, and digested with proteinase K (Roche Diagnostics) before hybridization with the 35S-labeled riboprobes (3–4×105 c.p.m./section, Amersham Biosciences). Hybridization reactions were carried out using antisense probes, as previously reported (Juillerat-Jeanneret et al. 2004). Sense probes were used as controls. Sections were counterstained with toluidine blue.

Cell culture

Cell culture experiments were conducted as previously described (Bertherat et al. 2005). Briefly, explants of the tumoral tissue were enzymatically dispersed. Adrenocortical cells were cultured at 37 °C in 5% CO2. Incubation experiments of cells were conducted for 24 h after 2 days in culture with fresh Dulbecco’s modified Eagle’s medium (DMEM) (control experiments) or DMEM with ACTH (1–24; Synacthen, Novartis Pharma), Ang II (Sigma), or 5-HT (Sigma) in the absence or presence of SB 269970 (Sigma) and GR 113808 (Glaxo Group Research). Cells were incubated with each secretagogue for 24 h at 37 °C. Renin (Schering-Cis Bio International, Gif/Yvette, France), cortisol, and aldosterone (Immunotech-Beckman Coulter, Marseille, France) concentrations in culture medium were measured using RIA procedures. Results are expressed as mean ± S.E.M. and statistical significance was assessed by Bonferroni test after one-way ANOVA.

Results

Histological examination of the resected mass

The resected tumor presented as a capsulated ovoid mass weighing 430 g and measuring 13×9×5 cm. Histologically, the tumor was composed of multiple spherical lobules infiltrating the capsule and the peritumorous adrenal cortex (Fig. 1A). Invasion of blood vessels by tumor cells was also observed. Tumor lobules were delimited by thin capsules and separated by fibrovascular stroma (Fig. 1B). Incubation of tissue slices with 17α-hydroxylase antibodies produced weak and focal immunostaining in the residual normal cortex and intense immunolabeling in tumor lobules (Fig. 1B). 17α-hydroxylase-like immunoreactivity was detected in clusters of large spongiocytic cells containing abundant lipid droplets in peritumorous adrenocortical tissue.
(Fig. 1E) and the majority of small compact cells in carcinoma parenchyma (Fig. 1F). Semiquantitative evaluation of immunoreactivity for 17α-hydroxylase suggested that steroidogenesis was more active in the tumor tissue than in the residual cortex (Table 2). Groups of 17α-hydroxylase negative cells were also observed in the neoplastic tissue (Fig. 1B3). Immunohistochemical experiments performed with antibodies against different markers of neurons and neuroendocrine cells showed that tumor lobules were immunopositive for synaptophysin (Fig. 1C and Table 2) and neuron specific enolase (Table 2), but were negative for protein S100 and chromogranin A (Fig. 1D; Table 2). By contrast, 17α-hydroxylase negative cells were labeled by both synaptophysin and chromogranin A antisera, indicating that they corresponded to chromaffin cells (Fig. 3C and D; Table 2).

**Figure 1** Histological characterization of the resected mass. (A) Low magnification view (HES coloration) showing the histological structure of the adrenal lesion. The tumor tissue (TT) was composed of multiple lobules (L). Inset, infiltration of the tumor capsule (TC) and peritumorous adrenocortical tissue (PAT) by carcinoma cells was observed (arrows). The localization of the microscopic fields presented in B–F is shown by empty squares (B–D (1–3), E and F). AC, adrenocortical capsule; M, medulla. (B–D) Immunohistological localization of 17α-hydroxylase (B; 17α-OH), synaptophysin (C; SYN) and chromogranin A (D; CGA) in tumor lobules. Labeling was revealed by permanent red in B and by diaminobenzidine (brown coloration) in C and D. Interlobular capsules and stroma are designated by arrows (B1–3). (E and F) Higher magnification views showing the cellular distribution of 17α-hydroxylation immunoreactivity. Labeled spongiocytic cells in peritumorous tissue (E) and compact cells in tumor tissue (F).
Immunohistological detection of 5-HT in the tumor tissue

Mast cells are the unique source of 5-HT in the normal human adrenal gland (Lefebvre et al. 1992, 2001). We have therefore investigated the presence of mast cells in the adrenocortical carcinoma by immunohistochemistry using specific antibodies to the mast cell marker tryptase and RAL 555 coloration. Clusters of tryptase-positive mast cells were detected in both the subcapsular region of the peritumorous adrenal cortex and the tumor tissue itself (Fig. 2B and C; Table 2). RAL 555-stained mast cells were observed in the vicinity of stromal vascular structures in close contact with spongiocytic and compact cells (Fig. 2D and E; Table 2). Incubation of carcinoma slices with 5-HT antibodies produced heterogeneous labeling in both peritumorous and tumor tissues (Fig. 2F). In the carcinoma tissue, the semiquantitative histological score varied from 1 to 3 (mean score: 2) among tumor areas (Table 2). As expected, 5-HT-like immunoreactivity was detected in mast cells located in the vicinity of blood vessels (Fig. 2G). In addition, the labeling was observed in subpopulations of spongiocytic cells in the peritumorous adrenal cortex and compact cells in the tumor tissue (Fig. 2G and H; Table 2). Conversely, no labeling could be observed in adrenal medulla (Table 2).

Effect of 5-HT on cortisol secretion by cultured tumor cells and immunohistochemical detection of 5-HT7 receptor

Cultured tumor cells were found to secrete cortisol (Fig. 3A and B). By contrast, aldosterone was undetectable in cell culture supernatant after incubation with DMEM alone or DMEM with 5-HT \((10^{-5} \text{ M})\), ACTH \((10^{-8} \text{ M})\) or Ang II \((10^{-7} \text{ M})\); data not shown. We have tested whether 5-HT could modulate cortisol production. Graded concentrations of 5-HT \((10^{-9} \text{ M}–10^{-6} \text{ M})\) provoked a dose-dependent increase in cortisol synthesis with modest potency \((\text{pEC}_{50} = 8.4 \pm 0.2; n = 4)\) and efficacy \((E_{\text{max}} = + 31.1 \pm 2.7\%; n = 4; \text{Fig. 3A})\) while ACTH \((10^{-9} \text{ M})\) did not significantly modify cortisol release (Fig. 3A, inset). The 5-HT4 receptor antagonist GR113808 \((10^{-7} \text{ M})\) weakly shifted the 5-HT response curve to the right yielding a mean \(\text{pEC}_{50}\) of \(7.8 \pm 0.1\) \((n = 4; P < 0.001)\), but did not significantly modify the maximum stimulatory effect of 5-HT on cortisol secretion \((E_{\text{max}} = +28.4 \pm 1.1; n = 4; P > 0.05; \text{Fig. 3A})\). SB269970 \((10^{-7} \text{ M})\), a specific 5-HT7 receptor antagonist exhibiting inverse agonistic properties (Rauly-Lestienne et al. 2007), induced a significant inhibition of cortisol production \((P < 0.001)\) and abolished the cortisol response to 5-HT \((10^{-6} \text{ M}; \text{Fig. 3B})\). The results of the cell incubation studies, suggesting the involvement of 5-HT7 receptors in the cortisol response to 5-HT, prompted us to investigate the presence of the 5-HT7 receptor protein in the tumor tissue. We observed that compact steroidogenic tumor cells were intensely stained by antibodies to 5-HT7 receptor (Fig. 3C).

Synthesis and release of renin by the tumor tissue

The expression of the renin gene was investigated by in situ hybridization. An intense hybridization signal was detected in tumor endothelial cells (Fig. 4A). The presence of renin mRNA was also observed in clusters of compact tumor cells (Fig. 4B). Labeling of tissue slices with the renin monoclonal antibody revealed the occurrence of immunoreactive cells, either isolated or arranged in small clusters disseminated in the carcinoma tissue (Fig. 4C). We have also investigated the production of renin by cultured tumor cells. Detectable amounts of immunoreactive renin were measured in culture medium (Fig. 4D). Application of 5-HT \((10^{-5} \text{ M})\) to cultured cells reduced renin release by 59.3\% \((P < 0.0001)\). By contrast, Ang II \((10^{-7} \text{ M})\) and ACTH \((10^{-8} \text{ M})\) did not significantly modify renin secretion (Fig. 4D).

Discussion

We have previously shown that, in benign adrenocortical carcinoma by immunohistochemistry using specific antibodies to the mast cell marker tryptase and RAL 555 coloration. Clusters of tryptase-positive mast cells were detected in both the subcapsular region of the peritumorous adrenal cortex and the tumor tissue itself (Fig. 2B and C; Table 2). Immunohistological experiments assessing the presence of mast cells in the adrenocortical carcinoma showed heterogeneous labeling in both peritumorous and tumor tissues. Graded concentrations of 5-HT (10^{-9} M–10^{-6} M) provoked a dose-dependent increase in cortisol synthesis with modest potency (pEC_{50} = 8.4 \pm 0.2; n = 4) and efficacy (E_{max} = +31.1 \pm 2.7\%; n = 4; Fig. 3A). ACTH (10^{-9} M) did not significantly modify cortisol release (Fig. 3A, inset). The 5-HT4 receptor antagonist GR113808 (10^{-7} M) weakly shifted the 5-HT response curve to the right yielding a mean pEC_{50} of 7.8 \pm 0.1 (n = 4; P < 0.001), but did not significantly modify the maximum stimulatory effect of 5-HT on cortisol secretion (E_{max} = +28.4 \pm 1.1; n = 4; P > 0.05; Fig. 3A). SB269970 (10^{-7} M), a specific 5-HT7 receptor antagonist exhibiting inverse agonistic properties (Rauly-Lestienne et al. 2007), induced a significant inhibition of cortisol production (P < 0.001) and abolished the cortisol response to 5-HT (10^{-6} M; Fig. 3B). The results of the cell incubation studies, suggesting the involvement of 5-HT7 receptors in the cortisol response to 5-HT, prompted us to investigate the presence of the 5-HT7 receptor protein in the tumor tissue. We observed that compact steroidogenic tumor cells were intensely stained by antibodies to 5-HT7 receptor (Fig. 3C).
eutopic 5-HT$_4$ or ectopic 5-HT$_7$ receptors (Cartier et al. 2003, Bertherat et al. 2005, Louiset et al. 2006). However, the effect of 5-HT on malignant adrenocortical tissues had never been investigated. We report a case of adrenocortical carcinoma responsible for severe Cushing’s syndrome associated with high plasma levels of renin. In vitro studies revealed the presence of 5-HT-like immunoreactivity in mast cells, as formerly described in the normal adrenal gland (Lefebvre et al. 1992). 5-HT was also detected in some steroidogenic cells disseminated in both the adrenocortical peritumorous and tumor tissues. Since

Figure 2 Immunohistological localization of 5-HT. (A) Low magnification view (HES coloration) showing the localization of the microscopic fields (empty squares) presented in B–H. L, tumor lobule; PAT, peritumorous adrenocortical tissue. (B and C) Immunohistochemical localization of tryptase (TRY). Tryptase-positive cells, i.e. mast cells, were observed in the peritumorous (B) and tumor (C) tissues. AC, adrenocortical capsule; C, capsule. (D and E) RAL 555 coloration showing the perivascular distribution of mast cells (arrows) in peritumorous (D) and tumor (E) tissues (*, blood vessels). (F–H) Immunohistochemical localization of 5-HT. (F) 5-HT-positive cells were observed in the peritumorous and tumor tissues. (G) Detection of 5-HT-like immunoreactivity in cells harboring the morphological characteristics of mast cells in adrenocortical peritumorous tissue (arrows). (H) 5-HT-positive compact cells in the tumor tissue.
adrenocortical cells do not physiologically contain 5-HT (Lefebvre et al. 1992), it seems highly probable that peritumorous 5-HT-positive cells corresponded to tumor cells. This hypothesis is supported by the observation that the tumor capsule and peritumorous adrenal cortex were clearly invaded by carcinoma cells. The occurrence of 5-HT in a subpopulation of tumor cells likely reflects neuroendocrine differentiation of the adrenocortical carcinoma. In agreement with this assumption, the tumor was found to express synaptophysin and NSE, as previously noticed in published series of malignant tumors of the adrenal cortex (Miettinen 1992, Haak & Fleuren 1995). The detection of 5-HT in the carcinoma tissue also suggested that the indolamine may have controlled the secretory activity of tumor cells through autocrine/paracrine mechanisms, in very much the same way as in the normal adrenal cortex and benign adrenocortical secreting lesions (Contesse et al. 2000, 2005, Bertherat et al. 2005).

We have therefore investigated the action of 5-HT on the secretory activity of cultured tumor cells. 5-HT was found to provoke a significant increase in cortisol production by tumor cells. Interestingly, the efficacy of 5-HT to stimulate cortisol secretion from tumor cells (i.e. +31%) was similar to that previously observed in a benign adrenocortical cortisol-producing tumor associated with an exaggerated in vivo cortisol response to serotonergic agonists (i.e. +36.5%; Contesse et al. 2005), suggesting that 5-HT could play a role in vivo in the regulation of cortisol production by the tumor tissue. Although the heterogeneity of the distribution of 5-HT-like immunoreactivity indicates that the influence of intra-adrenal 5-HT on cortisol secretion may be negligible in some areas of the tumor, we cannot exclude a physiopathological role of 5-HT released by platelets after local aggregation evoked by vascular damage due to blood vessel invasion by tumor cells. We also observed that ACTH did not modify cortisol secretion consistently with the well demonstrated lack of expression of functional ACTH receptors in adrenocortical carcinomas (Beuschlein et al. 2001).

5-HT-evoked stimulation of cortisol secretion was only slightly influenced by the specific 5-HT4 receptor antagonist GR113808, indicating that the eutopic serotonergic adrenal receptor was weakly involved in the steroidogenic action of 5-HT on tumor cells. By contrast, the specific 5-HT7 receptor antagonist and inverse agonist SB269970 inhibited cortisol production.

**Figure 3** Effect of 5-HT on adrenocortical carcinoma cells. (A) Effect of graded concentrations (from $10^{-9}$ to $10^{-6}$ M) of 5-HT on cortisol secretion by cultured cells derived from the adrenocortical carcinoma tissue in the absence (■) or in presence of the 5-HT4 receptor antagonist GR 113808 ($10^{-7}$ M; □). Inset, absence of effect of ACTH ($10^{-8}$ M) on cortisol secretion. (B) Effect of 5-HT ($10^{-6}$ M), the 5-HT7 receptor antagonist and inverse agonist SB269970 ($10^{-7}$ M), and 5-HT + SB269970 on cortisol secretion by cultured cells derived from the adrenocortical carcinoma tissue. *P<0.05; **P<0.01. Basal cortisol concentrations in culture medium were 2.98±0.35 nmol/l. (C) Immunohistochemical localization of 5-HT7 receptor in the adrenocortical carcinoma tissue. Inset, High magnification microphotograph showing 5-HT7 receptor immunoreactivity in the cytoplasm and at the periphery of a tumor cell. TT, tumor tissue; PAT, peritumorous adrenocortical tissue; TC, tumor capsule.
and abolished the glucocorticoid response to 5-HT. These results indicate that the stimulatory action of 5-HT on cortisol secretion by tumor cells was mainly mediated by ectopic 5-HT$_7$ receptors, as already observed in bilateral macronodular adrenal hyperplasias causing Cushing’s syndrome (Louiset et al. 2006). The presence of 5-HT$_7$ receptors in the tumor tissue was further established by the detection of 5-HT$_7$ receptor immunoreactivity in tumor cells.

Several mechanisms could be involved in the high preoperative plasma renin levels as well as their dramatic decrease after surgical removal of the tumor. Compression of the left renal artery by the tumor visualized by CT-scan and hypercortisolism could have favored renin hypersecretion by juxtaglomerular apparatus (Sacerdote et al. 2005, Magiakou et al. 2006). It was also conceivable that carcinoma may have synthesized and secreted substantial amounts of renin, as formerly reported (Iimura et al. 1986, Racz et al. 1992, Yamanaka et al. 2000). In agreement with this latter hypothesis, we were able to detect the occurrence of mRNAs encoding renin and the renin protein in the tumor tissue by use of in situ hybridization and immunohistochemistry, respectively. In addition, we observed the presence of substantial amounts of immunoreactive renin in tumor cell culture supernatant. By contrast with its effect on cortisol production, 5-HT was found to inhibit renin secretion by tumor cells, whereas ACTH and Ang II had no effect. This observation indicates that the carcinoma tissue expressed other ectopic serotonergic receptors than the 5-HT$_7$ receptor that is known to be positively coupled to adenylyl cyclase (Thomas & Hagan 2004). These receptors, that could not be characterized, may belong to the 5-HT$_1$ or 5-HT$_5$ types that are known to be negatively coupled to adenylyl cyclase (Lanfumey & Hamon 2004, Nelson 2004). The paradoxical inhibitory action of 5-HT on renin secretion by tumor cells also indicates that illegitimate membrane receptors can sometimes

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**Figure 4** Synthesis and release of renin by the tumor tissue.
(A) Localization of mRNAs encoding renin in blood vessel walls. An intense hybridization signal was detected in tumor endothelial cells (arrows). BV: blood vessel. (B) Localization of mRNAs encoding renin in tumor cells. The hybridization signal was observed in small groups of compact steroidogenic cells (arrows). (C) Immunohistochemical localization of renin in the carcinoma tissue. Renin-positive cells were scattered (arrows) or arranged in clusters disseminated in the adrenocortical tissue. TT, tumor tissue; PAT, peritumorous adrenocortical tissue; TC, tumor capsule. (D) Effect of 5-HT, angiotensin II (Ang II) and ACTH on renin secretion by cultured tumor cells. Inhibitory effect of serotonin (5-HT; $10^{-9}$ M) contrasting with the absence of effect of Ang II ($10^{-8}$ M) and ACTH ($10^{-8}$ M) on renin release. ***$P<0.001$. 

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contribute to limit the secretory activity of functional adrenocortical neoplasms. Collectively, our results show that 5-HT exerted complex actions on tumor cells, including both a stimulatory effect on cortisol production and an inhibitory effect on renin release that involved multiple types of receptors.

Hypokalemia observed both preoperatively and at the time of recurrence of the tumor was probably the result of severe hypercortisolism. Indeed, decreased plasma potassium level is a rather common observation in this condition (Niemann 2001). Hypokalemia is then classically attributed to the mineralocorticoid action of cortisol which becomes clinically apparent when cortisol levels are very high, owing to saturation of 11β-hydroxysteroid dehydrogenase type 2 by glucocorticoid excess (Stewart 1999). Co-secretion of mineralocorticoid precursors, such as deoxycorticosterone, by the tumor would be another potential explanation, as formerly observed (Messer et al. 2007).

A clear dissociation between renin and aldosterone levels was surprisingly observed during both preoperative and postoperative periods in the absence of any treatments capable of influencing the renin–angiotensin–aldosterone system. Indeed, preoperative plasma aldosterone levels were within the normal range in spite of elevated circulating renin and, consequently, high Ang II levels. The discrepancy between aldosterone and renin concentrations could have resulted from autonomous secretion of aldosterone by the tumor (Iimura et al. 1986, Messer et al. 2007). The slight postoperative decrease in plasma aldosterone levels and the absence of detectable aldosterone amounts in tumor cell culture medium allow to exclude this hypothesis and indicate that plasma aldosterone originated from the contralateral normal adrenal gland and not from the carcinoma. Another explanation would be that the tumor may have secreted inactive renin. However, we could not verify this potential mechanism since plasma renin activity was not measured. It is also possible that the lack of elevation of plasma aldosterone concentration was the consequence of hypokalemia which is known to lower in vivo the stimulatory effect of Ang II on aldosterone secretion by the zona glomerulosa (Vallotton et al. 1995). During the postoperative period, the moderate decrease in aldosterone levels (−27%) observed postoperatively did not parallel the important decline in renin concentrations (−88%). It is conceivable that the decrease in aldosterone levels induced by the fall of renin concentration may have been attenuated by a concomitant stimulation of aldosterone production evoked by both the increase in kaliemia and diminution in circulatory volume secondary to the regression of hypercortisolism. Finally, plasma hormone assays revealed that the patient’s adrenal carcinoma was producing androgens in addition to renin and cortisol.

In conclusion, our results provide the first demonstration of ectopic serotonin receptors, i.e. 5-HT7 receptors, in an adrenocortical carcinoma tissue. The present report also shows that 5-HT exerted complex effects on the secretory activity of the malignant adrenocortical tumor, combining stimulatory and paradoxical inhibitory actions on cortisol and renin secretions, respectively.

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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