CASE REPORT

Major hyperestrogenism in a feminizing adrenocortical adenoma despite a moderate overexpression of the aromatase enzyme

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Abstract

A 30-year-old male was referred for the rapid development of gynecomastia, and dramatic hyperestrogenemia was assessed: plasma estrone, estradiol but also cortisol were not suppressed by high-dose dexamethasone, while gonadotropin pulsatility was completely abolished. A 60-mm right adrenal tumor was evidenced on computed tomography-scan, and the patient underwent adrenalectomy. The tumor was found to express a moderate increase in aromatase activity compared with adjacent non-neoplastic adrenal tissue. Quantitative RT-PCR also showed a weak and non-significant increase in total aromatase mRNA in the tumor compared with normal adrenal tissue. Aromatase transcripts were mainly promoter PII-derived, but different patterns of aromatase minor transcripts were found: promoter I.3- and I.6-derived transcripts were identified in the tumor, while only promoter I.4-derived transcripts were found in normal adrenal. This case report demonstrates that a sharp aromatase overexpression is not a prerequisite for clinical and biochemical hyperestrogenism, and further characterizes the aromatase promoter utilization in this feminizing adrenocortical tumor and in the normal adrenal cortex.

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Introduction

Feminizing adrenal tumors are rare (1, 2) and few cases were extensively studied in order to characterize the adrenal origin of estrogen production and the involvement of aromatase P450 overexpression. Recent case reports have demonstrated the presence of aromatase immunoreactivity (3), high aromatase enzyme activity (3–5) and overexpression of CYP19 mRNA (5, 6) in adrenocortical feminizing tumors. In the present study, the hormonal, enzymatic and molecular characterization of one feminizing adrenal tumor occurring in a young adult male allowed us to observe a moderate overexpression of aromatase activity together with a mild parallel increase in aromatase transcripts, opening discussion on the mechanism of the marked estrogen overproduction evidenced by the plasma steroid pattern in this patient. Aromatase promoter utilization was further characterized within the tumoral and the normal adrenal cortex.

Case report

A 30-year-old man was admitted with a complaint of severe progressive bilateral gynecomastia associated with a 12 kg weight gain within the last 18 months, without other clinical hallmarks of Cushing's syndrome. The patient denied any hormone, drug or alcohol consumption. Physical examination and testicular echography revealed testis of normal size (45 × 25 mm) and structure, normal virilization, and Tanner IV breasts. Blood pressure was 130/80 mmHg. Abdominal computed tomography revealed a 6-cm right adrenal mass. Hormonal studies (Table 1) showed high plasma estrone (E1) (6- to 10-fold) and estradiol (E2) (3- to 6-fold) levels, high delta-4-androstenedione (Δ 4-A) and 17-hydroxyprogesterone (17OHP) levels but low testosterone and low apulsatile gonadotropin levels unresponsive to gonadotropin-releasing hormone (GnRH) stimulation (data not shown). Human chorionic gonadotropin (hCG) was undetectable. Free urinary cortisol was normal high (110 μ g/day), but a high dose dexamethasone test

Table 1 Plasma hormone levels before and one month after adrenalectomy (AdrX).

	Before AdrX	After AdrX	Normal values
E1 (pg/ml)	466	63	15–90
E2 (pg/ml)	120	24	8–45
Testosterone (ng/ml)	3	8.8	3–8
SHBG (nmol/l)	43	31	16–38
Δ4-androstenedione (ng/ml)	6	0.75	0.8–2.5
17-OH-progesterone (ng/ml)	74	2.4	0.6–2
DHEA (ng/ml)	2.1	nd	1–10
DHEA-S (ng/ml)	1200	nd	700–3900
11 Deoxycortisol (ng/ml)	6	0.69	0.2–1.1
Cortisol (ng/ml)	130	130	100–250
Aldosterone (pmol/l)	78	82	30–300
β-hCG (mU/ml)	<0.1	<0.1	(<0.1)
Basal LH (mU/ml)	<0.3	1.9	(3.5–10)
LH/GnRH (mU/ml)	4	32	9–21
Basal FSH (mU/ml)	0.4	1.5	2–6.5
FSH/GnRH (mU/ml)	0.4	4	3.5–10

SHBG, sex hormone binding globulin; DHEA, dehydroepiandrosterone; DHEA-S, DHEA sulfate; LH, luteinizing hormone; FSH, follicle-stimulating hormone; nd, not determined.

(8 mg/day for 2 days) failed to suppress plasma cortisol (160 ng/ml), E1 (1066 pg/ml) and E2 (210 pg/ml) plasma levels. Exogenous adrenocorticotropin (ACTH) and corticotropin-releasing hormone (CRH) failed to stimulate plasma cortisol, testosterone, E1 and E2. The patient underwent a right coelioscopic adrenalectomy which revealed an adrenocortical tumor of 55 × 60 × 50 mm size and 100 g weight. Pathological examination revealed a proliferation of pseudo-follicular large cells with sinusoidal but no vascular invasion and no evidence of atypical mitotic figures. No hemorrhage or necrosis were identified on the cut surface of the tumor. The Weiss and Hough score was 4/9. Immunocytochemistry was positive for chromogranin and KL-1 (epithelial marker). One month after surgery, the patient exhibited normal E1 and E2 levels, normal androgen levels and recovery of gonadotropin pulsatility (data not shown) and response to GnRH (Table 1). Within a 13-month follow-up period, no recurrent disease was identified by biological markers, thorax and abdominal magnetic resonance imaging and computed tomography.

Materials and methods

Tissue steroid concentrations

One tumor fragment and one normal adrenal fragment were homogenized in a phosphate buffer solution (0.1 mol/l, pH 7.4) and centrifuged at 800 *g* for 5 min. The supernatant was centrifuged at 100 000 *g* for 60 min. Supernatants from both tumor fragments were pooled, and E1, E2, Δ4-A and 17OHP were measured by RIA. Aromatase activity was determined in the pellet.

Aromatase activity assay

Aromatase activity was determined in fragments from the adrenal tumor, from the non-neoplastic adrenal tissue adjacent to the tumor, and from three normal human adrenals and rabbit granulosa cells as control. The aromatase assay of the particulate fraction was estimated based on the incorporation of tritium from [^{14} C]-androst-4-ene-3,17-dione (24 Ci/mmol, New England Nuclear, NEN Life Science Products, Paris, France) into ^{3} H₂O as described by Conley *et al.* (7). A reagent blank was set up by substituting 1 mg/ml BSA (bovine serum albumin) in the particulate fraction's buffer. The aromatase activity in tissue was inhibited by letrozole (Novartis Pharma), a non-steroid aromatase inhibitor, in order to assess the specificity of the measured aromatase activity.

Aromatase mRNA quantification by RT-PCR

Aromatase transcripts were measured in fragments from the adrenal tumor, from the non-neoplastic adrenal tissue adjacent to the tumor, and from three normal human adrenals. Total RNA was isolated from the tumor and from normal adrenal tissues as described by Chomczynski & Sacchi (8). The cDNAs obtained by retrotranscription with oligo-dT₁₂₋₁₈ were amplified using a polymerase chain reaction with the primers described in Table 2. DNA bands were visualized after staining with ethidium bromide and the intensity of the bands was evaluated with a camera (Vilber Lourmat, France). The sequences of PCR products were controlled by direct sequencing with ABI377 sequencer (Applied

Table 2 Oligonucleotides used as primers for PCR amplifications.

ARO EX10F	5' end sense oligo from exon X 5' TTT GAC CCT TGC TGT GAA TTA AGC CC 3'
ARO EX10R	3' end antisense oligo from exon X 5' CAT GGG AAG AAA GAG GGA GAA AAT GTC G 3'
ARO P.II	5' end sense oligo from exon P.II 5' GGG GAT TCT GTA ATT CTG TCC CT 3'
ARO I.3	5' end sense oligo from exon I.3 5' ATG ATA AGG TTC TAT CAG ACC AAG CGT C 3'
ARO I.4	5' end sense oligo from exon I.4 5' GGC TCC AAG TAG AAC GTG ACC AAC TG 3'
ARO COM	3' end antisense oligo from coding exon II 5' CAG CCC AAG TTT GCT GCC GAA 3'
GAPDH F	5' end sense oligo from GAPDH 5' GTG CTC AGT GTA GCC CAG GAT GC 3'
GAPDH R	3' end antisense oligo from GAPDH 5' TCA TCC ATG ACA ACT TTG GTA TCG TG 3'

Biosystems, Courtaboeuf Cedex, France). The quantitation of aromatase transcripts was performed as described by Breard *et al.* (9). PCR products were visualized on a 3% non-denaturing agarose Metaphore gel with ethidium bromide staining, and the quantitation of individual cDNA was based on the assumption that bands of equal amounts of PCR products from an individual cDNA and from the corresponding internal standard exhibited identical photometric intensity. The quantitation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed in all samples and the results were expressed as the amount of aromatase transcript/amount of GAPDH transcript.

Statistical analysis

The comparison of mRNA levels measured in the tumor and in the adjacent non-neoplastic adrenal fragments was performed by Student's *t*-test. The level of significance was $P < 0.05$.

Results

Steroid measurements in adrenal tissues showed that E2 and E1 concentrations were respectively 14-fold and 7-fold higher in the tumor than in normal adrenal. 17OHP and testosterone concentrations were respectively twofold and threefold higher in the tumor than in normal adrenal, while $\Delta 4$ -A concentrations were comparable in both tissues (Table 3).

Enzymatic experiments showed that aromatase activity was sevenfold higher in the tumor compared with the adjacent non-neoplastic adrenal cortex and only weakly enhanced in comparison with three normal human adrenal controls. Aromatase activity measured in granulosa cells used as a positive control was threefold higher than in the adrenal tumor (Table 3). Addition of letrozole blunted aromatase activity in all adrenal extracts.

Molecular studies identified aromatase mRNA in all adrenal fragments from tumor and control human adrenals. Quantitation of aromatase mRNA was thus performed by quantitative RT-PCR analysis, which

showed a weak and non-significant increase in the total aromatase mRNA in the tumor compared with the adjacent non-neoplastic adrenal, while the former was not different from three normal human adrenal controls which also expressed low levels of aromatase mRNA (Table 4). Promoter analysis was then performed after a 30-cycles RT-PCR, identifying PCR products from the tumor and from normal human adrenal cortex mainly derived from exon PII (Fig. 1A). After a second 30-cycles amplification, nested PCR revealed the presence of low levels of secondary PCR products, with different profiles in both tissues: the tumor expressed low PI.4-derived transcript levels compared with normal human adrenal cortex (ratio 1/30 to 1/70), while PI.3- and PI.6-derived transcripts were weakly expressed in the tumor but not expressed in the normal human adrenal cortex (Fig. 1B).

Discussion

In this study, a feminizing adrenal adenoma in a man exhibiting very high circulating levels of estrogens was extensively analyzed. Biochemical, enzymatic and molecular experiments surprisingly revealed only moderate enhancement of aromatase expression in this tumor when compared with the low measurable aromatase expression detected in the normal human adrenal cortex.

Previous investigations on feminizing adrenocortical tumors have emphasized the very high aromatase activity measured in these tumors, indeed at levels in the order of magnitude of placental aromatase activity (3–6, 10). The protein expression was detected within estrogen-producing tumors by Western blot and by immunohistochemistry (3, 10), with the demonstration of an immunoreactivity in the tumor cells but not in the adjacent non-neoplastic adrenal cells (3). Molecular studies by RT-PCR techniques have confirmed the expression of aromatase mRNA in an estrogen-producing adrenal tumor (5), and allowed the measurement of high levels of aromatase mRNA by quantitative RT-PCR (6, 10). Normal human adrenal was previously considered to express very low, if any, aromatase mRNA concentrations (4, 6, 10). Our results

Table 3 Steroid concentrations and aromatase activity in adrenal tumor, non-neoplastic adrenal (control) and rabbit granulosa cells. Aromatase activity was measured before and after addition of the aromatase inhibitor letrozole.

	Tumor	Adjacent adrenal	Control adrenal	Rabbit granulosa
E2 (ng/g protein)	55		4	
E1 (ng/g protein)	109		17	
$\Delta 4$ -A (ng/g protein)	43 250		53 570	
Testosterone (ng/g protein)	3250		1178	
17OHP (ng/g protein)	15 750		8930	
Aromatase activity (fmol/mg protein/h)	750	105	326 $\pm$ 109*	2350
+ Letrozole 10 nmol/l	47	15	44 $\pm$ 26*	89

* Results expressed as mean of three controls $\pm$ S.E.M.

Table 4 Aromatase mRNA quantification. mRNA was measured as attomoles aromatase mRNA and attomoles GAPDH mRNA ($\pm$ s.d.). Measurements were performed in triplicate for adrenal tumor and adrenal adjacent to the tumor, and in duplicate for healthy human adrenals.

Aromatase/GAPDH (mean $\pm$ s.d.)	
Adrenal tumor	0.12 $\pm$ 0.09
Adjacent adrenal	0.05 $\pm$ 0.13
Normal adrenal 1	0.14
Normal adrenal 2	0.16
Normal adrenal 3	0.03

Figure 1 After reverse transcription, P450 aromatase complementary DNA samples were amplified using primers specific for unique untranslated 5' ends of P450 aromatase transcripts. (A) Aromatase exon 10 (Arom EX 10) and promoter PII-specific transcripts amplified with 30 cycles of PCR. GAPDH transcripts were amplified in total RNA samples to confirm the use of comparable amounts of intact RNA. (B) Promoters PI3-, PI4- and PI6-specific transcripts amplified with nested PCR (two times 30 cycles of PCR). 1, normal adrenal; 2, adrenal tumor; 3, granulosa cells; Blc, blank PCR; STD, 50 bp DNA ladder.

are in contrast, since aromatase activity measured by the same tritium water-releasing method was only 0.7 pmol/mg protein/h in the present tumor, a level much lower than those reported previously, which ranged between 30 and 6000 pmol/mg protein/h (3, 4, 6, 10). Accuracy of our enzymatic study was assessed by the level of aromatase activity in granulosa cells which was in the same order of magnitude as previously reported in the rat ovary (11). Despite this low aromatase activity, we were able to compare the tumor and the non-neoplastic adjacent adrenal tissue, and found a sevenfold higher aromatase activity in

the former. Estrogen concentrations were higher in the tumor compared with the control adrenal, in the same order of magnitude as for the enzyme activities. Using a quantitative RT-PCR protocol, we found a weak but not significant increase in aromatase transcripts in the tumor compared with the adjacent non-neoplastic adrenal. The contrast between an increased aromatase activity in the tumor compared with adjacent non-neoplastic adrenal and the control adrenals, and the absence of an overexpression of aromatase mRNA in the tumor suggests translational modifications such as an increased translational level or an increased half-life of the translated protein. Although we cannot exclude a non-homogenous expression of the aromatase enzyme within the tumor due to putative endogenous aromatase inhibitors, the present results suggest that hyperestrogenemia may result from the tumor production of the precursor Δ 4-A which provides substantial amounts of estrogens from a huge mass of steroidogenic tumoral tissue exhibiting a moderate aromatase activity. Despite high circulating levels of Δ 4-A, its concentration in the tumor was similar to normal adrenal, which suggests that Δ 4-A was more rapidly metabolized in the tumor than in the normal adrenal. We cannot exclude the possibility that a part of the circulating estrogens may issue from extra-adrenal aromatization (12) which may be enhanced by inductive factors for aromatase from the tumor, such as eicosanoids or cytokines (13–15). Our findings, based on enzymatic studies and quantitative PCR techniques, also suggest that aromatase is weakly expressed in the normal human adrenal, as observed by Kimura *et al.* (4). These data are in accordance with previous studies based on adrenal vein catheterization (16, 17) and cell culture studies (18) which suggested production of estrogens by the human adrenal glands. As a whole, our results suggest that a moderate aromatase activity exists in normal human adrenal cortex as in the adrenal tumor investigated in this case report. A mass effect from this steroidogenic tumor which maintains a capacity for aromatization may explain the actual clinical and biochemical hyperestrogenism.

Another concern was to further characterize at the molecular level the regulation of aromatase expression in this adrenal tumor. Previous reports have highlighted that the gonadal promoter PII is mainly responsible for the expression of aromatase in feminizing adrenal tumors, with an exclusive PII pattern (5) or with the co-expression of PII and the minor proximal PI.3-derived transcript normally expressed in the adipose tissue (6, 10). In the present work, we were able to confirm the presence of the major transcript PII in the tumor as in the adjacent non-neoplastic adrenal and in normal adrenals, but we also detected specific patterns for minor transcripts in both tissues: in the tumor, PI.3-, PI.4- and PI.6-derived transcripts were detected at very low levels, while in normal adrenal,

only PI.4 was detected at higher levels than in the tumor. Although the significance of such patterns of aromatase promoter utilization remains unknown, the switching of the alternative exon I may result from the expression of regulatory factors in tumoral tissues, as previously observed in breast cancer (19).

In conclusion, a low level expression of the aromatase enzyme and of aromatase transcripts was demonstrated in this feminizing tumor as in the normal adrenal cortex, with a switch in promoter utilization characterizing non-neoplastic to neoplastic transition. This work provides a clue for an expression of aromatase in the normal human adrenal cortex, which needs to be confirmed by further studies.

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