CLINICAL STUDY

Aromatase expression in the normal human adult adrenal and in adrenocortical tumors: biochemical, immunohistochemical, and molecular studies

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Abstract

Objective: The aromatase enzyme catalyzes the final stage of estrogen biosynthesis pathway from androgens. Its expression in the adrenal is poorly studied except for the rare estrogen-producing adrenocortical tumors. In order to further characterize aromatase expression in the adrenal, we evaluated the aromatase enzyme activity, Cyp19a1 gene expression level, and promoter utilization in normal adrenal tissues and in adrenocortical secreting tumors.

Design and methods: Six normal adult adrenals (NA), 2 feminizing adrenal tumors (FT), 10 cortisol-producing adrenomas with overt (CS, n ≤ 4) or sub-clinical Cushing syndrome (SCS, n = 6) and 3 aldosterone-producing adrenomas (AP A) were studied. Tissue aromatase activity was determined by the tritiated (3H)-water method. Total aromatase mRNA were measured by a competitive RT-PCR. Promoter regions PII and PI.4-derived transcripts were also studied in NA, FT, and other steroid-producing tumors by a semi-quantitative comparative RT-PCR. Immunofluorescence analysis was performed in normal human adrenal tissues.

Results: Aromatase activity was detected in NA tissues and in all tumor subtypes; at high levels in both FT. In NA, aromatase immunofluorescence was detected in the cytoplasm of steroidogenic cells, mainly from zona reticularis. Compared with NA, aromatase transcript levels were similar in CS and AP A, lower in SCS and similar or higher in FT. Promoter analysis suggested predominant PII utilization in NA, AP A, and SCS, but similar PII and PL.4 utilization in CS tumors.

Conclusion: Aromatase is expressed at similar levels in normal adrenal and in adrenocortical tumors, but at variably high levels in FT. Different promoter utilization patterns are found among tumor subtypes.

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Introduction

The enzyme cytochrome aromatase P450 is a microsomal protein that catalyzes the final stage of estrogen biosynthesis pathway from its androgen substrate. The aromatase is encoded by the Cyp19a1 gene characterized by several untranslated exons expression of which relies on alternative promoters submitted to a tissue-specific regulation (1). The enzyme is widely expressed in steroidogenic tissues such as gonads and placenta but also in adipose tissue, brain, and bone which are able to synthesize estrogens (2). There is indirect evidence that the normal human adrenal cortex is also a source for estrogen production (3, 4) but the molecular mechanisms assigned in such biosynthesis are still poorly characterized. Aromatase is expressed in the human adrenal medulla at all stages from postnatal until pubertal, but only the zona glomerulosa expresses the enzyme in the post-adrenarche period (5). Aromatase is overexpressed in feminizing adrenocortical tumors (6–10) but its expression has not yet been investigated, to our knowledge, in non-feminizing tumors of adrenal origin. The aim of the present study was to evaluate the aromatase enzyme activity and Cyp19a1 gene expression level in the normal adrenal cortex and in adrenal feminizing tumors together with other steroid-producing adrenocortical tumors, in order to further characterize its expression.

Design and methods

Ethical considerations

The protocol for tissue collection and the experimental procedure were approved by the regional ethics committees, and written informed consent was obtained from the patients.
Adrenocortical tissue materials

Normal adult adrenal (NA) tissues were obtained from patients undergoing expanded nephrectomy for kidney cancer (n=6, sex ratio 1:1). All layers of the adrenal cortex were included in the molecular study, and were histologically normal. Tumor tissues were obtained from 15 adult patients with either an adrenal feminizing tumor (FT, n=2, M/F sex ratio 1:1), a cortisol-producing adenoma associated with overt (CS, n=4, M/F sex ratio 1:3) or subclinical Cushing’s syndrome (SCS, n=6, M/F sex ratio 2:1) or an aldosterone-producing adenoma (APA, n=3, M/F sex ratio 0:3). Tumor tissues were obtained by surgery and immediately dissected by the pathologist. Adrenocortical fragments were rapidly frozen and stored at -80°C until enzyme assay or RNA extraction.

Patients

Patient FT1 was a 30-year-old male presenting with severe gynecomastia related to a 6×5.5×5 cm right adrenal tumor secreting estrone and estradiol at plasma concentrations respectively 5- and 2.5-fold higher than normal. The clinical and biochemical characteristics of patient FT1 were previously reported (10). Tumor mass was 100 g. Weiss score staging was 3 (11), and McFarlane–Sullivan staging was II (12). Patient FT2 was a 26-year-old female presenting with irregular menses, obesity, and hirsutism related to a 5-cm left adrenal tumor secreting cortisol (free urinary cortisol 1761 nmol/day, normal range 55–275), testosterone (3.8 nmol/l, normal range 0.347–2.43), and estrogens (estrone 32 081 pmol/l, normal range 56–330, estradiol 36 000 pmol/l, normal range 30–170). Tumor mass was 50 g. Weiss score staging was 3, and McFarlane–Sullivan staging was I. 17p13 loss of heterozygosity was not found in the tumor tissue.

Patients from CS group exhibited the typical features of Cushing’s syndrome and high-plasma and urinary cortisol levels together with suppressed plasma ACTH levels. Patients from SCS group had no signs of Cushing’s syndrome. Normal basal plasma and urinary cortisol levels but exhibited non-suppressible plasma cortisol level after 1 mg dexamethasone administration, morning ACTH plasma level in the low normal range and/or ipsilateral uptake on 131I-6β-iodomethynorchol-esterone adrenal scintigraphy. The patients from APA group presented with hypertension, hypokalemia, elevated plasma and/or urinary aldosterone concentrations, and elevated aldosterone to active renin ratio.

Aromatase activity assay

We used the technique for aromatase activity assay previously described by Conley et al. (13). Microsomes from each tissue were prepared by successive ultracentrifugations. All procedures were carried out at 4°C in buffer 0.1 M phosphate, MgCl2 5 mM, pH 7.4. Protein concentrations in the preparations were determined according to Bradford et al. (1976). Microsomal aromatase activity was evaluated by measuring [3H]2O released from 300 nM (1β-N-3H) androst-4-ene-3,17-dione (24 Ci/mmol, New England Nuclear, PerkinElmer, Waltham, MA, USA) at 37°C for 3 h. The microsomal protein was resuspended and diluted to 2 mg protein/ml in 0.1 M phosphate buffer. MgCl2 5 mM (pH 7.4) containing 22 mM ATP, 22 mM NADP, 110 mM glucose-6-phosphate, 25 IU glucose-6-phosphate dehydrogenase, 33 mM NAD, and 0.8 nmol/ml androstenedione. The reaction was stopped by adding 3 ml of ice cold chloroform in test tube and shaking vigorously. After centrifugation at 2000 g for 5 min. the chloroform layer was transferred in tube and the aqueous layer was re-extracted with further 3 ml chloroform. Duplicate 0.4 ml of aqueous phase was transferred into tubes containing 0.8 ml of 5% dextran-coated charcoal suspension. Tubes were vortex mixed and incubated at 4°C for 10 min then centrifuged at 2000 g for 15 min. One milliliter supernatant from one tube was transferred into counting vial containing 3 ml of liquid scintillator. Supernatant of the other tube was transferred into counting vial and evaporated before adding 3 ml of liquid scintillator and the radioactivity value was taken as control and subtracted. The vial’s radioactivity was measured using a Tri-carb 1900CA liquid scintillation analyzer (Packard Instrument Co.). For all experiments, 1 mg BSA was taken as blank and the value was subtracted.

Aromatase mRNA quantification by RT-PCR

Aromatase transcripts were measured in fragments from adrenal tumors and from four normal human adrenals. Total RNA were isolated according to the usual technique (14). The cDNAs obtained by retrotranscription with oligo[dT]12-18 were amplified by PCR using the primers described in Table 1. One of the primers was fluorescence labeled with the fluorophor FAM. PCR products were separated on an acrylamide gel and intensity of fluorescence was measured with the ABI377 sequencer (Applied Biosystem, Foster City, CA, USA). The quantitation of aromatase transcripts was performed as previously reported (15). The quantitation of GAPDH was performed in all samples and results were expressed by the amount of aromatase transcript divided by that of GAPDH transcript.

PII and PI.4 aromatase promoters analysis

PII and PI.4-derived transcripts were detected in the tumors by a qualitative comparative RT-PCR. The cDNAs were obtained using a specific antisense primer hybridizing on exon III of aromatase gene. Sense primers AroPII and AroPI.4 corresponding respectively to exon II and exon I.4 were used with the same

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antisense primer AroCom as PCR primers to highlight transcripts depending on promoters II and I.4. Primers are detailed in Table 1. Promoter analysis of PCR products derived from exon PII and PI.4 were performed after a 30 and 40 cycle amplification respectively. PCR products were visualized on a 3% non-denaturing agarose gel.

Semi-quantitative comparison of PII and PI.4-derived transcripts

A semi-quantitative comparison of PII and PI.4-derived transcript levels was made. Sense primers AroPII and AroPI.4 were used with the same antisense primer AroCom as PCR primers to quantitate the transcripts depending on promoter II and I.4. PII and PI.4-derived transcripts were co-amplified with the primers (Aro-Com-fluo; Aro-PII) and (AroCom-fluo; Aro-PI.4) respectively. AroCom-fluo corresponds to the primer AroCom labeled with the fluorophor F AM. PCR products migrated on acrylamide gel to allow measurement of fluorescence intensity of PCR products corresponding to transcript PII and transcript PI.4 using ABI377 (Applied Biosystem).

Immunofluorescence analysis of human adrenal cortex

Paraffin sections of 3 μm thickness from adrenal tissues and ovarian follicle control tissues were dewaxed in toluene, then rehydrated through graded alcohols to water. Sections then underwent microwave antigen retrieval at full power in four 4 min cycles in 0.1 M sodium citrate. After standing for 20 min at room temperature, the slides were washed in PBS for 3 × 2 min. The sections were incubated overnight at 4 °C with the monoclonal mouse anti-human aromatase (Serotec, France) diluted 1:50 in 3% BSA in PBS as described previously (16). On the following day, the sections were washed with PBS and then incubated with texas red-labeled goat anti-mouse immunoglobulins (Santa Cruz, France) diluted 1:200 in PBS for 45 min at room temperature. After being washed with PBS, sections were coverslipped with mounting medium with DAPI (Santa Cruz, France). Control staining was performed on adjacent serial sections and consisted in replacing the primary antibody with PBS–BSA 3%. Phase contrast electron microscopy allowed the localization of the different structural zones of the human adrenal cortex.

Statistical analysis

The enzyme activity and the mRNA levels measured in the different adrenocortical fragments were compared by a non-parametric Mann–Whitney test. Level of significance was P < 0.05. Linear correlation analysis was performed by Spearman test.

Results

Aromatase enzyme activity

Microsomal aromatase activity was detected in NA and in all tumor subtypes. In comparison with NA, the two FT showed high aromatase activity level, with a 7- and 40-fold enhancement respectively (Fig. 1). In comparison with human follicle granulosa cell microsomes, aromatase activity detected in the FT1 (780 fmol/mg prot/h) and FT2 (4656 fmol/mg prot/h) feminizing tumors were respectively 1/35 and 1/6th (data not shown). Aromatase activity was detected at low level in all NA fragments (mean ± S.E.M. 103 ± 30.5 fmol/mg prot/h), and at a comparable level in adrenal samples from the CS, SCS, and APA secreting tumors (Fig. 1).

Figure 1 Aromatase activity in normal adrenal tissues, feminizing, and non-feminizing tumors. NA, normal adult adrenals; FT, feminizing tumors; CS, cortisol-producing adrenomas with overt Cushing’s syndrome; SCS, cortisol-producing adrenomas with subclinical Cushing’s syndrome; APA, aldosterone-producing adrenomas.

Table 1 Sequence of the primers used for CYP 21 polymerase chain reaction.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Oligo Type</th>
<th>Exon / Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aro5fluol</td>
<td>5 end sense oligo from exon X</td>
<td></td>
</tr>
<tr>
<td>Aro3</td>
<td>5 GCTTTGAGAAGGATAGGCTCTTCATTAAC 3</td>
<td>exon X</td>
</tr>
<tr>
<td>Bene1fluol</td>
<td>5 end sense oligo from GAPDH 3</td>
<td></td>
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<tr>
<td>Bene2</td>
<td>5 GCAAGTGCTGAGGCTCTTCATTAAC 3</td>
<td>exon X</td>
</tr>
<tr>
<td>AroRev</td>
<td>5 TCATCCATGACAACCTTTTGGGCTGA 3</td>
<td>exon IV</td>
</tr>
<tr>
<td>AroPII</td>
<td>5 CAGCAGCTTTTCATAAGGGAAGGCTGA 3</td>
<td>exon II</td>
</tr>
<tr>
<td>AroPI.4</td>
<td>5 GGGATTCTGTATTATTGTCTCCCT 3</td>
<td>exon I.4</td>
</tr>
<tr>
<td>AroCom</td>
<td>5 CAGCCCAAGTTTGTCTGCGCAAA 3</td>
<td></td>
</tr>
</tbody>
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Quantitative PCR analysis of Cyp19a1 mRNA

Aromatase mRNA expressed as a ratio to GAPDH mRNA were detected in all samples from NA fragments and from adrenocortical tumor fragments. A large overexpression of aromatase mRNA was observed in FT2 but not in FT1 in comparison with NA fragments (respectively 135 and 0.12 vs 0.12±0.05 (ARNm aromatase in amol/ARNm GAPDH in amol)×10⁵; Fig. 2). In the non-feminizing tumors, variable transcript levels were observed in comparison with NA: a 2-fold increase of aromatase mRNA was measured in APA but the difference did not reach significance, and the CS group had a mean aromatase mRNA level comparable with that from NA. By contrast, the SCS group showed a significantly lower aromatase mRNA level in comparison to NA (P<0.03; Fig. 2). A strong and significant correlation (r=0.67, P=0.039) was observed between aromatase activity and aromatase mRNA level in the tissue samples (data not shown).

Aromatase promoter analysis

PII and PL.4-derived transcripts were identified in both FT samples. FT1 expressed low levels of PL.4 derived transcripts. By contrast, FT2 expressed PII-derived transcripts at 5000-fold the level of PL-4. In the non-feminizing tumors, PII-derived transcripts were identified in all tumor groups while PL.4-derived transcripts were found in all except APA tumors (Fig. 3). The semi-quantitative comparison of PII and PL.4-derived transcript levels was established in a subset of normal adrenals (n=2) and in different tumor subtypes. PII-derived transcripts were predominant in normal tissues, but also in SCS (n=3) and APA (n=3) tumors. By contrast, CS tumors (n=2) exhibited a 1:1 PII/PI4 ratio (Fig. 4).

Immunofluorescence of normal human adrenal cortex

Incubation of slices from normal human adult adrenocortical tissue with the aromatase antibody revealed the presence of immunoreactive material in adrenal steroidogenic cells with a weak and diffuse staining in the zona glomerulosa and zona fasciculata cells (Fig. 5A), and a more sustained staining in the zona reticularis (Fig. 5B). Aromatase immunostaining was also observed in control tissue from human ovarian follicle (data not shown). No immunostaining was observed in the medulla (data not shown). Control sections without primary antibody showed no staining (data not shown). The comparison of DAPI nuclear staining and aromatase staining sections showed a cytoplasmic localization of aromatase (Fig. 5A and B).

Discussion

The present study demonstrates not only the expression of aromatase in adrenal tissues from the normal gland but also from different adrenocortical tumors. Such expression was detected by two methods, i.e. enzyme activity and mRNA quantitation by RT-PCR. Estrogen production by the adrenal was suggested from studies performed in the 1970s demonstrating the adrenal contribution to circulating estrogens by adrenal vein sampling in normal women and in patients with congenital virilizing adrenal hyperplasia (3, 4, 17). Cell culture experiments also suggested that normal human adrenal cells may produce estrone in vitro (18). More recent studies showed the presence of aromatase activity and/or aromatase transcripts in the normal adrenal gland (5, 6, 9) but such results were not found by others (8, 19). Aromatase mRNA lacking exon five was also identified in normal human adrenal (20). The present study has

Figure 2 Aromatase transcripts expression in normal adrenal tissues, feminizing, and non-feminizing tumors.

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shown the presence of aromatase activity, although at low level, in normal adrenal tissues. Moreover, immunofluorescence experiments confirmed the presence of aromatase in the cytoplasm of steroidogenic cells from the different adrenocortical zonas, with a stronger expression in the zona reticularis than in both zona fasciculata and zona glomerulosa. We observed no aromatase immunostaining in the medulla from adult adrenal. The aromatase antiserum used in these experiments is a well characterized antibody that previously allowed identification of aromatase expression in various mammalian gonadal and placental tissues, including the human ovarian follicle (16). Our results contrast with those from Baquedano et al. who found a marked aromatase immunostaining in prepubertal human adrenal medulla and in peripubertal zona glomerulosa (5).

Two FT were shown to exhibit a high aromatase activity in comparison with normal adrenal tissues. Previous studies utilizing the same technique for measuring aromatase activity demonstrated a marked aromatase overexpression in FT (6–10, 19). At the molecular level, aromatase transcripts were overexpressed in FT2 but not FT1, while moderately high level of enzyme activity was observed in the latter, a result possibly explained by a short half-life of aromatase mRNA in FT1. Weak aromatase overexpression in FT1 may explain the moderately high-circulating estrogen level that is possibly driven by androgen substrate excess and/or by a mass effect of the tumor. The level of aromatase expression in this FT probably reflects a lower degree of differentiation.

Promoter activity was also characterized in normal adrenocortical tissues and in estrogen-producing tumors. In both, promoter PII was the major transcript while P1.4 was weakly expressed confirming previous studies (8–10, 19).

The present study also questioned the presence of aromatase in adrenal tumors apart from the rare estrogen-producing tumors, which was suggested by studies based on adrenal vein catheterization in aldosterone and cortisol-producing tumors (21). We observed in a set of various steroid-secreting tumors that the enzyme activity and aromatase transcripts were detected in all tumors samples. Aldosterone and cortisol-producing adenomas showed similar aromatase expression levels compared with normal adrenal tissues. By contrast, adenomas associated with subclinical autonomous hypercortisolism had a lower level of aromatase expression. Such difference does not deserve an obvious explanation and may be confirmed in a larger group of tumors. The influence of gender and menopausal status on aromatase expression in adrenal tissues could not be investigated in the present study due to the small size of the tumor sample.

Promoter utilization was not uniformly distributed among non-estrogen tumors. Promoter PII was predominant in adenomas associated with aldosteronism or subclinical autonomous hypercortisolism. By contrast, both PII and P1.4 promoters were equally used in
adrenocortical tumors. Aromatase expression is dramatically enhanced in the stromal cells of female breast carcinoma suggesting that locally synthesized estrogens may act in a paracrine way to promote tumor growth (27). β-Estrogen receptors were identified in steroid-secreting adrenocortical tumors, and estrogens may thus induce adrenocortical cell proliferation (28). One may thus speculate on the implication of estrogen receptor expression in adrenocortical tumors, which should favor estrogen-induced proliferation. In this perspective, our study is limited by the small number of tumors that were investigated and by the absence of estrogen receptor quantitation.

In conclusion, our data demonstrate the presence of a functional aromatase enzyme in the normal adrenal gland but also in feminizing- and non-feminizing-producing adrenocortical tumors. The differential aromatase expression level and promoter utilization between tumor subtypes suggests complex regulating mechanisms that have to be further analyzed.

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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