Incomplete Testicular Feminization Syndrome: Studies on Androgen Receptor (AR) Function, AR Gene Analysis, and Aromatase Activities at Puberty and Long-Term Observations of Clinical and Hormonal Features From Infancy to Puberty

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Abstract. A female infant with partial androgen insensitivity (PAIS) was first seen at 4 months of age with slight virilization of the genitalia and externally palpable testes. Sex chromosome was 46,XY. She received left orchidectomy and exploratory laparotomy at 2 yr of age. At exploratory laparotomy, neither a uterus nor fallopian tubes were found. The right testis was preserved by fixing it at the external inguinal ring expecting spontaneous pubertal maturation. After discharge, serum levels of LH, FSH, testosterone (T) and estradiol (E2) were measured annually, and the steroid responses to hCG stimulation were examined every two yr. At the age of 10 yr, she developed breasts and a very feminine body habitus. At 12 yr, she received a clitoroplasty and right orchidectomy. The fibroblast cultures were made from the genital skin whereby androgen receptor (AR) binding was assessed by radioreceptor assay using \(^{3}H\)-DHT as the ligand, and thermoinstability of AR was noted despite normal maximum binding (B\(_{\text{max}}\)) and dissociation constant (Kd) at 22 °C. But another binding experiment with \(^{3}H\)-Mibolerone resulted in the lack of receptor binding. AR gene analysis with direct sequencing of coding exons of the gene revealed no abnormality of the AR gene. 5α-reductase activity was normal. Aromatase activity appeared to be enhanced in the genital skin fibroblast (GSF) cells as well as in the testicular tissue. The results of these studies indicated that the patient had PATS with impaired AR functions and increased aromatase activity. After the discharge, the patient has maintained feminine phenotype, receiving estrogen therapy with mestranol 0.02 mg/day po.

Key words: Testicular feminization syndrome, Incomplete testicular feminization, Partial androgen insensitivity, Androgen receptor, Testosterone, Aromatase activity

TESTICULAR feminization syndrome is known to be caused by inborn abnormalities of the androgen receptor (AR) function leading to androgen insensitivity and male pseudohermaphroditism [1]. The complete and incomplete types of androgen insensitivity are known for the familial occurrence, and the mode of inheritance is thought to be an X-linked recessive trait [2]. Gynecomastia occurs at puberty, unless bilateral orchidectomy is performed.
prepubertally. There appears to be little response to exogenously administered androgen preparations.

Recent development and availability of genetic analysis and receptor studies in subjects with sexual differentiation disorders have made it possible to diagnose androgen insensitivity in childhood, when the feminine pubertal manifestations are not apparent. Cases of androgen insensitivity with deletions or point mutations in the coding regions of the AR gene have been reported [1, 3].

Clinical problems remain to be solved in treating such patients. The most suitable hormonal manipulation and the optimal timing of orchidectomy in these patients are not yet established. The present experience of a girl with partial androgen insensitivity will provide a valuable information on growth, pubertal development and hormonal changes throughout the observation period from infancy to puberty.

Materials and Methods

The patient was born on September 14th, 1981 as the first child of parents who had no family history of sexual disorders. The father was 30 yrs old and the mother 26 yr old, and their marriage was not consanguineous. At the age of four mos, the patient was seen by a pediatrician who noticed slight enlargement of the clitoris with bilateral inguinal masses and performed sex chromosome analysis and steroid hormone measurements. The karyotype was 46, XY. Basal serum testosterone (T) and 5a-dihydrotestosterone (DHT) levels were 1.22 ng/ml and 0.57 ng/ml, respectively. Human chorionic gonadotropin (hCG) stimulation test resulted in stimulated peak levels of T and DHT of 7.16 and 1.67 ng/ml, respectively.

In February, 1984 when the patient was two yrs old, she was hospitalized for detailed evaluation. The external genitalia showed slight clitoromegaly and bilaterally palpable gonadal masses in the labial region. At exploratory laparotomy, neither a uterus nor fallopian tubes were found. Genitography revealed a blindly ending vaginal pouch. The left testis was removed but the right testis was preserved by fixing it outside the right external inguinal ring expecting gonadal hormone secretion at puberty. Clitoroplasty was not performed at this time. Microscopic findings of the resected left testis were compatible with features of immature prepubertal testicular tissue (Fig. 4a).

After discharge, the patient was regularly followed up and observation of the physical development and hormonal measurements were performed at regular intervals. At the age of 10 yrs, the patient developed breasts (Fig. 1) and pubic hair.

In August, 1994, when the patient was 12 yrs and 10 months old, she was admitted for the third time. The patient weighed 49 kg and her height was 159 cm. Reduction clitoroplasty [4] and right orchidectomy were performed. Genital skin of the clitoral prepuce and the labia majora was cultured to obtain genital skin fibroblast (GSF) for analyses of AR and other enzyme activities. Postoperatively convalescence was uneventful and the patient was discharged and has maintained feminine phenotype, while receiving estrogen replacement therapy with mestranol 0.02 mg/day po.

Hormone measurements and stimulation tests

Serum LH and FSH were determined by solid-phase radioimmunoassay. Serum T, free testosterone (free T) and estradiol (E2) were also determined by solid-phase radioimmunoassay. 5α-DHT was measured by radioimmunoassay after
LH-20 column chromatographic separation of the steroid from T [5]. Luteinizing hormone-releasing hormone stimulation test (LH-RH test) was done by im injection of 100 μg of synthetic LH-RH (gonadrelin di-acetate, Tanabe), followed by blood sampling at 0, 15, 30, 60 and 120 min for measurement of LH and FSH. hCG test was performed with im injection of hCG (Gonatropin™, Teikoku Hormone Mfg. Co., Ltd., 3,000 iu/day at 0900 h) for three successive days. Blood samples were withdrawn immediately before each hCG injection and at 0900 h the day after the last injection for steroid hormone assays.

**GSF cultures**

Tissues were cultured in Eagle’s minimum essential medium (E-MEM) solution including 20% fetal bovine serum (FBS) under 5% CO₂ atmosphere at 37 °C for 14 days. The cultured cells were seeded in a 225 cm² flask (Falcon) with 0.1% trypsin in 0.02% EDTA-in phosphate-buffered saline (EDTA-PBS), and kept in culture until the cells became very confluent.

**AR assay with ³H-DHT as the ligand**

AR assay was performed on the cultured GSF by the method described by Shima et al. [6]. Cells were incubated with 2 nmol/l ³H-DHT in the presence or absence of a 500-fold molar excess of R-1881 (methyltrienolone) at 22 °C for 40 min. Another set of cell suspension was pre-incubated at 22 °C for 20 min, and then further incubated at 42 °C for 20 min. After incubation, the cells were cooled in an ice-cold water bath, then washed and radioactivity was counted. The remaining level of the binding of ³H-DHT to the AR (thermolability) was expressed as the ratio (%) of the level of specific binding of the ligand after incubation at 42 °C to that after preincubation at 22 °C.

**AR assay with ³H-Mibolerone**

Radioligand receptor assay was performed with ³H-Mibolerone (7α, 17α-dimethyl-19-nortestosterone) as the labeled androgen [7]. Incubation was done at 4 °C for 18 h, and dextran-charcoal was used for separating the bound and unbound fractions of the mixture. The specific binding was expressed as femtomoles of steroid bound per mg of protein. Cultured cells from normal males and also from a human prostatic cancer cell line (Ln-Cap) were studied as the controls.

**AR gene analysis**

Genomic DNA for the polymerase chain reaction (PCR) was prepared from peripheral leukocyte chromosomes. Oligonucleotides flanking each exon of the AR gene used as PCR primers were the same as those reported by Nakao et al. [8]. The purified PCR products were sequenced directly by the method of Kusukawa et al. [9].

**Aromatase activity and aromatase m-RNA**

Aromatase activity of GSF and testicular tissue were performed by the method described by Berkovitz et al. [10], quantifying ³H-water released from 1-³H-androstenedione after aromatization. Aromatase mRNA was measured by the reverse transcriptase-polymerase chain reaction (RT-PCR) method, utilizing the fluorescent primer in the presence of internal standard RNA [11].

**5α-reductase activity**

5α-reductase activity was determined by measuring 5α-reduced metabolites produced by T in the cultured GSF preparation, with ³H-T as the tracer, and a thin layer chromatography (TLC) to separate the steroid metabolites [12].

**Light and electron microscopy of the testicular tissue**

For light microscopy, the testicular tissue was fixed with 10% formalin and embedded in paraffin. Sections 4 μm thick were stained with hematoxylin and eosin. For electron microscopy, the cut tissue pieces were fixed in 3% glutaraldehyde buffered with 0.1 M, pH 7.3 s-collidine followed by osmification, and embedded in epoxy resin. Thin sections were double-stained with uranyl acetate and lead citrate, and viewed through a JEM-100 SX electron microscope (JEOL, Tokyo).
Results

The height growth was recorded and compared with the normal male and female standard growth curves. The height growth of the patient has been within the normal range either as a girl or a boy, during the age span observed.

Basal serum hormone levels were determined every yr. Serum levels of LH, FSH and T were followed up (Fig. 2). Serum LH and FSH remained low at prepubertal levels until 10 yr old, when LH started to rise. Serum T was increased at 120 ng/dl at the age of four mos, but thereafter the T level remained low. After 10 yr of age the basal level of T rose rapidly. Serum E₂ and free T levels also gradually rose (data not shown). 5α-DHT was determined only twice. In infancy (4 mos old), it was 0.57ng/ml and at 12 yr the level was 0.54 ng/ml.

Standard hCG test was repeated at 4 mos, 4 yr, 6 yr, 8 yr, 10 yr, and 13 yr, as shown in Fig. 3a. It is noted that at 4 mos the T response was linearly increased with time reaching a high level of > 700 ng/dl, while slight but appreciable T responses were observed in subsequent yrs before puberty. The response pattern was rather stunted at the pubertal age of 13 yr, even though the basal level was that of a normal adult male.

Regarding E₂ responses to hCG, increases were negligible at the ages of 6 and 8 yr but at 13 yr the response was enhanced reaching the maximum level on the first day of stimulation (Fig. 3b).

Patterns of LH and FSH response to LH-RH stimulation tested at the age of 10 yr were normal (data not shown).

5α-reductase activity was normal considering the basal levels of DHT and their response to hCG. The amount of 5α-reduced products formed from T in the GSF preparation of the labial tissue was 6.198 pmol/h/mg protein which was within normal limits for the male [12].

AR assay of the cultured GSF with ³H-DHT as the ligand revealed a normal number of binding sites (Bmax) and a dissociation constant (Kd) at 22 °C but thermolability of the AR tested at 42 °C resulted in values less than 50% binding (Table 1).

AR assay with ³H-Mibolerone was performed on the cultured GSF from the patient, two normal males and a human prostatic cancer cell line (LnCap), and the results are shown in Table 2. AR binding was highest in LnCap and detected at 3-9 fmol/mg protein in the GSF of normal male sub-

Fig. 2. Results of hormone determinations [LH (■), FSH (●) and (▲) T] studied each yr from 4 mos to 13 yr of age.

Fig. 3. Responses of serum testosterone (T; 3a, top) and estradiol-17β (E₂; 3b, bottom) to human chorionic gonadotropin (hCG) test at various ages of the patient. 5,000 IU of hCG was injected im on 3 successive days from day 0 through day-2.
Aromatase activities and aromatase-mRNA of the cultured GSF cells from the patients and those from normal male subjects are shown in Table 3. Aromatase activities were consistently measurable in the patient's GSF and especially 50-100 fold stronger in the testicular tissue. On the other hand, aromatase activities and aromatase-mRNA were hardly measurable in the two normal subjects. In these normal subjects aromatase activities were enhanced by preincubation with dexamethasone, and aromatase-mRNA appeared to be stimulated after preincubation with T as well as dexamethasone. In the GSF preparations of the patient, no remarkable increases, either in aromatase activity or mRNA values were observed after preincubation with T or dexamethasone.

Histologically, the left testis resected at the age of 2 yr showed features of immature seminiferous tubules of small diameter containing Sertoli cells and a few spermatogonia without lumen formation. No mature Leydig cells were identifiable (Fig. 4a). The right testis resected at 12 yrs, consisted of two portions with distinct histological characteristics: The predominant portion was represented by seminiferous tubules of larger diameter than those of the immature left testis, but still lacked lumen formation without differentiation of germ cells beyond spermatogonia (Fig. 4b). A few clusters of tubules consisting of degenerated Sertoli cells and lacking spermatogenetic cells were noted. Leydig cells containing a moderate amount of cytoplasm were localized singly or in clusters around the seminiferous tubules. Another portion of the specimen, which macroscopically appeared to be whitish nodules 2-3 mm in diameter, was composed of even smaller-sized tubules, densely arranged and completely lacking spermatogonia (Fig. 4c). No Leydig cells were observed in these foci. Electron microscopic features of the Leydig cells showed the presence of less-well developed smooth endoplasmic reticulum, than in the mature Leydig cells after puberty, and the absence of Reinke crystalloids (Fig. 5). Giant mitochondria were also seen.

**Discussion**

The present case was diagnosed as an incomplete form of testicular feminization syndrome due to partial androgen insensitivity. 5α-reductase activity was normal. One testis was spared orchidectomy and was fixed at the inguinal posi-

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**Table 1.** Androgen receptor measurements with DHT as the ligands in GSF cells from the patients

<table>
<thead>
<tr>
<th>Samples (Skin sites of GSF*)</th>
<th>Kd** (x 10^{-10}M)</th>
<th>Bmax*** (sites/cell)</th>
<th>Receptor (fmol/mg protein)</th>
<th>Thermolability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labia majora tissues</td>
<td>1.78</td>
<td>2328</td>
<td>13.15</td>
<td>26.4</td>
</tr>
<tr>
<td>Clitoral prepuse tissues</td>
<td>2.01</td>
<td>2846</td>
<td>16.08</td>
<td>48.3</td>
</tr>
</tbody>
</table>

*GSF, Genital skin fibroblasts; **Dissociation constant; ***Maximum binding capacity.

**Table 2.** Androgen receptor measurements with Mibolerone as the ligands in various cells including the GSF cell from the patient

<table>
<thead>
<tr>
<th>Sample sources</th>
<th>Tissue sources</th>
<th>Receptor (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LnCap</td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>No. 1 (4-yr normal male)</td>
<td>preputial skin GSF</td>
<td>9</td>
</tr>
<tr>
<td>No. 2 (31-yr normal male)</td>
<td>preputial skin GSF</td>
<td>3</td>
</tr>
<tr>
<td>No. 3 (Patient)</td>
<td>clitoral prepuse GSF</td>
<td>0</td>
</tr>
<tr>
<td>No. 4 (Patient)</td>
<td>labial skin GSF</td>
<td>0</td>
</tr>
</tbody>
</table>

LnCap, a human prostate cancer cell line; GSF, genital skin fibroblasts.
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**Table 3. Aromatase activities and aromatase m-RNA measurement in patient and normal male subjects**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Tissue</th>
<th>Preincubation</th>
<th>Aromatase activities (pmol/min/mg protein)</th>
<th>mRNA (amol/µgRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>0.001</td>
<td>0.0231</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testosterone</td>
<td>0.0013</td>
<td>0.025</td>
</tr>
<tr>
<td>Patient</td>
<td>Preputial skin</td>
<td>Dexamethasone</td>
<td>0.0014</td>
<td>0.0272</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>0.0005</td>
<td>0.0076</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testosterone</td>
<td>0.0004</td>
<td>0.0066</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dexamethasone</td>
<td>0.001</td>
<td>0.0113</td>
</tr>
<tr>
<td></td>
<td>Labial skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testis</td>
<td></td>
<td>0.0513</td>
<td>0.4215</td>
</tr>
<tr>
<td>Normal male (31 yr)</td>
<td>Preputial skin</td>
<td>Control</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testosterone</td>
<td>N.D.</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dexamethasone</td>
<td>0.0026</td>
<td>0.0342</td>
</tr>
<tr>
<td>Normal male (4 yr)</td>
<td>Preputial skin</td>
<td>Control</td>
<td>N.D.</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testosterone</td>
<td>N.D.</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dexamethasone</td>
<td>0.0019</td>
<td>0.0276</td>
</tr>
</tbody>
</table>

N.D.: not detectable.

Fig. 4. (a) Left testis removed at 2 yr of age. Seminiferous tubules contain spermatogonia (arrows). There is no recognizable Leydig cells in the loose interstitial tissue. (b) Right testis at 12 yr of age. Seminiferous tubules in most part contain spermatogonia (arrows), and are larger in diameter, compared to Fig 8a. (c) The tubules lacking germ cells are densely packed in several white nodules. No Leydig cells are recognized (× 200).

...tion, expecting natural occurrence of pubertal feminization. Indeed, at the age of 10 yr, feminine breast development was noted. Most of patients with testicular feminization syndrome had been subjected to early bilateral orchidectomy to prevent malignant changes but recent reports indicate that the occurrence of malignant neoplasms in feminizing testes is not higher than that in cryptorchid males [13]. If the child is raised as a girl, at least one testis may reasonably be preserved and spontaneous pubertal femininity be expected, provided that the patient’s compliance to visit for a complete follow-up is granted.

AR binding studies on cultured GSF of the patient with 3H-DHT as the ligand yielded decreased binding at 42 °C, indicating thermolability of the
AR, whereas another study with $^3$H-Mibolerone as the ligand disclosed total lack of AR binding in the GSF derived from the patient. The latter finding indicates a receptor negative type of androgen insensitivity but slight virilization of the external genitalia makes the possibility unlikely.

The discrepancy may have resulted not only from the use of different ligands but from different experimental conditions, i.e., AR binding studies with $^3$H-DHT were performed at 22 °C for 20 min, whereas the method with $^3$H-Mibolerone as the ligand employed a temperature of 4 °C and an incubation time of 18 h. In the latter experiment, failure to detect AR binding in the GSF from the patient was substantiated by a Western blot analysis to quantify AR protein by using an antibody to AR (NH-27) [14]. Preparations from LnCap cells and those from normal male subjects revealed distinct AR bands, and no obvious band was detected in specimens from the patient (data not shown). Although one cannot draw any definite conclusions from this single observation, AR protein may possibly be subject to complex variation in this syndrome. Noteworthy is a report on the presence of isoforms of AR in human GSF [15].

Unexpectedly, AR gene analysis revealed no alterations in the entire coding region of the gene. A variety of deletions or point mutations, mostly in the steroid responsive or DNA binding domains of the AR gene, have been reported in cases of androgen insensitivity presenting with complete or incomplete testicular feminization syndrome [16]. The failure to detect any alterations in the entire exons of the AR gene suggests the possibility of other regulatory mechanisms of AR gene expression. Recent studies indicate the involvement of promoter regions upstream of the AR gene, which may be activated by reactions generated by a cyclic AMP responsive element included in the promoter [17].

Aromatase activities and expression of aromatase mRNA were rather enhanced in the patient under basal conditions in comparison to normal male subjects in the present study but stimulation of the enzyme by T or dexamethasone was not remarkable. Aromatase activities in the cultured GSF from normal individuals are increased several fold by coincubation with androgens. Androgen-induced stimulation of aromatase activities are reportedly inhibited in the complete form of testicular feminization syndrome, but in the partial form of the syndrome (PAIS) the stimulation was intermediate [18]. Preincubation with T in the present study did not induce appreciable stimulation of aromatase activity or mRNA expression in the patient. It may perhaps be that in patients with androgen insensitivity, aromatase is already fully expressed to the upper limit of stimulation or up-regulation by hitherto unknown mechanisms, even in the control situation. Whether failure of androgens in inducing the stimulation of aromatase activity in complete or incomplete forms of testicular feminization is related to underlying mechanisms of androgen insensitivity remains to be elucidated.

References

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