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Genetics

Androgen insensitivity in man: Evidence for genetic heterogeneity

(testicular feminization/*tfm* locus/skin fibroblasts/dihydrotestosterone receptor)

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ABSTRACT **We have studied ten phenotypically similar patients with complete androgen insensitivity. All of the patients tested had significantly elevated serum luteinizing hormone and plasma androgens within or above the normal adult male range. On the basis of specific dihydrotestosterone binding by skin fibroblasts, we identified two subgroups. Six patients from five different families had undetectable dihydrotestosterone binding, while four patients from two families had normal binding activity. Our results indicate that within the clinical syndrome of androgen insensitivity there are at least two distinct genetic variants. These variants may result from allelic mutations of the same X-linked gene specifying the dihydrotestosterone receptor or, alternatively, from mutations of separate genes both being essential for androgen action in responsive cells.**

Recently we reported the presence of a receptor protein specific for dihydrotestosterone (DHT) in the cytoplasm and nucleus of cultured human skin fibroblasts (1, 2). In contrast, cells from two male siblings with complete androgen insensitivity syndrome (AIS) showed no detectable specific DHT binding (2, 3). The androgen insensitivity in this family apparently resulted from a mutation of an X-linked gene specifying the DHT receptor (3).

In order to determine whether other patients with this clinical syndrome demonstrate a similar defect in androgen binding, we have studied additional patients with the AIS phenotype. On the basis of the DHT binding characteristics of their fibroblasts, two distinct groups were identified, those with undetectable DHT binding and those with normal androgen binding activity. These findings suggest that there is genetic heterogeneity among patients with androgen insensitivity.

MATERIALS AND METHODS

Subjects. Ten subjects with complete AIS were studied. Subjects no. 1 and 2 are nonidentical twins who were previously reported to have absent DHT binding activity in their skin fibroblasts (2). Subject no. 5, described by Wilkins (4), had a similarly affected sibling. Subjects no. 7, 8, and 9 (individuals IV-2, IV-4, and IV-10 in Fig. 1) are from a family described in detail elsewhere (5, 6). The pedigree shown in Fig. 1 has been revised from that previously reported (6). The remaining four subjects were sporadic cases with no family history of similar disorder.

All 10 patients had an XY karyotype, normal female external genitalia, and bilateral testes. With the exception of subject no. 10, who was gonadectomized prior to puberty, and subject no. 4, who had not yet reached puberty, all subjects had spontaneous breast development. There was considerable variation in the amount of sexual hair present in post-pubertal subjects. Subject no. 5 had a complete absence of pubic and axillary hair; subjects no. 1, 2, 3, 9, and 10 have very sparse pubic and axillary hair; while subjects no. 6, 7, and 8 had a normal female quantity and distribution of pubic and axillary hair.

Skin specimens from each subject were obtained at the time of surgery or by biopsy. Skin samples from normal newborns undergoing circumcision were used as normal controls. Informed consent was obtained.

DHT Receptor Assay. Fibroblast cultures were established from all specimens, and cells were grown in minimal essential medium with Earle's salts supplemented with nonessential amino acids and 15% fetal calf serum.

Whole cell DHT binding was measured as previously reported (1, 2). Confluent monolayers of fibroblasts in 100 mm petri dishes were incubated for 20 min at 37° with [^{3}H]DHT (0.2 to 1.5 × 10^{-9} M) dissolved in minimal essential medium without fetal calf serum. After incubation, the cells were harvested using 0.25% trypsin and washed at 4° in Tris-sucrose buffer (0.02 M Tris·HCl, pH 7.5, 0.32 M sucrose, 1 mM $MgCl_2$) containing 1 mg/ml of bovine gammaglobulin. All subsequent procedures were carried out at 4°. After centrifugation at 800 × *g* for 15 min, the cells were suspended in 1.0 ml of Tris·KCl buffer (0.02 M Tris·HCl, pH 7.5, 0.5 M KCl, 1.5 mM EDTA) and lysed in an ultrasonic cleaner. The sonicate was centrifuged at 1600 × g for 20 min, and 0.25 ml of the supernatant was taken for DNA measurement. Another 0.7 ml was chromatographed on a Sephadex G-25 column (6 × 0.9 cm) in Tris·KCl buffer. The void column was collected and assayed by liquid scintillation to determine the total protein-bound radioactivity. Samples of the incubation medium were also assayed to determine the concentration of [^{3}H]DHT that had been added to the cells.

To quantitate specific DHT binding, a cell aliquot was incubated with [^{3}H]DHT plus an amount of nonradioactive DHT corresponding to 100 times that of [^{3}H]DHT. Bound radioactivity measured in this way was considered nonspecific. Specifically bound DHT was calculated by subtracting the nonspecific activity from the total protein-bound counts (1, 2). Increasing [^{3}H]DHT concentrations in the incubation medium were used to produce a saturation curve. The binding capacity (B_{max}) and apparent dissociation constant (K_d) were determined by linear regression analysis of a double-reciprocal plot (7).

To study the retention of DHT by purified nuclei, confluent monolayer cultures of fibroblasts were incubated with 2 × 10^{-9} M [^{3}H]DHT for 30 min at 37°. Similar cultures were incubated in parallel with 2 × 10^{-9} M [^{3}H]DHT plus a 100-fold excess of nonradioactive DHT. The cells were harvested at 37° in a 0.25% trypsin–0.05% EDTA solution. Sub-

Abbreviations: DHT, dihydrotestosterone; AIS, androgen insensitivity syndrome; LH, luteinizing hormone; FSH, follicle stimulating hormone.

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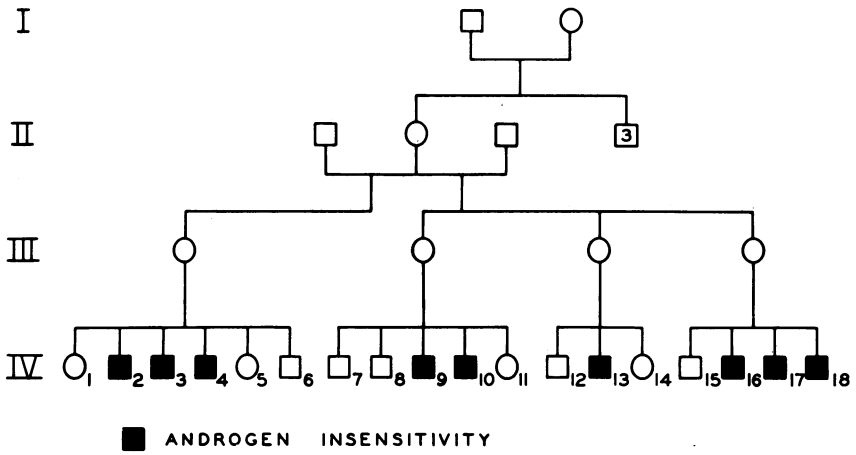

FIG. 1. Pedigree showing transmission of androgen insensitivity.

sequent isolation procedures were carried out at 4°. The cells were collected by centrifugation at 800 × *g* for 15 min, washed with Tris-sucrose buffer, and resuspended in 1 volume of hypotonic buffer (0.02 M Tris·HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$). After 10 min the cells were ruptured by passing them back and forth 10 times through a 25 gauge needle. Exactly 1/7 volume of hypertonic buffer (0.02 M Tris·HCl, pH, 7.5, 2.2 M sucrose, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$) was added to return the suspension to isotonicity. The nuclei were collected by centrifugation at 1600 × *g* for 20 min. The crude nuclear pellets were washed with Tris-sucrose buffer and purified by centrifuging at 100,000 × *g* in a SW50.1 rotor for 1 hr through a 1.8 M sucrose solution. Nuclei prepared in this manner are intact and free from cytoplasmic tags by light microscopy. The purified nuclei were washed once with Tris-sucrose buffer, resuspended in 1 ml of Tris·KCl buffer, and assayed for bound radioactivity and DNA.

To investigate the reproducibility of the assay, seven replicate dishes of wrist fibroblasts from a normal subject were incubated with the same DHT concentration (2×10^{-9} M) and seven different sonicates were prepared and analyzed. Specific DHT binding had a mean value of 223 ± 19 SD moles × $10^{-18}/\mu g$ of DNA. A similar experiment, utilizing replicate sonicates of fibroblasts from subject no. 1, yielded specific binding values ranging from −21 to +16 moles × $10^{-18}/\mu g$ of DNA with a mean −7.5 ± 11.6 SD (3).

Analytical Procedures and Reagents. [*1,2*-^{3}H]dihydrotestosterone (80 Ci/mmol) was obtained from New England Nuclear Corp., and dihydrotestosterone was obtained from Sigma Chemical Co. Tissue culture medium components, trypsin, and EDTA were obtained from Grant Island Biological Co.

Protein was determined by the method of Lowry *et al.* (8), with bovine serum albumin as the standard. DNA was measured by the method of Burton (9), with calf thymus DNA as the standard.

Plasma testosterone and DHT concentrations were determined by a competitive protein-binding method published elsewhere (10); mean ± SD value for testosterone and DHT in our laboratory for normal adult men are 575 ± 150 and 53 ± 16 ng/100 ml, respectively. Androstendione concentrations were evaluated by a double isotope dilution method described previously (11); mean ± SD value for normal adult men are 109 ± 20 ng/100 ml. Serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) were determined by a double antibody radioimmunoassay (12). The Second International Reference Preparation of Human Menopausal Gonadotropin (2nd IRP-HMG) was used as a standard. Normal ranges for adult values in our laboratory are 5.5–18 mIU/ml for LH and 8.8–18 mIU/ml for FSH.

RESULTS

The concentrations of androgens and gonadotropins in blood of seven postpubertal subjects are shown in Table 1. Serum FSH levels were normal or slightly high, but serum LH was significantly elevated in all patients tested. Similar to previous reports (12, 13), all of the subjects in the present study had levels of testosterone and DHT within or above the range for normal adult males. In addition, blood production rates of testosterone for subjects no. 1 and 7 have previously been shown to be within the normal adult male range (subjects no. 2 and 3 of ref. 14). These data indicate that there is no defect in androgen synthesis in this group of patients.

Whole cell DHT binding activity was assessed in fibroblasts from all 10 subjects with AIS (Table 2). Subjects no. 1–6 had essentially undetectable specific DHT binding. However, subjects no. 7–10 demonstrated binding activity within the normal range (1–3). As expected, members of the same family yielded similar results. Subjects no. 1 and 2, nonidentical twins, had undetectable binding; whereas subjects no. 7, 8, and 9 (individuals IV-2, IV-4, and IV-10 in Fig. 1) showed normal DHT binding activity.

Nuclear retention of DHT was next investigated in the four subjects with normal whole cell binding to determine

Table 1. Concentration of androgens and gonadotropins in blood of patients with androgen insensitivity

Subjects	Age (years)	Plasma androgens (ng/100 ml)			Gonadotropins (mIU/ml)	
		T	DHT	Δ	LH	FSH
1*	14-1/2	637	63	161	57.0	10.7
2*	16	661	32	229	36.4	10.0
3	18	1090				
6	15	981			62.2	38.8
7*	15	2370	78	295	42.0	21.7
8*	24	640	84	366	31.5	19.4
10	22	978			109.6	19.4

T, testosterone; Δ, androstendione.
* Previously reported values (12).

Table 2. DHT binding characteristics of skin fibroblasts from patients with androgen insensitivity

Subjects	Age (years)	Origin of fibroblasts	Apparent K_d ($M \times 10^{-9}$)	B_{max} (mol $\times 10^{18}$/ μg of DNA)
1*	19	Wrist	Unmeasurable	<10
		Pubis	Unmeasurable	0
2*	19	Wrist	Unmeasurable	<20
		Labia	Unmeasurable	<10
3†	18	Abdomen	Unmeasurable	<20
4	0.1	Arm	Unmeasurable	<10
5	59	Wrist	Unmeasurable	0
6	15	Inguinal	Unmeasurable	<10
7 (IV-2‡)	25	Wrist	1.03	146
8 (IV-4‡)	39	Wrist	0.92	303
9 (IV-10‡)	19	Labia	1.01	394
10	22	Pubis	0.60	321

* Siblings whose values were previously reported (2).
† Previously reported value (4).
‡ Refers to Fig. 1.

whether cytoplasmic DHT-receptor complex was effectively translocated to the nucleus. As shown in Table 3, there was no difference in specific nuclear retention of labeled DHT between androgen-insensitive subjects and neonatal foreskin controls. In all cases, nuclear bound DHT accounted for approximately 50% of the whole cell binding. This is similar to our previously published data on nuclear binding in normal cells (1, 2).

The wide range in binding values among individuals in Table 3 is mainly attributable to the site of origin of the skin fibroblasts. In our assay, cells from wrist skin have generally lower androgen binding capacity than those from genital or pubic skin (1–3).

DISCUSSION

All of the patients in the present study have clinical phenotypes characteristic of complete AIS (5, 8, 15, 16). In addition, all seven of the postpubertal patients tested had plasma androgen levels within or above the range for normal adult males. The finding of elevated plasma testosterone in phenotypic females with no evidence of virilization suggests insensitivity to the action of androgens at the tissue level (12–14). This is further substantiated by the abnormally high serum LH levels, which indicate relative inability to hypothalamic centers to respond to plasma androgens in the feedback regulation of LH secretion (12).

Utilizing a skin fibroblast assay for androgen binding capacity, we were able to identify two distinct subgroups within the syndrome of androgen insensitivity. Six patients representing five different families had undetectable specific DHT binding, while four patients from two families demonstrated normal binding activity.

The finding of undetectable androgen binding in fibroblasts from subjects no. 1–6 confirms and extends our previous observations (2, 3). Using a cell-free system, we recently demonstrated that the primary cellular abnormality in this group of patients is deficient cytoplasmic androgen binding, leading to an inability to transport DHT to its acceptor site in the nucleus (unpublished data). A similar defect has been observed in the androgen insensitive (*tfm*, testicular feminization) mouse kidney (17–20) and rat preputial gland (17). The deficiency of cytoplasmic androgen binding in these cases may be due either to a decreased quantity of a normal receptor protein or to a structural alteration of the receptor such that there is loss of affinity for the steroid.

The cause of the androgen insensitivity in subjects no. 7–10 with normal DHT binding capacity is more difficult to explain. We found no abnormality in cytoplasmic binding or nuclear uptake and retention of DHT. However, normal uptake and retention by nuclei of these patients do not necessarily imply normal receptor–chromatin interactions. At least two basic mechanisms can be invoked to explain their androgen unresponsiveness. The region of the DHT receptor that interacts with nuclear chromatin to initiate gene transcription may be altered. Alternatively, the DHT receptor may be normal, with the mutation involving an alteration of other protein(s) necessary for androgen-mediated transcription.

Recently Sibley and Tomkins (21) and Gehring and Tomkins (22), using cultured glucocorticoid-responsive mouse lymphoma cells, were able to isolate a number of steroid-resistant mutant clones. They found that steroid unresponsiveness could result from three possible mechanisms: an alteration in cytoplasmic steroid binding activity; a defect in association of the steroid-receptor complex with the nucleus; or some still unidentified abnormality in which cytoplasmic binding and nuclear retention of steroid were normal. Our patients no. 1–6 appear to have a defect similar to the first

Table 3. Nuclear retention of DHT by fibroblasts from androgen insensitive subjects with normal whole cell binding

Subjects	Origin of fibroblasts	Specific DHT binding*	
		Whole cell	Nuclei
Control	Neonatal foreskin	577	262
Control	Neonatal foreskin	353	192
7	Wrist	160	109
8	Wrist	106	58
9	Labia	779	269
10	Pubis	497	269

* Moles $\times 10^{18}$/μg of DNA.

mutant of Tomkins *et al.* (21, 22), whereas patients no. 7–10 could be considered analogous to their third mutant.

The present study demonstrates that within the clinical phenotype of complete AIS there are at least two distinct genetic results from a mutation of an X-linked gene specifying the DHT receptor (3). The pattern of transmission of the second variant (Fig. 1) is compatible with either X-linked or sex-limited autosomal dominant inheritance. This latter mutation could also involve the gene coding for the DHT receptor, in this case being allelic with that in the patients with deficient cytoplasmic androgen binding. Alternatively, the mutation may be in a separate gene, possibly also on the X chromosome. Thus it is conceivable that more than one gene is required for androgen expression in responsive cells. In any event, studies of cells from androgen insensitive individuals are ideal for elucidating the mechanism of androgen action.

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