Male Pseudohermaphroditism Presumably Due to Target Organ Unresponsiveness to Androgens

DEFICIENT 5α-DIHYDROTESTOSTERONE BINDING IN CULTURED SKIN FIBROBLASTS

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ABSTRACT Maximum specific 5α-dihydrotestosterone (DHT) binding activity (B_{max}) has been measured in intact confluent monolayers representing fibroblast strains derived from nongenital and genital (labium majus) skin of normal individuals and of 11 patients fulfilling the clinicogenetic criteria of complete testicular feminization (TF). Nine labium majus strains from adult females had a mean B_{max} value three times greater than that of seven nongenital strains from adult females (33 vs. 11 fmol/mg cell protein). The B_{max} results for 13 adult nongenital strains varied from 5.6 to 23.3 fmol/mg protein; the values for males and females had very similar means and ranges. The variation could not be correlated with the chronologic age of adult skin explant donors or with the in vitro age (mean population doubling level) of the cultures assayed. The B_{max} activities of three nongenital strains from normal infants (two male, one female) did not exceed 5 fmol/mg protein. Seven of eight nongenital TF strains had B_{max} values below 2 fmol/mg protein; the value for the eighth coincided with the lower limit of normal adults. The lower limit of DHT binding in normal labium majus strains was 15 fmol/mg protein. Three of five labial strains from patients with TF had B_{max} values close to zero; the other two fell between 10 and 15 fmol/mg protein. It is apparent that labial skin fibroblast strains from clinically homogeneous patients with TF have highly variable degrees of DHT binding deficiency, and that they permit a more reliable diagnosis of severe and intermediate degrees of DHT binding deficiency than do strains of nongenital skin fibroblasts.

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INTRODUCTION

Target cell unresponsiveness to androgen has been identified as the cellular fault in three types of human male sexual maldevelopment caused by mendelian gene mutation. The prototype disorder is X-linked (1) complete testicular feminization (TF) [1] in which there is neither normal male differentiation of the Wolffian ducts, urogenital sinus or genital tubercle, folds and swellings prenatally, nor genital or somatic virilization at puberty. These patients are resistant to testosterone and its immediate derivative, 5α-dihydrotestosterone (DHT, 5α-androstan-17β-ol-3-one) (2). Familial incomplete male pseudohermaphroditism (FIMP), type 1 is like TF in pattern of genetic transmission, the universality of target organs affected, and presumably in concordant resistance to testosterone and DHT, but its defect is incomplete in severity and variable in expression (3). All patients with TF and most with FIMP, type 1 have female breast development because of elevated plasma estradiol levels secondary to defective

[1] *Abbreviations used in this paper:* B_{max}, maximum binding capacity; DHT, 5α-androstan-17β-ol-3-one; FIMP, familial incomplete male pseudohermaphroditism; K_d, apparent dissociation constant; MP, male pseudohermaphroditism; 5α-reductase, Δ^4-3-ketosteroid-5α-oxidoreductase; TF, testicular feminization.

TABLE I

Clinical Features of Patients Referred with the Diagnosis of Testicular Feminization

Patients	Age	Cell strains	Unambiguous female external genitalia at birth	Spontaneous pubertal feminization with sparse/absent sexual hair, without hypokalemia/hypertension	Family history		
					Fully affected		Delayed menarche and (or) decreased sexual hair
					Siblings	Maternal relatives	
	yr						
1	25	JJX*	+	+	+	?	?
2	21	JAX	+	+	+	?	?
3	16	JRX	+	+	+	?	?
4	3/12	TMN, TML	+	?	−	+ (×2)	?
5	22	CVL	+	+	+	−	+ (×3)
6	17	TRA, TRL	+	+	(adopted)		
7	17	SFL	+	+		+ (×8)	+
8	18	RDL	+	+	+ (×3)	?	+
9	29	TOD	+	+	−	−	−
10	10	HCD	+	?	−	−	+
11	19	GHA	+	+	+	?	?

* The final letter of the cell strain codes denotes the anatomic source of the skin explant from which the strain was developed: A abdominal (suprapubic); D, deltoid area of the arm; L, labium majus; N, nongenital (source unknown); X, axilla.

feedback regulation of luteinizing hormone secretion at the pituitary-hypothalamic level (3). FIMP, type 2 resembles the most severe expression of the type 1 disorder from the external genital viewpoint, but differs from the other two in having an autosomal recessive etiology, normal Wolffian duct differentiation, no gynecomastia, and prominent somatic and genital virilization at puberty (4, 5). The phenotype of this disorder is explainable by reduced conversion of testosterone to DHT, due to deficient Δ^4-3-ketosteroid-5α-oxidoreductase (5α-reductase) activity, in DHT-dependent targets such as the urogenital sinus and external genital primordia before birth, and the prostate and facial hair follicles at puberty (5).

Fibroblasts cultured from normal human skin metabolise testosterone to DHT as well as other 5α-reduced products (6, 7) and they synthesize a specific receptor protein that binds DHT in the cytoplasm and translocates it into the nucleus (8). Skin fibroblasts cultured from genital skin (prepuce, labium majus, and scrotum) perform both functions to a greater extent than do those from various nongenital skin sites (7–10). The preservation of such differentiative properties in serially subcultured skin fibroblasts predicts that these cells will express the mutations responsible for the three known types of androgen-unresponsive male sexual maldevelopment. The prediction has been fulfilled. Thus, preputial skin fibroblasts of patients with type 2 FIMP have deficient 5α-reductase activity (7), and skin fibroblasts (both genital and nongenital) from patients with TF lack or have very low levels of the specific receptor protein(s) responsible for binding DHT (10, 11).

The purpose of this study was to measure DHT binding activity in genital and nongenital skin fibroblast cell strains derived from a group of 11 patients referred with the diagnosis of TF. It is important to note that a majority of the cell strains studied represent patients whose testes were removed either before the advent of accurate radioreceptor or radioimmunoassays for the sex hormones and gonadotropins, or, before experience with these assays for accurate differential diagnosis of male pseudohermaphroditism (MP) was widely disseminated. Furthermore, none of the cell strains belongs to a patient whose anabolic unresponsiveness to androgen has been assessed. Nevertheless, all of the patients whose strains were chosen for study were considered extremely likely to have complete TF, according to exclusive clinicogenetic criteria defined below. In addition, five of the patients (or an affected relative) had normal adult male levels of plasma testosterone.

METHODS

Origin of cell strains

Testicular feminization. Essential clinical data on the 11 patients are summarized in Table I. Additional features common to the group were normal testis differentiation, absence of female internal genitalia, a blind (usually short) vagina, and absence of prepubertal adrenocortical failure. Spontaneous pubertal feminization (that is essentially normal except for primary amenorrhea and faulty develop-

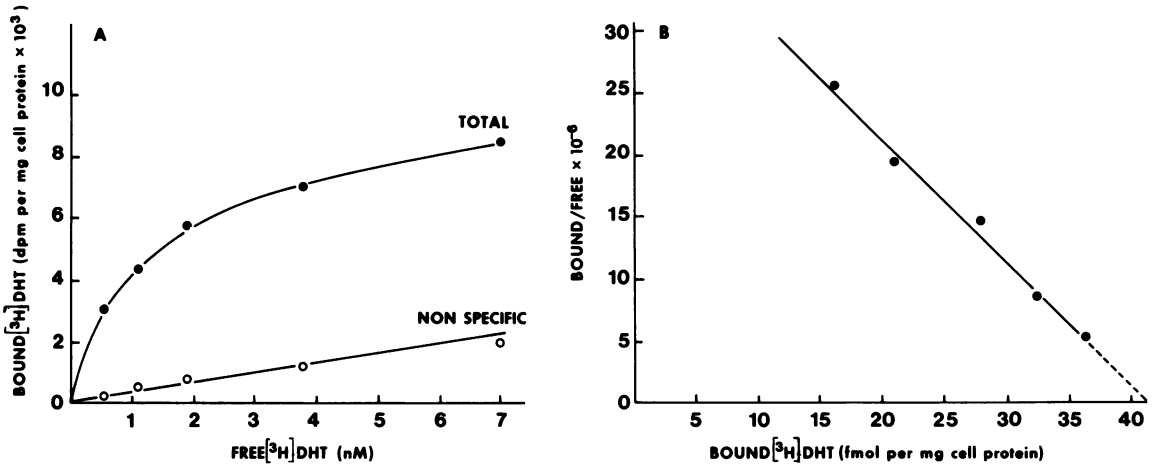

FIGURE 1 (A) A representative curve of [^{3}H]DHT binding in NEL, a normal adult female labium majus skin fibroblast strain. (B) Specific binding is plotted according to Scatchard (27). The intercept at the abscissa defines B_{max}; the reciprocal of the slope defines the apparent K_d.

ment of sexual hair, and that is not accompanied by hypokakalemic hypertension) in an XY individual born with unambiguously female external genitalia and absent or vestigial Wolffian duct derivatives, despite normal testis differentiation, was sufficient to discount all known genetic disorders of male sexual maldevelopment other than classical complete TF. This discriminator was applicable to 8 of the 11 patients; 2 of the other 3 (patients 4 and 10) were prepubertal at the time of referral but had fully or partially (12) affected adult maternal relatives. The remaining patient (patient 6, Table I) had a nonmosaic XYY karyotype in multiple tissues. It is likely that this was causally unrelated to her TF phenotype because this aneuploidy is relatively common (1.5 per 1,000) among newborn males without sexual anomalies (13), and her pituitary-hypothalamic resistance to androgen was documented (14). The full process of excluding other causes of MP with unambiguously female external genitalia at birth follows; normal testis differentiation excluded covert sex chromosome aneuploidy and pure XY gonadal dysgenesis (15). Congenital lipoid adrenal hyperplasia (16) and Δ^5-isomerase-3β-hydroxysteroid dehydrogenase deficiency (17) were discounted because, although each can result in males with unambiguously female external genitalia at birth, almost all such patients have life-threatening adrenocortical failure before puberty. 17, 20-Cholesterol desmolase deficiency (18) has been described in only one family, and the three affected males had ambiguous external genitalia. The lack of virilization or hypokalemic hypertension, respectively, among our postpubertal patients indicated that none had 17-ketoreductase (19) or 17α-hydroxylase (20–22) deficiency. 5α-reductase deficiency was excluded because of normal Wolffian duct differentiation, external genital ambiguity at birth, and significant genital and somatic virilization at puberty.

Controls. Most of the control strains were developed in our laboratory from small skin samples obtained with informed consent. These explants were obtained from volunteers by full-thickness punch biopsy (4 mm) of skin in the deltoid or upper medial regions of the arm, or from skin incisions made at the time of surgery. Four strains were obtained from the stock of control strains in "The Human Genetic Mutant Cell Respository" maintained at the Institute for Medical Research, Camden, N. J. (Dr. A. Greene). The age of nongenital skin donors varied from infancy to 60 yr. The labial skin donors were all postpubertal. None of the controls was known to have a disorder of sex steroid hormone metabolism or responsiveness.

Cell culture

The materials and methods used for developing cell strains from small samples of skin and maintaining them in serial subculture have been described previously (23, 24). Our cultures were screened for mycoplasma by a sensitive test (25) capable of revealing such contamination when standard microbiological culture methods fail to do so. Occasional cultures were found to be contaminated. This did not affect the results obtained on control or patient cell strains because uncontaminated sister strains yielded the same results as their contaminated partners, and purposeful contamination induced no change from results obtained on uncontaminated replicates. To avoid the possible influence of population density of a culture on its specific DHT binding activity, all monolayers were grown to confluence before their DHT binding was determined. The cell strains used in this study had population doubling times of 24–36 h. The population doubling time of a strain is one measure of its in vitro age (26). There was no obvious relationship between the population doubling time or the calculated in vitro age of a strain and its DHT binding activity, an observation made by others (8).

DHT binding assay

The principle of the assay is to use a set of replicate confluent monolayers for measuring "total" [^{3}H]DHT binding in the absence of excess (200 ×) cold DHT, and "nonspecific" [^{3}H]DHT binding in the presence of excess cold DHT. The latter is low-affinity, high-capacity binding attributable to various macromolecules in the cell. The difference between "total" and "nonspecific" [^{3}H]DHT represents "specific" DHT binding at that concentration. The maximum binding capacity (B_{max}) of the specific DHT binding activity in a strain and the apparent affinity with which it binds DHT (K_d, dissociation constant) were deter-

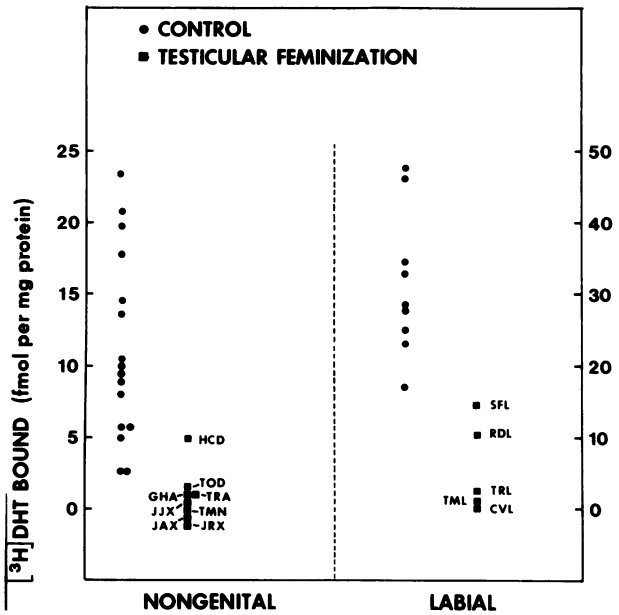

FIGURE 2 The distribution of mean specific dihydrotestosterone binding maxima in fibroblast strains derived from nongenital and labial skin of controls and males with complete TF. Notice that the results for nongenital and labial skin strains are plotted on axes which differ by a factor of 2. TRL and TRA, TML and TMN are pairs of labial and nongenital strains, respectively, derived from single patients. All of the patient strains and many of the control strains were assayed three or more times. Each assay was performed at least in quadruplicate. Replicates seldom deviated from the mean by more than 10 percent. The use of probe sonication to effect total cell disruption in place of gentle cell disruption by Dounce homogenization has enabled us to detect small amounts of DHT binding (30) in some control nongenital strains that had previously appeared to lack it (10). Nevertheless, some TF strains had so little specific DHT binding that negative mean values resulted when nonspecific was subtracted from total binding.

mined by exposing a series of replicate monolayers to varying concentrations of [^{3}H]DHT, each in the presence and absence of a 200-fold excess of cold DHT, and subjecting the data to Scatchard analysis (27) (Fig. 1). Specific DHT binding activity is high-affinity ($K_d \sim 1$ nM), low-capacity (5–70 fmol/mg cell protein) binding attributable to one or more specific cytoplasmic proteins synthesized by the cells (8).

The assay procedure used was that described by Keenan et al. (8) with four modifications: (*a*) the serum-free medium used for incubating the monolayers with DHT was additionally buffered (to pH 7.4) with 15 mM Hepes; (*b*) bovine gamma globulin was excluded from the solution used to wash the monolayers before scraping them from the dishes with rubber policemen; this allowed us to determine cellular protein content accurately and to express DHT binding activity per milligram cell protein; (*c*) the cells were ruptured by direct exposure for 10 s to the 1-cm probe of a sonicator (Measuring and Scientific Equipment Co., London, England) set to an amplitude of 2 μm; and (*d*) dextran-coated charcoal adsorption of "unbound" (free) DHT rather than molecular sieving was used to isolate it from "bound" DHT. Pellets of dextran-coated charcoal were prepared by centrifuging (2,000 *g*, 20 min, 4°C) 1 ml of a suspension of 0.5% charcoal and 0.05% dextran T-70 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in 0.02 M Tris-HCl (pH 7.5, 4°C), 0.4 M KCl, 1.5 mM EDTA, and 2 mM mercaptoethanol. The cell extract supernates (0.5 ml) were added to the pellets and the mixture was resuspended by vigorous vortexing for 3 s. After storage at 4°C for 10 min, the suspensions were centrifuged as above, and portions of the supernate, containing bound [^{3}H]DHT (80 Ci/mmol), were removed for protein determination (28) and scintillation counting (29).

RESULTS

The mean specific dihydrotestosterone B_{max} for the individual skin fibroblast cell strains of various classes are shown in Fig. 2.

Controls. The values for 16 control nongenital strains (column 1) varied ninefold and had a mean of 11.2 fmol/mg cell protein. This large range of normal variation was not obviously related to either the precise anatomical origin of the nongenital skin sample from which a strain was developed, or the in vitro age (mean population doubling level) of a strain at the time of its assay. Nor could the variation be related to the sex or simply correlated with the chronologic age of a skin donor. Thus, while it is notable that the three strains with the lowest values were derived from infants of both sexes at approximately 1 yr of age, the fourth and fifth lowest values represent strains derived from a 60-yr-old man and a 51-yr-old woman, respectively. Seven adult female strains ranged from 6 to 21 ($\bar{x} = 11.7$) fmol/mg protein, while six adult male strains ranged from 6 to 23 ($\bar{x} = 14.2$).

The B_{max} values for nine adult labial strains (column 3, Fig 2) varied from 17 to 53 and had a mean of 33.2 fmol/mg cell protein. As above, neither donor age nor in vitro age could account for the variation observed among the strains. The difference between the labial and nonlabial strains of adult females had a $P < 0.001$ (31).

Testicular feminization. Data on nongenital strains from eight patients referred with the diagnosis of TF are shown in column 2 of Fig. 2. Of the seven strains whose B_{max} values were below normal, three (JJX, JAX, JRX) were from adult siblings with typical TF. The nongenital TF strain (HCD) whose B_{max} value was in the normal range was derived from deltoid skin of a 10-yr-old girl whose maternal grandmother never developed axillary hair.

Results of labial strains from five patients referred with the diagnosis of TF are given in column 4 of Fig. 2. Three of the strains had values clearly below the range of controls and two were intermediate. It is noteworthy that two (TRL and TML) of the three strains well below the controls could be paired with nongenital strains (TRA and TMN) developed simultaneously from the same patients. In both cases the

values for the nongenital strains fell below the lower limit of the controls. Unfortunately, we do not have nongenital strains from the two patients whose labial strain results (SFL and RDL) were close to the lower limit of the controls.

DISCUSSION

As a group serially subcultured adult labial skin fibroblast strains have significantly more specific DHT binding than strains derived from nongenital skin of both sexes varying in age from 1 to 60 yr. Six of nine labial strains had B_{max} values exceeding 25 fmol/mg cell protein, whereas none of the nongenital strains did, including seven from adult females. Keenan et al. (8) have previously alluded to the tendency of foreskin strains to have more DHT binding than nongenital strains.

The difference between genital and nongenital strains in respect to DHT binding recalls the fact that genital strains metabolise testosterone more rapidly than those from nongenital skin (7, 9). The variability among strains within each class in respect to DHT binding is surpassed by their variability in rates of testosterone metabolism (7) and amounts of 5α-reductase activity (32). The origin of this variability is not known, but one clue is derived from studies indicating that single-cell clones isolated from one strain can differ in their patterns of testosterone metabolism (33) and in their amounts of DHT binding (1).

The lower limit of DHT binding in our control nongenital strains is so close to the lowest detectable level that the diagnosis of even a severe DHT binding deficiency would carry a significant risk of error. In contrast, the spread between the lower limit of our control labial strains and the lowest detectable level is large enough to permit not only a confident diagnosis of complete DHT binding deficiency but also the strong suspicion of intermediate degrees of such a deficiency. A decisive precedent for this situation is found in the use of cultured skin fibroblasts to diagnose 5α-reductase deficiency in patients with type 2 FIMP (4). Here, the enzyme activity in nongenital fibroblasts from the patients falls within the range of normal nongenital fibroblasts so that the phenotype can be recognized only in genital (foreskin) fibroblasts (7).

It is clear from the present study that two of the labial strains derived from patients with the phenotype of complete TF have a lesser degree of DHT binding deficiency than the three others (column 4, Fig. 2). The existence of such heterogeneity has been affirmed by Amrhein et al. (34), who have recently discovered a family with complete TF whose cultured skin fibroblasts have quantitatively normal DHT binding. Of the eight nongenital TF strains that we have studied, the one (HCD, column 2, Fig. 2) with normal DHT binding may represent a patient of the latter type.

If those patients who have the typical phenotype of TF despite quantitatively normal fibroblast DHT binding can be shown to have cytoplasmic DHT receptors that are qualitatively normal, then a defect in target cell responsiveness to DHT centripetal to the step of cytoplasmic binding is possible. It is important to realize, however, that defective nuclear localization or chromatin binding may be secondary to selective dysfunction of cytoplasmic receptors that retain a normal ability to bind DHT (35).

Measurement of specific DHT binding in cultured genital skin fibroblasts should be an early step in the diagnosis and management of individuals or families with MP. The result can indicate the priorities that should be attached to standard tests for evaluating the testis-pituitary-hypothalamic axis, may influence gender assignment when anatomic considerations permit a choice, and will inevitably accelerate the accumulation of knowledge on the heterogeneity of those forms of male sexual maldevelopment that are due to deficits or defects in the molecular apparatus that underlies normal androgen responsiveness.

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