Cultured human skin fibroblasts: a model for the study of androgen action

Terry R. Brown and Claude J. Migeon
Dept. of Pediatrics, Division of Pediatric Endocrinology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, U.S.A.

Abstract

Human skin may be considered as a target organ for androgens, as are male sex accessory organs, since all events involved in testosterone action have been observed in this tissue. As a corollary, the mechanism of androgen action can be studied *in vitro* in cultured skin fibroblasts. The advantages of this system are that studies can be performed with intact human cells under carefully controlled conditions, differentiated genetic and biochemical characteristics of the cells are faithfully preserved and the biological material is renewable from a single biopsy specimen. The metabolism of androgens, in particular the 5α-reduction of testosterone to the active metabolite, dihydrotestosterone, the intracellular binding of androgen to its specific receptor protein and its subsequent translocation to the nucleus have been studied in skin fibroblasts. The intracellular androgen receptor content of genital skin fibroblasts is higher than that from nongenital skin sites. In addition, the androgen receptor has been characterized as a specific macromolecule with properties of high affinity and low capacity similar to that of other steroid hormone receptors.

The pathophysiology of three genetic mutations which alter normal male sexual development and differentiation has been identified in the human skin fibroblast system. In 5α-reductase deficiency, an autosomal recessive disorder in which dihydrotestosterone formation is impaired, virilization of the Wolffian ducts is normal but the external genitalia and urogenital sinus derivatives are female in character. At least two types of X-linked disorders of the androgen receptor exist such that the actions of both testosterone and dihydrotestosterone are impaired and developmental abnormalities may involve both Wolffian derivatives and the external genitalia as well. These two forms of androgen insensitivity result from either the absence of androgen receptor binding activity (receptor (–) form) or apparently normal androgen receptor binding with absence of an appropriate biological response (receptor (+) form). In addition, studies with human skin fibroblasts may also be of value in defining the cellular mechanisms underlying the broad spectrum of partial defects in virilization.

In summary, we have correlated our studies of the molecular mechanism of androgen action in human genital skin fibroblasts with those of other investigators as these studies contribute to our understanding of male sexual development and differentiation.

Abbreviations

The following abbreviations and trivial names for steroids have been used: dihydrotestosterone (DHT), 17β-hydroxy-5α-androstan-3-one; androsterone, 3α-hydroxy-5α-androstan-17-one; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; androstanediols, 5α-androstan-3α, 17β-diol and 5α-androstan-3β, 17β-diol; androstanedione, 5α-androstan-3, 17-dione; methyltrienolone (R1881), 17β-hydroxy-17α-methylestra-4, 9, 11-trien-3-one; RU-23908, 5, 5-dimethyl-3-[4-nitro-3-(trifluoromethyl)-

Molecular and Cellular Biochemistry 36, 3–22 (1981). 0300–8177/81/0361–0003/$02.40.

phenyl]-2, 4-imidazolidinedione; R2956, 17-hydroxy-2, 2′, 17α-trimethylestra-4, 9, 11-trien-3-one; cyproterone acetate, 6-chloro-17α-acetoxy-1, 2α-methylene-4, 6-pregnadiene-3, 20-dione; triamcinolone acetonide, 9-fluoro-11β, 16α, 17, 21-tetrahydroxy-pregna-1, 4-diene-3, 20-dione-6, 17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β, 17α, 21-trihydroxypregna-1, 4-diene-3, 20-dione.

I. Introduction

Cultures of human genital skin fibroblasts have evolved as a system for the study of genetic and biochemical abnormalities of androgen action and sexual differentiation (1–3). The obvious values of this system derive from the human origin of the cells and the renewable nature of cultured biological material. In addition, fibroblasts reflect genetically determined characteristics of the donor. Experimental observations from cultured skin fibroblasts also parallel results obtained with fresh specimens of skin and/or other tissues. Presently, several important steps in the mode of action of androgens can be evaluated in human skin fibroblast cultures. These include the intracellular conversion of testosterone to its active metabolite, 5α-dihydrotestosterone (DHT), as catalyzed by the presence of the enzyme NADPH: $\triangle^4$-3-ketosteroid 5α-oxidoreductase (5α-reductase). Furthermore, the presence of an androgen receptor protein has been demonstrated in skin fibroblasts derived from various anatomical sites for both male and female subjects. The study of DHT binding to its specific receptor protein and characterization of the macromolecular steroid complex has been pursued in several laboratories. Within this review, we will attempt to correlate our experience and that of others with cultured human skin fibroblasts as a model system for the study of androgen action and male sexual differentiation.

II. Androgen physiology: sexual development and differentiation

Androgens of fetal testicular origin serve as obligatory triggers, during restricted and critical periods of embryonic and early neonatal life, for the initial differentiation and growth of many organs of the male reproductive tract such as the Wolffian ducts, urogenital sinus and external genital primordia (4, 5). Androgens also serve to permanently imprint regions of the central nervous system that control the masculine modes of gonadotropin secretion and sexual behavior. At the time of puberty, they promote the appearance of the secondary male sex characteristics, which include growth of the external genitalia, development of the prostate and seminal vesicles, male distribution of body hair and increase in total muscle mass. Androgens are also essential for spermatogenesis and they exert a feedback control on the output of gonadotropins by the hypothalamic-pituitary axis. They can produce masculinization in the female and also have specific effects on hematopoiesis. This astonishing diversity of morphogenetic and physiologic effects by androgens makes it extremely difficult to define 'target' versus 'nontarget' tissues for these hormones and underscores the need for caution in framing any simple unitary hypothesis to account for all biological actions of androgens in molecular terms.

At the present, a general concept of steroid hormone action at the cellular level has evolved (6–8). In the case of androgens, either testosterone or its peripheral and intracellular metabolite, dihydrotestosterone, may serve as the active hormone. Early events in steroid action include uptake of the hormone by passive diffusion into the target cell, binding to a specific cytoplasmic receptor protein, translocation of the steroid-receptor complex to the nucleus and binding of this complex to specific acceptor sites on the chromatin. These events lead to activation of the transcriptional apparatus, appearance of specific mRNA species resulting in the synthesis of new proteins and the manifestation of the hormone-mediated effect.

III. Cultured human skin fibroblasts as a model system

In the early 1970s, we began studies of cultured human skin fibroblasts as a model for the study of the molecular mechanisms of androgen action (9). Our initial premise for this work was based upon skin as a target organ for the action of male hormones. The stimulation of sebaceous gland activity and hair growth in genital areas of skin represent two examples of the androgen-depen-

dence of this tissue. In addition, early investigations had demonstrated that human skin could metabolize androgens *in vitro* (10, 11).

Monolayer cultures of human fibroblasts are easily propagated from explants of small skin biopsy specimens maintained in minimal essential medium (MEM) enriched with 10–15% fetal bovine serum at 37 °C in an atmosphere of 95% air: 5% CO_2 (12). It is advantageous that fibroblasts represent intact human cells which can be maintained under carefully controlled conditions. Another assumption was that cultured skin fibroblasts maintained the function and the genetically determined molecular properties of the donor individuals.

IV. 5α-Reductase activity in skin fibroblasts

Dihydrotestosterone (DHT), the 5α-reduced metabolite of testosterone, serves as the effective intracellulr mediator of certain androgen actions both in male phenotypic differentiation during embryogenesis and in differentiated tissues in the postnatal state (4, 5). In particular, DHT induces the virilization of the indifferent urogenital sinus and urogenital tubercle of the male fetus into the prostate gland, male urethra and male external genitalia. From observations on the metabolism of testosterone by human skin *in vitro* (10, 11, 13), the assumption that cultured skin fibroblasts maintained this function was readily testable. In accord with two previous reports on the metabolism of testosterone by skin fibroblasts (14, 15), our initial publication (9) in 1974 observed that over 50% of the original testosterone added to the fibroblast monolayers was reduced to DHT following incubation at 37 °C for 45 min.

1. Methodology

Our method for the measurement of testosterone metabolism assays the products released into the media following cellular uptake and metabolism of the substrate by intact fibroblasts in a confluent monolayer (Fig. 1). Following removal of the growth medium from the culture plates, the cells are washed with Hanks balanced salt solution. In a typical assay, serum-free MEM containing testosterone in a final concentration of 200 nM (2nM ^{3}H-testosterone) is added to each 60 mm culture plate and returned to the incubator at 37 °C. After 30 and 60 min of incubation, aliquots of the media are removed to tubes containing methylene chloride and ^{14}C-steroids (testosterone, DHT and androstenedione) as corrections for extraction recoveries. Steroids are extracted from the aqueous media into methylene chloride, taken to dryness, redissolved in methanol and separated by thin-layer chromatography in the system, chloroform: ether (90:10). The following spots are identified by exposure to iodine vapor through corresponding Rfs to authentic steroid standards: androstanediols, DHT and androsterone, testosterone, androstenedione and androstanedione. The plates are scraped and the steroids are eluted from the silica gel and quantitated by scintillation spectrophotometry. The 5α-

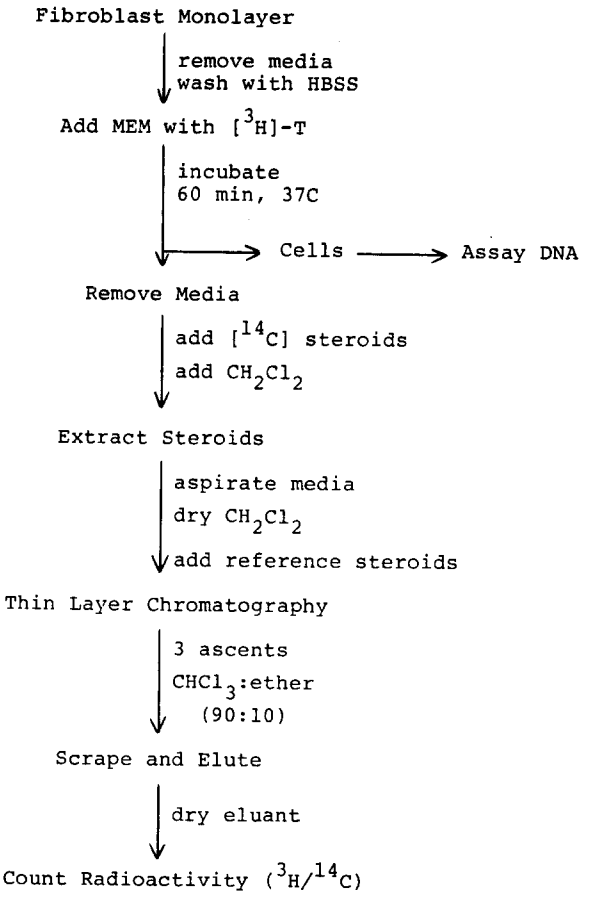

Fig. 1. Methodologic sequence for the assay of 5α-reductase activity in cultured human skin fibroblasts as described in the text.

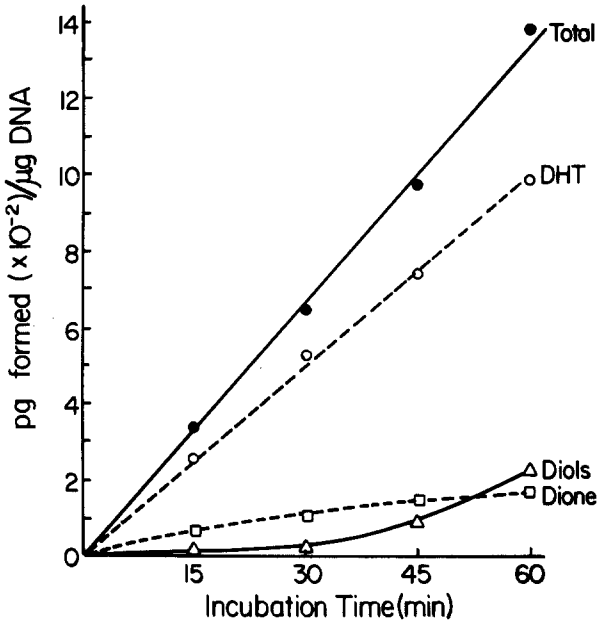

Fig. 2. An example of the time-course for formation of 5α-reduced products from testosterone by cultured human newborn foreskin fibroblasts incubated at 37 °C. The total (●) 5α-reduced products represent the sum of 5α-dihydrotestosterone (DHT, o), 5α-androstane-3α- and 3β-diols (DIOLS, △) and d5α-androstanedione (DIONE, □). Each point represents the mean of 4 independent determinations on the same cell strain.

reduced products are represented by the sum of androstanediols, DHT and androstanedione, with DHT being by far the dominant product (Fig. 2). Following incubation and removal of the radioactive media, the cell monolayers are washed, harvested by scraping, sonicated and assayed for DNA content per plate. The sum of 5α-reduced products formed from testosterone is expressed as pg/µg DNA/h. Other investigatiors have followed similar assay methods with intact fibroblast monolayers (16–18) or have quantitated 5α-reductase activity in a cell-free system (19).

2. 5α-Reductase activity in normal skin fibroblasts

In recent studies from our laboratory (20) with tissues from 8–22 week old human fetuses, low, but detectable 5α-reductase activity was observed at eight weeks gestation in nongenital skin fibroblasts and was present in fibroblasts propagated from a variety of tissues from older fetuses, including testis, kidney, lung and clitoris. Earlier, Wilson (16) had observed that the rate of DHT formation from testosterone was greater in fibroblast monolayers grown from foreskin and scrotal skin than in fibroblasts from nongenital skin sites. The finding of this specialized metabolic function in skin fibroblasts of different anatomical origin was also reported by Pinsky *et al.* (21), Mulay *et al.* (15) and by our laboratory (22) (Fig. 3). However, the decrease in DHT formation with age that had been observed for human foreskin homogenates by Wilson and Walker (13), was not apparent with fibroblasts grown from foreskins of subjects at various ages (16).

Recent studies with cultured human skin fibroblasts have suggested that 5α-reductase activity may be androgen-dependent (23), similar to that

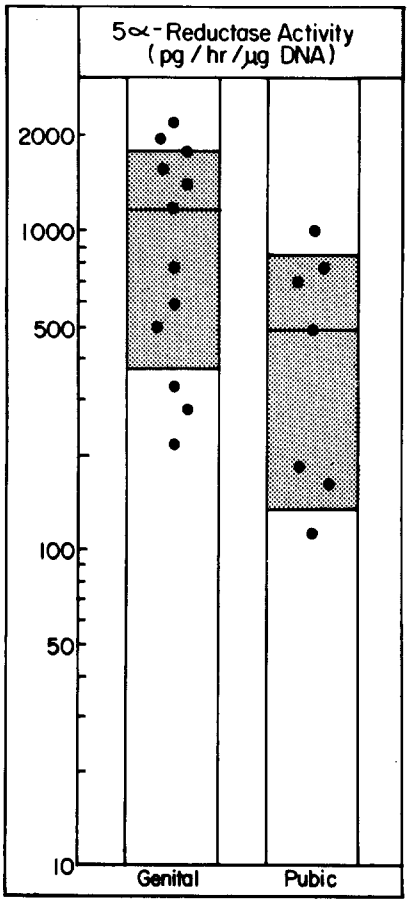

Fig. 3. 5α-reductase activity in cultured skin fibroblasts propagated from genital or pubic skin specimens of normal subjects. Shaded areas represent the mean ± SD for genital (1063 ± 697) and pubic skin (487 ± 353).

reported for pubic skin homogenates by the same investigators (24, 25). Enzyme activity from male foreskin fibroblasts was higher than from female pubic skin fibroblasts, with intermediate levels observed for pubic skin fibroblasts from hirsute women (23). The difference between males and females in this report might be related to the site of the skin specimen from which the fibroblasts originated, since it has been reported by several laboratories that genital skin fibroblasts have higher 5α-reductase activity than those from pubic skin (15, 16, 21, 22, 26). Our laboratory (27) and Pinsky *et al.* (17) have not observed significant differences in 5α-reductase activities of pubic skin fibroblasts from men and women. However, it is important to note, as demonstrated by Kaufman *et al.* (28, 29) with cloned cell lines, that fibroblasts derived from the same skin region display different patterns of steroid metabolism. In fact, two recent reports (18, 23) indicated that 5α-reductase activities in skin fibroblast strains increased with serial subculture, which may be due to selective outgrowth of particular cell types or to alterations of the original characteristics of the cells. In our laboratory, we have not observed any significant pattern of change in 5α-reductase activity in four separate strains of foreskin fibroblasts examined in each of 22 consecutive cell passages (T. Brown, unpublished data).

Since human genital skin fibroblasts contain an active 5α-reductase, the potential for various compounds to inhibit this enzyme activity can be readily tested. Two such compounds are the secosteroids, 5, 10-secoestra-4, 5-diene-3, 10, 17-trione (I) and 5, 10-seco-19-norpregna-4,5-diene-3, 10, 20-trione (II) which we demonstrated to be noncompetitive type inhibitors of 5α-reductase activity in foreskin fibroblasts (30). Compound I ($K_i = 1.6 \times 10^{-6}$M) was a less potent inhibitor than compound II ($K_i = 0.53 \times 10^{-6}$M) in fibroblast cultures. Neither compound was an effective inhibitor of DHT receptor binding since their relative affinities were on the order of 10^3 times less than DHT. Therefore, the human genital skin fibroblast system can be of benefit in identifying the mechanism of action for potential antiandrogenic compounds, as well as evaluating their efficacy.

The enzyme from genital skin fibroblasts exhibits maximal activity in a narrow pH range with an optimum of 5.5 in cell-free assays (19). The activity is NADPH-dependent and is localized to the 100 000 × g particulate (microsomal) fraction of cell sonicates (19). Nongenital skin fibroblasts do not exhibit a sharp peak of maximal activity at pH 5.5 but rather exhibit lower total activity spread over a wide (pH 6–9) pH range (19). Although testosterone is presumed to be the principal physiologic substrate of the enzyme, 5α-reductase activity is greater with the substrate, 20α-hydroxypregn-4-en-3-one (31). The metabolism of this latter pregnene derivative has been used by Moore & Wilson (31) to suggest the existence of two separate enzyme activities in genital skin fibroblasts: one with a pH optimum in the normal range at pH 5.5 which may be deficient due to a genetic abnormality in 5α-reductase activity and the other with activity in the pH range from 7–9 which is lower, but similar in both normal and mutant cells. The overall significance of this finding at the present time is unclear and awaits purification of this enzyme. The authors have suggested that two different enzymes might exist or that some form of post-translational control of a common precursor occurs, the latter of which might involve regulation by a separate gene locus. It remains clear that the physiologic consequence of 5α-reductase enzyme deficiency is reflected in the absence of measureable enzyme activity at the pH optimum of 5.5 in cell-free preparations or in whole cells at physiologic pH.

3. Pathophysiology of 5α-reductase deficiency

In 1974, Imperato-McGinley *et al.* (32) and Walsh *et al.* (33) reported the first cases of familial male pseudohermaphroditism associated with a deficiency of 5α-reductase. This is an autosomal recessive disorder, the consequences of which are seen only in 46, XY subjects even though the biochemical abnormality is present in both sexes. This is related to the fact that 5α-reductase activity is required during fetal life for the formation of DHT which is necessary for the normal masculinization of the external genitalia of male subjects (34–36). Affected male patients have bilateral testes with normal plasma gonadotropins, normal male testosterone production and plasma levels and normal virilization of the external genitalia. Later, in puberty, there is virilization of the external genitalia and normal development of axillary and pubic hair, increases in testosterone production within the male range but low plasma dihydro-

testosterone levels accompanied by normal gonadotropin levels and no gynecomastia develops. Elevated ratios of urinary 5β-reduced (etiocholanolone) to 5α-reduced (androsterone) steroids and decreased *in vivo* conversion of testosterone have been demonstrated in affected individuals. In these patients, 5α-reductase activity is virtually undetectable in biopsies from the male genital tract (33) and in fibroblasts cultured from genital skin (16) as compared to normal controls.

The molecular features of the mutation have been studied in cultured human skin fibroblasts of affected patients using either intact cells in a monolayer or in a cell-free system following cellular disruption (16, 19, 31, 37–39). Several variants of 5α-reductase deficiency have evolved from the efforts of Wilson and co-workers indicating genetic heterogeneity among families with similar clinical presentations (Table 1). In cell-free extracts of fibroblasts from patients of two families, 5α-reductase activity was markedly deficient when measured at the normal pH optimum of 5.5 for the enzyme (19, 31). In a third family, 5α-reductase activity was extremely low in fresh tissue measurements but was only in the low normal range of assays with cultured fibroblasts (37, 38). In further contrast to the other two families, a normal pH optimum and a normal K_m for testosterone was demonstrated for the enzyme from fibroblasts of affected members of the third family. However, affinity for NADPH, the enzyme cofactor, was reduced and the enzyme was unstable, with a rapid turnover in the presence of cycloheximide. Recently a fourth family was described in which the enzyme from the fibroblasts of the affected individual exhibited intermediate levels and intermediate stability of 5α-reductase, with altered affinity for both testosterone and NADPH (39). Our experience (40) with documented deficiency of 5α-reductase activity from studies with genital skin fibroblasts has been limited to a single case without rigorous classification according to the methods of Wilson *et al.* (2). In summary, cultured human skin fibroblasts have been of significant value in elucidating the molecular mechanisms which underlie the presentation of familial male pseudohermaphroditism associated with deficient conversion of testosterone to DHT.

V. Androgen receptor in skin fibroblasts

Our laboratory has made its most significant contributions toward defining the mechanism of androgen action mediated by the intracellular androgen receptor (1, 41, 42). In particular, studies in our laboratory and in others with cultured human skin fibroblasts have contributed greatly to the understanding of the biochemical and genetic mechanisms associated with the androgen insensitivity syndrome. At least two variants exist for the complete form of the syndrome: (a) a mutation(s) involving the cytoplasmic receptor protein which interferes with binding of testosterone and DHT

Table 1. Heterogenous properties of 5α-reductase enzyme from cultured skin fibroblasts of families exhibiting 5α-reductase deficiency.[1]

Family	Activity at pH 5.5	Km for testosterone	Km for NADPH	Stability after exposure to cycloheximide
	pmol/mg protein/h	μM	μM	%
Normal[2]	33	0.08 ± 0.01[3]	40 ± 8[3]	>95
Dallas	0.2	1.80	250	>95
Dominican Republic	0.2	3.40	97	>95
Los Angeles	4.5	0.16	1760	< 5
New York	0.6	2.20	4.25	75

[1] Data taken from References 2, 37, 39.
[2] N = 12.
[3] Mean ± SEM.

and (b) a mutation(s) which interferes with steroid-receptor complex activation of transcription and/or translation. We will present the characteristics of androgen receptor binding in cultured human skin fibroblasts and discuss investigations of the aforementioned mutations in androgen action.

1. Methodology

The general methodology for the androgen receptor binding assay as performed in our laboratory is outlined in Fig. 4. Replicate fibroblast monolayers are incubated with serum free media containing various concentrations of radiolabeled steroid alone or combined with an excess of radioinert ligand. In most of the studies to be described,

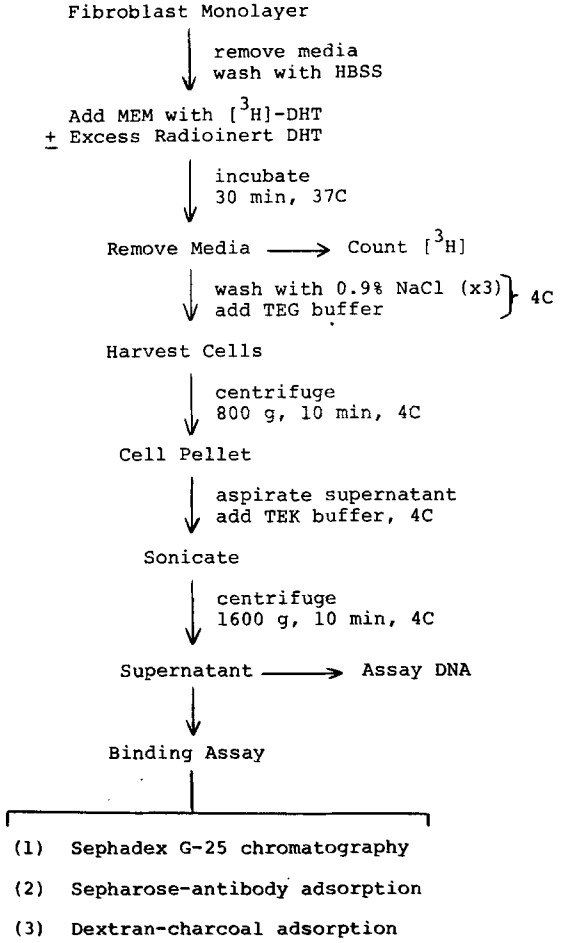

Fig. 4. Methodologic sequence for the assay of androgen receptors in cultured human skin fibroblasts as described in the text.

we have used ^{3}H-dihydrotestosterone (DHT) since this androgen is considered to be the predominant intracellular hormone of most target tissues. Additionally, as previously mentioned, testosterone is rapidly converted to DHT within normal skin fibrobasts and retained intracellularly, as well as being released into the media. Only in 5α-reductase deficient fibroblasts can testosterone binding be studied. After a short exposure (30–60 min) to the radioligand the media is removed, the cell monolayers are washed, the cells are manually scraped into an aqueous buffer solution and collected by low-speed centrifugation. The cell pellet is routinely sonicated in high salt buffer, centrifuged at low-speed and the lysate is assayed by one of several procedures available for separation of bound and free steroid. These procedures include: (1) adsorption of unbound steroid onto dextran-gelatin coated charcoal (0.05% dextran T70, 0.1% calfskin gelatin, 0.5% activated charcoal) and removal by centrifugation (43, 44); (2) binding of unbound steroid to an anti-testosterone antibody covalently linked to sepharose 4B and sedimentation by centrifugation (45, 46); and (3) separation of unbound steroid from macromolecular bound steroid by G-25 column chromatography (9, 12). By the first two procedures, protein-bound steroid remains in the final supernatant fraction and in the third technique protein-bound steroid is collected in the void volume eluant from the column. Specific receptor binding is determined either as the difference between total bound steroid (incubation with ^{3}H-DHT only) and nonspecific binding (incubation with ^{3}H-DHT plus excess unlabeled DHT) or as the difference between bound steroid in duplicate aliquots from the same sample, one of which is heated to 80 °C for 5 min to destroy the receptor (nonspecific binding) while the other is maintained at 0 °C (total binding), prior to removal of free steroid by the antibody or dextran-charcoal adsorption procedures. The latter techniques which utilize heat have two distinct advantages: (a) only one fibroblast monolayer need be incubated at each concentration of ^{3}H-DHT since a second replicate culture need not be incubated with an excess of radioinert DHT to determine nonspecific binding and (b) both total (0 °C) and nonspecific (80 °C, 5 min) binding are determined with aliquots from the same cell pool rather than two separate cell pools incubated in the presence and absence of excess

radioinert steroid. The validity of these procedures for assay of specific receptor binding have been verified for the antibody adsorption method (46) and the dextran-charcoal adsorption method (T. Brown, unpublished observation). In all cases, the binding data are plotted by the method of Scatchard (47) following a linear regression analysis.

These methods provide differing degrees of sensitivity, versatility and expediancy to adapt to the use of various steroids and experimental conditions. The dextran-coated charcoal adsorption of free steroid from cell pools incubated with ^{3}H-DHT in the presence or absence of excess radioinert DHT is followed by Kaufman *et al.* (48) for fibroblast monolayers and by Eil *et al.* (49) for the dispersed cell assay recently described. The method of Griffin & Wilson (50) differs in the following respects: (a) cells are harvested by exposure to trypsin: EDTA; (b) total and nonspecific binding are determined as total radioactivity in cells exposed to ^{3}H-DHT and ^{3}H-DHT plus 0.5 μM nonradioactive DHT, respectively, following several washes of the dispersed cells but without further efforts, such as dextran-coated charcoal, to remove free steroid from the final cell sonicate; and (c) following a plot of bound DHT *vs* DHT concentration, their maximum specific binding, B_{max}, is determined by extrapolation of the linear portion of the curve plotted for total binding at higher concentrations of ^{3}H-DHT to the y-axis, rather than by Scatchard analysis of the specific (total minus nonspecific) DHT binding.

2. Androgen receptor studies in normal fibroblasts

The saturation kinetics of specific androgen binding in genital skin fibroblasts assessed with various concentrations of ^{3}H-DHT (0.1–2.0 nM) is shown in Fig. 5. A linear regression best fit of the data in the form of a Scatchard plot provides an estimation of the affinity (K_d) and the number of sites available (B_{max}) for steroid binding to the receptor. The characteristic properties, high af-

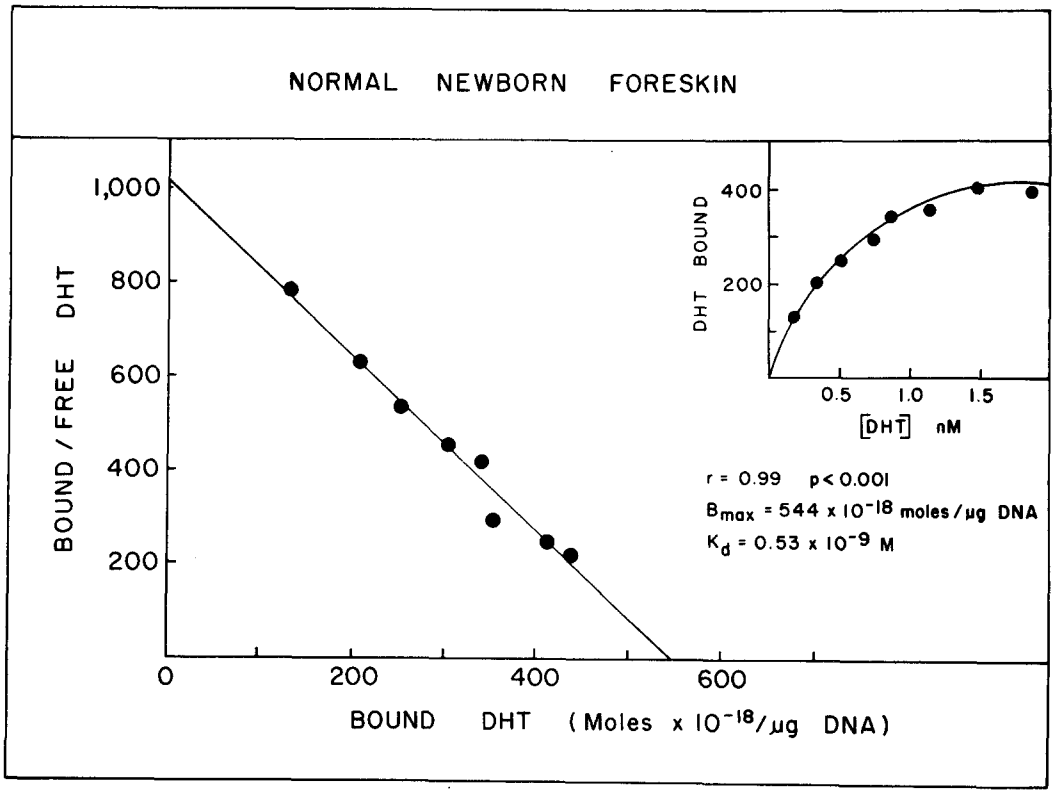

Fig. 5. Saturation analysis of dihydrotestosterone (DHT) binding in cultured newborn foreskin fibroblasts from a normal subject. The binding capacity (B_{max}) and binding affinity (K_d) were calculated from a Scatchard plot of the specific binding (inset) as a function of DHT concentration.

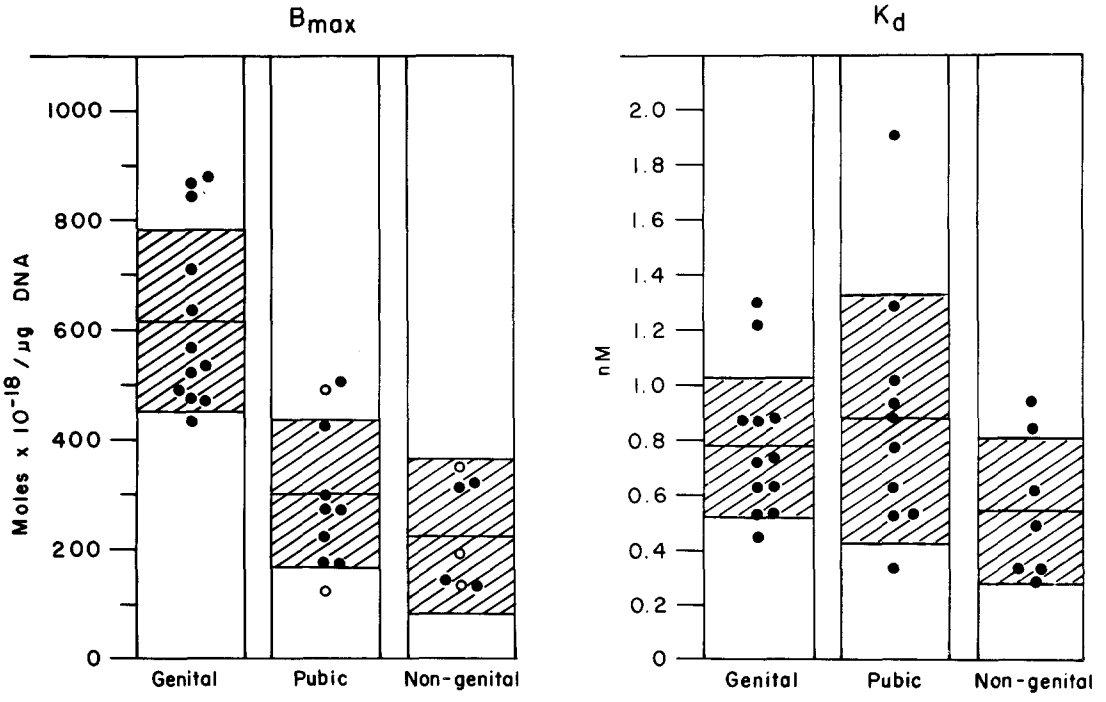

Fig. 6. Binding capacity (B_{max}) and binding affinity (K_d) for dihydrotestosterone (DHT) in cultured fibroblasts from genital, pubic and nongenital skin specimens of normal subjects. B_{max} values are significantly greater in genital skin (labial fold, scrotal, foreskin or preputial) fibroblasts than in pubic or nongenital skin fibroblasts, whereas, the K_d values are similar.

finity and low capacity, of steroid receptors is demonstrated in these experiments. The binding capacity is estimated to be 2500 to 15 000 binding sites per cell with an affinity in the range of 10^{-10} M. We have observed a higher binding capacity for DHT in fibroblasts derived from genital skin (foreskin, labia) than pubic or nongenital skin (abdomen, forearm) without a coincident and definitive difference in the binding affinity (Fig. 6) (12, 22, 27). These differences in fibroblast binding capacities, dependent upon the anatomical site of origin, have been confirmed by other workers (51, 52). Furthermore, we demonstrated the presence of low, but detectable levels of androgen receptor in nongenital skin from human fetuses as early as eight weeks of age (20). In the 8–22 week old fetuses studied, the highest quantity of DHT binding (B_{max} = 1006 moles $\times$ $10^{-18}/\mu$g DNA) was observed in scrotal skin fibroblasts from a 17-week old male fetus, with lower levels found in skin fibroblasts from the buttocks, clitoris, back, pubic and inguinal areas.

Maximum binding is obtained within 15–30 min when cells are incubated at 37 °C, but the rate of ligand receptor association is much slower at 4 °C (12). Subcellular fractionation of cells containing bound DHT demonstrates that approximately 50% of the intracellular specifically bound steroid is localized to the nucleus (12). Translocation of the cytoplasmic androgen receptor-steroid complex to the nucleus occurs very rapidly at 37 °C and is nearly maximal after 15 min of incubation (12). Although other workers (53) have confirmed our observations on the distribution of the intracellular steroid-receptor complex, a recent report (48) indicated that variable levels (44–100%) of the total cellular bound steroid may be present in the nuclear fraction from different fibroblast strains. In addition, we observed differential extraction of the nuclear steroid-receptor complex dependent upon the concentration of salt in the buffer. In our studies, 65–70% of the total nuclear bound steroid is extracted by sonication in 0.5 M KCl buffer (Fig. 7). The salt-resistant radioactivity remaining in the

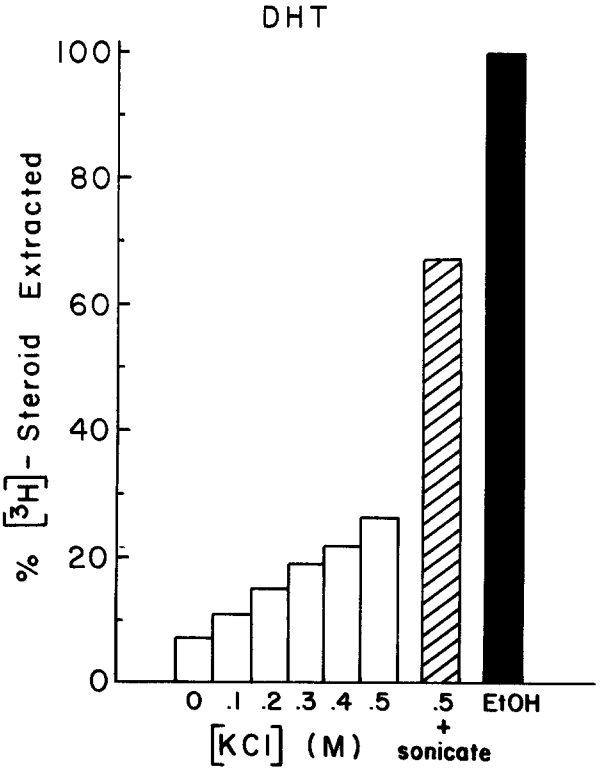

Fig. 7. Extraction of nuclear bound androgen. Replicate newborn foreskin fibroblast monolayers were incubated with 2nM [^{3}H]-DHT for 30 min at 37 °C. Aliquots of purified nuclei were pelleted and resuspended in Tris-EDTA buffer containing various concentrations of KCl (0–0.5 M) at 0 °C for 30 min, one of which included sonication at 0.5 M KCl. Radioactivity was measured in the supernatant following extraction and recentrifugation of the residual nuclear components. Results are the mean of triplicate determinations expressed as the percentage of nuclear radioactivity extracted by ethanol at 25 °C for 30 min (= 100%).

residual nuclear pellet after salt extraction is quantitatively recovered by reextraction in ethanol. In another study by Kaufman *et al.* (48), the percentage of total nuclear bound steroid resistant to high salt (0.4 M KCl) extraction ranged from 27 to 87 among various fibroblast strains, but the salt-extractable fraction appeared to be unrelated to the total cell androgen binding present in the nucleus.

In order to further characterize the androgen receptor, the steroid-receptor complex was chromatographed on a Sephadex G-150 column (10) (Fig. 8). The first peak (N) corresponds to high

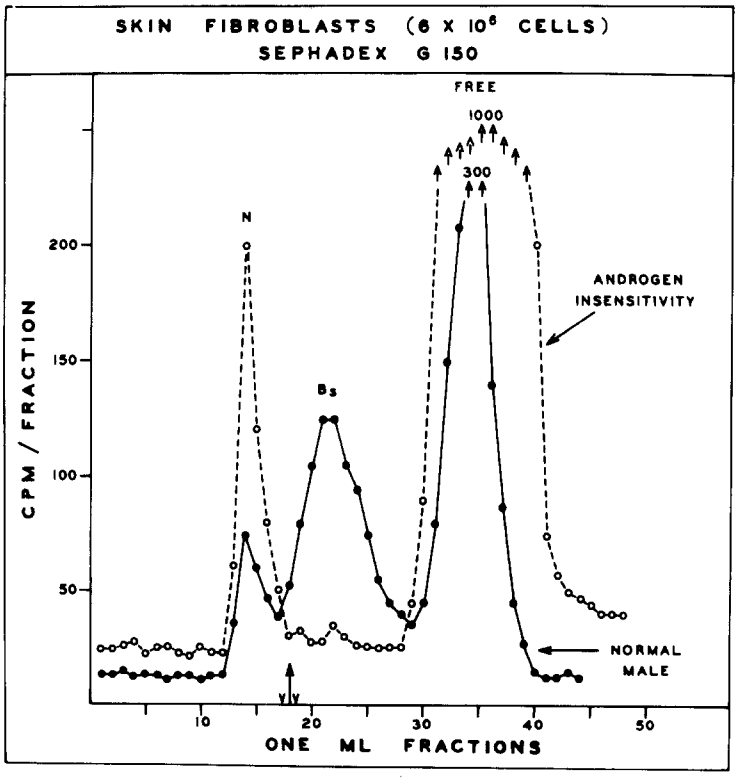

Fig. 8. Sephadex G-150 column chromatography of genital skin fibroblast sonicates following incubation of cells with [^{3}H]-DHT. The solid line (—) represents the chromatographic profile from a normal male subject and the dashed line (---) from a subject with complete androgen insensitivity, receptor (-). Note the absence of peak B_s in the subject with androgen insensitivity.

molecular weight material which is eluted with the void volume. This probably represents an aggregated form of steroid-binding protein(s), which although more likely to occur in low ionic strength buffer, persists even in the presence of high salt (0.5 M KCl). The second peak (B_s) represents the specific androgen receptor protein complex as evidenced by the ability of excess radioinert DHT to compete with ^{3}H-DHT for binding. Following sephacryl S-200 gel filtration, Kaufman and coworkers (54) have observed a single peak from the cytosol fraction eluting with the void volume and two peaks from the nuclear fraction, one eluting with the void volume and the other eluting much later. They estimated the molecular weight of the void volume species to be greater than 185 000 for both cytosol and nuclei and the second nuclear peak to be approximately 20 000. Although they suggest that the high molecular weight species represents the receptor specific binding, they do not offer any proof of the binding specificity through competition with excess nonradioactive DHT. Furthermore, the preparation and elution of the cytosol fraction with low ionic strength buffer favors formation of protein aggregates of high molecular weight which may not be representative of the receptor protein. By contrast, the nuclear fractions were prepared and eluted from the column in high salt (0.4 M KCl) buffer. A critical investigation of these findings still must be performed.

Cell sonicates subjected to sucrose density gradient centrifugation under high salt (0.4 M KCl) conditions exhibited a peak of specifically bound ^{3}H-DHT with a sedimentation coefficient of 3–4 S (10) (Fig. 9). Similar results have been reported by other investigators with some further studies described under low salt conditions as well as for cytosol and nuclear fractions (51, 54). Griffin *et al.* (51) observed small but diffuse peaks in both the 8 S and 4 S regions of gradients under low salt conditions. Kaufman *et al.* (54) demonstrated the presence of two sedimentation peaks in the 4–7 S range with cytosol fractions centrifuged through low salt gradients and a single 3–5 S cytosol peak with high

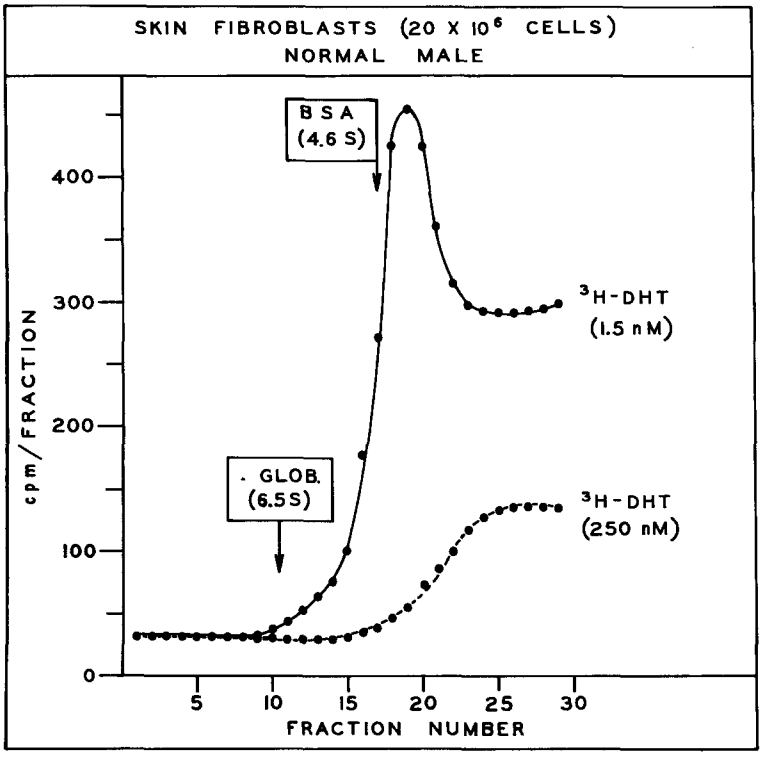

Fig. 9. Sucrose density gradient analysis of the macromolecular DHT-receptor complex from genitals skin fibroblast sonicates (0.4 M KCl) following incubation of cells with [^{3}H]-DHT. The solid line (—) represents the gradient profile with [^{3}H]-DHT only and the dashed line (– – –) with [^{3}H]-DHT + 200-fold excess radioinert DHT.

salt (0.4 M KCl) gradients. These results were indicative of other experiments in which cytosol (low salt) displayed two peaks of radioactivity and nuclear extracts (0.4 M KCl) showed evidence of a single 3–5 S peak. As an extension of these findings, no difference was observed in the thermostability of the cytosol and nuclear binding activities, whereas the thermostability of a given nuclear binding activity varied directly with its sedimentation coefficient (54). The heterogeneity of receptor binding moieties under these *in vitro* conditions for sucrose density gradient analysis and thermostability, may be useful for defining qualitative aberrations of the androgen receptor in various forms of androgen insensitivity. A summary of the characteristics of the androgen receptor in skin fibroblasts is provided in Table 2.

DHT has been the ligand of choice for most studies of androgen receptor binding in skin fibroblasts. The reasons for this are essentially two-fold: (1) DHT is an endogenous androgen as opposed to a synthetic compound and (2) endogenous plasma testosterone is rapidly converted intracellularly to DHT in many androgen target organs. The latter principle is also true in normal human genital skin fibroblasts as we and others have described the rapid intracellular 5α-reduction of testosterone to DHT (15, 16, 21, 22). As a result, predominantly DHT is bound to the cytosolic and nuclear androgen receptor following incubation of fibroblasts with testosterone (12). Lamberigts *et al.* (18) have reported that higher estimated binding capacities (B_{max}) were observed after incubation with DHT than after incubation with testosterone in 9 of the 18 cell lines studied with a single saturating concentration of ^{3}H-steroid. However, the opposite relationship of steroid to B_{max} was observed in 3 fibroblast strains. In the same studies, a distinct correlation was observed between 5α-reductase activity and receptor binding estimated from incubations with testosterone.

As alternatives to the study of DHT binding, we have investigated androgen receptor binding of: (a) testosterone (T), in a fibroblast strain derived from a patient with an inherited deficiency in 5α-reductase activity and (b) methyltrienolone (R1881), a potent synthetic androgen, in fibroblast strains from normal and complete androgen insensitive patients (55).

Both T and DHT were found to bind to a specific protein in genital skin fibroblasts of a patient with 5α-reductase deficiency (40). By saturation kinetic analysis of T and DHT binding, the maximum number of binding sites (B_{max}) were similar for both androgens, whereas the apparent dissociation constant (K_d) of the androgen receptor for T was greater than for DHT. In competition studies of ^{3}H-T bound to the receptor with unlabeled T or DHT, the inhibitor constant (K_i) for T was 2–3 fold greater than the K_i for DHT (Fig. 10). In addition, the dissociation rate constant (K_d) for ^{3}H-T bound to the receptor was greater than for ^{3}H-DHT ($t_{1/2}$ for T = 10 h and $t_{1/2}$ for DHT = 74.5 h. From these results, we suggest that T can play a major role in the sexual differentiation of male patients with 5α-reductase deficiency during their fetal life and at puberty. The lesser degree of masculinization during fetal life in these patients could be explained by the low affinity and faster turnover rate of the T-receptor complex relative to the DHT-receptor complex. Furthermore, a hypothesis which has previously pervaded the literature suggests that some androgen-responsive cells are endowed with specific DHT-receptors while other such cells harbor separate 'testosterone' receptors. In fact, the relative 5α-reductase enzymatic activity in a given target tissue may be of ultimate importance in determining whether the androgen receptor binds T or DHT and the affinity is, therefore, an inherent property of a single receptor type.

The synthetic steroidal androgen, methyltrienolone (R1881), associates with the androgen receptor of genital skin fibroblasts following binding kinetics similar to DHT (55). R1881 binding reaches a plateau after 30 min incubation at 37 °C with B_{max} ($= 695 \times 10^{-18}$ moles/μg DNA) and K_d ($= 0.56 \times 10^{-9}$ M) values determined by Scatchard analysis which were not significantly different from DHT

Table 2. Characteristics of DHT receptor from human skin fibroblasts.

1. Low capacity (2500–15 000 binding sites/cell)
2. High specificity for DHT
3. High affinity ($K_d \cong 10^{-9}$ M)
4. Found in cytosol and nuclei
5. Heat labile
6. Protease sensitive
7. Not affected by DNAse or RNAase
8. Sedimentation coefficient (0.4 M KCl) = 3–4 S

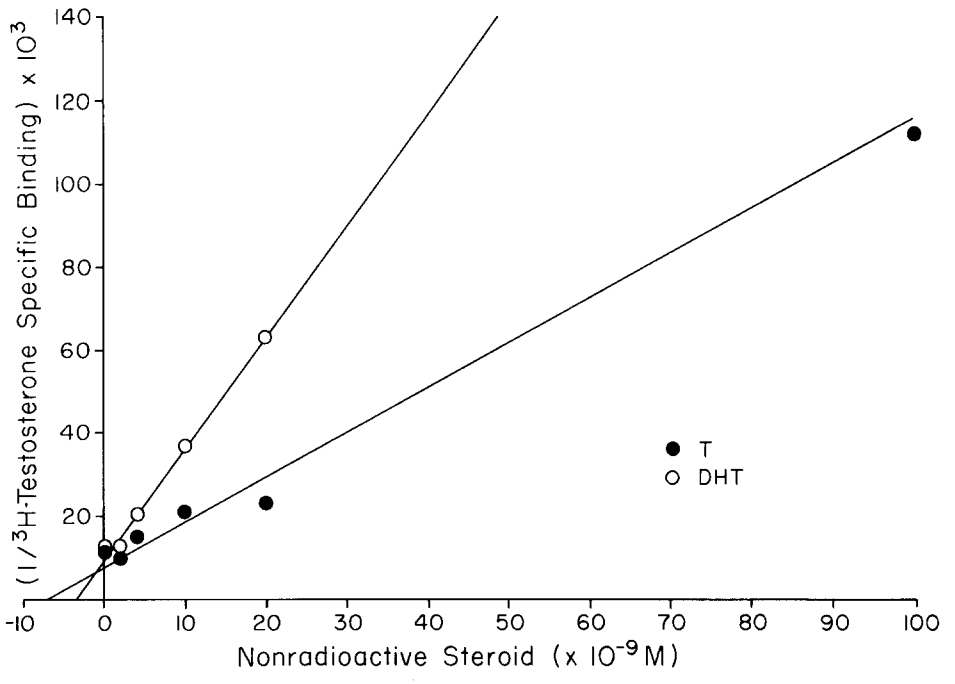

Fig. 10. Dixon plot of competition by radioinert testosterone (T) or dihydrotestosterone (DHT) for [^{3}H]-T binding to the androgen receptor from foreskin fibroblasts of a subject with 5α-reductase deficiency. The ratio of the apparent inhibiting constants, K_i(T); K_i (DHT) was 2.3, indicating a lower affinity of the receptor for T (●) than for DHT (○).

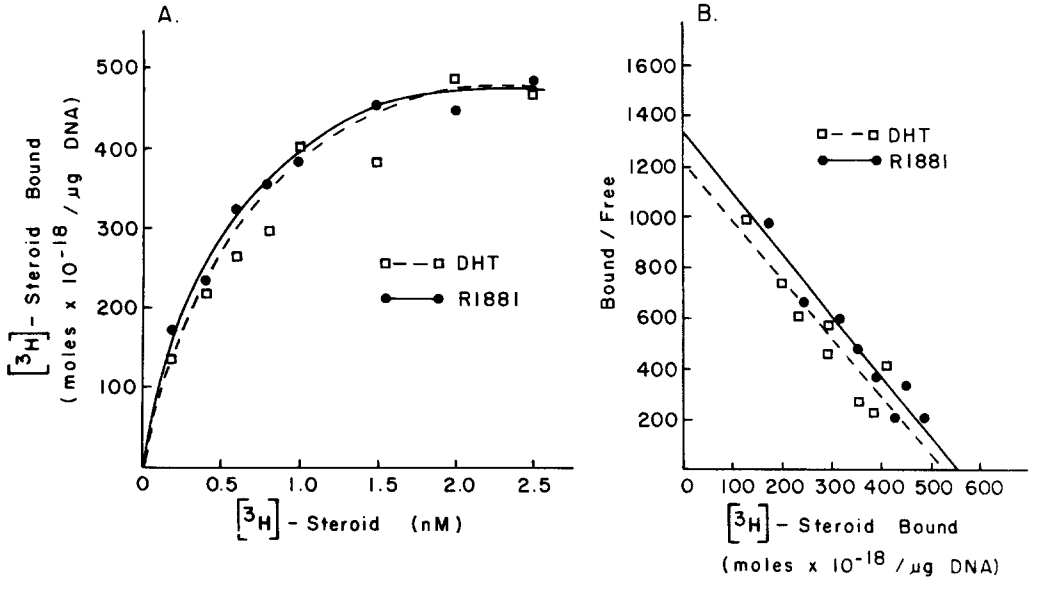

Fig. 11. Saturation analysis of dihydrotestosterone (DHT) and methyltrienolone (R1881) binding to the androgen receptor of cultured newborn foreskin fibroblasts.

A. Specific binding of DHT (□) and R1881 (●) with increasing concentrations of [^{3}H]-steroid (0.2–2.5 nM) was determined as the difference between total (^{3}H-steroid alone) and nonspecific binding (^{3}H-steroid plus 100-fold excess nonradioactive steroid) in whole cell lysates by G-25 sephadex column chromatography.

B. Scatchard analysis of the data from specific binding of DHT (□) or R1881 (●) as described above. The maximum binding capacity (B_{max}; moles $\times 10^{-18}$/µg DNA) for DHT = 536 and for R1881 = 551. The binding affinity (K_d; $\times 10^{-9}$ M) for DHT = 0.41 and for R1881 = 0.42.

binding in normal newborn foreskin fibroblasts (Fig. 11). R1881 has also been reported to bind to the progestin receptor (56–59), however, we observed no effect of added progesterone on the saturation kinetics of R1881 binding in skin fibroblasts. This finding also implies that genital skin fibroblasts do not contain a progestin receptor. Competitive binding assays of ^{3}H-R1881 and ^{3}H-DHT with various unlabeled steroids indicate that R1881 has a 1.5–2.0 fold greater affinity for the androgen receptor than DHT, with the synthetic antiandrogens, R2956 and cyproterone acetate, next in order of decreasing relative affinities. Estradiol, progesterone, dexamethasone and triamcinolone acetonide have very low relative affinities compared to either R1881 or DHT. R1881 is not appreciably metabolized by foreskin fibroblasts since greater than 90% of the original substrate concentration is recovered as R1881 after 4 h incubation at 37 °C. A single peak of radioactivity is observed coincidently for both R1881 and DHT, sedimenting at approximately 3.2 S, following sucrose density gradient centrifugation of fibroblast cell lysates in high salt (0.5 M KCl) buffer. Subcellular distribution of R1881 binding is similar to that of DHT, with approximately 50% of the total cellular bound androgen associated with the nuclear fraction. Only 67% of the total ethanol extractable nuclear bound androgen is extracted by high salt (0.5 M KCl) buffer and sonication. In summary, R1881 shares many of the same properties as DHT in binding to the androgen receptor of genital skin fibroblasts with one notable exception, R1881 can be studied under steady state conditions since it is not metabolized.

As alluded to above, the fibroblast system is also useful toward the evalution of various compounds as antiandrogens (T. Brown, submitted for publication). Compounds such as cyproterone acetate, inhibit the intracellular binding of DHT to the androgen receptor with a relative affinity approximately 1/3 that of DHT itself. The synthetic steroidal derivative, R2956, also intereferes with DHT binding with about the same potency as cyproterone acetate. By contrast, another nonsteroidal antiandrogenic compound, RU23908, is not an effective inhibitor of intracellular androgen binding to the receptor in fibroblasts. Furthermore, our studies also demonstrated that an active metabolite of RU23908 is not formed in fibroblasts which might in turn inhibit DHT binding. In conclusion, cultured human skin fibroblasts provide an intact cellular system for the study of androgen receptor binding and potential inhibitors of the receptor mediated mechanism of androgen action.

3. Pathophysiology: complete androgen insensitivity

One of the most thoroughly studied of the congenital defects contributing to male pseudohermaphroditism is that of insensitivity of reproductive tissues to either endogenous or exogenous androgens. The complete form of the androgen insensitivity syndrome, which is frequently but rather inappropriately designated 'testicular feminization', is transmitted via a recessive gene localized on the X-chromosome (41). This condition has been described in mice (Lyon-Hawkes mutation) (60, 61), rats (Stanley-Gumbreck mutation) (62, 63) and man (64, 65), as well as other mammalian species. Its characteristics in man include the following: (1) abdominal or inguinal testes that frequently secrete testosterone in amounts that approximate its output by the scrotal testes of normal adult males; (2) no significant abnormality in the metabolism of testosterone by liver or other tissues, or in the excretion of its major metabolic inactivation products; (3) abnormally high circulating levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH); (4) an external female phenotype frequently associated with higher levels. of circulating estrogens than in normal males, with female external genitalia and breast development at puberty, but no pubic and axillary hair; (5) a grossly normal appearing 46,XY karyotype; and (6) an absence of internal sexual organs that normally can arise from either the Wolffian or the Müllerian ducts (i.e. lack of oviducts, uterus and upper vagina, as well as epididymis, vas deferens, seminal vesicles and prostate gland). These individuals are refractory to the masculinizing actions of large doses of exogenous testosterone or other androgenic steroids.

From studies of androgen binding in human skin fibroblasts, the absence of functional androgen receptors was established as a biochemical basis for the syndrome of complete androgen insensitivity (AIS) (9) (Fig. 12). This defect is analogous to that

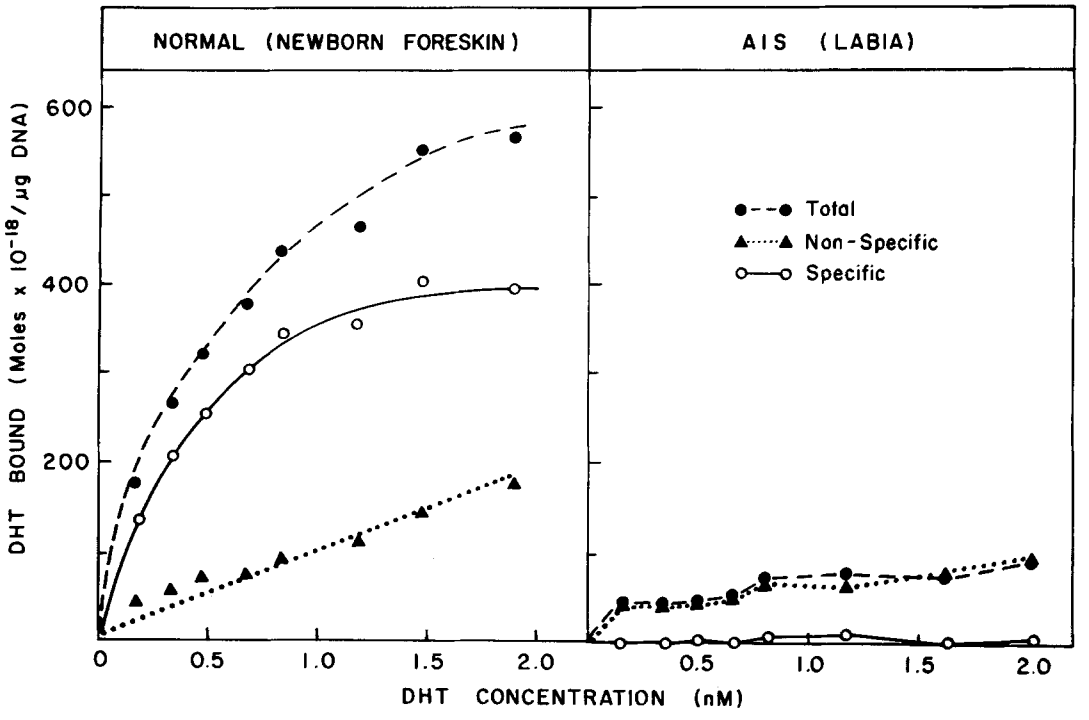

Fig. 12. Saturation curves for dihydrotestosterone binding to the androgen receptor from genital skin fibroblasts of a normal male subject (left) and a subject with complete androgen insensitivity, receptor (-) (right). The curves represent the total (●) and nonspecific (▲) binding plotted from the experimental data and the calculation of the specific receptor binding (○) as the difference.

demonstrated in the androgen insensitive (*Tfm*/Y) mouse mutant (66). We have now studied a total of 15 patients from 11 families with AIS coincident with a lack of DHT binding activity in skin fibroblasts taken from various anatomical sites (1, 41) (Fig. 13). The absence of DHT receptor binding in patients with androgen insensitivity has been confirmed by the studies of other investigators (51, 54, 67).

More recently, genetic heterogeneity has been observed in human patients exhibiting the complete form of the androgen insensitivity syndrome (1, 67). Among 20 subjects seen in our clinic with the complete form of androgen insensitivity syndrome, five were observed to have normal levels of androgen binding activity in skin fibroblasts and no abnormality of nuclear uptake or retention of DHT. Two other laboratories have reported a total of seven additional patients with similar characteristics (48, 69). We have elected to categorize these patients as complete androgen insensitive, receptor (+).

In patients with absence of cytoplasmic DHT binding, (receptor (-)) the androgen insensitivity may be due either to a lack of the receptor protein or to a structural alteration of the receptor, such that there is a loss of affinity for the steroid (Fig. 14). The category of complete androgen insensitivity with normal DHT binding capacity (receptor (+)) is more difficult to explain. Although no quantitative abnormality has been observed in cytoplasmic binding or nuclear uptake of DHT, this does not rule out the existence of a qualitatively abnormal receptor protein. We recently observed that fibroblasts from the labia majora of a patient with AIS, receptor (+), had androgen receptors with lower affinity for DHT when compared to normal controls (T. Brown, unpublished observation). This conclusion was derived from determinations of the apparent dissociation constant (k_d), the inhibitory constant (k_i) and the dissociation rate constant (K_d). Furthermore, the DHT receptor complex of the AIS patient was less stable at 23 °C than the androgen receptor from normal genital

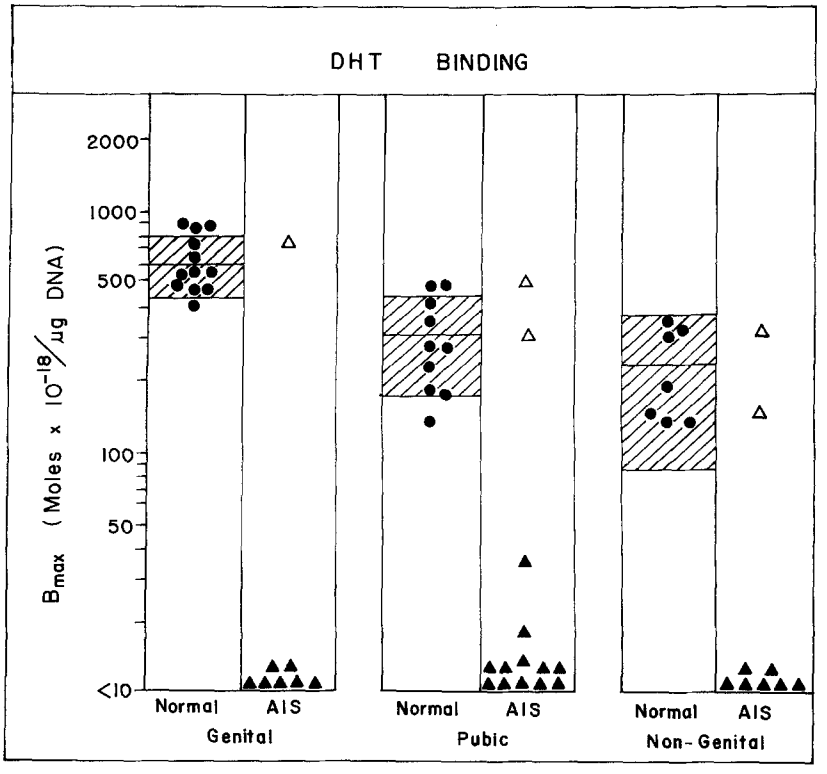

Fig. 13. Dihydrotestosterone binding capacity (B_{max}) in skin fibroblasts from normal male subjects and in subjects with complete androgen insensitivity. Heterogeneity is demonstrated among patients with complete androgen insensitivity syndrome (AIS) since 15 subjects had no receptor binding, receptor (-) (▲), whereas, 5 subjects had normal receptor binding, receptor (+) (△). The shaded areas represent the mean ± SD for normal subjects (●).

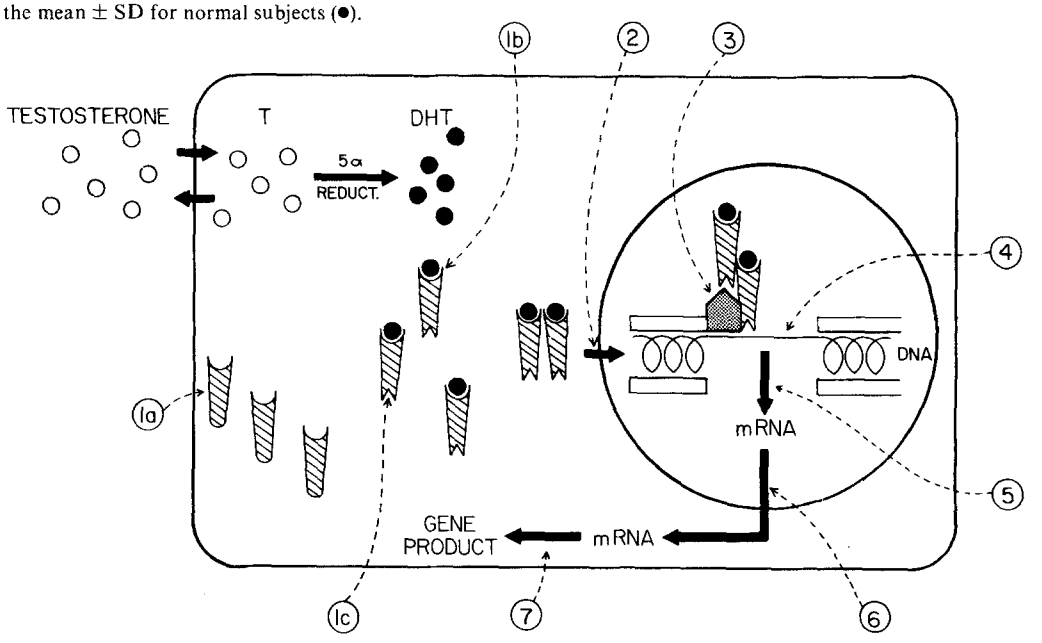

Fig. 14. Conceptual diagram for the mode of action of androgens within a target cell with enumeration of potential sites for mutations which might cause androgen insensitivity.

(1) mutation affecting the cytosolic receptor: (a) absent receptor; (b) abnormal receptor binding site for steroid; (c) abnormal receptor site for recognition of the nuclear acceptor. (2) defect in nuclear translocation of steroid-receptor complex. (3) abnormal nuclear acceptor site. (4) abnormal regulatory molecule(s). (5) abnormal mRNA transcription. (6) abnormal mRNA processing. (7) abnormal translation of androgen dependent protein(s).

skin fibroblasts. Further evidence for a qualitative abnormality in the androgen receptor was obtained through temperature sensitivity studies by Griffin (69) and by Pinksy *et al.* (70). They independently reported that incubation of cultured fibroblasts with DHT at 42 °C resulted in significant reductions in steroid binding with cells from AIS, receptor (+) patients, when compared to cells from normal individuals which exhibited no differences in binding capacity at 37 °C and 42 °C. Further studies are indicated to identify the biochemical implications of this apparent genetic alteration in the tertiary structure of the androgen receptor protein which may affect its interaction with nuclear chromatin acceptor sites to initiate gene transciption.

Partial androgen insensitivity

The clinical phenotype of hereditary male pseudohermaphroditism covers a spectrum of features representing defective virilization. These consist of a small phallus, perineal hypospadias, bifid scrotum, pseudovagina and, at puberty, gynecomastia with incomplete virilization and azoospermia. These manifestations are seen in the syndrome described by Reifenstein (71, 72), Rosewater (73), Gilbert-Dreyfus (74) and Lubs (75) and their associates. Wilson *et al.* (76) provided evidence that the abnormality in one family with Reifenstein syndrome was due to diminished end-organ sensitivity to androgen, rather than decreased androgen secretion as previously suggested. We concurred with this conclusion following the study of eight patients presenting clinically with Reifenstein syndrome and proposed the term 'partial androgen insensitivity' to characterize these conditions of partially defective androgen action (22).

DHT binding studies in skin fibroblasts demonstrated two genetic variants similar to those reported in complete androgen insensitivity syndrome: several patients had a partial deficiency of cellular DHT binding, while four others had DHT binding activity in the normal range (22). The cause of the androgen insensitivity in the latter four individuals is unknown, but analogous in general principle to the AIS, receptor (+) individuals. In all of the subjects we tested, DHT receptors were found in both cytosol and nuclei of skin fibroblasts. Griffin & Wilson (50, 69) have reported five additional patients with Reifenstein syndrome, associated deficient androgen receptor binding in scrotal skin fibroblasts. No qualitative abnormalities in the residual androgen receptor were demonstrated in their studies and unlike the patients they studied with AIS, receptor (+), Reifenstein patients had receptors that were not thermolabile. By contrast, Pinsky *et al.* (70) have reported that a patient with incomplete androgen insensitivity but normal receptor binding displayed an intermediate level of receptor thermostability between the less stable AIS, receptor (+) species and the receptor species present in normal fibroblasts.

Genetics of androgen insensitivity

Absent androgen receptor binding activity due to the lack of the receptor or a modification of the receptor which precluded steroid binding was demonstrated in tissues from animals with known X-linked androgen insensitivity (66). Evidence suggesting homology between X-chromosomal genes of all mammals (77) and the presence of a *Tfm* locus on the mouse X-chromosome associated with androgen insensitivity (60, 61) presented the possibility for a corresponding gene on the human X-chromosome. Indeed, the distribution of affected cases of complete androgen insensitivity within families has been consistent with a mode of inheritance associated with a sex-linked recessive mutation (1, 41). However, the pedigrees could not rule out the possibility of a male-limited autosomal dominant mode of transmission for the disorder. Studies in our laboratory with skin fibroblasts from human patients with complete AIS, receptor (-) (9), provided the means to determine if the human locus was indeed X-linked.

The androgen receptor phenotype of an obligate heterozygote (the mother of three affected individuals) permitted us to discriminate between the alternative modes of transmission. If the androgen receptor deficiency were attributable to a single dose of a mutant autosomal gene, then one would predict that both males and females with one dose of the gene should have an identical receptor deficiency. By contrast, if the defect was related to an X-chromosome mutation, the mother of affected genetic males would have only a partial deficiency. The experimental results demonstrated that uncloned maternal skin fibroblasts had DHT binding activity within the normal range (78), indicating that the mutation was not autosomal

and, therefore, must be X-linked. Furthermore, in clonal populations of skin fibroblasts of maternal origin, two subpopulations of normal and undetectable androgen binding activities were observed (Fig. 15). This latter finding is compatible with X-inactivation and further confirms the X-linked mode of inheritance for the human androgen insensitivity syndrome, receptor (–).

Although it has not been possible to study in a similar fashion the families of AIS, receptor (+) patients, present evidence suggests a similar mode of inheritance (1, 41). Furthermore, it has been suggested that variants with receptor (–) and receptor (+) are allelic forms. However, several other abnormalities in the mode of action of androgens could account for the AIS of subjects with the receptor (+) form and genetic heterogeneity could be present in this group of patients.

Conclusion

From studies of cultured human genital skin fibroblasts, at least three different classes of genetic mutations of androgen action have been identified in man. Abnormal male sexual differentiation and development can result from deficiency of 5α-reductase enzyme activity, absence of androgen binding to its intracellular receptor and inability of the androgen receptor complex to initiate specific biological responses. The first defect in enzyme activity appears to be an autosomal recessive trait, whereas the latter two abormalities involving the androgen receptor are apparently X-linked recessive disorders. Whereas the gene product is absent or grossly abnormal to the extent that normal androgen action is precluded in the cases of 5α-reductase and receptor (–) androgen insensi-

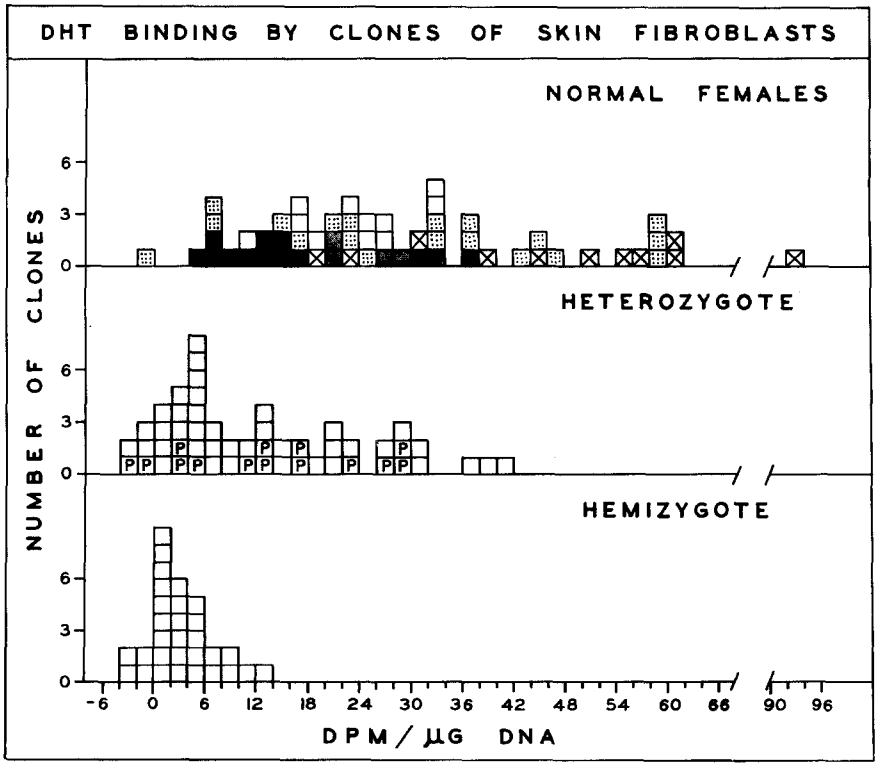

Fig. 15. Dihydrotestosterone binding by skin fibroblast clones. Clones were derived from normal females (top), a heterozygous female carrier of the gene for androgen insensitivity, receptor (–) form (middle) and a hemizygous XY subject with androgen insensitivity, receptor (–) form (bottom). All clones were derived from wrist skin except those labeled P which were from the pubis. A different symbol is used within the blocks to distinguish clones from each of four normal adult females.

tivity, the crucial site to account for receptor (+) androgen insensitivity has yet to be identified.

Cultured human fibroblasts have also been shown to be an excellent model for the screening of compounds which might inhibit 5α-reductase activity or which might block the expression of androgen action at the cellular level by competing for the androgen receptor. In addition, one must keep an open mind as to the possible existence of auxiliary mechanisms within cells which determine and regulate steroid hormone action beyond what is presently known about receptor-mediated activities. Future study and elucidation of the pathophysiology underlying various forms of androgen insensitivity will undoubtedly contribute to our better understanding of the intricacies of hormone action, cellular control and sexual development and differentiation. Cultured human genital skin fibroblasts may provide a biological medium by which to define these biochemical events.

Acknowledgements

We thank Ms. Jacqueline Kolb and Mr. Stephen Rothwell for expert technical assistance and Ms. Barbara S. Mace for excellent secretarial work.

We would also like to recognize the dedicated efforts of the Fellows who have worked in our laboratory over the years and whose work is summarized in this review: Drs. Bruce Keenan, James Amrhein, Walter Meyer, Didier DeWailly, Marc Maes, Nadia Zerhouni, Charles Sultan and Kaye Fichman.

This work was supported by U.S. Public Health Service Research Grant AM-00180, Traineeship Grant T32-AM07116, and Research Career Award 5-K06-AM-21855 (CJM).

References

1. Migeon, C. J., Brown, T. R. & Fichman , K. R., 1980. In: Monographs on endocrinology, The intersex child (Josso, N., ed.), Springer-Verlag, Berlin-Heidelberg, in press.
2. Griffin, J. E. & Wilson, J. D., 1980. N. Engl. J. Med. 302: 198–209.
3. Pinsky, L., 1978. In: Numerical taxonomy of birth defects and polygenic disorders (Bergsma, D. & Lowry, R. B., eds.), Vol. XIV, pp. 73–95, Alan R. Liss, New York.
4. Wilson, J. D., Griffin, J. E. & George, F. W., 1980. Biol. Reprod. 22: 9–17.
5. Wilson, J. D., 1978. Annu. Rev. Physiol. 40: 279–306.
6. Chan, L. & O'Malley, B. W., 1976. N. Engl. J. Med. 294: 1322–1328.
7. Liao, S., 1975. Int. Rev. Cytol. 41: 87–172.
8. Mainwaring, W. I. P., 1977. In: The mechanism of action of androgens, pp. 1–178, Springer-Verlag, New York.
9. Keenan, B. S., Meyer, W. J., Hadjian, A. J., Jones, H. W. & Migeon, C. J., 1974. J. Clin. Endocrinol. Metab. 38: 1143–1146.
10. Wotiz, H. H., Mescon, H., Doppel, H. & Lemon, H. M., 1956. J. Invest. Dermatol. 26: 113–121.
11. Gomez, E. C. & Hsia, S. L., 1968. Biochemistry 7: 24–32.
12. Keenan, B. S., Meyer, W. J., Hadjian, A. J. & Migeon, C. J., 1975. Steroids 25: 535–5 52.
13. Wilson, J. D. & Walker, J. D., 1969. J. Clin. Invest. 48: 371–379.
14. Shanies, D. D., Hirschhorn, K. & New, M. I., 1972. J. Clin. Invest. 51: 1459–1468.
15. Mulay, S., Finkelberg, R., Pinsky, L. & Solomon, S., 1972. J. Clin. Endocrinol. Metab. 34: 133–143.
16. Wilson, J. D., 1975. J. Biol. Chem. 250: 3498–3504.
17. Pinksy, L., Kaufman, M., Straisfeld, C., Zilahi, B. & St.-G. Hall, C., 1978. Am.J. Med. Genet. 1: 407–416.
18. Lamberigts, G., Dierickx, P., DeMoor, P. & Verhoeven, G., 1979. J. Clin. Endocrinol. Metab. 48: 924–930.
19. Moore, R. J., Griffin, J. E. & Wilson, J. D., 1975. J. Biol. Chem. 250: 7168–7172.
20. Sultan, C., Migeon, B. R., Rothwell, S. W., Maes, M., Zerhouni, N. & Migeon, C. J., 1980. Pediatr. Res. 14: 67–69.
21. Pinsky, L., Kaufman, M., Straisfeld, C. & Shanfield, B., 1974. J. Clin. Endocrinol. Metab. 39: 395–398.
22. Amrhein, J. A., Jones-Klingensmith, G., Walsh, P. C., McKusick, V. A. & Migeon, C. J., 1977. N. Engl. J. Med. 297: 350–356.
23. Mowszowicz, I., Kirchhoffer, M.-O., Kuttenn, F. & Mauvais-Jarvis, P., 1980. Molec. Cell. Endocrinol. 17: 41–50.
24. Kuttenn, F. & Mauvais-Jarvis, P., 1975. Acta Endocrinol. 79: 164–176.
25. Mauvais-Jarvis, P., 1977. In: Androgens and antiandrogens (Martini, L. and Motta, M., eds.). pp. 229–245, Raven Press, New York.
26. Pinksy, L., Finkelberg, R., Straisfeld, C., Zilahi, B., Kaufman, M. & Hall, G., 1972. Biochem. Biophys. Res. Commun. 46: 346–349.
27. Amrhein, J. A., Meyer, W. J., Danish, R. K. & Migeon, C. J., 1977. J. Clin. Endocrinol. Metab. 45: 732–738.
28. Kaufman, M., Straisfeld, C., Shanfield, B., Zilahi, B. & Pinsky, L., 1974. Am. J. Hum. Genet. 26: 47A (Abstract).
29. Kaufman, M., Pinsky, L., Straisfeld, C., Shanfield, B. & Zilahi, B., 1975. Exp. Cell. Res. 96: 31–36.
30. Zerhouni, N., Maes, M., Sultan, C., Rothwell, S. & Migeon, C. J., 1979. Steroids 33: 277–285.
31. Moore, R. J. & Wilson, J. D., 1976. J. Biol. Chem. 251: 5895–5900.
32. Imperato-McGinley, J., Guerrero, L., Gautier, T. & Peterson, R. E., 1974. Science 186: 1213–1215.
33. Walsh, P. C., Madden, J. D., Harrod, M. J., Goldstein, J. L., MacDonald, P. C. & Wilson, J. D., 1974. N. Engl. J. Med. 291: 944–949.
34. Imperato-McGinley, J. & Peterson, R. E., 1976. Am. J. Med. 61: 251–272.

35. Peterson, R. E., Imperato-McGinley, J., Gautier, T. & Sturla, E., 1977. Am. J. Med. 62: 170–191.
36. Imperato-McGinley, J., Peterson, R. E., Gautier, T. & Sturla, E., 1979. N. Engl. J. Med. 300: 1233–1237.
37. Leshin, M., Griffin, J. E. & Wilson, J. D., 1978. J. Clin. Invest. 62: 685–691.
38. Fisher, L. K., Kogut, M. D., Moore, R. J., Goebelsmann, U., Weitzmann, J. J., Issacs, H., Griffin, J. E. & Wilson, J. D., 1978. J. Clin. Endocrinol. Metab. 47: 653–664.
39. Imperato-McGinley, J., Peterson, R. E., Leshin, M., Griffin, J. E., Cooper, G., Draghi, S., Berenyi, M. & Wilson, J. D., 1980. J. CLin. Endocrinol. Metab. 50: 15–22.
40. Maes, M., Sultan, C., Zerhouni, N., Rothwell, S. W. & Migeon, C. J., 1979. J. Steroid. Biochem. 11: 1385–1390.
41. Fichman, K. R., Migeon, B. R. & Migeon, C. J., 1980. In: Advances in human genetics (Harris, H. & Hirschhorn, K., eds.), Vol. 10, pp. 333–377, Plenum Press, New York.
42. Migeon, C. J., Amrhein, J. A., Keenan, B. S., Meyer, W. J. III & Migeon, B. R., 1979. In: Genetic mechanisms of sexual development (Vallet, H. L. and Porter, I. H ., eds.), pp. 93–128, Academic Press, New York.
43. Korenman, S. G. & Dukes, B. A., 1970. J. Clin. Endocrinol. Metab. 30: 639–645.
44. McGuire, W. L. & deLaGarza, M., 1973. J. Clin. Endocrinol. Metab. 37: 986–989.
45. Castañeda, E. & Liao, S., 1974. Endocr. Res. Commun. 1: 271–281.
46. DeWailly, D., Maes, M., Amrhein, J. A., Rothwell, S. R. & Migeon, C. J., 1979. J. Lab. Clin. Med. 94: 106–113.
47. Scatchard, G., 1949. Ann. N. Y. Acad. Sci. 51: 660–672.
48. Kaufman, M., Pinsky, L., Baird, P. A. & McGillivray, B. C., 1979 Amer. J. Med. Genet. 4: 401–411.
49. Eil, C., Lippman, M. E. & Loriaux, D. L., 1980. Steroids 35: 389–404.
50. Griffin, J. E. & Wilson, J. D., 1977. J. Clin. Endocrinol. Metab. 45: 1137–1143.
51. Griffin, J. E., Punyashthiti, K. & Wilson, J. D., 1976. J. Clin. Invest. 57: 1342–1351.
52. Kaufman, M., Straisfeld, C. & Pinsky, L., 1977. Somatic Cell Genet. 3: 17–25.
53. Collier, M. E., Griffin, J. E. & Wilson, J. D., 1978. Endocrinology 103: 1499–1505.
54. Kaufman, M., Pinsky, L., Kubski, A., Straisfeld, C., Dobrenis, K., Shiroky, J., Chan, T. & MacGibbon, B., 1978. J. Clin. Endocrinol. Metab. 47: 738–745.
55. Brown, T. R., Rothwell, S. W. & Migeon, C. J., 1981. J. Steroid Biochem., in press.
56. Sirett, D. A. N. & Grant, J. K., 1978. J. Endocrinol. 77: 101–110.
57. Asselin, J., Labrie, R., Gourdeau, Y., Bonne, C. & Raynaud, J. P., 1976. Steroids 28: 449–459.
58. Shain, S. A., Boesel, R. W., Lamm, D. L. & Radwin, H. W., 1978. Steroids 31: 541–556.
59. Menon, M., Tananis, C. E., Hicks, L. L., Hawkins, E. F., McLaughlin, M. G. & Walsh, P. C., 1978. J. Clin. Invest. 61: 150–162.
60. Lyon, M. F. & Hawkes, S. G., 1970. Nature 227: 1217–1219.
61. Ohno, S. & Lyon, M. F., 1970. Clin. Genet. 1: 121–127.
62. Stanley, A. J. & Gumbreck, L. G., 1964. The Endocrine Society Program, p. 40 (Abstract).
63. Bardin, C. W., Bullock, L. P., Schneider, G., Allison, J. E. & Stanley, A. J., 1970. Science 167: 1136–1137.
64. Morris, J. M., 1953. Amer. J. Obstet. Gynecol. 65: 1192–1211.
65. Wilkins, L., 1950. In: The diagnosis and treatment of endocrine disorders in childhood and adolescence, pp. 297–341, Charles C. Thomas, Springfield, Ill.
66. Bardin, C. W., Bullock, L. P., Sherins, R. J., Mowszowicz, I. & Blackburn, W. R., 1973. Rec. Prog. Horm. Res. 29: 65–109.
67. Kaufman, M., Straisfeld, C. & Pinsky, L., 1976. J. Clin. Invest. 58: 345–350.
68. Amrhein, J. A., Meyer, W. J. III, Jones, H. W. & Migeon, C. J., 1976. Proc. Natl. Acad. Sci. USA 73: 891–894.
69. Griffin, J. E., 1979. J. Clin. Invest. 64: 1624–1631.
70. Pinksy, L., Kaufman, M., Feldstein, W. & Nudo, J., 1980. Pediatr. Res. 14: 483 (Abstract).
71. Reifenstein, E. G. Jr., 1947. Proc. Amer. Fed. Clin. Res. 3: 86.
72. Bowen, P., Lee, C. S. N., Migeon, C. J., Kaplan, N. M., Whalley, P. J., McKusick, V. A. & Reifenstein, E. C., 1965. Ann. Intern. Med. 62: 252–270.
73. Rosewater, S., Gwinup, G. & Hamwi, C. J., 1965. Ann. Intern. Med. 63: 377–385.
74. Gilbert-Dreyfus, S., Sebaoun, C. I. A. & Belaisch, J., 1957. Ann. Endocrinol. (Paris) 18: 93–101.
75. Lubs, H. A. Jr., Vilar, D. & Bergenstal, D. M., 1959. J. Clin. Endocrinol. Metab. 19: 1110–1120.
76. Wilson, J. D., Harrod, M. J. Goldstein, J. L., Hemsell, D. L. & MacDonald, P. C., 1974. N. Engl. J. Med. 290: 1097–1103.
77. Ohno, S., 1967. In: Sex chromosomes and sex-linked genes, Springer-Verlag, New York.
78. Meyer, W. J. III, Migeon, B. R. & Migeon, C. J., 1975. Proc. Natl. Acad. Sci. USA 72: 1469–1472.

Received November 17, 1980.