ANDROGEN RECEPTORS AND METABOLISM IN CULTURED
HUMAN FETAL FIBROBLASTS

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SUMMARY

Testosterone metabolism and dihydrotestosterone (DHT) receptor activity were studied in fibroblasts cultured from genital and non-genital tissues of 8- to 22-week old human fetuses.

As early as the eighth week of gestation, DHT receptor activity was detected in non-genital skin. The binding capacity (B_{max}) was greater in genital skin fibroblasts (mean $\pm$ SD = 474 ± 32 moles $\times 10^{-18}$ /ug DNA) than non-genital skin (mean $\pm$ SD = 124 ± 42 moles $\times 10^{-18}$ /ug DNA). DHT receptor binding (B_{max}) was found in fibroblasts derived from testes (112 moles $\times 10^{-18}$ /ug DNA), but not intestine (< 10 moles $\times 10^{-18}$ /ug DNA). The DHT receptor activity of fetal skin fibroblasts of genital origin was similar to that of fibroblasts derived from the foreskin of normal newborns. DHT receptors from fetal and newborn fibroblast cultures had similar sedimentation coefficients in sucrose density gradient centrifugation, but there were small differences in their relative affinities for 17 β -estradiol and cyproterone.

Low, but detectable 5 α -reductase activity was observed at 8 weeks gestation in non-genital skin fibroblasts and was present in fibroblasts from a variety of tissues of older fetuses, including testes, kidneys and lungs. The highest 5 α -reductase activity of 210 pg/hour/ug DNA was found in fibroblasts cultured from clitoral tissue from a 10-week old fetus. In all but one specimen, the 5 α -reduced products were either DHT or 5 α -androstanedione.

The demonstration of 5 α -reductase activity and specific DHT receptors in fetal tissues suggests that the intracellular mechanism for androgen action is present in the fetus, similar to that after birth.

SPECULATION

We have observed the conversion of testosterone into dihydrotestosterone (DHT) as well as DHT binding to a specific receptor in human fibroblasts cultured from a variety of tissues of fetal origin as early as 8 weeks gestation. It is speculated that the concentration of testosterone, the rate of formation of DHT and the concentration of androgen receptors each play a role in the differentiation of the external genitalia.

INTRODUCTION

The action of androgens on their target cells is mediated by specific receptors. Dihydrotestosterone (DHT) combines with a cytosolic binding protein to form a hormone-protein complex which is translocated into the nucleus where it exerts its action on chromatin (5). Fibroblasts raised from the genital skin of normal subjects have receptors which bind DHT with high affinity ($K_d = 0.55 \pm 0.23 \times 10^{-9}$ M) and high specificity, but low capacity ($B_{max} = 518 \pm 260 \times 10^{-18}$ moles/ug DNA) (12). Although K_d and B_{max} values are similar for fibroblasts derived from male and female individuals, the B_{max} is higher in cells originating from genital skin than in cells from non-genital skin (11).

Development of the Wolffian ducts and masculinization of the external genitalia in the male fetus is known to be under the influence of testicular androgens. However, very little work has been done to characterize the androgen receptor in human fetuses because of the difficulty in obtaining tissues (10, 24, 26). In the present paper, we have studied human fetal fibroblasts for their ability to transform testosterone into DHT and for the presence of receptors capable of binding DHT.

MATERIALS AND METHODS

Tissue Samples: The materials of this study were the foreskins of normal newborn males obtained at circumcision and fetal tissues obtained for another study from hysterectomies according to a protocol approved by the Joint Committee on Clinical Investigation and the Joint Committee on Fetal Tissues of the Johns Hopkins Medical Institutions. Informed consent was obtained in all cases. Estimates of fetal age were based on the data of the last menstrual period, fetal foot size and crown-to-rump length. Fetal sex was determined by chromosome analysis (16).

Cell Cultures: Fibroblasts were established from the explants from these tissues. After the initial passage they were stored in liquid nitrogen, to be thawed and used as needed. The cells were plated on 100 mm Petri dishes in GIBCO's minimum essential medium with Earl's salts, supplemented with non-essential amino acids, 15% fetal calf serum, 27 mM sodium bicarbonate, penicillin (100 U/ml), amphotericin B (2.5 ug/ml), streptomycin (25 ug/ml) and gentamycin (4 ug/ml). Cells used for assays were in subcultures 5 to 10.

DHT Receptor Assay: Confluent monolayers of fibroblasts in 100 mm Petri dishes were incubated for 15 min at 37°C, with serum-free medium containing ^{3} H-DHT (specific activity = 123 Ci/mmol) in concentrations varying from 0.2 to 2.0×10^{-9} M. After incubation the medium was removed and an aliquot was counted to determine unbound radioactivity. The fibroblasts were washed three times with 5 ml 0.9% NaCl, at 4°C, to remove the bulk of the residual unbound ^{3} H-DHT. Then, 2.5 ml Tris-KCl buffer (0.02M Tris-HCl, 1.5 mM Na₂ EDTA and 0.5M KCl, pH 7.9 at 4°C) were added to the plates and the cells were removed with a rubber policeman, collected by centrifugation, and sonicated in 0.8 ml Tris-KCl buffer. The sonicates were centrifuged at 1,600 x g for 20 min and the supernatants were used for the assay. A 0.25 ml aliquot was obtained for DNA determination (3). Two other 0.25 ml aliquots were used, one of them being heated for 3 min at 60°C to denature the receptor protein. Then, both were incubated with 0.25 ml of a solution containing a testosterone antibody coupled to sepharose. The testosterone antibody, which had 98% cross-reactivity for DHT, was coupled to CNBr-activated sepharose 4B as described by Castañeda and Liao (4). After 2 hours shaking in an ice-bath, the mixture was centrifuged. The sepharose-antibody complexed the free DHT and sedimented to the bottom of

the tubes while 0.5 ml of the supernatant was removed, added to 5 ml Formula 947 (Yorktown Research), and counted for radioactivity.

Specific binding was determined by subtracting the heated sample from the non-heated, the validity of this technique having been demonstrated previously (6). The maximum binding capacity (B_{max}) and the apparent dissociation constant (K_d) of the DHT receptor were derived from Scatchard plots using linear regression analysis (21).

5 α -Reductase Activity: Reduction of testosterone to 5 α -reduced metabolites was assayed as previously described (1). Briefly, confluent monolayers of fibroblasts were incubated with 4 ml of serum deficient medium containing 4 nM ^{3} H-testosterone (specific activity = 98.8 Ci/mmol; New England Nuclear). After 30 and 60 min, 0.5 ml of the medium was removed and ^{14} C-testosterone, ^{14} C-DHT and ^{14} C-androstenedione were added for recovery purposes. Steroids were extracted from the mixture with carbon tetrachloride. Silica gel thin layer chromatography was then used to separate the following steroids: testosterone, androstene-3,17-dione, DHT, androsterone, 5 α -androsterone-17 β -diol (3 α + 3 β) and 5 α -androstane-3,17-dione. They were visualized with iodine vapors, eluted and the radioactive content was determined by liquid scintillation counting (1). The 5 α -reductase activity was expressed as picograms of 5 α -reduced products generated per hour, per ug DNA.

Sucrose Density Gradient: Fibroblast monolayers were incubated for 30 min at 37°C with media containing 1.5×10^{-9} M ^{3} H-DHT. Cell sonicates were prepared in 0.3 ml Tris-KCl buffer as described above, and respun for 1 hour at 5,000 x g. A 20 μ l sample from the supernatant was retained for protein determination, while a 200 μ l sample was layered on top of a linear sucrose gradient. The continuous gradient, ranging from 5 to 20% sucrose in Tris-KCl buffer with 10% (vol/vol) glycerol, was prepared using a Buchler gradient maker in 5 ml nitro-cellulose tubes and allowed to diffuse overnight. Linearity was checked with a refractometer. The sample was centrifuged for 18 hours at 167,000 x g at 0°C in a Beckman L5 Ultracentrifuge (SW 50.1 rotor). Fractions of 200 μ l were collected through the bottom of the tubes and counted in 5 ml of Formula 947 for radioactive content. Bovine-globulin (8.0S) and bovine serum albumin (4.6S) were used as markers and their peaks in the gradients were determined by the Lowry protein assay (14). The coefficients of sedimentation of the ^{3} H-DHT peaks were calculated by the method of Martin and Ames (15).

Competition Studies: Confluent monolayers from the same strain of fibroblasts were incubated for 15 min at 37°C with 2×10^{-9} M ^{3} H-DHT and simultaneously with various concentrations (0 - 400 $\times 10^{-9}$ M) of one of the following unlabeled steroids: DHT, 5 α -androstane-3 β ,17 β -diol, 17 β -estradiol, progesterone, dehydroepiandrosterone, and cyproterone acetate. Specific binding was determined by the antibody method (6). Data were analyzed by Dixon (7) and Logit-log plots (19) using linear regression analysis.

RESULTS

Receptor Binding Studies: Saturation curves performed on fibroblasts derived from 12 newborn foreskins yielded a maximum binding capacity (B_{max}) for DHT of $689 \pm 387 \times 10^{-18}$ moles/ug DNA (mean $\pm$ SD). The mean $\pm$ SD apparent dissociation constant (K_d) for these newborn specimens was $0.85 \pm 0.37 \times 10^{-9}$ M, with a range of 0.37 to 1.36×10^{-9} M (Table 1).

Table 1. Characteristics of the binding of DHT to receptors in cultured skin fibroblasts of human fetuses

#	Age (weeks)	Sex	Origin	B_{max} *	K_d †
K 1	8	M	non-genital skin	89	0.47
K 12	10	F	buttocks	107	0.36
			clitoris (n=3)	453	0.42
JK	14	M	back (n=3)	112	0.75
Fe 0	17	F	pubis	459	1.98
			clitoris	511	2.04
Fo	17	M	scrotum (n=2)	1006	0.85
			inguinal	379	1.14
Fe 16	22	M	non-genital skin (n=3)	185	0.57
Normal newborns		M	foreskin	689 ± 387	0.85 ± 0.37

*($\times 10^{-18}$ moles/ug DNA)

†($\times 10^{-9}$ M)

Three of the fetuses, K 1 (8 weeks), JK (14 weeks) and Fe 16 (22 weeks) provided non-genital skin. DHT receptors were present in non-genital skin fibroblasts of all three. The youngest one was an 8-week old male with a B_{max} of 89×10^{-18} moles/ug DNA and a dissociation constant of 0.47×10^{-9} M (Fig. 1). The K_d values were in the same range for the three subjects examined; however, the B_{max} was greatest in the oldest fetal specimen studied (22 weeks) (Table 1).

When fibroblasts cultured from genital areas of male and female fetuses were assayed, both sexes had B_{max} values similar to that found in the male newborn foreskins (Table 1). As with adult subjects, fibroblasts from genital skin had greater numbers of DHT receptors than those from non-genital skin (Fig. 2).

Tissues other than skin were also cultured and examined for specific androgen binding (Fig. 3, Table 2). DHT receptors were present in fibroblasts derived from testes and lung. The K_d values were in the range of those found for skin fibroblasts. The number of binding sites was similar to that found for non-genital skin fibroblasts but lower than those of genital target skin. One specimen from intestine was analyzed and no significant receptor binding was detected.

Table 2. Characteristics of the binding of DHT to receptors in cultured fibroblasts derived from various fetal organs

#	Age (weeks)	Sex	Origin	B _{max} *	K _d †
K 12	10	F	kidney	63	0.28
JK	14	M	kidney	52	0.25
Fe 16	22	M	testes (n=2)	108	0.71
JK	14	M	testes (n=2)	116	0.84
Fe 19	14	F	lung	80	2.0
JK	14	M	lung	128	0.81
Fe 16	22	M	intestine (n=3)	< 10	-

*($\times 10^{-18}$ moles/ μ g DNA)

†($\times 10^{-9}$ M)

The cultures of fetal kidney cells consisted of mixtures of fibroblasts and epithelial cells. In two cases, cells of renal origin contained only a small number of DHT receptors ($B_{max} = 52$ and 63×10^{-18} moles/ μ g DNA), but their apparent dissociation constants were low ($K_d = 0.25$ and 0.28×10^{-9} M), suggesting a high affinity of the receptors for DHT.

Sucrose Density Gradient: The cytosol prepared from genital skin fibroblasts previously incubated with ^{3} H-DHT was submitted to sucrose gradient centrifugation in high ionic strength buffer (0.5M KCl). A peak of bound ^{3} H-DHT was observed in the 4S region (Fig. 4). A similar sedimentation profile was observed in gradients run with cytosol prepared from fibroblasts from newborn foreskins.

Competition Studies: The steroid specificity of the DHT receptor of fibroblasts from androgen target tissues of fetuses and newborns was determined by measuring the specific binding of ^{3} H-DHT in the presence of various unlabeled steroids. The concentration of steroid competitor needed to displace 50% of the specific ^{3} H-DHT binding is presented in Table 3.

Table 3. Ligand specificity of the DHT receptor in fetal clitoris and newborn foreskin

Steroid Competitor	Competitor concentration ($\times 10^{-9}$ M) needed to displace 50% of maximal specific ^{3} H-DHT binding	
	K 12 Clitoris	Newborn Foreskin
Dihydrotestosterone	49	$4.24 \pm 2.0^*$
17 β -estradiol	125	49 ± 16
Cyproterone Acetate	2.8	9.3 ± 3.4

*Mean $\pm$ SD

5 α -Reductase Activity: Fetal skin fibroblasts transformed testosterone into 5 α -reduced products (Table 4). This activity was present in the fibroblasts of the youngest fetal specimen tested, the 8-week old male. However, the rate of transformation was slow compared to fibroblasts from normal non-genital adult skin (mean $\pm$ SD = 504 ± 222 pg/hour/ μ g DNA). Although the formation of 5 α -reduced compounds was faster in fibroblasts from clitoris of fetus K 12 than those from non-genital fetal skin, a pattern observed in adult subjects, the fetal values were still at the lower limit of the normal adult range.

The activity of 5 α -reductase was also detected in testes, lung and kidney. DHT was the major 5 α -reduced steroid produced for all of the samples except for the two lung specimens in which androstenedione predominated. Androstenediol was not produced by any of the fetal cultures.

DISCUSSION

It is now well established that DHT receptors play an important role in the mode of action of androgens in human subjects and animals (1, 2, 5, 9-13, 20). The tissues of male and female individuals contain similar amounts of DHT receptor (1), and it is the different concentration of androgens available to the target cells that determines the level of androgenic activity. In the presence of abnormally high amounts of androgens, the genitalia of female fetuses assume a male phenotype. In contrast, male fetuses in whom the production of testicular androgens is impaired (as in congenital adrenal hyperplasia due to 3 β -hydroxy-steroid dehydrogenase deficiency), present an ambiguous or female external genitalia. Androgen uptake by the urogenital tract of male and female rabbit fetuses has been demonstrated by Wilson (26), and a testosterone binding protein has been observed in the reproductive tract of rat fetuses (10). To our knowledge, only preliminary data have been reported re-

Table 4. The 5 α -reductase activity in cultured fibroblasts derived from human fetal skin and various organs

#	Age (weeks)	Sex	Origin	Products formed* pg/ μ g DNA/60 min		
				5 α -reduced steroids	DHT	5 α -androstane-dione
K 1	8	M	non-genital skin	31	27	4
K 12	10	F	buttocks	32	31	1
			clitoris	210	140	70
			kidney	133	84	49
JK	14	M	testes	13	12	1
			lung	35	13	22
Fe 19	14	F	lung	23	5	18
Fo	17	M	scrotum	189	141	16

*No 5 α -androstenediols were formed except for Fo which formed 32 pg/ μ g DNA/60 min

garding testosterone uptake in two human fetuses (24).

Our data demonstrate that DHT receptors are present in non-genital skin fibroblasts as early as the eighth week of gestation. The number of binding

sites is low, tending to increase with fetal age. Furthermore, similar to observations on fibroblasts from adult subjects (12), fibroblasts from genital skin of fetuses yielded much greater number of binding sites for DHT than fibroblasts from non-genital skin.

DHT receptors may also be important in tissues other than skin. It has been shown that steroids can stimulate collagen biosynthesis in the lung (18). This effect might be mediated by steroid receptors since such receptors have been described in rat lung cytosol (17). The presence of DHT receptors in fetal lung suggests the potential for an androgen mediated response in the physiology of the lung.

Testosterone receptors have been described in mouse kidney cytosol (2), whereas DHT receptors have been described in the rat kidney (25). Based on indirect evidence, it has been suggested that the human kidney has a DHT binding cytosolic receptor (9). Our studies demonstrate that fibroblasts from human fetal kidneys have DHT receptors. Although there was little DHT bound, the apparent dissociation constant was lower than in skin fibroblasts, implying a specific role for the receptors in the kidney cells.

Sucrose density gradient characterization showed gross similarity between newborn and fetal DHT receptors. However, many receptor proteins sediment in the same region, and density gradient analysis cannot be expected to detect small differences in physical properties of receptors.

To probe for small differences, competition studies were carried out on genital skin fibroblasts. These are useful for detecting differences in the specificity of the receptor for various steroids. We found that the receptor in fetal cells had a lower affinity for DHT, 17 β -estradiol but higher affinity for cyproterone acetate than that in newborn cells.

Several investigators have reported that testosterone is converted to its 5 α -reduced metabolites more extensively in androgen target tissue than in non-target tissue (8, 13, 22, 23). Similarly, it has been shown that 5 α -reductase activity is greater in fibroblasts from genital skin than in those from non-genital skin of infants and adults (11). Fibroblasts from fetal genitalia, when assayed for 5 α -reductase activity, were found to have levels lower than those of the newborn foreskin. The presence of low 5 α -reductase activity in lung, testes and kidney fibroblasts along with the presence of DHT receptor activity suggest that androgens may have specific actions on these organs.

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