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Androgen Inactivation in Human Lung Fibroblasts: Variations in Levels of 17β-Hydroxysteroid Dehydrogenase Type 2 and 5α-Reductase Activity Compatible with Androgen Inactivation

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Androgens delay lung maturation through their action on lung fibroblasts. Knowing that testosterone is secreted by the lung epithelial-like cell line A-549, we have studied the metabolism of androgens by several human lung diploid fibroblasts cells lines. No 17-ketosteroid reductase activity was detected. In contrast, testosterone was transformed mainly into androstenedione and androstanedione with no 5α-dihydrotestosterone formed, indicating the presence of 17β-hydroxysteroid dehydrogenase (HSD) type 2 and 5α-reductase activities. The eight cell lines analyzed had either a low or high 17β-HSD type 2 activity level. No correlation between these levels and the sex or age stage of cells was established, but Northern blot analysis of human lung RNA samples of five adult subjects revealed very similar variations between subjects in the level of 17β-HSD type 2 mRNA. The 5α-reductase activity had a marked substrate preference for androstenedione, the product of 17β-HSD type 2. When tritiated testosterone was used as substrate, only barely detectable levels of 5α-dihydrotestosterone were observed by HPLC in the presence of the 17β-HSD type 2 inhibitor EM-919. The use of unlabeled testosterone or of the antiandrogen hydroxyflutamide demonstrated that the tritiated testosterone substrate itself had no effect on levels of 5α-reduction. In fact, in these cells, 5α-reductase has no significant activity on testosterone, but it further converts the product of 17β-HSD type 2, thus playing a role with 17β-HSD type 2 in androgen inactivation. Because androgens delay lung maturation and surfactant synthesis by their action on lung fibroblasts, it is of particular interest to find that the steroid metabolism of these lung fibroblast cells is oriented toward androgen inactivation. Because lung fibroblasts of subjects with low 17β-HSD type 2 expression levels are likely to be exposed to higher levels of androgens, an allelic variation of the 17β-HSD-2 gene is suspected, which would result in familial incidence of respiratory distress. This is in line with reported cases of familial incidence of respiratory distress. (*J Clin Endocrinol Metab* 87: 3883–3892, 2002)

DURING LUNG DEVELOPMENT there is pressure by positive and negative regulators of fibroblast-epithelial communication in the control of epithelial type II (PTII) cell maturation that will end by the formation of surfactant synthesis by PTII cells (1). An improper regulation of the factors that govern these processes can delay surfactant production (2), thus increasing the risk for respiratory distress (RD), which is more frequent in male infants (3, 4). The risk for RD increases with the degree of prematurity of the baby, and, interestingly, this condition is more frequent in male than in female infants. Some growth factors such as epithelial growth factor or TGFβ1 antagonize lung maturation processes (5, 6), whereas other factors such as the stromal-derived growth factor neuregulin-1 stimulates surfactant synthesis, as seen in the mouse cell line MLE12 (7). Steroids are known to take part in regulating the processes. For example, glucocorticoids stimulate fibroblast/PTII cells communication, which in turn will stimulate production of pulmonary surfactant by PTII cells. *In vitro*, exposure of fibroblasts to androgens decreased their ability to stimulate the production of surfactant by PTII cells through fibroblast/PTII cell communication (8). Current findings indicate that these steroidal effects are mediated through binding of androgens to androgen receptors (9, 10), which are present in both male and female lung tissues (11).

Studies of laboratory animals also indicate that androgens play a role of negative modulator on surfactant production. When 5α-dihydrotestosterone (5α-DHT) is administered to pregnant females, surfactant production in the lung of female fetuses is decreased to the levels of male fetuses from untreated pregnant females (9, 12). Moreover, male mice carrying the *Tfm* gene (male with testicular feminization), which have no functional androgen receptors, have surfactant levels comparable with that of the normal female at comparable developmental time point (13). All these results suggest that the sex difference in surfactant production is related to the metabolism of androgens. In addition, these results obtained *in vivo* validate the *in vitro* models in which androgens negatively modulate surfactant production.

Because testes do not secrete testosterone during late ges-

Abbreviations: DHEA, Dehydroepiandrosterone; 5α-DHT, 5α-dihydrotestosterone; E_1, estrone, 1,3,5-estratrien-3-ol-17-one; FBS, fetal bovine serum; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of nicotinamide adenine dinucleotide; PTII, epithelial type II cell; RD, respiratory distress.

tation, how could androgens modulate lung maturation at that time? *In situ* metabolism of androgens by the lung itself can answer this question. Tissue- and cell-specific expression of steroidogenic enzymes allows for local formation of sex steroids from circulating C19 steroid precursors of adrenal origin, namely, dehydroepiandrosterone (DHEA) and androstenedione (14). 17β-Hydroxysteroid dehydrogenase (17β-HSD) exists in multiple isoforms, each encoded by a specific gene and each having either a predominant reductase or a dehydrogenase activity *in vivo*. Thus, the presence of specific 17βHSDs expressed in the cells will have as consequences to produce either active 17β-hydroxyandrogens (types 1, 3, 5, or 7) or inactive 17-ketosteroids (types 2, 4, 6, or 8). Although testosterone can efficiently bind the androgen receptor, its 5α-reduction by the 5α-reductase leads to the formation of 5α-DHT, the most potent active androgen.

Early studies report that 3α-HSD and 17β-HSD are the major enzymatic activities detected in lung slices, while low 5α-reductase and 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) activities were also detected (15–17). Recently, we studied the steroidogenic capacity of the male lung adenocarcinoma epithelial cell line A-549. These are PTII-like cells that synthesize disaturated phosphatidylcholines like normal lung PTII cells do. We demonstrated that these cells produce active androgens from androstenedione. A-549 steroid metabolism involves 17β-HSD type 5 and 3α-HSD type 3 as predominant enzymes, and a low 5α-reductase activity (Fig. 1). Consequently, in this model the testosterone produced is stabilized, whereas 5α-DHT is rapidly inactivated into androstane-3α,17β-diol. Therefore, androgens are thought to be produced by PTII cells *in vivo*, at least in the male lung.

Knowing that androgens delay lung maturation by their action on lung fibroblasts and that A-549 cells have the potential to synthesize and secrete testosterone, we studied steroid metabolism in lung fibroblast cells. This study is important because the capacity of fibroblasts to metabolize androgens is a key step in determining their sensitivity to androgens present in their environment. Thus, the aim of this study is to determine whether fibroblasts can control their intracellular levels of androgens obtained *via* PTII cells. If so, this androgen metabolism should contribute to decrease the risk of RD. We compared the steroid metabolism of eight lung and one skin fibroblast cell lines, all normal, diploid cells of human origin. We found no capacity for androgen formation in these cells, but we did observe a pattern of steroid metabolism leading to androgen inactivation without formation of 5α-DHT. The two enzymes that allow for this specific pattern of inactivation are 17β-HSD type 2 for androgen inactivation and 5α-reductase type 1, which has no significant activity on testosterone in these lung fibroblasts. In addition, these cell lines can be separated into two groups having high or low 17β-HSD type 2 activity. Moreover, we also present a Northern blot study in which levels of 17β-HSD type 2 mRNA also vary from subject to subject with either a high or a low expression profile. Thus, our results suggest the existence of an allelic variation in the 17β-HSD type 2 gene that is likely to determine the predisposition of subjects to RD.

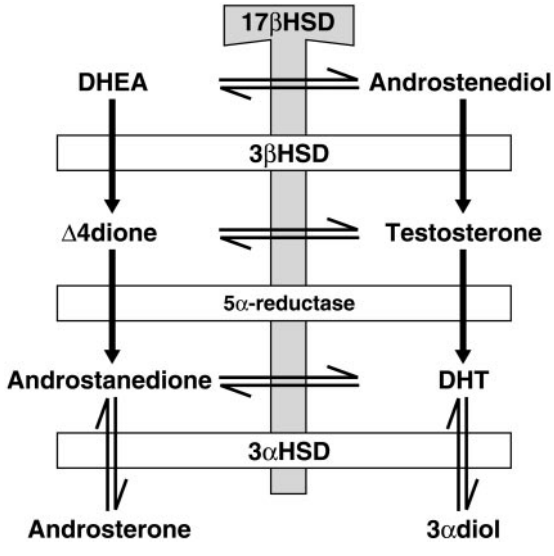

FIG. 1. General pathway of synthesis and inactivation of androgens in peripheral target tissues. 17β-HSD, 3β-HSD, 5α-reductase, and 3α-HSD activities are encoded by more than one gene, each being tissue and cell type specific. The 17β-HSD activity is not actually in equilibrium. Although both directions are possible, each isoform catalyzes predominantly one reaction. 17-Ketosteroids: DHEA, androstenedione, androstanedione, and androsterone. 17β-hydroxysteroids: androstenediol, testosterone, 5α-DHT, and 3α,17β-diol.

Materials and Methods

Chemicals

The following reagents were purchased from NEN Life Science Products (Boston, MA): [1,2,6,7-^{3}H]T (95.0 Ci/mmol), [1,2,6,7-^{3}H]-androstenedione (74.0 Ci/mmol), [1,2-^{3}H]5α-DHT (60 Ci/mmol), [1,2,6,7-^{3}H]DHEA (100 Ci/mmol), [2,4,6,7-^{3}H]estrone (114 Ci/mmol), and [6,7-^{3}H]E2 17β (51.0 Ci/mmol). HEPES, BICINE [N,N-bis(2-hydroxyethyl)glycine], nicotinamide adenine dinucleotide phosphate NAD(P) and its reduced form NAD(P)H, and BSA were obtained from Sigma (St. Louis, MO); fetal bovine serum (FBS) from Hyclone Laboratories, Inc. (Logan, UT); unlabeled steroids from Steraloids (Wilton, NH); and hydroxyflutamide and EM-919 from Drs. Fernand Labrie and Donald Poirier (Laboratoire d'Oncologie et d'Endocrinologie Moléculaire, Québec, Canada).

Cell cultures

CCD-32Lu, CCD-34Lu, CCD-34Sk, LL 24, MRC-5, and IMR-90 cells [respectively, CRL-1485, CRL-1491, CRL-1497, CCL-151, CCL-171, and CCL-186; American Type Culture Collection (ATCC), Manassas, VA] were grown in MEM with Earle's buffered salt solution containing 1.5 g/liter $NaHCO_3$, nonessential amino acids, 10% (vol/vol) FBS [except LL 24; without nonessential amino acids and with 15% (vol/vol) FBS]. For MRC-5 and IMR-90, 1.0 mM sodium pyruvate was added as recommended by ATCC. Hs 907.Lu, FHs 738Lu, and Hs 389(B).Lu cells (respectively, CRL-7657, HTB-157, and CRL-7266, ATCC) were cultured in DMEM containing 25 mM HEPES, 4.5 mg/liter glucose, and 3.7 g/liter $NaHCO_3$, and supplemented with 10% (vol/vol) FBS according to ATCC specifications. Penicillin (50 IU/ml) and streptomycin (50 μg/ml) were added in all media. Serum complement was heat inactivated at 56 C for 20 min. All cell lines were cultured at 37 C in 5% CO_2 atmosphere.

Steroid metabolism in intact cells

Cells (50,000 to 10^5) were incubated in 24-well plates with 1 ml of the appropriate culture media containing either tritiated testosterone (2×10^6 dpm; 9.6 nM) or androstenedione (2×10^6 dpm; 12.3 nM) for the indicated time periods. When indicated, FBS was treated with dextran-coated charcoal as described (18) to remove steroids and other small molecules. At the end of incubation, culture media were frozen until

steroid extraction, and 100 μl methanol were added to each well to fix cells before determination of DNA amounts using 3,5-diaminobenzoic acid as described (19, 20). Steroids were extracted twice with 5 ml diethylether, evaporated, and applied to silica gel-coated plates and resolved in toluene-acetone (4:1, vol/vol). Plates were revealed using a Storm apparatus (Molecular Dynamics, Inc., Sunnyvale, CA) to localize the radioactive signals then spots were scraped and disintegrations per minute were determined by scintillation counting (model LS3801, Beckman, Mississauga, Ontario, Canada). Values in disintegrations per minute were used to determine the percentage of each steroid. Amounts of femtomoles of substrate and products were calculated using these percentage values and total amount of steroid added per well. Each time point was assayed in triplicate and values were normalized per microgram of DNA. Rates of conversion were determined from the average of three slopes (femtomoles per microgram per hour). Enzymatic activities were calculated using the following percentage values: 17β-HSD type 2, [(androstenedione + androstanedione + androsterone (if any))/total steroid] × 100; 5α-reductase, [(androstanedione + androsterone + 5α-DHT (if any))/total steroid] × 100.

HPLC analysis

Samples of 5α-DHT (see Fig. 8) were analyzed by HPLC. The very weak spots corresponding to the positions of 5α-DHT on silica gel-coated plates were scraped and samples of triplicate were pooled to increase sensitivity. Samples were extracted twice with 5 ml diethylether, dried, and resuspended in 125 μl of mobile phase (H_2O/acetonitrile/tetrahydrofurane 65:23:12, vol/vol/vol). Samples were run on an Ultrasphere ODS column (4.6 × 150 mm). Tritiated testosterone, 5α-DHT, and androsterone were used as standards.

Cell fractionation

Cells were homogenized in ice-cold buffer containing 20% (vol/vol) glycerol, 1.0 mM EDTA, and 4 mM potassium phosphate (pH 7.0). Samples were homogenized by hand in an all-glass Dounce homogenizer fitted with a B-pestle, centrifuged at 1,000 × *g* for 10 min to remove cell debris and again at 105,000 × *g* for 60 min. Supernatants were saved as cytosol. The microsome-enriched pellets were washed, subjected to a second round of ultracentrifugation, suspended in buffer, and saved as microsomes. Completeness of homogenization was assessed by light microscopy, as previously described (18). Protein was measured by the method of Markwell *et al.* (21) with BSA as the standard.

Enzymatic assays on cytosol and microsomes

17β-HSD, 3β-HSD, and 3α-HSD were assayed as previously described (18, 22, 23). Briefly, a 10-μl aliquot of cytosol or microsomes was combined with 10 μl reaction mixture containing 1.0 mM nicotinamide nucleotide cofactor, 2.0 μM [^{3}H]-labeled E_2, testosterone, DHEA, or 5α-DHT in 0.08 M BICINE [N,N-bis(2-hydroxyethyl)glycine] (pH 9.0), for dehydrogenase activities, or 2.0 μM [^{3}H]-labeled E_1 (estrone, 1,3,5-estratrien-3-ol-17-one), androstenedione, DHEA, or 5α-DHT in 0.08 M HEPES (pH 7.2), for reductase activities. Assays were run at 37 C. Reaction mixtures were transferred in total to the preadsorbant layer of silica gel HL plates and fractionated into substrate and product by TLC with benzene: acetone (4:1, vol/vol) as the solvent. Following TLC, substrate and products were localized by a light misting with water and scraped into 10 ml Ecolume for liquid scintillation counting. Percentage conversion was calculated as the ratio of counts per minute in product/counts per minute in substrate plus product. This value was converted to picomoles per milligram protein per 30 min as previously described (24).

RNA extraction and Northern blot analyses

Total RNA was extracted from 4 × 10^6 cells using Tri-reagent, a mixture of phenol and guanidine thiocyanate in a monophasic solution (Molecular Research Center, Cincinnati, OH) as described (22). RNA (15 μg) was glyoxalized, resolved by 1.2% (wt/vol) agarose gel electrophoresis, and transferred to Hybond-XL membrane (Amersham/Pharmacia Biotech, Baie d'Urfée, Québec, Canada). Membranes were successively hybridized with the following cDNA probes: full-length 17β-HSD type 2 fragment (22); 5α-reductase type 1 (25); 5α-reductase type 2 (26); and γ-actin 2-kb segment (27). All of the probes were labeled by random priming (28). Hybridizations were performed under high stringency conditions (50% formamide, 42 C), and membranes were washed in 1× saline sodium citrate, 0.1% SDS at 60 C (1× saline sodium citrate: 150 mM NaCl, 15 mM CH_3COONa). Relative RNA amounts were determined directly from membranes using a Strom apparatus and the ImageQuant software (both from Molecular Dynamics, Inc., Sunnyvale, CA), or autoradiographs were quantified by densitometric scanning (BioImage Visage 110s, Genomic Solution; RMLuton, Jackson, MI) with the Whole Band Analysis software (WBA; RMLuton).

Results

Steroid metabolism by intact CCD-32Lu and Hs 907.Lu lung fibroblast cell lines

Two human lung fibroblast cell lines, namely CCD-32Lu and Hs 907.Lu, were first selected to initiate the study of steroid metabolism. These cell lines were established from male and female neonates, respectively. To study the capacity of these cells to produce androgens from steroid precursors present in the circulation, intact cells in culture were exposed to tritiated DHEA and androstenedione for 24 h. Barely detectable levels of androstenediol and testosterone were respectively obtained, indicating that there was no or very low 17-ketosteroid reductase activity in these cells (data not shown). In CCD-32Lu cells, low but significant levels of newly formed androstenedione were detected with tritiated DHEA, which is indicative of 3β-HSD activity, and barely detectable amounts were observed for Hs 907.Lu. For both cell lines, more than 96% of the DHEA used as substrate was recovered after a 24-h incubation.

The capacity of fibroblast cells to metabolize testosterone was also evaluated. After a 24-h incubation, the major product was androstenedione (Fig. 2A). A small amount of androstanedione was detected and no 5α-DHT was observed. These results show that the major steroidogenic activity in these lung fibroblast cells is a 17β-HSD type 2-like activity and that 5α-reductase activity is also present. Interestingly, a time course study using values normalized per microgram of DNA revealed that the oxidative 17β-HSD activity was clearly higher in CCD-32Lu, compared with Hs 907.Lu (Fig. 2B). Using tritiated 5α-DHT as substrate, 3α-HSD activity was also detected in both cell lines (data not shown).

Knowing that exposure of fibroblasts to glucocorticoids stimulates the communication between these cells and PTII cells leading to surfactant synthesis by PTII cells (1), the effect of glucocorticoids on steroid metabolism by fibroblasts was investigated. FBS was treated with activated charcoal, and CCD-32Lu and Hs 907.Lu cells were cultured with either normal FBS or charcoal-treated FBS, both in the presence or absence of 1 μM dexamethasone. Treatment of serum and the presence of dexamethasone had no effect on the 17β-HSD type 2-like activity in both cell lines (data not shown).

17β-HSD, 3β-HSD, and 3α-HSD activities in CCD-32Lu and Hs 907.Lu cytosol and microsomes

To better characterize the steroidogenic enzymes present in these lung fibroblast cells, the substrate and cofactor preferences of the 17β-HSD and other steroidogenic activities were studied in cell extracts of CCD-32Lu and Hs 907.Lu cells. As shown in Fig. 3, 17β-HSD specific activity levels

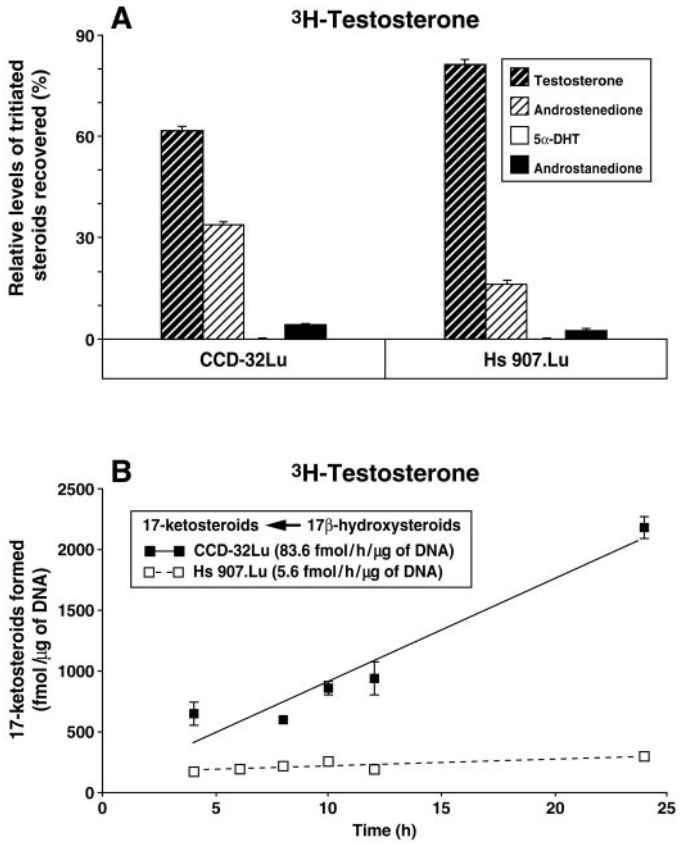

FIG. 2. Major steroidogenic activities of the CCD-32Lu and Hs 907.Lu human lung fibroblast cell lines. A, Relative levels of tritiated steroids recovered from the medium after a 24-h incubation of CCD-32.Lu and Hs 907.Lu cells with tritiated testosterone. B, Time course of 17-ketosteroids formation (androstenedione + androstanedione) from the initial substrate testosterone for CCD-32Lu and Hs 907.Lu cells. Values are normalized per microgram of DNA. A and B, values are the mean (±SD) of triplicate cultures. Rates presented in B are the mean (±SD) of three slopes.

were higher in CCD-32-Lu cells than in Hs 907.Lu cells. Dehydrogenase activity with E_2 and testosterone exceeded reductase activity with E_1 and androstenedione and the highest specific activities were associated with microsomes. Nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH) were the preferred cofactors. With regard to their potential for DHEA metabolism, CCD-32Lu cells also had the highest level of activity (Fig. 4, A and B). The principal activity was microsomal conversion of DHEA to androstenediol with NADH as the preferred cofactor. Putative 3β-HSD activity based on the conversion of DHEA to androstenedione was at the limit of detection. 5α-DHT was converted to androstanedione and 3α,17β-diol with NAD and NADH as the preferred cofactors by both cells types (Fig. 4, C and D).

Steroid metabolism in a wide selection of human lung fibroblast cell lines

Higher 17β-HSD type 2 activity in CCD-32Lu, compared with Hs 907.Lu, is suggestive of sex-specific levels of expression. The effect of the sex or age stage of cells on levels of steroidogenic activities was addressed. Steroid metabolism in eight human lung fibroblast cell lines was compared. Characteristics of these cell lines are presented in Table 1. Cells were incubated in the presence of tritiated testosterone or androstenedione and levels of 17β-HSD type 2 and 5α-reductase activities were respectively determined. Although 5α-reductase activity with androstenedione was very similar in all cell lines, 17β-HSD type 2 activity showed large variations (Fig. 5). In fact, the lung fibroblast cell lines can be divided into two groups, the first having a very low 17β-HSD type 2 activity (Hs 907.Lu, Hs 389(B).Lu, and LL-24) and the second presenting a strong 17β-HSD type 2 activity, significantly higher than the 5α-reductase activity estimated with [^{3}H]-androstenedione (CCD-32Lu, MRC-5, IMR-90, FHs-738Lu, and CCD-34Lu). Because the relative level of these two activities may influence the fate of testosterone entering in the cell, the 17β-HSD type 2 to 5α-reductase activity ratio was calculated (Table 1). No correlation between the 17β-HSD type 2 to 5α-reductase ratio and either the sex or age stage of cells could be established.

The human fibroblast cell line CCD-34Sk was included in this study because it was isolated from skin of the same subject as the lung CCD-34Lu cell line. Interestingly, 17β-HSD type 2 activity was very low in these skin cells in contrast to the lung cells of the same individual (Fig. 5 and Table 1).

For all experiments, cells were cultured under conditions recommended by ATCC, which differ slightly between cell lines. To exclude the culture conditions as the cause of the observed variations of 17β-HSD type 2 activity, each of the CCD-32Lu, MRC-5, Hs 907.Lu, and Hs-389(B).Lu cell lines was cultured under both the CCD-32Lu (high 17β-HSD type 2 activity) and the Hs 907.Lu (low activity) culture conditions. The variability of the 17β-HSD type 2 activity between cell lines was reproduced under each culture conditions (data not shown).

Substrate preference of the 5α-reductase in lung fibroblast intact cells

The two substrates for 5α-reductase enzyme are the substrate (testosterone) and the product (androstenedione) of the 17β-HSD type 2 enzyme. Consequently, in the presence of 17β-HSD type 2, the value obtained for 5α-reductase activity may vary, depending on the tritiated substrate used, if the 5α-reductase has a substrate preference in intact cells. To address this issue, the 5α-reductase activity of the fibroblast cell lines was determined using both androstenedione and testosterone as substrates. The first observation of interest, shown in Fig. 6, is that the rate of 5α-reduction is different, depending on the substrate used. This occurs in all of the analyzed cell lines. Our data indicate that the preferred substrate is androstenedione, which was converted, depending on the cell line, from 1.5- to 26.2-fold more efficiently than testosterone. The second observation is that the difference in the rate of 5α-reduction for the two substrates is higher in cell lines with a low 17β-HSD type 2 activity, compared with cells with a high 17β-HSD type 2 activity. This suggests that the 5α-reductase activity value estimated from [^{3}H]-testosterone in cell lines with a high 17β-HSD type 2 activity is overestimated because of conversion of testosterone into androstenedione.

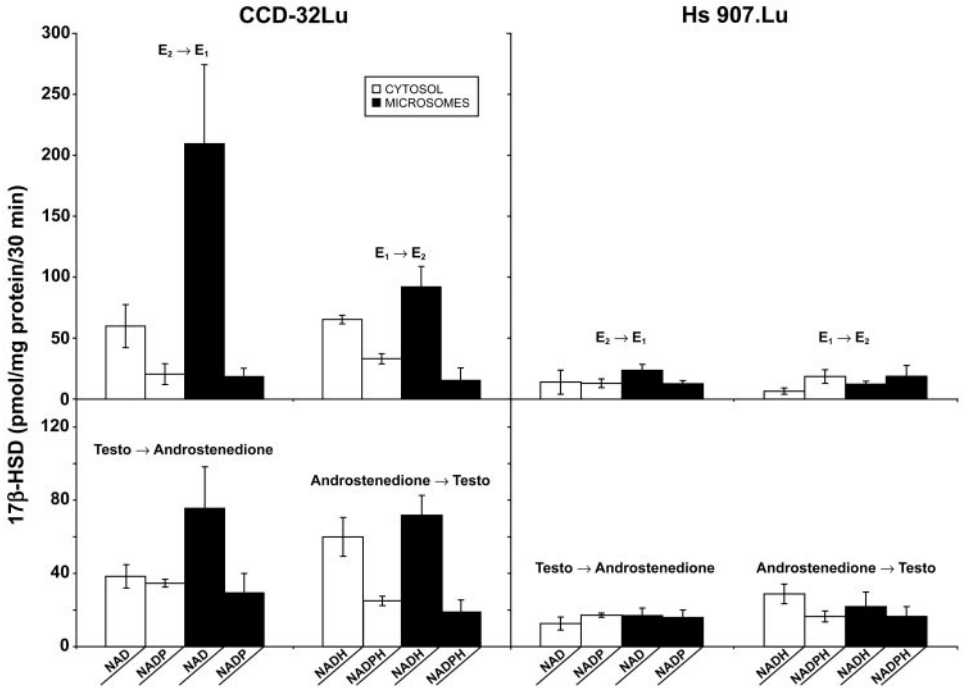

FIG. 3. Enzymatic 17β-HSD activity of cytosol and microsomes from CCD-32Lu and Hs 907.Lu cells. Dehydrogenase activity was assayed with E_2 and testosterone (Testo) with NAD and NADP as the cofactor. Reductase activity was assayed with E_1 and androstenedione with NADH and NADPH as the cofactor. Experimental conditions were as described in *Materials and Methods*. The values are the mean (±SD) of cytosol or microsomes from triplicate cultures.

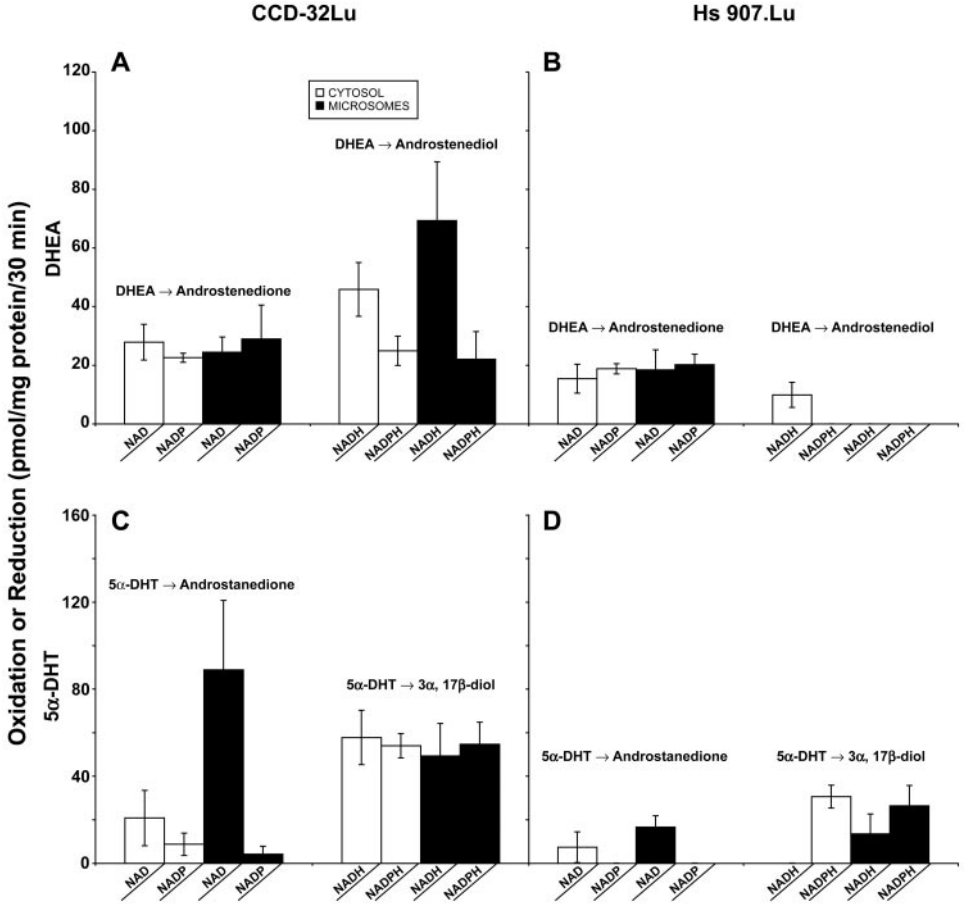

FIG. 4. Oxidation and reduction of DHEA and 5α-DHT by cytosol and microsomes from CCD-32Lu and Hs 907.Lu cells. Assay conditions were as described in Fig. 3 with [^{3}H]-DHEA (A and B) or [^{3}H]-5α-DHT (C and D) as the substrate.

To demonstrate that the above observations are not due to down-regulation of the 5α-reductase gene caused by the added tritiated testosterone, fibroblast cells were incubated with tritiated androstenedione in the presence of unlabeled testosterone and tritiated androstenedione or testosterone in the presence of the antiandrogen hydroxyflutamide. The 5α-

TABLE 1. Ratio of 17β-HSD type 2 / 5α-reductase activities in human fibroblast cell lines

Cell lines	Cell origin	Age stage[a]	Gender	17β-HSD2 / 5α-reductase activity ratio[b]
LL 24	Lung	5 yr	M	0.2
CCD-34Sk[c]	Skin	N (2.5 wk)	F	0.2
Hs 389(B).Lu	Lung	F (16 wk)	F	0.4
Hs 907.Lu	Lung	N (7 wk)	F	0.4
FHs 738Lu	Lung	F (2nd trimester)	M	2.4
CCD-34Lu[c]	Lung	N (2.5 wk)	F	2.9
CCD-32Lu	Lung	N (1 month)	M	3.1
IMR-90	Lung	F (16 wk)	F	4.4
MRC-5	Lung	F (14 wk)	M	4.9

[a] N, Newborn; F, fetal.
[b] Tritiated testosterone and androstenedione were, respectively, used for measurement of 17β-HSD type 2 and 5α-reductase activities.
[c] These two cell lines were isolated from the same subject.

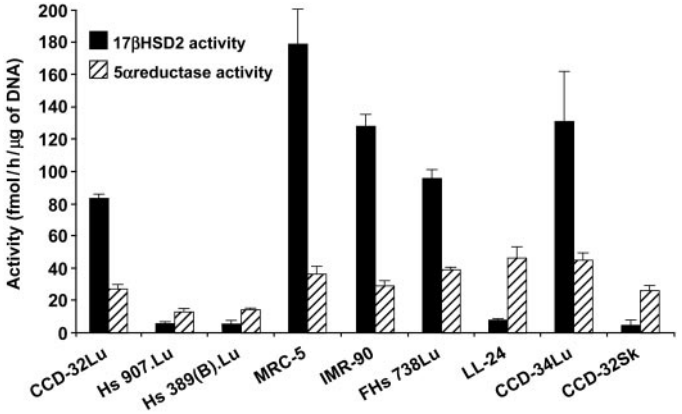

FIG. 5. Comparison of 17β-HSD type 2 and 5α-reductase activities among eight human lung fibroblast cell lines and one human skin fibroblast cell line. 17β-HSD type 2 activity was determined from a time-course study as described in Fig. 2B using [^{3}H]-T as substrate. 5α-Reductase activity represents the rate of formation of androstanedione and androsterone from [^{3}H]-androstenedione and was calculated from values obtained at 24-h incubation from triplicate cultures. All the values are normalized per microgram of DNA.

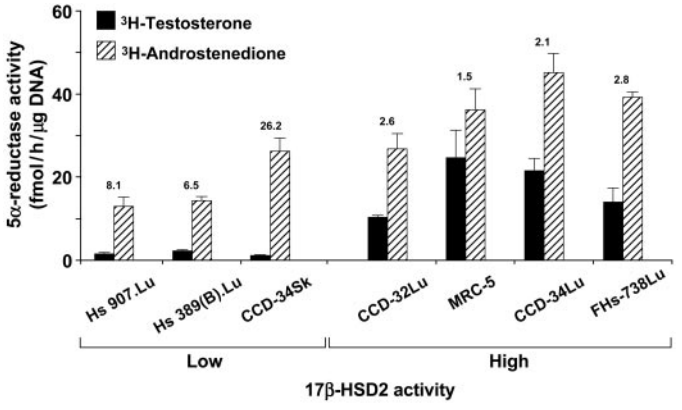

FIG. 6. Difference in the measured rates of 5α-reductase activity according to the tritiated substrate used for the assay. Cells were incubated either with [^{3}H]-T or [^{3}H]-androstenedione for 24 h. Values are the mean (±SD) of triplicate cultures and represent total 5α-reduced steroids produced per hour [androstanedione + androsterone + 5α-DHT (if any)] after normalization per microgram of cell DNA. The *numbers above the bars* represent the ratio of 5α-reductase activities from [^{3}H]-androstenedione/[^{3}H]-T.

reductase activity was greater with androstenedione and was not affected by the presence of hydroxyflutamide or unlabeled testosterone (Fig. 7). To determine whether 5α-DHT is produced by the 5α-reductase expressed in lung fibroblast cells, CCD-32Lu cells were incubated with the 17β-HSD type 2 inhibitor EM-919 (29). This inhibitor had no effect on the 5α-reduction of [^{3}H]-androstenedione (Fig. 8A), but it completely abolished the 17β-HSD type 2 activity with [^{3}H]-T (Fig. 8B). HPLC analysis clearly showed that 5α-DHT was produced from [^{3}H]-T in CCD-32Lu cells but only at very low levels. However, the nearly complete inhibition of 17β-HSD type 2 activity clearly failed to increase the 5α-reduction of [^{3}H]-T to the levels observed with [^{3}H]-androstenedione. In fact, the levels of 5α-DHT synthesized from [^{3}H]-T in the absence of 17β-HSD type 2 activity was at least 65-fold lower then levels of androstanedione synthesized from [^{3}H]-androstenedione.

17β-HSD type 2 and 5α-reductase mRNA levels in lung fibroblast cell lines

Northern blot analyses were performed with RNA from fibroblast cell lines using 17β-HSD type 2, 5α-reductase type 1, 5α-reductase type 2, and actin cDNA probes. RNA from the lung epithelial type II-like cell line A-549 was included for comparison purposes. The 17β-HSD type 2 gene is expressed by the fibroblasts but not by the A-549 cells (Fig. 9A). The 5α-reductase type 1 cDNA probe produced a hybridizing signal for all the analyzed fibroblast cell lines and A-549 cells at quite similar levels as estimated after normalization with actin expression levels (Fig. 9C and data not shown). The 5α-reductase type 2 probe was negative for all cell lines (data not shown). A linear relationship was found between 17β-HSD type 2 mRNA and activity levels for most of the lung fibroblast cell lines (Fig. 9B). Exceptions are the CCD-32Lu cells, which have a very high mRNA level relative to the activity, and the IMR-90, which exerted the higher activity to mRNA ratio.

17β-HSD type 2 mRNA levels in human lung tissues

Samples of total RNA isolated from lung of five adult humans were tested for the presence of 17β-HSD type 2 mRNA. This transcript was found in all of the lung tissues (Fig. 10). Interestingly, subjects can be divided into two groups with low (1-fold) or high (3.3- to 4.7-fold) 17β-HSD type 2 mRNA levels (Fig. 10).

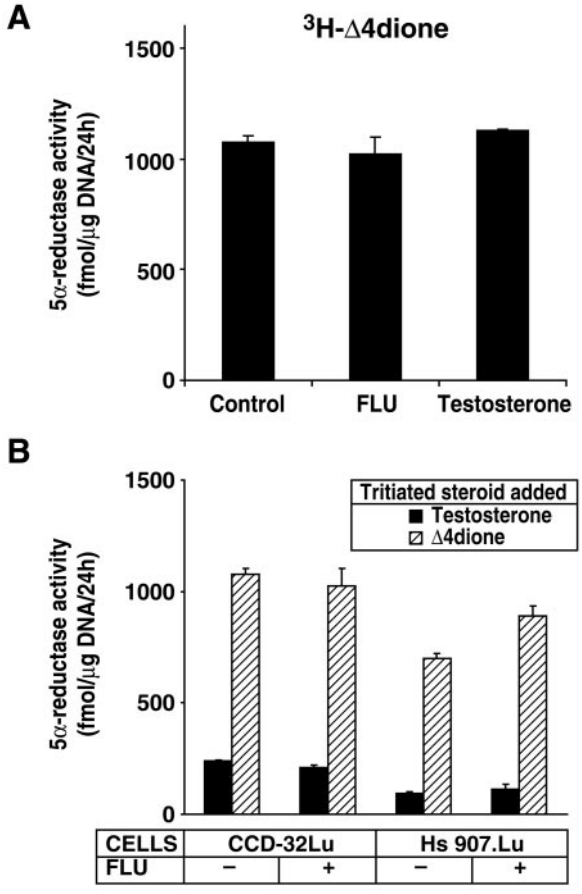

FIG. 7. Effect of hydroxyflutamide and unlabeled testosterone on levels of 5α-reductase activity. A, 5α-reductase activity from tritiated androstenedione was determined for CCD-32Lu cells after a 24-h incubation in the presence of 1 μM hydroxyflutamide (FLU), 10 nM unlabeled testosterone, or none of these compounds (control). B, CCD-32Lu and Hs 907.Lu cells were incubated with tritiated testosterone or androstenedione, both in the presence or absence of 1 μM hydroxyflutamide during a 24-h period. Results of both panels are the mean (±SD) of triplicate cultures.

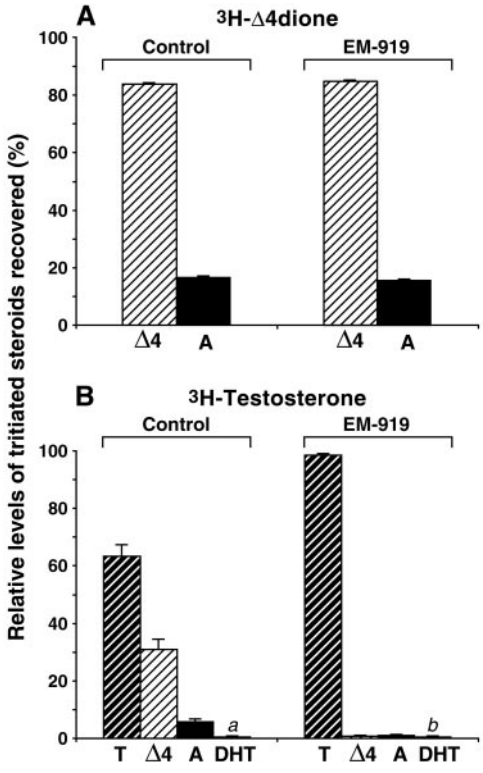

FIG. 8. Effect of the 17β-HSD type 2 inhibitor EM-919 on 5α-DHT synthesis in CCD-32Lu cells. CCD-32Lu cells were preincubated for 3 h in the presence or absence of 1 μM EM-919, the product identified as number 10 in (29). Tritiated androstenedione (A) or testosterone (B) was then added and incubation pursued for 30 h. All values are the means of triplicate cultures (± SD) and were determined by scintillation counting except for 5α-DHT that were determined by HPLC. The values corresponding to one sample are: *a*, 0.1%; *b*, 0.3%. Δ4, Androstenedione; A, androstanedione; DHT, 5α-DHT.

Discussion

We had recently reported that the lung type II-like epithelial cell line A-549 expresses a pattern of steroid metabolism in which 17β-HSD type 5 and 3α-HSD type 3 are the predominant enzymes. As a result, these cells converted androstenedione into testosterone, which was detected in the culture media. In contrast, 5α-DHT was rapidly converted into 3α,17β-diol and did not accumulate in the medium (18). We now report that normal human lung fibroblast cell lines have an opposite pattern of steroid metabolism. Indeed, lung fibroblasts inactivate active androgens into their 17-ketosteroid counterparts through expression of the 17β-HSD type 2 gene. Knowing that androgens modulate fibroblast/epithelial cell communication leading to cell differentiation and the beginning of surfactant production during pregnancy, our findings of an opposite steroid metabolism in the two cell types is of particular interest.

Although the study of steroid metabolism in intact cells revealed the presence of 17β-HSD dehydrogenase activity (active androgens → 17-ketosteroids) higher in CCD-32Lu cells than in Hs 907.Lu cells, the study of steroid metabolism following cell fractionation into cytosol and microsomes has allowed to define the biochemical characteristics of the relevant enzyme. CCD-32Lu and Hs 907.Lu cells were assayed for 17β-HSD activity with E_2, E_1, testosterone, and androstenedione. The observations that activity was significantly higher in CCD-32Lu cells, the highest specific activities were microsomal, and the E_2 to testosterone activity ratio was approximately 2 are consistent with the presence of 17β-HSD type 2 (24). That NAD and NADH were the preferred cofactors is also characteristic of the type 2 isoform. The microsomal conversion of DHEA to androstenediol and 5α-DHT to androstanedione with NADH and NAD as the preferred cofactors, respectively, is also consistent with the presence of 17β-HSD type 2. All these observations concur with results from Northern blot analyses showing higher levels of the 17β-HSD type 2 mRNA in CCD-32Lu cells than in Hs 907.Lu cells. Other steroidogenic activities were also detected in the cell fractions. The conversion of DHEA to androstenedione and 5α-DHT to 3α,17β-diol are indicative of the presence of 3β-HSD and 3α-HSD, respectively. These observations concur with the study of intact cells in which 3α-HSD activity was observed for CCD-32Lu and Hs 907.Lu, and a very low 3β-HSD activity was detected with only

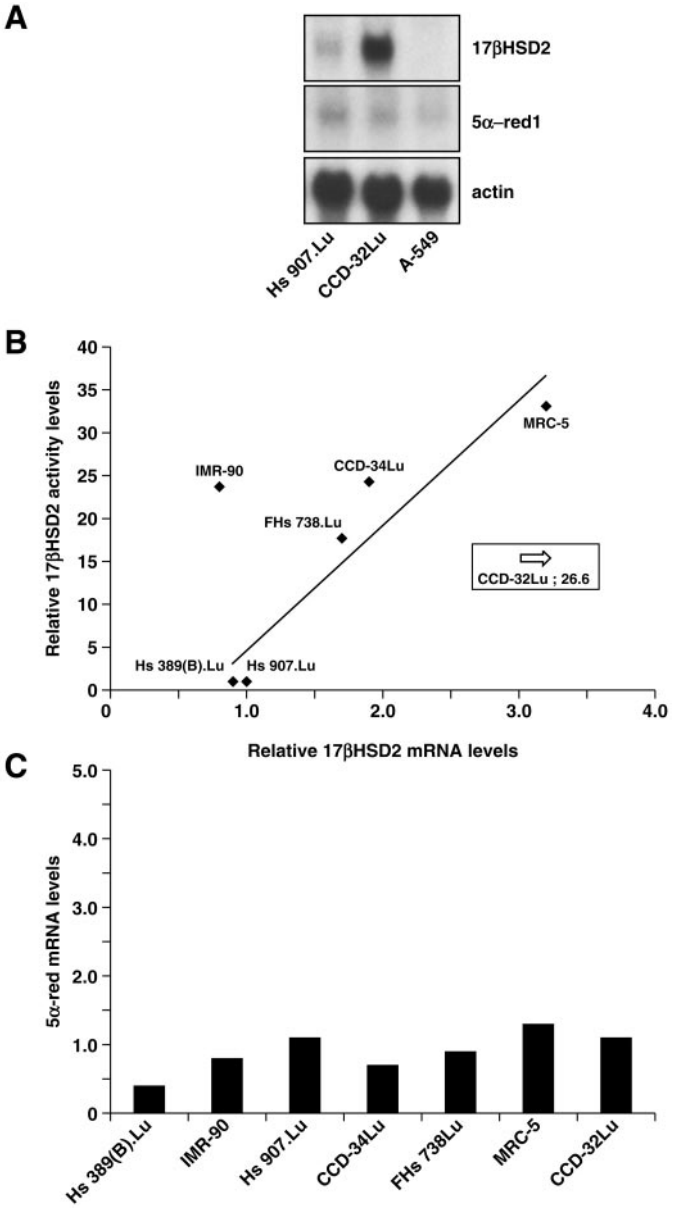

FIG. 9. Comparison of mRNA levels of 17β-HSD type 2 and 5α-reductase between human lung fibroblast cell lines. A, Example of Northern blot hybridization with the indicated probe (*right*) for 15 μg total RNA from three cell lines. B, Relative 17β-HSD type 2 mRNA levels are normalized with actin hybridization. 17β-HSD type 2 activity levels are relative values calculated from results of Fig. 5, compared with the value of Hs 907.Lu, which was arbitrarily fixed to 1. C, Relative 5α-reductase mRNA levels as determined by hybridization with the 5α-reductase type 1 cDNA probe. The values are normalized with actin hybridization. 17β-HSD2, 17β-HSD type 2; 5α-red1, 5α-reductase type 1.

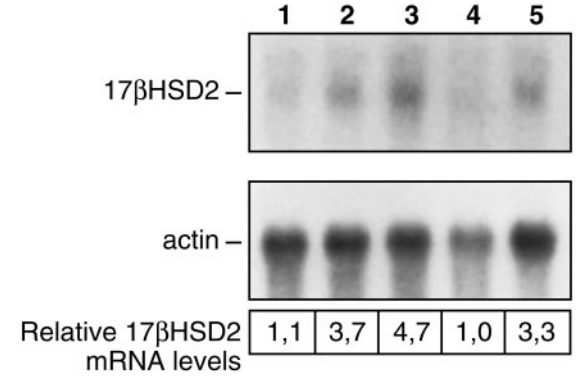

FIG. 10. Levels of 17β-HSD type 2 mRNA in human lung tissues. Total RNA samples (20 μg) isolated from lung tissues are from women aged 57, 41, 43, and 60 yr (*samples 1–4*) and from a 52-yr-old subject of unknown sex (*sample 5*). The relative 17β-HSD type 2 (17β-HSD-2) mRNA levels are normalized with actin hybridization. The lower value (*sample 4*) was arbitrarily fixed to 1.0.

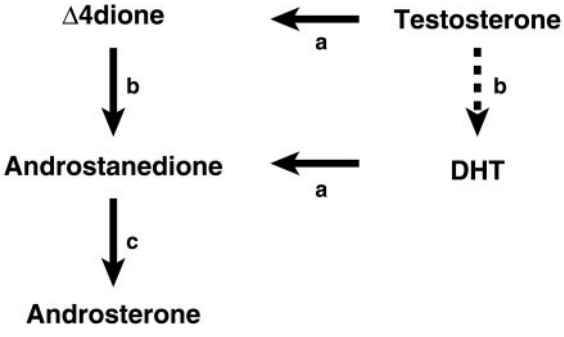

FIG. 11. Steroid metabolism in human lung fibroblast cells. a, 17β-HSD type 2; b, 5α-reductase type 1; c, 3α-HSD activities. *Dotted arrow* represents barely detectable activity.

CCD-32Lu. The human lung fibroblast steroidogenic pathway is summarized in Fig. 11.

Steroid metabolism by human lung fibroblasts having a high 17β-HSD type 2 activity is particularly useful to restrict the occupancy of the androgen receptor. Not only does the 17β-HSD type 2 activity allow for the inactivation of testosterone into androstenedione, but the 5α-reductase activity also shows a substrate preference that favors the 5α-reduction of androstenedione over that of testosterone. Consequently, the product of 17β-HSD type 2 is further converted into other metabolites without significant conversion of testosterone into 5α-DHT, which is the most potent androgen. The efficiency of cell lines to inactivate testosterone instead of increasing its androgenic potential is a function of the 17β-HSD type 2 to 5α-reductase activity ratio. The data in Table 1 allow a division of the cell lines into two groups having a high or low value for this ratio. However, it should be noted that the values were calculated using the 5α-reductase activity determined from tritiated androstenedione. This value was selected to calculate the ratio because it is not affected by 17β-HSD type 2 activity. However, when calculated using tritiated testosterone, the 5α-reductase activity cannot be compared between cell lines because the conversion of testosterone into androstenedione results in overestimation of the 5α-reductase activity for cell lines having high 17β-HSD type 2 activity. Consequently, the 5α-reductase activity values measured with tritiated androstenedione are, in fact, relative values that do not represent the actual level of testosterone 5α-reduction.

We have clearly demonstrated that the 5α-reductase expressed in human lung fibroblast cell lines exerts a strong substrate preference for androstenedione over testosterone. The addition of unlabeled testosterone did not alter the conversion of [^{3}H]-androstenedione to androstanedione and the

use of the antiandrogen hydroxyflutamide failed to increase the conversion of [^{3}H]-T into 5α-DHT, thus showing that the presence of [^{3}H]-T did not modulate 5α-reductase expression. Moreover, levels of [^{3}H]-T 5α-reduction were at least 65-fold lower than those of [^{3}H]-androstenedione 5α-reduction in the presence of EM-919. Thus, it is clear that the 5α-reductase expressed in human lung fibroblast cells exerts a strong substrate preference for androstenedione. Positive signal was obtained by Northern blots with the 5α-reductase type 1 probe. It was demonstrated that 5α-reductase type 1 had an apparent Km value for androstenedione that was 6 times lower, and a Vmax that was 2 times higher, than that of testosterone in cell homogenates prepared from Chinese hamster ovary cells transfected with recombinant 5α-reductase type 1 (30). This means that the catalytic efficiency of the enzyme for androstenedione as substrate was 12 times higher than that of testosterone. In our experiments, the difference in the catalytic efficiency of the enzyme between the two substrates was at least 5 times more pronounced. This could be explained by the fact that the Km and Vmax values were determined from cell homogenates, although in our experiments, the difference in the rate of conversion between the two substrates was determined in intact cells. In fact, our results are very informative about the role that plays 5α-reductase type 1, at least in human lung fibroblasts. In these cells, the endogenous 5α-reductase type 1 activity on testosterone is low enough, and its activity on androstenedione is high enough, to participate in testosterone inactivation (by removing the product of the 17β-HSD type 2 enzyme) without formation of significant amount of 5α-DHT. Thus, in these cells, the 5α-reductase type 1 might play an active role in minimization of the androgen potential.

Knowing that results obtained from cell line models have to be considered with circumspection in regard to the actual *in vivo* situation, we propose a model of lung steroid metabolism (Fig. 12) that would be informative and useful for a better comprehension of the mechanisms regulating fibroblast/epithelial cell communication in which androgens play a role of negative modulator. We have to keep in mind that androgens delay lung maturation and surfactant production by their action on lung fibroblast cells and that the steroid metabolism of these cells, which is oriented toward androgen inactivation, is particularly adapted to reduce the risk of RD by decreasing the intracellular level of androgens. The androgen receptor has been detected in both lung fibroblasts (11) and PTII-like A549 cells (31). The occupancy of this receptor depends on local steroid metabolism, which differs between cell types. The age stage of cell lines used in this study enables us to suggest that the pattern of steroid metabolism presented in Fig. 11 might be present in fibroblasts at the time of stimulation of surfactant synthesis because the same enzymes are expressed in both cells isolated during the second trimester of pregnancy and in neonates. The first question is, What is the source of androgens in the fetal lung? In the human, there is no testosterone synthesis by the gonads at the time of stimulation of surfactant synthesis. The major source would be local formation of androgen from circulating steroid precursors of adrenal origin. In light of the results obtained from the study of the A-549 cell line (18), we can suggest that androstenedione entering from the circulation is converted into testosterone by the 17β-HSD type 5 expressed in PTII cells. The low 5α-reductase and the high 3α-HSD type 3 activities present in these cells allow accumulation and secretion of testosterone but not that of 5α-DHT, which is rapidly converted into 3α,17β-diol. As a paracrine factor, testosterone can reach fibroblasts in which it would be rapidly inactivated into 17-ketosteroids (androstenedione) by the 17β-HSD type 2. The strong substrate preference of the 5α-reductase type 1 expressed in fibroblasts allows further conversion of androstenedione in androstanedione without formation of a significant level of 5α-DHT. Thus, the occupancy of the androgen receptor in fibroblast cells (11) might be a function of the rate of testosterone formation by PTII cells and the rate of testosterone inactivation by fibroblasts, both events being candidates for modulation.

It is clear from our results that the normal lung fibroblast cell lines can be segregated in two groups regarding the basal level of 17β-HSD type 2 activity. No relationship between basal level of this activity and the sex of the fetus or age stage of cells at time of isolation was observed. The linear relationship between 17β-HSD type 2 mRNA and activity observed in our assays suggests that this difference may not

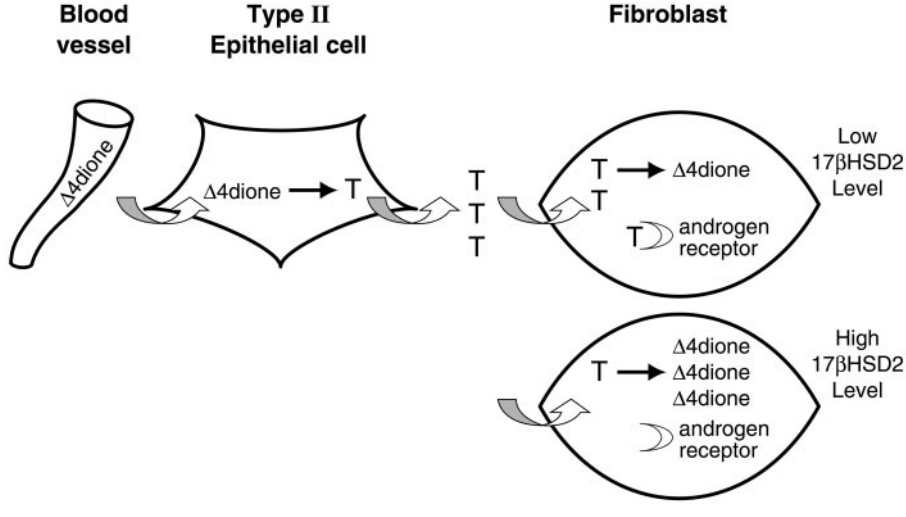

FIG. 12. The occupancy of the androgen receptor in lung fibroblasts is determined by paracrinology and the capacity of these cells to inactivate androgens. The model is described in the text. Adione, Androstenedione; T, testosterone.

arise from a mutation affecting the enzyme activity. Among the hypotheses that can be postulated to explain this variation are a mutation affecting either the 17β-HSD type 2 promoter or any region of a related regulating gene or the existence of two subpopulations of lung fibroblast cells. It is also not excluded that some cell lines could have been altered over time in culture following their isolation, even if all are normal diploid cells. However, we report here variations in levels of 17β-HSD type 2 mRNA between lung samples obtained from adult human subjects. Although such a variation remains to be demonstrated for samples obtained from neonates, these results open the way to further studies aiming to determine whether subjects with low levels of 17β-HSD type 2 activity are predisposed to RD. Indeed, subjects with low 17β-HSD type 2 activity in lung fibroblasts should have higher androgen receptor occupancy, compared with subjects with high 17β-HSD type 2 activity (Fig. 12). Because it is expected for the male to produce more androgens than the female, males would be more affected than females by a low 17β-HSD type 2 expression profile. Nagourney *et al.* (32) reported that "preterm infants born to women with a previous preterm infant affected by RDS are at an increased risk of RDS, which suggests an important genetic (or other familial) tendency in its origin." Our results suggest that the 17β-HSD-2 gene could be a candidate to conciliate these observations.

We have demonstrated that human lung fibroblast cells have the potential to inactivate exogenous androgens through the expression of 17β-HSD type 2 and 5α-reductase type 1 genes. In our cell model, 5α-reductase type 1 has no significant activity on testosterone, but it further converts the product of 17β-HSD type 2, thus playing an important role in androgen inactivation. Finally, variation in 17β-HSD type 2 activity and mRNA from cell line to cell line and mRNA from subject to subject open the way to further studies aiming to explore the possibility that subjects with the low 17β-HSD type 2 expression profile could be predisposed to RD.

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