

The Human Keratinocyte Cell Line HaCaT: An In Vitro Cell Culture Model for Keratinocyte Testosterone Metabolism

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INTRODUCTION

In recent years, the skin has been shown to contain a broad spectrum of enzymes capable of metabolizing a wide range of topically applied drugs and endogenous substrates. A major part of this metabolic activity is located within the epidermis (1).

The metabolic capacity of the skin may have consequences for the topical as well as the transdermal delivery of drugs, resulting in a probably reduced bioavailability for a transdermally delivered substance. For example, 16–21% of transdermally applied glyceryl trinitrate was reported to be metabolized in the skin (2). Cutaneous metabolism may also lead to reactive metabolites, having the potential for contact sensitization, an important problem in transdermal delivery.

For these reasons, there is a growing interest in methods for studying human skin metabolism. Since it is difficult to differentiate skin from systemic metabolism under in vivo conditions, there is need for suitable in vitro models. The spontaneously immortalized human keratinocyte cell line HaCaT represents a readily available in vitro model and has already been used as a model for skin toxicity studies (3). The full epidermal differentiation capacity of HaCaT cells was demonstrated by transplantation onto nude mouse skin (4).

The aim of our study was to investigate the metabolism of the androgen testosterone (T) in HaCaT cells. T is extensively used in TDS for the treatment of male hypogonadism. Furthermore, cutaneous metabolism of T is of general interest, because the skin has been recognized as a major site for endogenous androgen metabolism as well as a target organ for these steroids. T is reduced to 5 α -dihydrotestosterone (DHT) in the extracellular compartment by two distinct isoforms of the membrane bound steroid 5 α -reductase (5 α -R) (EC 1.3.1.22) at the target cell site (5). DHT represents the most potent androgen and is thought to be involved in acne and other androgen-related disorders, such as male pattern baldness and hirsutism (6). We investigated the metabolism of T in HaCaT cells to determine the suitability of this in vitro model for steroid metabolism in human skin.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) with and without HEPES and fetal calf serum were obtained from Gibco BRL-Life Technologies, Paisley, UK. Cell culture dishes and six-well multidishes (35 mm) were purchased from Nunc, Roskilde, Denmark. Twelve-well multidishes (22.5 mm) were obtained from Costar, Cambridge, USA.

Testosterone (T), dihydrotestosterone (DHT), androstenedione (Ae), androstenedione (Aa), androsterone (Andro) and epiandrosterone were a gift of Schering AG, Berlin, Germany. Androstan-3 α ,17 β -diol (Adiol), Finasteride and MTT were obtained from Sigma Chemical Co., St. Louis, USA. MK 386 was donated by MSD GmbH, Haar, Germany. [1,2,6,7-³H]testosterone (spec. activity 98.0 Ci/mmol, radiochemical purity: 97.8%) was purchased from Amersham Life Science, Braunschweig, Germany.

Cell Culture

HaCaT cells were cultured according to well described standard methods (7). The cells were used between the 35th and 43rd passage. The protein contents of different passages grown on various culture dishes were determined by the method of Lowry (8).

Cell Viability Using Different Media

For the different incubation conditions, it was necessary to use other media than DMEM, because this medium interfered with specific assays. The effects of DMSO, HEPES, PBS supplemented with glucose and EBSS on the mitochondrial activity were determined using a colorimetric assay (MTT test), which is described elsewhere (7). The MTT transformation of cells in complete DMEM was set as 100% viability.

Incubation with ³H-Testosterone

HaCaT cells were seeded into 12-well plates at a density of 2×10^4 cells/cm². After 3 weeks medium was removed, the cells were washed and incubated with 0.5 ml of a solution of [1,2,6,7-³H]-T (³H-T) together with unlabeled substrate in DMEM with HEPES (pH 7.4). At the end of each incubation period, the medium was removed and the cells were washed with 0.5 ml PBS. Combined media and wash solutions were extracted twice with 1 ml of diethylether. The solvent was evaporated to dryness and the residue was redissolved in 100 μ l ether. The yield of radioactivity in the organic phase was measured by scintillation counting of control incubations (TRI-CARB 2100 TR liquid scintillation analyzer, Canberra-Packard GmbH, Dreieich, Germany) and was found to be $93 \pm 4\%$ (n = 3).

Separation of Tritium Labeled Metabolites

The whole etheric solution was applied to 20 \times 20 cm silica-TLC plates (Polygram SIL G, Machery-Nagel, Dueren, Germany), which were then developed in dichloromethane-diethylether (4 + 1). Plates were scanned for radioactivity using an automatic linear β -scanner (Berthold, Wildbad, Germany)

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and the Chroma software (Berthold). The radioactive metabolites were identified by comparison with ^3H -steroid standards run in parallel. Steroids were quantified as a percentage of total radioactivity by integration of the peaks.

Incubations with Unlabeled Testosterone

HaCaT cells were seeded into 6-well plates at a density of 2×10^4 cells/cm². After 3 weeks medium was removed, the cells were washed and 1 ml of a solution of T in DMSO and EBSS (pH 7.4) was added. The final DMSO concentration did not exceed 1% (v/v). At the end of each incubation period, the medium was removed and the cells were washed with 1 ml of water. Combined media and wash solutions were lyophilized and the residue was redissolved in 4 ml diethylether. After centrifugation 3 ml of the solution were taken to dryness, redissolved in 1 ml of methanol and analyzed by a LC/MS method. The recovery for this method was found to be $98 \pm 3\%$.

LC/MS Assay

Unlabeled T and metabolites were assayed by liquid chromatography/mass spectrometry (LC/MS) using a Waters™ LC Module I plus liquid chromatograph equipped with a Fisons™ MD 800 mass detector. A LiChrospher® 100 RP 18 (5 μm , 250×4.6 mm i.d., Merck KGaA) was used as an analytical column maintained at 30°C. The mobile phase consisted of methanol/water (65:35) at a flow rate of 1.0 ml/min. The identification of the steroids was based on comparison with authentic standards and on individual mass spectra. Quantification of T and metabolites was performed from peak areas at individual ion mass in comparison to authentic standards by the MassLab software (Fisons).

Effect of pH on 5 α -R Activity

The experimental conditions used for studying the effect of pH on 5 α -R activity were the same as outlined above for incubations with labeled testosterone, except that the cells were incubated for one hour in PBS adjusted to pH values from 5 to 8.5 containing 0.1 μM T. The pH of the incubation medium was constant during the reaction time. The activity of 5 α -R was expressed by the sum of DHT, Adiol, Andro and Aa.

Inhibition Studies

The experimental conditions used for studying the effect of two inhibitors, Finasteride and MK 386, on 5 α -R activity were the same as outlined above for incubations with labeled testosterone, except that the cells were incubated for one hour with 0.1 μM T and the specific inhibitor. Finasteride and MK 386 were used in final concentrations of 0-500 nM. Inhibitory activities were expressed as percentage of the sum of 5 α -reduced products formed in the absence of inhibitor.

RESULTS AND DISCUSSION

Properties of HaCaT Cells

Previously, it was shown that the specific activity of 5 α -R and other steroid metabolizing enzymes in keratinocytes increased with cell time in culture (9), indicating that specific

enzyme activity is directly related to terminal differentiation in these cells.

For HaCaT cells, it was demonstrated that the expression of terminal differentiation markers, such as cytokeratins K1 and K10, significantly increased with time in culture up to 3 weeks (10).

The protein content of HaCaT cells of different passages grown in multiwell-dishes for 21 days was 0.21 ± 0.025 mg \times cm⁻². There were no statistically significant differences, neither between different passages of cells, nor between the cell culture dishes used, indicating constant cell density. Therefore, all investigations were performed with HaCaT cells grown for 3 weeks.

Cytotoxicity Studies

To demonstrate cell viability under different cell culture conditions used for metabolism studies, the MTT cytotoxicity assay was conducted for cells maintained in different media, partially supplemented with DMSO. Supplementation of HEPES as well as the presence of the cosolvent DMSO up to 1.5% (v/v) did not reduce cell viability. The viability of HaCaT maintained in EBSS, the medium used for incubations with unlabeled T, decreased with time, but did not fall below 80% in 24 hours.

Metabolism of T in HaCaT

When HaCaT cells were incubated with ^3H -T, $93 \pm 4\%$ of the radioactivity added was found in the culture medium plus wash. Therefore, the amount of metabolites formed was measured only in these combined solutions. From incubations with 0.1 μM (physiologic plasma concentrations) up to 176 μM (upper limit of solubility) it was shown that the time course of appearance of various metabolites was strictly dependent on the substrate concentration (data not shown).

Representative results of chromatographic identification of T metabolites are shown in Figs. 1A and 1B. After the incubation with ^3H -T five metabolites were separated by TLC and identified against authentic standards: 5 α -androstane-3 α ,17 β -diol (Adiol), 5 α -dihydrotestosterone (DHT), androsterone (Andro), androst-4-ene-3,17-dione (Ae) and 5 α -androstane-3,17-dione (Aa) (Fig. 1A). Another peak was found with a greater polarity than Adiol (Rf: 0.1), which did not correspond with any of the available standards.

Qualitatively as well as quantitatively a close agreement to the above reported results was found with the incubation of unlabeled T, where Adiol, DHT, Andro and Ae were found by the LC/MS assay. The identity of the metabolites was confirmed by comparing mass spectra of metabolites (Fig. 1B) to those of authentic standards. Although Adiol and Andro had similar retention times, it was possible to separately quantitate both metabolites at the specific channel corresponding to the individual ion mass. In contrast to incubations with ^3H -T, no Aa was detected, which may be due to the higher detection limit.

An advantage of using the LC/MS assay is that identification of metabolites is not only based on the determination of retention times, but also on comparison of the individual mass spectra. However, when small, physiological, concentrations of T are investigated in the cell culture model, the use of radiolabeled substrate is indispensable, due to the lower detection limit of this method.

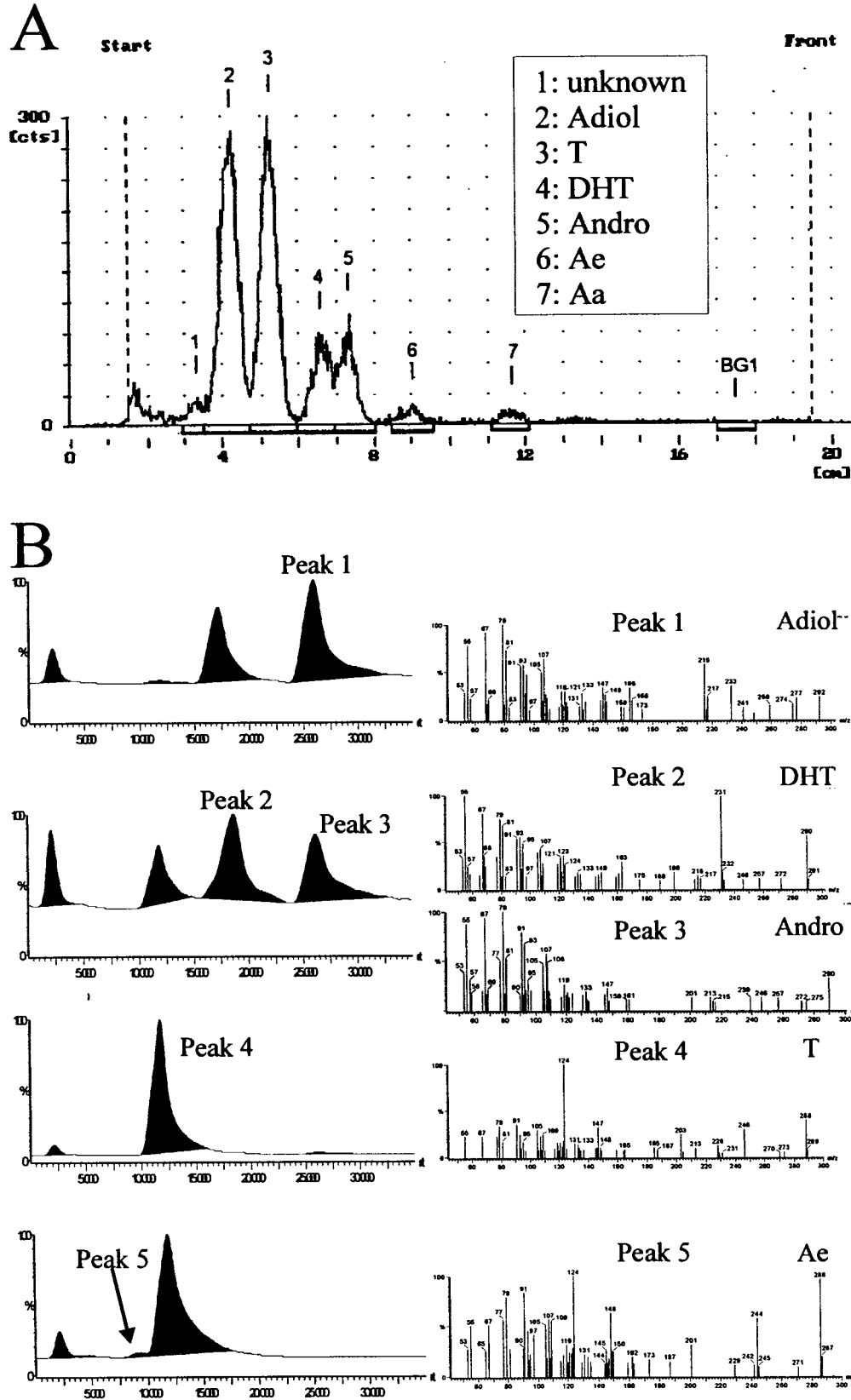


Fig. 1. A. Representative TLC chromatogram of extracts from the incubation of HaCaT cells with ³H-testosterone. (0.8 μM T; 6 h). B. Representative HPLC chromatogram of extracts from the incubation of HaCaT cells with unlabeled testosterone (176 μM; 24 h) including mass spectra of the isolated peaks. The area of the peaks was determined at the channel corresponding to the specific ion mass. left side: retention times in LC; right side: corresponding mass spectra.

Therefore, all subsequent investigations were performed with tritium labeled T. From the metabolic pattern observed it can be concluded, that the same enzymes, that were previously found in human skin, are also expressed in the HaCaT cell line. These enzymes are: 5 α -reductase (5 α -R), 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and 3 α -hydroxysteroid dehydrogenase (3 α -HSD).

Comparison with Other In Vitro Models

To determine whether the profile of metabolites derived from the HaCaT cells is similar to those obtained with other in vitro methods, we compared our data to studies performed with excised human skin (11), the so called Living Skin Equivalent (LSE™) (11) and human fibroblasts in culture (12). Therefore, results of an incubation were chosen, which yielded a relative amount of T unchanged comparable to the examined studies (Table 1).

It is obvious that the metabolic profile in HaCaT cells is similar to those obtained in human skin, LSE and skin fibroblasts. The major difference for the HaCaT cells is an increased amount of Adiol formed, indicating high activities of 3 α -HSD besides marked activities of 5 α -R, which is known to be predominant in human skin (13). The lower standard deviations for the HaCaT cells demonstrate higher reproducibility, while in human skin and primary cultures other variables, such as differences in anatomical site and inter-individual variability may contribute to differences in the metabolic pattern.

5 α -Reductase Activity

To further characterize the enzymatic activity in the HaCaT cell line, we investigated which of the two distinct isozymes of 5 α -R is expressed in these cells. Both subtypes of 5 α -R differ in their tissue distribution and biochemical properties. The type I isozyme (5 α -R1) has a broad optimal pH of 6-9 and is mainly located in the sebaceous glands and in epidermal and follicular keratinocytes of non-genital skin (13). This isozyme is inhibited by MK 386 (7- β -methyl-4-aza-cholestan-3-one), a new selective 5 α -R1 inhibitor (14).

The type 2 isozyme has a sharp pH optimum of about 5.5 and is located predominantly in the prostate and in genital skin (13). Finasteride, the first compound currently approved for treatment of benign prostatic hyperplasia, represents a high affinity inhibitor of 5 α -R2 and a slow binding, low affinity inhibitor of 5 α -R1 (15).

pH Optimum

To determine the pH optimum for 5 α -R in HaCaT cells, enzyme activity was measured over the pH range 5–8.5 using a substrate concentration of 0.1 μ M, which approximated the plasma level of T in adult male. From Fig. 2 it can be seen that the amount of 5 α -reduced metabolites increased from pH 5 to 8. Optimal activity was observed at pH 7.5–8, while there was minor transformation at pH 5. From this pH profile it was assumed that the activity of isozyme type 1 is markedly higher in

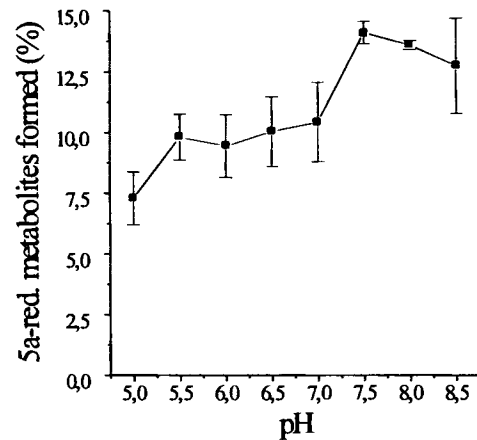


Fig. 2. The effect of pH on the 5 α -reductase activity in HaCaT cells. PBS buffer adjusted to the defined pH values from 5 to 8.5 was used as incubation medium. The concentration of testosterone was 0.1 μ M. Each data point represents the mean and standard deviation of three determinations.

Table 1. Comparison of the Metabolic Profile of Testosterone in Different In Vitro Models—Metabolites Found in HaCaT and Fibroblasts were Divided into Groups to Compare with Results of the Other Studies

Metabolites	Human leg skin ^a	Human abdominal skin ^b	LSE ^c	Cultured skin fibroblasts ^d	HaCaT ^e
Polar metabolites	23 \pm 1 ^f	12 \pm 1	13 \pm 5	—	7 \pm 1
Testosterone	45 \pm 2	64 \pm 6	54 \pm 11	44 \pm 26	45 \pm 1
5 α -androstane-3 α ,17 β -diol	—	13 \pm 3	6 \pm 2	23 \pm 21	31 \pm 2
androstenedione, epi-androsterone, 5 α -DHT	15 \pm 1	4 \pm 1	5 \pm 1	22 \pm 10	9 \pm 1
5 α -DHT, androsterone	15 \pm 1	4 \pm 1	14 \pm 2	21 \pm 7	13 \pm 1
Androstenedione	2 \pm 0	1 \pm 0	1 \pm 0	1 \pm 1	1 \pm 0

Note: Results are expressed as the mean percentage of radioactivity found \pm SD.

^a Data taken from Ref. 11, application of 5 μ g/cm² T in acetone for 24 h (n = 4).

^b Data taken from Ref. 11, application of 5 μ g/cm² T in acetone for 24 h (n = 3).

^c Data taken from Ref. 11, application of 5 μ g/cm² T in acetone for 24 h (n = 3).

^d Data taken from Ref. 12, incubation of cells in 60 mm petri dish with 0.05 μ Ci/2ml for 48 h (n = 10).

^e Incubation of HaCaT cells (passage 42) with 0.8 μ M T for 4 h (n = 6).

^f This fraction possibly contains Adiol, because all peaks with Rf values smaller than T were counted here.

HaCaT cells then the activity of 5α -R2. At higher pH, increased activity of 17β -HSD was observed, which is in accordance to investigations performed by others (6).

Inhibition Studies

To substantiate the results from the pH-dependent incubations, we investigated the effect of selective inhibitors on the 5α -R activity in the HaCaT cell line. As shown in Fig. 3, MK 386 exhibited a strong dose-dependent inhibition of 5α -R activity in HaCaT cells. The concentration of MK 386 required to inhibit 5α -R by 50% (IC_{50}) was estimated to be about 25 nM. This is in close agreement with the IC_{50} of 20 nM found for 5α -R1 expressed in human scalp skin (14). Finasteride showed markedly less inhibitory activity on 5α -R in HaCaT cells with an IC_{50} of about 150 nM, which is similar to the value of 200 nM found for hair follicles expressing 5α -R1, and much higher than the typical IC_{50} of 5nM in genital skin (5). 17β -HSD activity was not decreased by any of the inhibitors used, which is in accordance to results reported by others (5).

From these findings, it was assumed that the subtype I of 5α -R is predominantly expressed in HaCaT cells.

High levels of type I 5α -R expressed in human skin are thought to be important in controlling the function of sebaceous glands. Therefore, the development of new compounds that specifically and effectively inhibit the type I isozyme of 5α -R may be of importance for dermatological purposes in the near future (14). Since the HaCaT cell line mainly expresses this 5α -R subtype, our model may be useful in the process of screening for potential inhibitors for the treatment of androgen-dependent skin disorders.

CONCLUSIONS

From our investigations it was shown that the HaCaT cell line expresses the enzyme systems for the biotransformation of T. While a LC/MS assay allows clear identification of the metabolites by their mass spectra, the use of tritium labeled T is preferable when small concentrations of metabolites have to be quantitated.

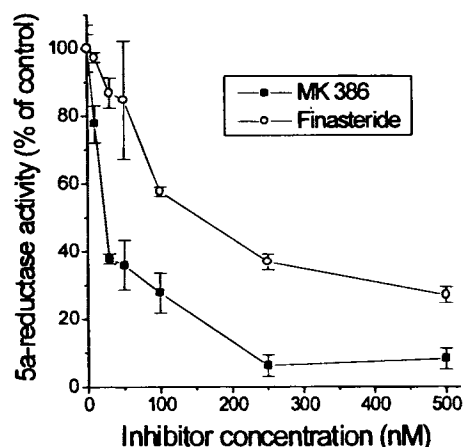


Fig. 3. Inhibition of 5α -reductase activity in HaCaT cells by MK 386 (—■—) and Finasteride (—○—): Cells were incubated with 0.1 μ M testosterone. Each data point represents the mean and standard deviation of three determinations.

The metabolite profile for T in HaCaT cells was similar to that seen in excised human skin and other in vitro models. However, the HaCaT cell line exhibits a higher degree of reproducibility.

Therefore, we suggest that the HaCaT cell line represents a suitable model for studying keratinocyte testosterone metabolism under controlled conditions.

Furthermore, the model is useful for the estimation of effects of xenobiotics on the endogenous steroid metabolism, as shown by 5α -R inhibition studies. For this purpose the main advantages of the cell line are the ability to work in intact cells, which is believed to be a more physiological model (15), besides the possibility to simultaneously study the effect of the potential inhibitors on other steroid metabolizing enzymes.

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