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 5α-dihydrotestosterone (DHT) in the extranuclear compartment by two distinct isoforms of the membrane bound steroid 5α-reductase (5α-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 multidishe (35 mm) were purchased from Nunc, Roskilde, Denmark. Twelve-well multidishe (22.5 mm) were obtained from Costar, Cambridge, USA.

Testosterone (T), dihydrotestosterone (DHT), androstenedione (Ac), androstenedione (Aa), androsterone (Andro) and epiandrosterone were a gift of Schering AG, Berlin, Germany. Androstan-3α,17β-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 × 10⁴ 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 (2H-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 µl ether. The yield of radioactivity in the organic phase was measured by scintillation counting of control incubations (TRICARB 2100 TR liquid scintillation analyzer, Canberra-Packard GmbH, Dreieich, Germany) and was found to be 93 ± 4% (n = 3).

Separation of Tritium Labeled Metabolites

The whole etheric solution was applied to 20 × 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 $^3$H-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 $2 \times 10^4$ 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 ± 3%.

**LC/MS Assay**

Unlabeled T and metabolites were assayed by liquid chromatography/mass spectrometry (LC/MS) using a Waters™ LC Module 1 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 5α-R Activity**

The experimental conditions used for studying the effect of pH on 5α-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 5α-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 5α-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 5α-reduced products formed in the absence of inhibitor.

**RESULTS AND DISCUSSION**

**Properties of HaCaT Cells**

Previously, it was shown that the specific activity of 5α-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 × 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 $^3$H-T, 93 ± 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 $^3$H-T five metabolites were separated by TLC and identified against authentic standards: 5α-androstan-3α,17β-diol (Adiol), 5α-dihydrotestosterone (DHT), androsterone (Andro), androst-4-ene-3,17-dione (Ac) and 5α-androstan-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 Aa 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 $^3$H-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.