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Characterization and modulation of sex steroid metabolizing activity in normal human keratinocytes in primary culture and HaCaT cells

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Abstract

Skin, the largest organ of the human body, synthesizes active sex steroids from adrenal C19 precursor steroids. Normal human breast epidermal keratinocytes in primary culture were used to evaluate the enzymatic activities responsible for the formation and degradation of active androgens and estrogens during keratinocyte differentiation. Enzymatic activities, including 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) were measured using [^{3}H] steroids as substrates. After 10–60 days in culture, no 3β-HSD activity was detected, but all other activities were measured, demonstrating the ability of keratinocytes to convert androstenedione (4-DIONE) into the potent androgen dihydrotestosterone (DHT). Furthermore, marked changes in enzymatic activity were observed during cell differentiation: 17β-HSD was first detected during the third week of culture, the level of activity reaching a peak during the fourth week. This peak was followed by a progressive decrease during keratinization. On the other hand, 5α-reductase and 3α-HSD activities were first detected during the fourth week of culture. The enzymatic activities involved in the formation and degradation of sex steroids were also characterized in the immortalized human keratinocyte cell line HaCaT. It was then found that HaCaT cells possess a pattern of steroid metabolizing enzymes similar to that of human epidermal keratinocytes in culture. Since glucocorticoids are known to exert potent pharmacological effects on the skin, the effect of dexamethasone (DEX) on cell proliferation and enzymatic activities was determined using HaCaT cells. DEX causes a 55% decrease in HaCaT cell proliferation (IC_{50}: 10 nM) whereas DEX caused a three- to five-fold stimulation of oxidative 17β-HSD activity in intact cells in culture (ED_{50}: 30 nM) and this stimulatory effect was competitively blocked by the glucocorticoid antagonist RU486. A four-fold increase in type 2 17β-HSD mRNA levels was also observed as measured by real-time PCR, correlating with the increase in oxidative activity. No effect of DEX on the other enzymatic activities (3β-HSD, 5α-reductase, and 3α-HSD) was observed. Since increased levels of inflammatory cytokines have been detected in some skin diseases then these cytokines might play a role in the differentiation of keratinocytes. In this regard, we found that interleukin-4 (IL-4) induced the expression of 3β-HSD in HaCaT cells, thus allowing the cells to produce a different set of sex steroids from adrenal C19 precursors. The present data thus indicate that HaCaT cells are a useful model to further study the regulation of the enzymes involved in the metabolism of sex steroids in keratinocytes.
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Keywords: Keratinocyte; Androgens; Estrogens; Glucocorticoids; Interleukin-4

Abbreviations: 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3α-DIOL, androstane-3α,17β-diol; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-DIOL, androstane-3β,17β-diol; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase 4-DIONE, androstenedione; 5-DIOL, androst-5-ene-3β,17β-diol; A-DIONE, 5α-androstane-3,17-dione; ADT, androsterone; DEX, dexamethasone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E1, estrone; E2, 17β-estradiol; EC_{50}, half-maximal stimulatory concentration; IC_{50}, half-maximal inhibitory concentration; EPI-ADT, epiandrosterone; RU486, RU38486 or mifepristone; TESTO, testosterone

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1. Introduction

Skin is the body's largest organ and is capable of performing a variety of metabolic functions, including those involved in the metabolism of drugs, carcinogens, and hormones, including their synthesis [1,2]. The importance of the skin in the biosynthesis of active sex steroids from inactive precursors is strongly supported by the expression in this tissue of the key steroidogenic enzymes, namely 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD) and aromatase [3–6]. The 3β-HSD enzyme is responsible for the oxidation and isomerization of 5-ene-3β-hydroxysteroid precursors into 4-ene-ketosteroids, thus catalyzing an essential step in the formation of all classes of active steroid hormones [7,8]. Two types of 3β-HSDs have been characterized in humans. Type 1 3β-HSD is predominantly expressed in the placenta and peripheral tissues, such as the skin and mammary gland, while type 2 3β-HSD is almost exclusively expressed in classical steroidogenic tissues such as the adrenals and gonads [9,3,10]. Type 1 displays approximately 10-fold higher activity than type 2 which could facilitate steroid formation from relatively low concentrations of substrates usually present in peripheral tissues. In the human skin, 3β-HSD is expressed primarily in the sebaceous glands, thus playing a key role in the conversion of inactive adrenal steroid precursors into potent androgens and/or estrogens [3,11]. In fact, a close correlation has been reported in humans between 3β-HSD activity and the rate of in vivo sebum secretion [11]. More recently, the presence of type 1 3β-HSD has also been reported in SZ95 sebocytes but not in HaCaT keratinocytes [12].

Epidermal keratinocytes in primary culture have been shown to metabolize C19- and C21-Δ^4-3-oxo steroids to the corresponding 5α-reduced metabolites, thus indicating a high level of 5α-reductase activity [13]. Two types of human 5α-reductases, namely type 1 and type 2, have been characterized. The 5α-reductase type 1 gene encodes an isoenzyme that is transiently expressed in newborn skin and scalp but continuously expressed in the skin from puberty onward [14]. The presence of type 1 5α-reductase has also been reported in SZ95 sebocytes and HaCaT keratinocytes [12]. 5α-reductase type 1 has been shown to be primarily responsible for virilization and male-pattern baldness. The 5α-reductase type 2 isoenzyme, on the other hand, is also transiently expressed in newborn skin as well as in scalp. However, this isoenzyme is predominantly found in fetal genital skin, male accessory sex glands and in the prostate [5,14]. Inherited defects in the type 2 isoenzyme lead to male pseudohermaphroditism where affected males have normal internal genitalia with female-type external genitalia.

The presence of 17β-HSD activity has also been reported in epidermal keratinocytes [13]. In the human, nine different types of 17β-HSDs have so far been described. 17β-HSD type 1 primarily catalyzes the reductive reaction converting estrone (E1) to 17β-estradiol (E2) [15,16]. Type 1 17β-HSD is highly expressed in the human placenta; but its expression is also found in the endometrium and ovary and in a long series of peripheral tissues [17]. Type 2 17β-HSD was cloned from the human prostate and has been shown to possess oxidative activity inactivating both androgens and estrogens [18]. This enzyme is expressed in a variety of peripheral tissues including the placenta, prostate, liver, kidney, small intestine and endometrium [19] as well as in SZ95 sebocyte and HaCaT keratinocyte cell lines [12]. This microsomal 17β-HSD also possesses a secondary 20α-HSD activity, which converts 20α-dihydroprogesterone to progesterone. Type 3 17β-HSD was cloned from a human testes library and is almost exclusively expressed in this tissue [20]. The main reaction catalyzed by this enzyme is the conversion of androstenedione to testosterone, but DHEA and E1 can also be converted to their reduced forms 5-DIOL and E2, respectively [20]. Mutations in type 3 17β-HSD lead to a rare autosomal deficiency called male pseudo-hermaphroditism [20]. A fourth member of the 17β-HSD family was identified in human liver [21]. This isoenzyme was described as a 17β-estradiol dehydrogenase which converts E2 into E1. Type 4 17β-HSD can also use 5-DIOL as substrate, but since the affinity is weak, conversion into DHEA is low. Its expression is found in a wide variety of tissues including the liver, heart, prostate, testis, ovary and placenta [21]. Inactivation of E2 in almost all tissues, including non-steroidogenic cell lines, provided insight about a probable housekeeping function for this enzyme [21]. Human type 5 17β-HSD is unique among the 17β-HSD enzymes since it belongs to the aldo–keto reductase family, whereas the others are members of the short chain alcohol dehydrogenases. The cDNA was originally cloned from human prostate as a 3α-HSD [22]. However, it was subsequently shown to possess a specific 17β-HSD activity, which is markedly decreased upon cell homogenization [23]. This property of type 5 17β-HSD explains why its activity was overlooked in initial studies. Moreover, in analogy with type 2 17β-HSD, type 5 17β-HSD possesses a secondary 20α-HSD activity. Type 5 17β-HSD activity catalyzes as well as the transformation of androstenedione (4-DIONE) to testosterone, the transformation of androstanedione into DHT. Type 5 17β-HSD is expressed in several tissues including the prostate, liver and adrenals [22–24]. Type 7 17β-HSD is also expressed in the prostate as well as in the liver, testis, placenta, breast and ovary and efficiently catalyzes the transformation of E1 to E2 [25]. Other 17β-HSDs have also been described, but their exact physiological role in steroid metabolism has not been definitively demonstrated [26–31].

Two major types of 3α-HSDs have been characterized in humans. Type 1 3α-HSD is expressed in the liver, while type 3 3α-HSD is expressed in several other tissues including prostate, brain, testis, adrenals as well as the HaCaT cell line. Type 3 3α-HSD is more widely expressed than type 1 and is found in many peripheral tissues that produce active steroids. It is thus likely that type 3 3α-HSD plays a role

in the intracrine control of active hormone levels in these tissues [32]. 3α-HSDs transform DHT to 3α-diol but also possess a 20α-HSD activity, which transforms progesterone into 20α-OH-progesterone. For both enzymes, the activity represents approximately two thirds of the 3α-HSD activity [32].

Aromatase, on the other hand, is a member of the cytochrome P450 superfamily and is responsible for the biosynthesis of estrogens (C18) from C19 steroids (androgens) [33]. Aromatase expression is found mainly in human placenta, but there are numerous tissues expressing this enzyme, including the liver, intestine, skin fibroblasts, sebaceous glands, brain, ovary and adipose tissue [11,34].

Since the local or intracrine formation of steroids [35] potentially plays an important role in diseases such as acne and seborrhea, we have investigated the characteristics of the enzymatic activities responsible for the formation and degradation of active androgens and estrogens in primary human breast epidermal keratinocytes as well as in HaCaT cells, a spontaneously immortalized aneuploid human keratinocyte cell line [36]. The modulation of the different enzymatic activities was studied during differentiation of the human breast epidermal keratinocytes, which occurs during long-term culture of these cells.

The potent anti-inflammatory effect of glucocorticoids in the skin is well recognized. On the other hand, some dermatological diseases, psoriasis being an example, are characterized by abnormal keratinocyte proliferation and terminal differentiation. The fact that T-cell infiltration precedes epidermal differentiation [37] along with the effectiveness of T-cell based therapeutics, suggests that T-cells play a role in triggering and perpetuating psoriasis [37–40]. There is also increasing evidence that cytokines play a crucial role in the pathogenesis of psoriasis and of other dermatological diseases [41]. Among these, Interleukin-4 (IL-4) is a cytokine produced by T-cells previously shown to induce proliferation of normal human keratinocytes [42]. We have also studied the effect of the synthetic glucocorticoid dexamethasone (DEX) and interleukin-4 on different enzymatic activities using HaCaT cells as in vitro model.

2. Materials and methods

2.1. Chemicals

[1,2-^{3}H] androst-5-ene-3β,17β-diol (5-DIOL) (52.4 Ci/mmol), [1,2-^{3}H] testosterone (TESTO) (41 Ci/mmol), [1,2-^{3}H] dihydrotestosterone (DHT) (43.7 Ci/mmol), [6,7-^{3}H] 17β-estradiol (E2) (43.7 Ci/mmol), and [1,2,6,7-^{3}H] androst-4-ene-3,17-dione (4-DIONE) (85 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). Dexamethasone (DEX) was purchased from Steraloids Inc. (Newport, RI). Organic solvents were obtained from BDH (Montreal, Canada). RU486 was kindly provided by Roussel-UCLAF (Romainville, France).

2.2. Maintenance of stock cell cultures

The keratinocytes (primary human breast epidermal keratinocytes and HaCaT cells) were kept under an atmosphere of 5% CO_2 at 37 °C. Normal human breast epidermal keratinocytes were isolated from breast skin of women undergoing reduction mammoplasty as previously described [43]. The standard culture medium for keratinocytes was a serum-free medium (SFM for keratinocytes) supplemented with bovine pituitary extract (0.05 mg/ml), recombinant epidermal growth factor (rEGF1-51) at 5 ng/ml, 100 IU penicillin/ml, 50 μg streptomycin sulfate/ml and 1 μg fungizone/ml. The SFM and supplements were obtained from Gibco Laboratories Co. (Grand Island, NY). Normal keratinocytes were cultured up to 70 days and subcultured weekly between days 19 and 55 using 0.05% trypsin/EDTA from Sigma (St. Louis, MO).

The HaCaT cell line, generously supplied by Dr. N.E. Fusening (German Cancer Research Center, Heiderberg, Germany), is derived from human skin keratinocytes that spontaneously transformed in vitro during long term incubation [36,44]. HaCaT cells were grown in RPMI-1640 medium (Sigma) containing 100 IU penicillin/ml, 50 μg streptomycin sulfate/ml, 2 mM L-glutamine and 3% fetal bovine serum (FBS) (Hyclone, Logan, UT). Subcultures were performed by treating the cells with 0.05% trypsin/EDTA solution [44].

2.3. Assays for enzymatic activities in intact cells in culture

Primary human breast epidermal keratinocytes or HaCaT cells were plated in triplicate at the indicated density in 24-well plastic culture plates containing RPMI-1640 medium supplemented as described above, but with 1% dextran-coated charcoal-treated serum for HaCaT cells and standard SFM for human breast epidermal keratinocytes. After 2 days, the medium was removed and cells were washed twice with PBS before adding 1 ml of fresh medium containing the indicated radioactive substrates (10 nM). Cells were cultured in parallel for determination of DNA content as previously described [45].

2.4. Assays for enzymatic activities in cell homogenates

After the indicated treatment, cells were collected using trypsin-EDTA, resuspended in a reaction buffer (100 mM KH_2PO_4; 20% glycerol; 1% EDTA; pH 7.4) and submitted to three cycles of sonication before rapid freezing at −80 °C. Protein concentration was determined in parallel using the micro-Bradford assay. For enzymatic assays, 100 μg of total protein was incubated in the presence of the indicated [^{3}H]

steroids in the reaction buffer described above containing 1 mM of NAD+. All incubations were performed in triplicate at 37 °C.

2.5. Analysis of [^{3}H] metabolites

At the end of incubation with the indicated [^{3}H] substrates, the medium was collected and steroids were extracted twice with two volumes of diethylether. The organic phase was then evaporated; the residue was dissolved in dichloromethane and then separated either by thin layer chromatography (TLC) using a mixture of 4:1 (v/v) toluene and acetone or by high-performance liquid chromatography (HPLC) using a System Gold Unit (Beckman) as previously described [46].

2.6. Real-time quantitative PCR assay

HaCaT cells were plated at a density of 32,000 cells/well in 6-well plates. After 2 days, cells were incubated in the presence or absence of 100 nM of DEX for 6 days with medium changes every 2 days. Total RNA was extracted from HaCaT cells using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocol. Five μg of total RNA for each cell sample (+ or − DEX) was reverse-transcribed using a partially degenerated oligo-dT (5′-TTTTTTTTTTTTVN-3′, where V = A, C or G and N = any nucleotide) primer and SuperScript™ II RNase H$^-$ reverse transcriptase (Gibco InVitrogen, Burlington, ON) according to the supplier's protocol. All PCR reactions were performed on an ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). For each PCR assay, a reaction mixture was prepared with 1× TaqMan master mix buffer (Applied Biosystems, Foster City, CA), which included Taq Gold polymerase, 0.9 μM of each primer, 0.2 μM of probe (TaqMan® PROBE, Applied Biosystems, Foster City, CA), and various concentrations of each cDNA sample in a final volume of 10 μl. A standard curve was constructed with two-fold serial dilutions of reverse-transcribed RNA–DNA hybrid from HaCaT control cells. The standard curve was composed of seven concentrations (i.e. 200, 100, 50, 25, 12.5, 6.25 and 3.125 ng). Primers and probes specific to type 2 17β-HSD were chosen with the assistance of the computer program Primer Express 1.5 (Applied Biosystems, Foster City, CA). The sequence of the primers and probe for type 2 17β-HSD were 5′-GTTTAAATTACCCACTGGA-3′ (sense), 5′-GTGAGTCTAGCGGGCAAAGG-3′ (antisense) and 5′-GCCAGTCGAAAGGG-3′ (probe), respectively. The thermal cycling parameters included an initial step at 50 °C for 2 min, a denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Data obtained from two separate experiments performed in triplicate was standardized by setting the value of type 2 17β-HSD expression levels in HaCaT control cells to 1.0. Data for type 2 17β-HSD in DEX treated cells was expressed relative to this standard (means ± S.E.M.).

2.7. Calculations and statistical analysis

Statistical analysis was performed according to the multiple-range test of Duncan–Kramer. Data are presented as means ± S.E.M. of triplicate dishes. Dose–response curves as well as EC_{50} or IC_{50} values were calculated using a weighted iterative nonlinear least squares regression as described [45]. When S.E.M. overlaps with the symbol, only the symbol is illustrated.

3. Results

In order to investigate the characteristics of the enzymatic activities responsible for the formation and degradation of active androgens and estrogens in human keratinocytes, normal human breast epidermal keratinocytes grown for 39 days in primary culture were incubated for up to 18 h with 10 pmol of the following [^{3}H] steroids: 5-DIOL, TESTO, DHT and E2. Analysis of the products formed enabled us to evaluate the expression of steroid metabolizing enzymes such as 3β-HSD, 17β-HSD, 5α-reductase, aromatase and 3α-HSD in these cells. When [^{3}H] 5-DIOL was used (Fig. 1A), 8.8 pmol were converted into [^{3}H] DHEA after 18 h of incubation, thus showing the presence of oxidative 17β-HSD activity. The absence of formation of any 4-ene-ketosteroid indicates that there is no detectable 3β-HSD activity in these cells. The presence of 17β-HSD activity is confirmed by incubation with [^{3}H] TESTO (Fig. 1B) where 7.2 pmol were converted into [^{3}H] 4-DIONE. The presence of 5α-reductase and 3α-HSD activities is indicated by the formation of 0.6 pmol [^{3}H] A-DIONE and 0.3 pmol [^{3}H] ADT (Fig. 1B).

There was no formation of E2 from [^{3}H] TESTO, thus indicating that there is no significant aromatase activity in these cells. When [^{3}H] DHT was used as substrate, [^{3}H] A-DIONE was found to be the main metabolite (Fig. 1C). It can also be seen in the same figure that 2.5 pmol of [^{3}H] ADT and 0.13 pmol of [^{3}H] 3α-DIOL were formed, thus confirming the presence of 3α-HSD activity. When [^{3}H] E2 was used as substrate, 8.8 pmol of E1 were formed at the end of the incubation period (Fig. 1D), thus indicating the presence, in normal human breast epidermal keratinocytes, of oxidative estrogenic 17β-HSD activity.

In order to determine if the major enzymatic activities identified were differently expressed during differentiation of human breast epidermal keratinocytes, the activities were measured at different incubation times. As illustrated in Fig. 2A–D, 17β-HSD activity levels were extremely low or undetectable up to day 14 which was followed by a rapid increase in 17β-HSD activity during the third week in culture, with a peak ranging from 10.3 to 13.5 pmol of product formed/μg DNA/6 h on day 28 for the four substrates tested.

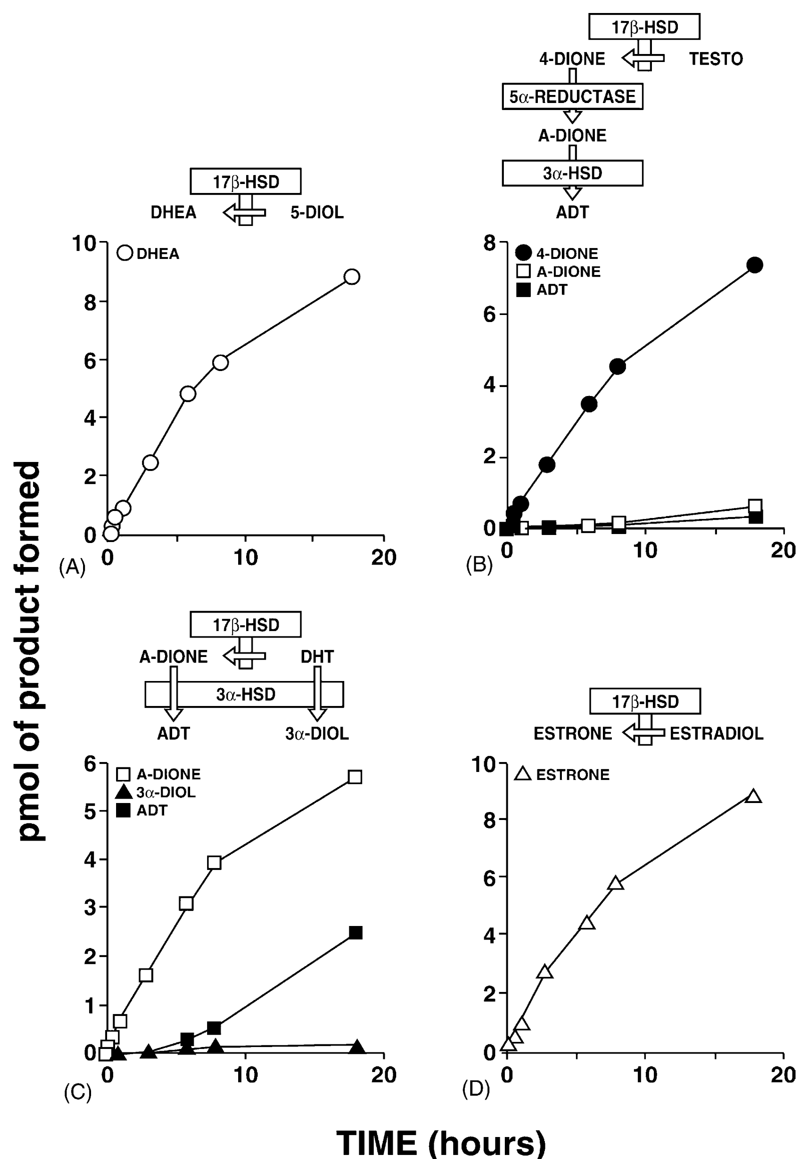

Fig. 1. Multiple steroid metabolizing activities are present in normal human breast epidermal keratinocytes. After 39 days in primary culture, cells were plated at a density of 75,000 cells/well in 24-well plates. After 2 days, the medium was changed for 1 ml of fresh standard SFM medium containing 10 nM of [^{3}H] 5-DIOL (A), [^{3}H] TESTO (B), [^{3}H] DHT (C), or [^{3}H] E2 (D). At the end of the indicated incubation periods, free steroids were extracted and analyzed as described in Section 2. Cell proliferation was determined by measuring DNA content. Results are expressed as means ± S.E.M. of pmol of formed product.

This maximal activity was followed by a sharp 75–80% decrease in 17β-HSD activity measured on day 34, with levels slowly decreasing thereafter to reach values ranging between 0.7 and 2.5 pmol of product formed/μg DNA/6 h on day 57 of culture (Fig. 2A–D). A comparable level of activity was measured with different substrates which correlates well with the substrate specificity of oxidative type 2 17β-HSD activity.

A different pattern was observed for 5α-reductase activity during differentiation of the human breast epidermal keratinocytes (Fig. 3). In fact, no 5α-reductase activity could be detected for the first 20 days in culture. However, between days 20 and 28 there was an abrupt increase in the transformation of [^{3}H] 4-DIONE into [^{3}H] A-DIONE, which then reached a plateau which was maintained up to the last time interval examined, namely day 57.

We then examined the steroid metabolizing activity of the immortalized human keratinocyte cell line HaCaT. As illustrated in Fig. 4A, when [^{3}H] 5-DIOL was used as substrate, only [^{3}H] DHEA was produced after 8 h of incubation. HaCaT cells thus possess oxidative 17β-HSD activity for androgens as do human breast epidermal keratinocytes. This activity is well illustrated in Fig. 4B by the efficient conversion of [^{3}H] TESTO into [^{3}H] 4-DIONE while the formation of [^{3}H] DHT and [^{3}H] A-DIONE indicates that these cells also possess relatively high levels of 5α-reductase

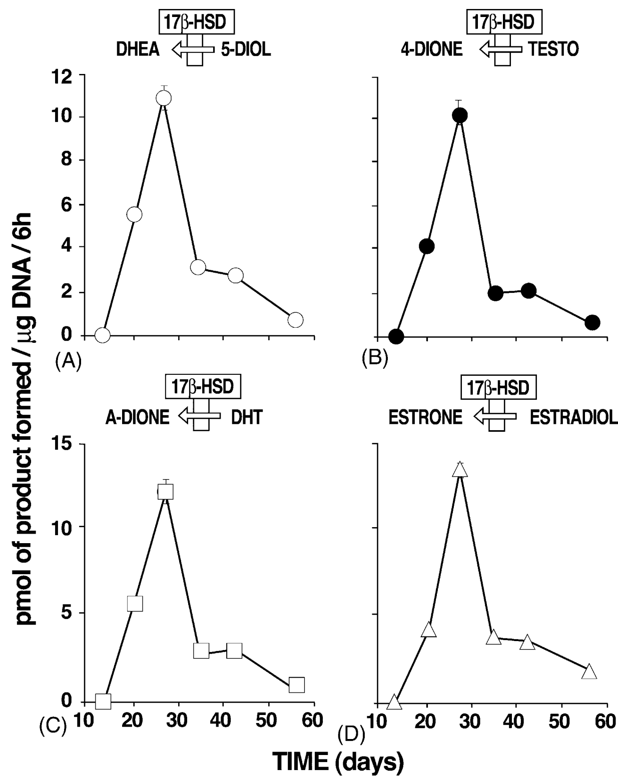

Fig. 2. Effect of time on oxidative 17β-HSD activity in primary cultures of human breast epidermal keratinocytes. After the indicated time in primary culture, normal human breast epidermal keratinocytes were plated at a density of 75,000 cells/well in 24-well plates. After 2 days, medium was changed for 1 ml of fresh standard SFM medium containing 10 nM of [^{3}H] 5-DIOL (A), [^{3}H] TESTO (B), [^{3}H] DHT (C), or [^{3}H] E2 (D) and incubated for 6 h. Free steroids were extracted and analyzed as described in Section 2. Cell proliferation was determined by measuring DNA content. Results are expressed as means ± S.E.M. of pmol of product formed/µg DNA/6h.

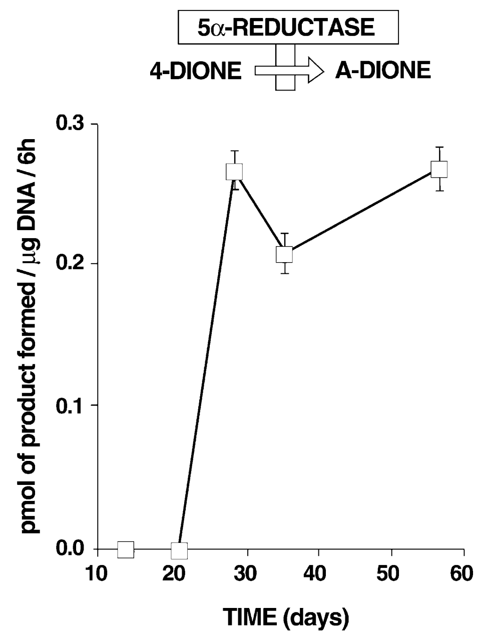

Fig. 3. Effect of time on 5α-reductase activity in primary cultures of human breast epidermal keratinocytes. After the indicated time in primary culture, normal human breast epidermal keratinocytes were plated at a density of 75,000 cells/well in 24-well plates. After 2 days, medium was changed for 1 ml of fresh standard SFM medium containing 10 nM of [^{3}H] 4-DIONE and incubated for 6 h. Free steroids were extracted and analyzed as described in Section 2. Cell proliferation was determined by measuring DNA content. Results are expressed as means ± S.E.M. of pmol of product formed/µg DNA/6h.

activity. When [^{3}H] DHT was used as substrate, [^{3}H] 3α-DIOL, [^{3}H] A-DIONE and [^{3}H] ADT were found at the end of the 8-h incubation period, thus also indicating the presence of 3α-HSD activity in HaCaT cells. The 3α-HSD activity is outlined by the exclusive formation of [^{3}H] ADT when [^{3}H] A-DIONE was used as substrate (Fig. 4E). The HaCaT cells also possess estrogenic 17β-HSD activity as demonstrated by the formation of [^{3}H] E1 from [^{3}H] E2 after the 8 h incubation period (Fig. 4F).

Since glucocorticoids are known to exert potent pharmacological effects on the skin, we studied the effect of DEX on the proliferation of HaCaT cells as well as on steroid metabolism in these cells. DEX caused a 55% decrease in HaCaT cell proliferation, the effect being exerted at an IC_{50} value of 10 nM (Fig. 5A). Concomitantly, it can be seen in Fig. 5B that DEX caused a maximal three-fold stimulation of oxidative 17β-HSD activity as reflected by the effective conversion of [^{3}H] 5-DIOL into [^{3}H] DHEA. This stimulatory effect of DEX was exerted at an EC_{50} value of 30 nM. Moreover, as measured by type 2 17β-HSD specific real-time PCR assay (Fig. 5C), DEX treatment of HaCaT cells in culture causes a four-fold increase in type 2 17β-HSD mRNA expression levels, which renders this enzyme a strong candidate for the oxidative 17β-HSD activity observed in these cells. Although the presence of other types of 17β-HSDs cannot be excluded, the substrate specificity, low affinity and activity in intact cells of other members of this enzyme family renders type 2 17β-HSD the most probable isoenzyme responsible for the observed oxidative activity. As illustrated in Fig. 6, the stimulatory effect of DEX on oxidative 17β-HSD activity was competitively blocked by increasing concentrations of the glucocorticoid antagonist RU486, thus suggesting that the effect of DEX is mediated by the glucocorticoid receptor. In addition, it should be mentioned that such incubation with DEX did not affect 5α-reductase or 3α-HSD activity (data not shown).

The effect of DEX on oxidative 17β-HSD activity was also assayed in cell homogenates. When [^{3}H] 5-DIOL (Fig. 7A), [^{3}H] TESTO (Fig. 7B) or E2 (Fig. 7C) were used as substrates, DEX had a marked stimulatory effect on oxidative 17β-HSD activity (six-, four-, and eight-fold stimulation, respectively), thus suggesting that DEX does not alter the bio-availability of the cofactor, but more likely regulates the expression of the type 2 17β-HSD enzyme.

We have recently shown that IL-4 induces a rapid and potent induction of 3β-HSD type 1 gene transcription in human breast cancer cell lines as well as in normal human

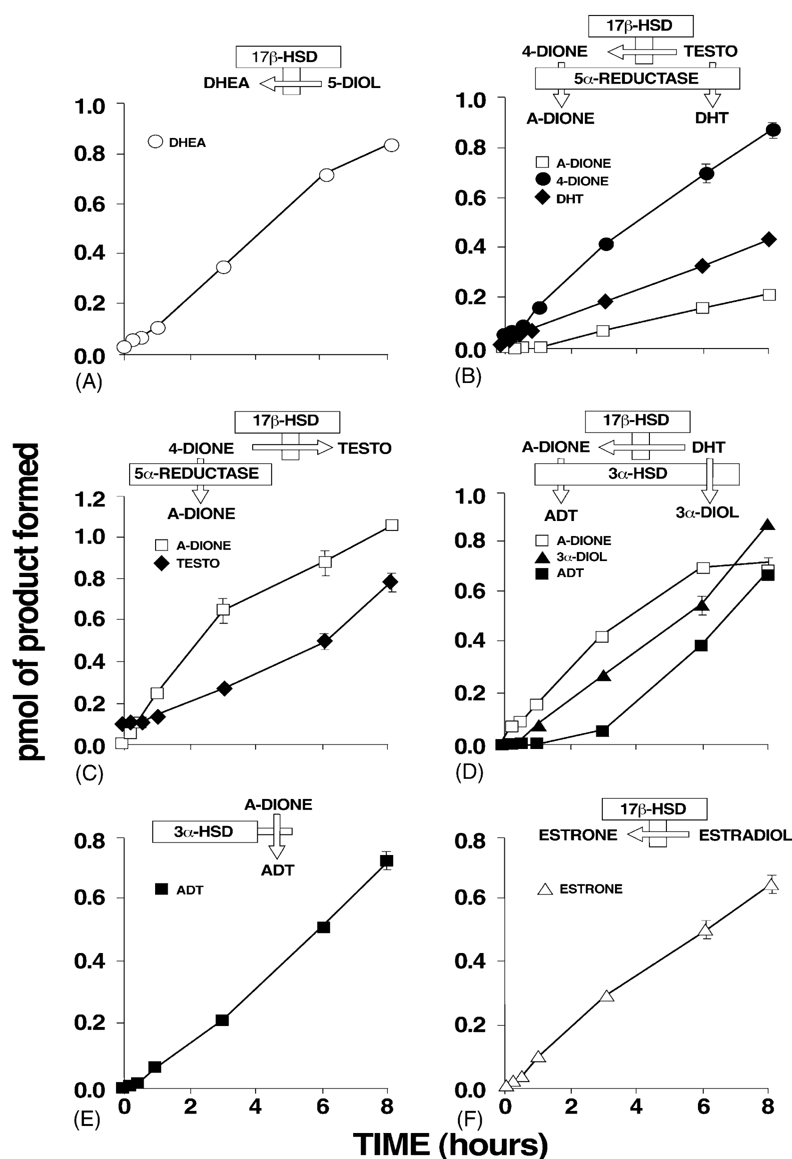

Fig. 4. Multiple steroid metabolizing activities are present in human keratinocyte HaCaT cells. HaCaT cells were plated at a density of 75,000 cells/well in 24-well plates. After 2 days, the medium was changed for 1 ml of fresh RPMI-1640 medium containing 10 nM of [^{3}H] 5-DIOL (A), [^{3}H] TESTO (B), [^{3}H] 4-DIONE (C), [^{3}H] DHT (D), [^{3}H] A-DIONE (E) or [^{3}H] E2 (F). At the end of the indicated incubation periods, free steroids were extracted and analyzed as described in Section 2. Cell proliferation was determined by measuring DNA content. Results are expressed as means ± S.E.M. of pmol of formed product.

mammary epithelial cells [10,47]. Since 3β-HSD type 1 is expressed in the skin [3,9], and human keratinocytes constitutively express the IL-4 receptor [48,49], the effect of IL-4 on type 1 3β-HSD expression was preliminary investigated in HaCaT human immortalized keratinocytes [10]. This effect was further characterized in the present study. HaCaT cells were incubated with increasing concentrations of IL-4 for 2 days. As indicated above, 3β-HSD activity was not detectable in HaCaT cells under basal growth conditions, as indicated by the absence of detectable conversion of [^{3}H] DHEA (Fig 8A). However, incubation with IL-4 induced a marked conversion of [^{3}H] DHEA into [^{3}H] 4-DIONE, thus indicating an induction of 3β-HSD activity in these cells. This potent up-regulation in 3β-HSD activity was observed at an EC_{50} value of 30 pM. Similarly, in control HaCaT cells under basal conditions, [^{3}H] 5-DIOL was only converted to [^{3}H] DHEA (Fig. 8B), thus resulting from the endogenous oxidative 17β-HSD activity present in these cells. It can also be seen in Fig. 8B that the induction of 3β-HSD activity by IL-4 was responsible for the conversion of [^{3}H] 5-DIOL into 4-ene-ketosteroids (TESTO and 4-DIONE). A-DIONE and DHT were also produced from 4-DIONE and TESTO by 5α-reductase activity. We could not however ascertain the identity of a sixth spot in experiments analyzed by TLC. Under these conditions of analysis, ADT, 3α-DIOL, EPI-ADT and 3β-DIOL, which could be produced from subsequent

metabolism of A-DIONE and DHT by 3α-HSD and 3β-HSD activity, also migrate at the same position as DHT. Thus, the values of A-DIONE and the sixth spot were added and generically referred to as 5α-reduced products.

In order to further characterize the predominant [^{3}H] 5-DIOL metabolic pathway(s) and to measure the relative contribution of 3β-HSD and 17β-HSD activities, we performed a time course experiment after a 2-day incubation in the presence or absence of 100 pM IL-4. In the absence of IL-4, [^{3}H] 5-DIOL is only converted to [^{3}H] DHEA by oxidative 17β-HSD activity (Fig. 9A), which is consistent with the data shown inFigs. 4A and 8B. On the other hand,

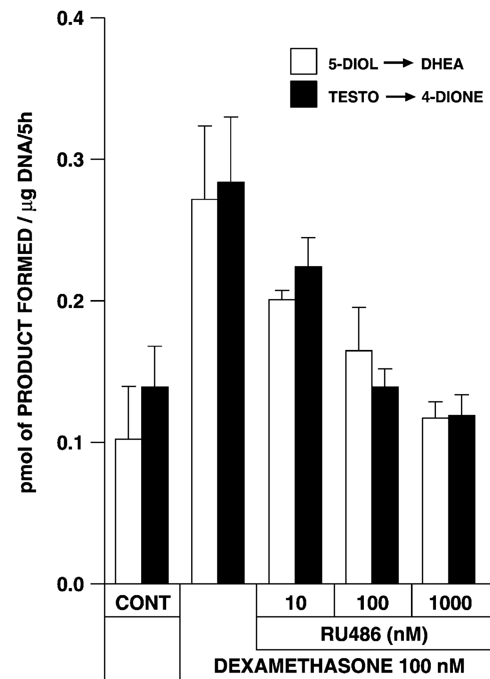

Fig. 6. The glucocorticoid antagonist RU486 blocks the effect of DEX on oxidative 17β-HSD activity in intact HaCaT cells in culture. HaCaT cells were plated at a density of 8000 cells/well in 24-well plates. After 2 days, cells were incubated with 100 nM of DEX and the indicated increasing concentrations RU486 for 6 days with medium changes every 2 days. Thereafter, the medium was changed for 1 ml of fresh RPMI-1640 medium containing 10 nM of [^{3}H] 5-DIOL or [^{3}H] TESTO and incubated for 5 h. At the end of the indicated incubation periods, free steroids were extracted and analyzed as described in Section 2. Cell proliferation was determined by measuring DNA content. Results are expressed as means ± S.E.M. of pmol of product formed/μg DNA/5 h.

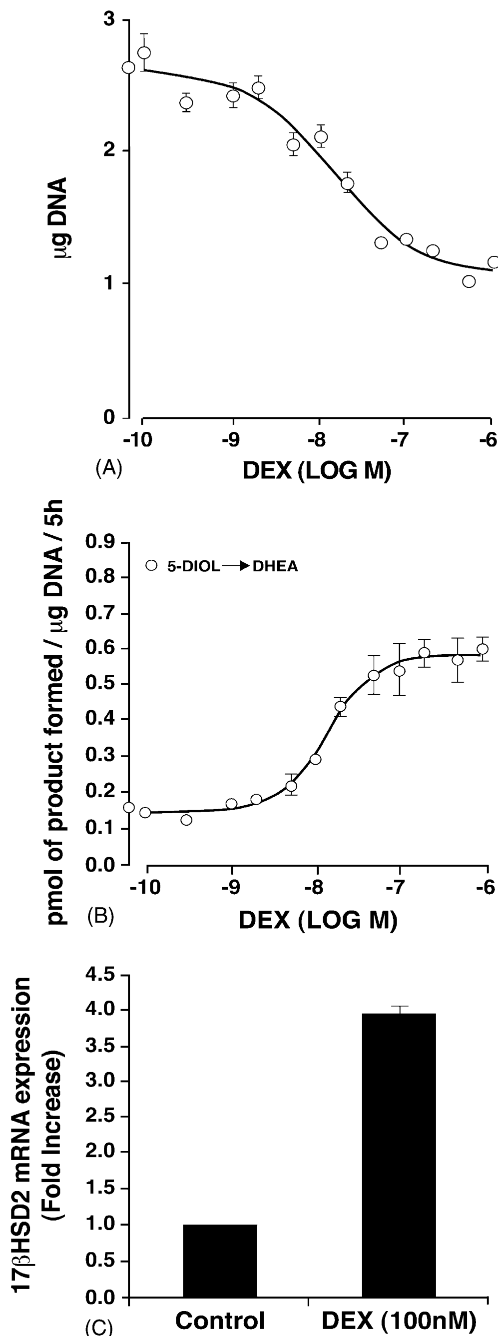

Fig. 5. Effect of DEX on HaCaT cell proliferation, as well as on oxidative 17β-HSD activity and mRNA expression of 17β-HSD type 2 in intact HaCaT cells in culture. (A) HaCaT cells were plated at a density of 8000 cells/well in 24-well plates. After 2 days, cells were incubated in the presence of the indicated concentrations of DEX for 6 days with medium changes every 2 days. Cell proliferation was determined by measuring DNA content. Data are expressed as means ± S.E.M. of DNA content. (B) HaCaT cells were plated at a density of 8000 cells/well in 24-well plates. After 2 days, cells were incubated in the presence of the indicated concentrations of DEX for 6 days with medium changes every 2 days. Thereafter, medium was changed for 1 ml of fresh RPMI-1640 medium containing 10 nM of [^{3}H] 5-DIOL and incubated for 5 h. At the end of the indicated incubation periods, free steroids were extracted and analyzed as described in Section 2. Cell proliferation was determined by measuring DNA content. Results are expressed as means ± S.E.M. of pmol of product formed/μg DNA/5 h. (C) HaCaT cells were plated at a density of 32,000 cells/well in 6-well plates. After 2 days, cells were incubated in the absence or presence of 100 nM of DEX for 6 days with medium changes every 2 days. Thereafter, total RNA was extracted from control and DEX treated HaCaT cells and relative expression levels of type 2 17β-HSD were determined by real-time PCR assays. Data was standardized by setting the value of type 2 17β-HSD expression levels in HaCaT control cells to 1.0. Data for 17β-HSD DEX treated cells was expressed relative to this standard (means ± S.E.M.).

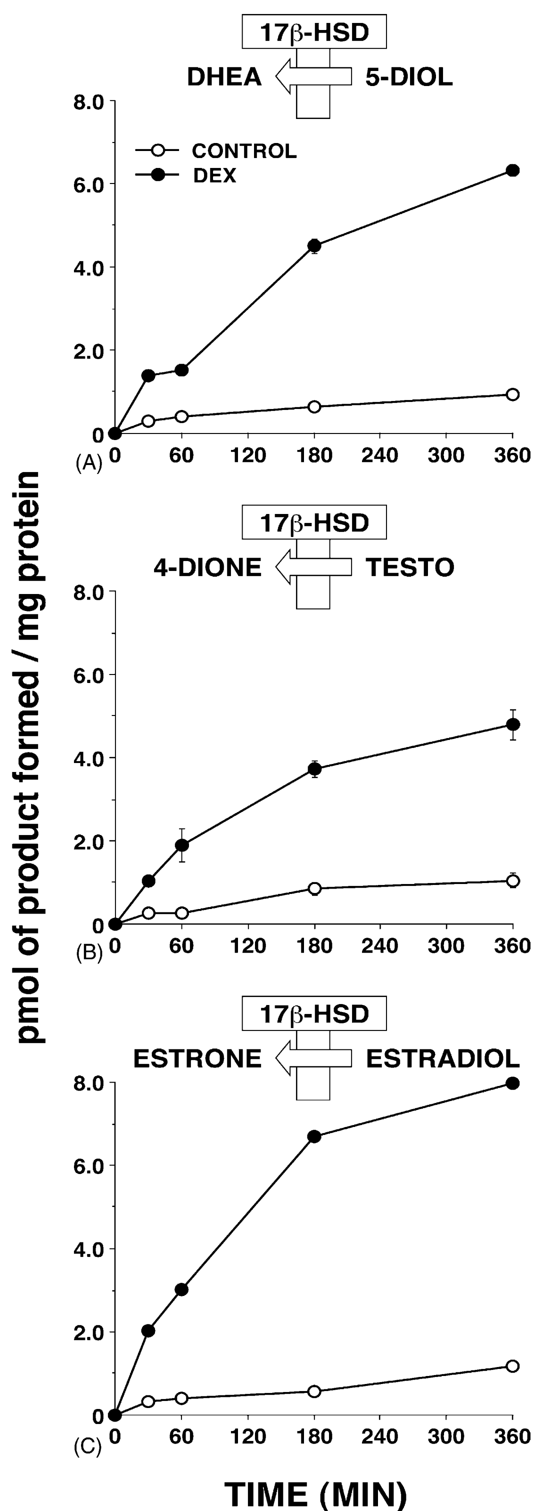

Fig. 7. DEX increases 17β-HSD activity in HaCaT cell homogenates. HaCaT cells were plated at a density of 1,000,000 cells in T-175 flasks. After 2 days, cells were incubated in the presence or absence of 100 nM of DEX for 6 days with medium changes every 2 days. Thereafter, cells were harvested as described in Section 2. Hundred micrograms of proteins was incubated up to 6 h in the presence of 10 nM of [^{3}H] 5-DIOL (A), [^{3}H] TESTO (B), or [^{3}H] E2 (C). Data are expressed as means ± S.E.M. of the product formed/mg of proteins.

in IL-4-treated HaCaT cells, there was no [^{3}H] DHEA formation, but rather a rapid conversion to [^{3}H] TESTO, which was further converted to [^{3}H] 4-DIONE by oxidative 17β-HSD activity. Thereafter, [^{3}H] TESTO and [^{3}H] 4-DIONE were converted to different [^{3}H] 5α-reduced products by 5α-reductase activity.

4. Discussion

Steroid metabolism studies using primary keratinocytes in culture must take into account the marked changes in the expression pattern of the steroidogenic enzymes throughout development and terminal differentiation of these cells in vitro. Moreover, cells obtained from different women lead to heterogeneous patterns of cell differentiation, which can affect the expression of the corresponding steroid metabolizing genes. It is thus of importance to be able to use a relatively stable cell line of immortalized keratinocytes (HaCaT) to facilitate and support studies on the regulation of steroid metabolism in an epidermal model. In fact, the present data show that HaCaT cells possess the steroid metabolizing enzymes present in human epidermal keratinocytes in culture.

In human keratinocytes in primary culture, 17β-HSD, 5α-reductase and 3α-HSD activities, but no 3β-HSD activity could be detected. As mentioned above, it is of interest that almost similar data on steroid metabolism were found in HaCaT cells. These observations are in agreement with recent findings that 3β-HSD expression in the human skin is primarily observed in the sebaceous glands but not in keratinocytes [3,4,12,35].

Based on the tissue-specific expression of the two types of 5α-reductase [5,13], type 1 5α-reductase is probably responsible for the relatively high level of conversion of TESTO into DHT and 4-DIONE into A-DIONE in both primary cultures of keratinocytes and HaCaT cells. The identity of the 17β-HSD enzymes responsible for the efficient conversion of 5-DIOL into DHEA, TESTO into 4-DIONE, DHT into A-DIONE, and E2 into E1 in both human keratinocytes in primary culture as well as in HaCaT cells remains to be determined, but type 2 17β-HSD is a strong candidate since it is the predominant, if not the only one, possessing an oxidative activity using as substrates both androgens and estrogens. On the other hand, the observed conversion 4-DIONE into TESTO in HaCaT cells may be due to the presence of type 5 17β-HSD [50].

Furthermore, the enzyme 17β-HSD displayed an interesting pattern of expression in primary cultures of keratinocytes: its expression was undetectable or very low for the first 2 weeks of culture. This was followed by a peak of expression around day 28 for all substrates tested and then, by a rapid decrease towards a lower steady-state expression of the enzyme through day 57 in culture. This pattern of expression is of particular interest since no other gene has yet been reported to follow this profile in primary cultures of keratinocytes. In addition, there are only a few examples of

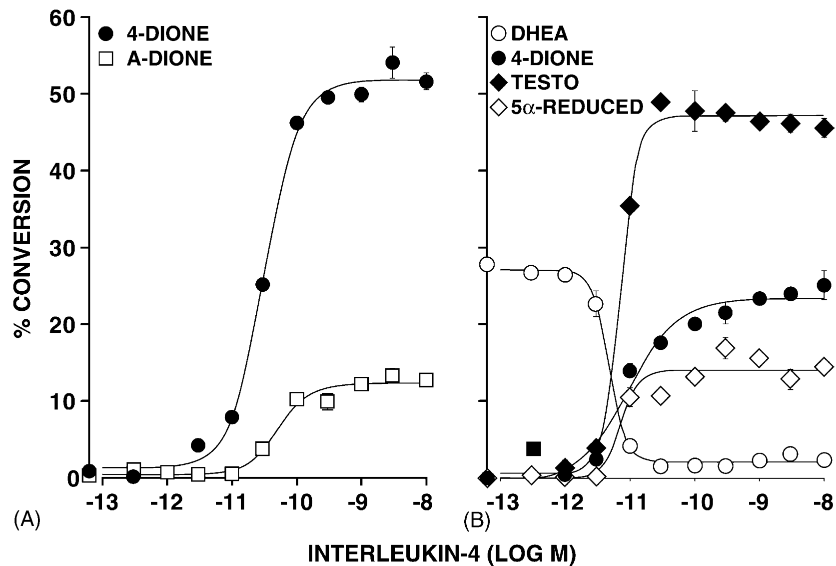

Fig. 8. Interleukin-4 induces 3β-HSD activity in intact HaCaT cells in culture. HaCaT cells were plated at a density of 20,000 cells/well in 24-well plates. After 2 days, cells were incubated for 2 days with the indicated concentrations of IL-4. Thereafter, 3β-HSD activity was assayed for 16 h using 10 nM [^{3}H] DHEA (A) or [^{3}H] 5-DIOL (B). Data are expressed as the means ± S.E.M. of the percentage of conversion.

genes whose expression has been shown to increase during differentiation of keratinocytes: minoxidil sulfotransferase [51] and cholesterol sulfotransferase [52] are markers that appear early in development, and their expression is increased through terminal differentiation. The keratin 1 and keratin 10 genes are also considered as good markers of terminal differentiation, as well as glycoprotein 38 [53]. However, these genes do not display a typical pattern of expression similar to that of 17β-HSD activity. The reason for the regulation of expression observed for 17β-HSD activity during aging of cultures is still unknown, but, as mentioned above, this particular pattern is the first to be identified in an epidermal cell model.

Since glucocorticoids are known to exert potent pharmacological effects in the skin, we used the stable cell line HaCaT to further characterize the regulation of oxidative 17β-HSD activity by the potent glucocorticoid DEX. After a 6-day treatment with DEX, intact HaCaT cells in culture as well as cell homogenates were studied for oxidative 17β-HSD activity. In intact cells, despite its effect on

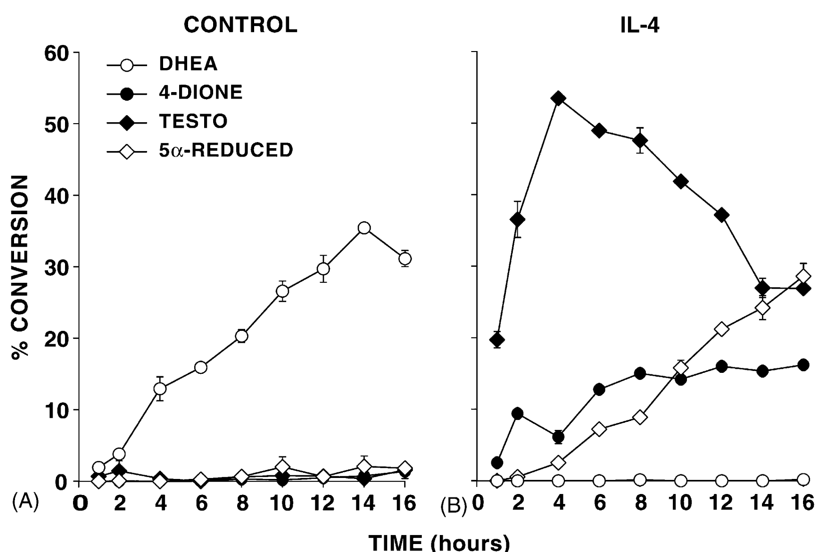

Fig. 9. Time course of IL-4 action on the conversion of 5-DIOL in intact HaCaT cells in culture. HaCaT cells were plated at a density of 20,000 cells/well in 24-well plates. After 2 days, cells were incubated for 2 days in the presence or absence of 100 pM IL-4. Thereafter, the medium was changed for 1 ml of fresh RPMI-1640 medium containing 10 nM of [^{3}H] 5-DIOL. At the end of the indicated incubation periods, free steroids were extracted and analyzed as described in Section 2. Data are expressed as the means ± S.E.M. of the percentage of conversion.

reducing cell proliferation, DEX was able to stimulate oxidative 17β-HSD activity in a dose-dependent manner. This effect was found to be specific since RU486, a glucocorticoid antagonist, was able to completely prevent the stimulatory effect of DEX. Real-time PCR experiments show that DEX caused a four-fold increase of type 2 17β-HSD mRNA levels, which correlates well with the observed increase of oxidative 17β-HSD activity in HaCaT cells (Figs. 5 and 6). It is of interest to note that in cell homogenates, DEX was able to stimulate oxidative 17β-HSD activity by four-fold (using [^{3}H] TESTO) to eight-fold the control value (using [^{3}H] E2), thus suggesting that DEX does not alter the bio-availability of the cofactor, but more likely regulates the expression of the type 2 17β-HSD enzyme.

An early study had demonstrated that human skin converted DHEA to TESTO, a process which required the expression of 3β-HSD [54]. Thereafter, it has been shown that 3β-HSD type 1 is predominantly expressed in the sebaceous glands where it could play a role in the conversion of inactive adrenal steroid precursors into potent androgens [3]. In this respect, it is interesting to mention that a close correlation between 3β-HSD activity and the rate of in vivo sebum secretion was reported in humans [11]. In the present study, we have demonstrated that even if no 3β-HSD activity was detectable in HaCaT cells under basal culture condition, IL-4, a cytokine secreted by activated T-cells caused the induction of this activity. We have previously reported such an induction of 3β-HSD type 1 expression in human breast cancer cells [10,47]. The present study thus confirms and extends our preliminary data showing that IL-4 induces the expression of this latter gene in HaCaT cells [10].

This effect of IL-4 in keratinocytes is in agreement with the fact that human keratinocytes constitutively express IL-4 receptor and that its expression is increased in some epidermal proliferative diseases such as psoriasis [48,49]. Moreover, it is known that infiltrating activated T-cells are present at the dermal–epidermal junction, and that the infiltration precedes the development of epidermal hyperproliferation [55]. Furthermore, the effectiveness of T-cell based therapeutics has suggested that T-cells play a role in triggering and perpetuating keratinocyte proliferative diseases such as psoriasis [37–40]. In view of the present findings, it would be interesting to investigate the expression of type 1 3β-HSD in keratinocytes of psoriatic lesions, and to determine if the expression of 3β-HSD correlates with the expression of IL-4 and IL-4 receptors. It would also be relevant to determine the contribution of sex steroids to the etiology of proliferative diseases of the skin.

The widespread pattern of steroidogenic enzyme expression shown in HaCaT cells and keratinocytes in primary culture puts the emphasis on the fact that these cells possess all the machinery to produce active sex steroids and would therefore represent an illustrative example of intracrinology. In accordance with this process, we demonstrated that at least two factors (DEX and IL-4) could regulate the expression of two different enzymes, thus resulting in the modulation of steroid metabolism in these cells. Finally, the present data show that HaCaT cells are a good model to further study the regulation of the enzymes involved in the metabolism of sex steroids in keratinocytes.

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