Suppression of Ornithine Decarboxylase Gene Expression by Retinoids in Cultured Human Keratinocytes

David R. Olsen, Ph.D., Noreen J. Hickok, Ph.D., and Jouni Uitto, M.D., Ph.D.
Departments of Dermatology, and Biochemistry and Molecular Biology, Jefferson Medical College, and Section of Molecular Dermatology, Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, U.S.A.

Modulation of ornithine decarboxylase (ODC) gene expression by retinoids was analyzed in human keratinocyte cultures maintained in serum-free medium containing 0.15 mM Ca^{++}. Cells were incubated with all-*trans*-retinoic acid, 13-*cis*-retinoic acid or arotinoid Ro15-0778 (10^{-10} to 10^{-5} M), total RNA was isolated, and mRNA transcripts for ODC were analyzed by Northern and slot blot hybridizations with a human ODC cDNA. Treatment of cells for 24 h resulted in a dose-dependent decrease in ODC mRNA levels, with an estimated IC_{50} of ~1×10^{-8} M for all-*trans*- and 13-*cis*-retinoic acid, while Ro15-0778 was somewhat less effective (IC_{50} ~$1-5 \times 10^{-7}$ M). The suppression of ODC mRNA levels by retinoids was detectable at ~3 h of incubation, with essentially a maximal inhibition at 12 h. Reduced ODC mRNA levels noted after 24 h of incubation with 5×10^{-7} M all-*trans*-retinoic acid were accompanied by a reduction in ODC enzyme activity. To determine if all-*trans*-retinoic acid was regulating ODC gene expression directly, or if protein synthesis was required, ODC expression was analyzed in cultures treated with protein synthesis inhibitors. In the presence of cycloheximide or puromycin, all-*trans*-retinoic acid did not suppress ODC mRNA levels. These findings suggest that suppression of ODC gene expression is not a direct effect of all-*trans*-retinoic acid, but depends on ongoing protein synthesis. *J Invest Dermatol 94:33–36, 1990*

Retinoids are powerful therapeutic agents that have been used either systemically or topically to treat a number of dermatologic disorders [1]. In proliferating normal keratinocytes, retinoids cause an arrest of cell growth in a predifferentiated state [2,3]. This growth suppression may be central to their therapeutic effects in hyperproliferative epidermal disorders, such as psoriasis and ichthyosis [1].

A number of model systems have been used to measure the efficacy of retinoids. These include the ability to induce differentiation of F9 embryonal carcinoma cells [4], and to inhibit ODC activity in mouse skin after tape stripping [5]. ODC is the first and, apparently, the rate-limiting enzyme in the biosynthesis of the polyamines, substances implicated in many cellular processes, such as transcriptional and translational efficiency [6]. There is a requirement for polyamines for cell growth [7]. ODC has one of the shortest half-lives of known mammalian enzymes ($t_{1/2}$ ~ 8–12 min in mouse kidney [8]), and the enzyme responds to a variety of hormones and trophic stimuli with a rapid induction of activity (see, e.g., [9,10]). Therefore, changes in ODC activity occur rapidly and have been well correlated with the growth state of cells [11].

In order to further understand the mechanism of inhibition of epidermal ODC activity by retinoids, we have examined their effects on ODC mRNA levels in cultured human epidermal keratinocytes, specifically, the effects of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and Ro15-0778. All three retinoids caused a dose-dependent suppression of ODC mRNA levels, and this effect could be abolished by inhibition of protein synthesis.

MATERIALS AND METHODS

Reagents All-*trans*-retinoic acid and 13-*cis*-retinoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Ro15-0778 was the kind gift of Dr. Stanley Shapiro (Hoffman-LaRoche, Nutley, NJ). All retinoids were stored at −20°C in the dark and dissolved in DMSO (Fisher Scientific, Philadelphia, PA) immediately before use. All other reagents were obtained from Sigma or Fisher.

Cell Cultures Normal human epidermal keratinocytes were purchased from Clonetics Corp. (San Diego, CA) at passage 2, and were grown, with daily medium changes, in medium containing 0.15 mM Ca^{++} (KGM, Clonetics Corp.), supplemented with 50 μg/ml bovine pituitary extract [12]. Experiments were performed on cell cultures (passage 5) in mid-to-late log phase of growth. The reti-

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Reprint requests to: Jouni Uitto, M.D., Ph.D., Department of Dermatology, Thomas Jefferson University, Rm. M-46 Jefferson Alumni Hall, 1020 Locust Street, Philadelphia, PA 19107.

Abbreviations:
- cDNA: complementary DNA
- CHX: cycloheximide
- α-DFMO: α-difluoromethylornithine
- DMSO: dimethylsulfoxide
- mRNA: messenger RNA
- ODC: ornithine decarboxylase
- RA: all-*trans*-retinoic acid
- SDS: sodium dodecyl sulfate
- 1 × SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 6.8

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noid, or vehicle ([DMSO] ≤ 0.1%), was added to culture medium in the dark.

RNA Isolation, Northern Hybridizations, and Slot Blots RNA was isolated using the guanidinium isothiocyanate/cesium chloride method [13,14]. For Northern hybridization [15], total RNA was fractionated on 1% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized with the human ODC cDNA, pODC 10/2H [16] or with human β-actin cDNA [17], which were labeled by nick translation with α[^{32}P]dCTP [18]. For slot blots, serial dilutions of total RNA were immobilized on nitrocellulose and hybridized with the ^{32}P-labeled cDNAs. All blots were washed in 0.1 × SSC, 0.1% SDS, at 65°C for 30 min. The filters were exposed with DuPont Cronex film at −70°C, and mRNA levels were quantitated using a He-Ne laser densitometer at 633 nm (LKB, Bromma, Sweden). The results were expressed as densitometric units (U) per μg RNA.

ODC Activity Cells were trypsinized, collected by centrifugation, and washed in phosphate-buffered saline. Cytosols were prepared by lysing the cells with three freeze/thaw cycles, followed by centrifugation at 150,000 × g for 30 min at 4°C. ODC activity was measured using the $^{14}CO_2$-release assay as described by Seely and Pegg [19]. Activities were expressed as cpm/mg cytosolic protein/h at 37°C. Background $^{14}CO_2$ release was determined using cytosol in the presence of 5 mM α-difluoromethylornithine (α-DFMO). Cytosolic protein was determined using the Bio-Rad Protein Assay kit.

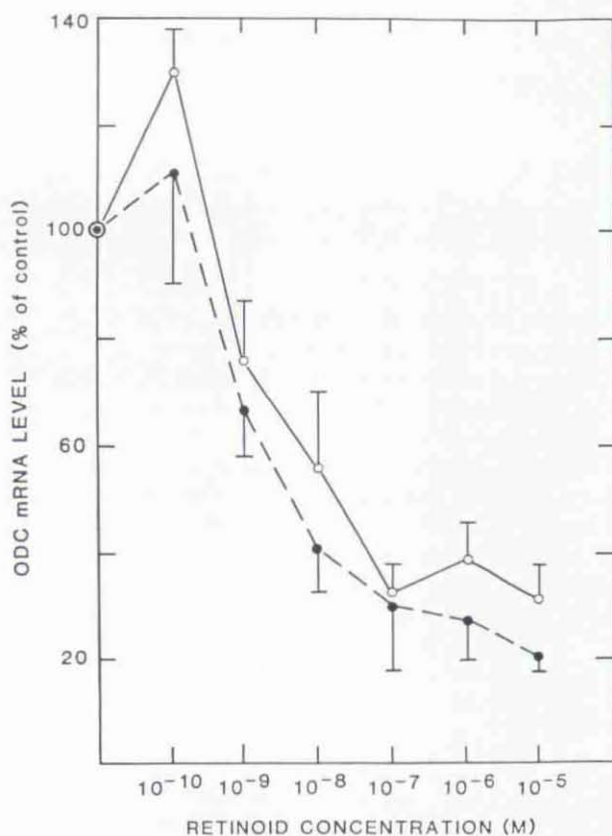
RESULTS

To compare the effects of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and Ro15-0778 on ODC mRNA levels, subconfluent proliferating keratinocytes were treated with 10^{-10} to 10^{-5} M retinoid for 24 h (Fig 1). Total RNA was isolated by the guanidinium isothiocyanate/CsCl [13,14] method and ODC mRNA levels determined by Northern blotting [15] and slot blot hybridization with the human ODC cDNA [16]. A dose-dependent decrease in ODC mRNA levels was observed with all three retinoids tested. All-*trans*- and 13-*cis*-retinoic acid produced qualitatively similar dose-response curves and were approximately ten-fold more potent at suppressing ODC mRNA levels than Ro15-0778 (IC_{50} ~1 × 10^{-8} M for 13-*cis*- and all-*trans*-retinoic acid; IC_{50} ~1–5 × 10^{-7} M for Ro15-0778). The same Northerns were re-probed with β-actin cDNA [17] to insure that equal amounts of RNA were used in the different experiments. No change in β-actin hybridization signal was measured between the different incubation conditions indicating that the suppression of ODC mRNA was due to a specific effect of the retinoids (data not shown).

The time course of suppression of ODC mRNA levels was measured using 5 × 10^{-7} M all-*trans*-retinoic acid over 48-h (Fig 2). No change was measured after 1 h of treatment; however, a significant decrease in ODC mRNA levels was observed at 3 h, with the suppression being essentially complete by 12 h (Fig 2B).

To correlate the changes observed in ODC mRNA with changes in ODC activity, evolution of $^{14}CO_2$ from [^{14}C] ornithine [18] was measured in extracts from keratinocytes that had been treated with DMSO (≤0.1%, control) or 5 × 10^{-7} M all-*trans*-retinoic acid dissolved in DMSO for 0 and 24 h. Controls at 0 and 24 h showed no significant difference in ODC activities, whereas keratinocytes treated with all-*trans*-retinoic acid for 24 h contained approximately 30% of the ODC activity of the control cells (Table I).

We then asked if the retinoids were causing the observed suppression of ODC mRNA levels directly, or if protein synthesis was necessary for this effect, by using the protein synthesis inhibitors, cycloheximide and puromycin. Keratinocytes, pre-incubated with 10 μg/ml cycloheximide for 1 h and then labeled with [^{3}H]leucine in the presence of cycloheximide for 6 h, showed a greater than 95% decrease in protein synthesis (data not shown). Therefore, proliferating keratinocytes were pre-incubated with 10 μg/ml cycloheximide, and then exposed to 5 × 10^{-7} M all-*trans*-retinoic acid, in the presence of cycloheximide, for 6 h. ODC mRNA levels were compared to DMSO-treated cultures containing cycloheximide (Fig 3). As shown above, treatment of keratinocytes with 5 × 10^{-7} M all-*trans*-retinoic acid caused a decrease in ODC mRNA levels. However, this decrease was not observed when cycloheximide was used in conjunction with retinoic acid treatment (Fig 3*A*,*B*). In fact, treatment of keratinocytes with cycloheximide caused an approxi-



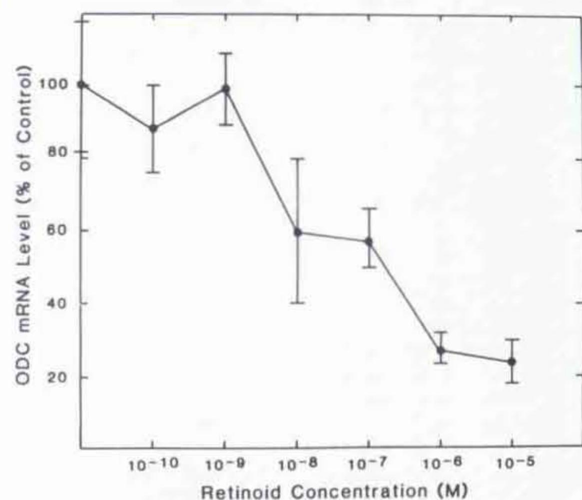


Figure 1. Dose-dependent suppression of ODC mRNA levels as a function of retinoid concentrations. Normal human keratinocytes were incubated for 24 h with the indicated concentrations of (A) all-*trans*-retinoic acid *(open circles)* or 13-*cis*-retinoic acid *(solid circles)*, or (B) Ro15-0778. ODC mRNA levels were determined by slot blot hybridizations and quantified by scanning densitometry. Control cultures received vehicle alone. The values are mean ± SD of three parallel cultures, each assayed using five different RNA concentrations in two separate experiments.

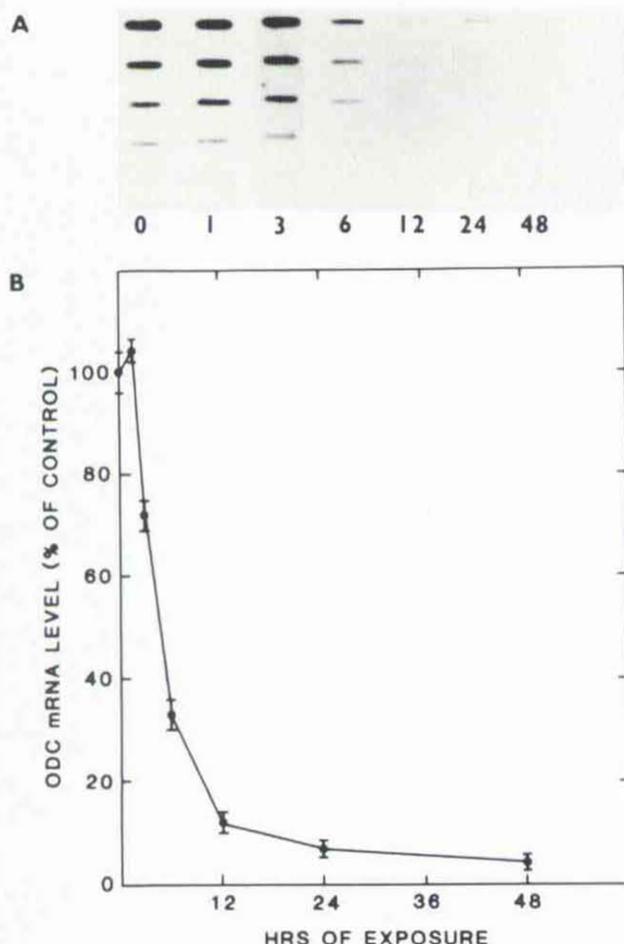


Figure 2. Time course of suppression of ODC mRNA levels by all-*trans*-retinoic acid. Keratinocytes were treated with 5×10^{-7} M all-*trans*-retinoic acid for the indicated times. ODC mRNA levels, shown as slot blot hybridizations of dilutions *(A)* were quantified by densitometry and expressed as percent of control *(B)*. The values in B are mean ± SD of five parallel determinants.

mately twofold increase in ODC mRNA levels over control. To insure that any effects noted in the presence of cycloheximide were due to its inhibition of protein synthesis, keratinocytes were also treated with 50 μg/ml puromycin or 50 μg/ml puromycin plus 5×10^{-7} M all-*trans*-retinoic acid. Like cycloheximide, puromycin blocked the suppression of ODC mRNA levels by retinoic acid and, when used alone, caused a small increase in ODC mRNA concentration (Fig 3*B*).

Table I. Effect of All-*Trans*-Retinoic Acid on ODC Activity in Human Keratinocytes

Treatment	ODC activity (cpm/mg protein)
Control, 0 h	9980.6 ± 3040.3 (9)[a]
Control, 24 h	12332.4 ± 3352.0 (9)
5×10^{-7} M all-*trans*-retinoic acid, 24 h	3725.0 ± 1240.5 (10)
5 mM α-DFMO[b]	847.4 ± 453.0 (15)

[a] Values shown are mean ± SD determined from two separate experiments. The number of flasks assayed is indicated in parentheses.

[b] Cytosols were incubated in the presence of 5 mM α-DFMO to determine background $^{14}CO_2$ release.

DISCUSSION

Changes in ODC activity followed by changes in polyamine levels have been shown to accurately reflect the proliferative state of many cell types [11]. In F9 embryonal carcinoma cells, retinoic acid induces differentiation [4,20,21] and suppresses ODC activity [22]. Inhibition of ODC by the suicide inhibitor α-DFMO is also capable of causing F9 cells to differentiate [23]. It was therefore concluded that polyamines must be present in F9 cells above a minimum concentration, or the cells will differentiate. However, other differentiation signals appear to function independently of polyamine concentration [21].

In this study, we asked if retinoic acid caused suppression of ODC mRNA levels in cultured human keratinocytes and whether this suppression was a direct effect of retinoic acid or whether an intermediate protein was required. Our results indicate that all-*trans*-ret-

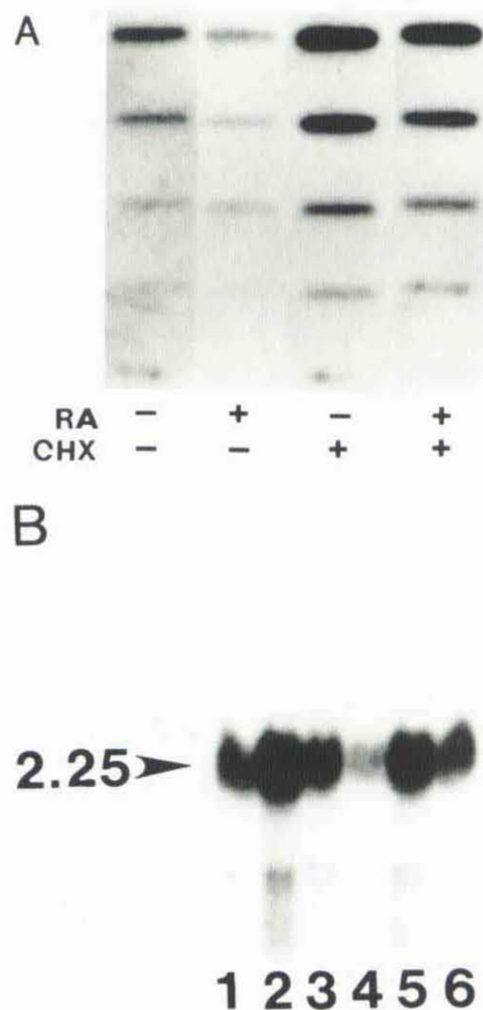


Figure 3. Effect of protein synthesis inhibitors on suppression of ODC mRNA levels by all-*trans*-retinoic acid. *(A)* Keratinocytes were pre-treated with 10 μg/ml cycloheximide followed by a 6-h treatment with 10 μg/ml cycloheximide (CHX) and/or 5×10^{-7} M all-*trans*-retinoic acid (RA) as indicated. ODC mRNA levels were determined by slot blot hybridizations. *(B)* Keratinocytes were treated with vehicle alone *(lane 1)*; pre-treated for 1 h, followed by a 6-h treatment with 10 μg/ml cycloheximide *(lane 2)*; pre-treated for 1 h, followed by a 6-h treatment with 50 μg/ml puromycin *(lane 3)*; treated for 6 h with 5×10^{-7} M all-*trans*-retinoic acid *(lane 4)*; pre-treated for 1 h with 10 μg/ml cycloheximide followed by treatment for 6 h with 10 μg/ml cycloheximide and 5×10^{-7} M all-*trans*-retinoic acid *(lane 5)*; or pre-treated for 1 h with 50 μg/ml puromycin, followed by treatment for 6 h with 50 μg/ml puromycin and 5×10^{-7} M all-*trans*-retinoic acid *(lane 6)*. ODC mRNA levels were visualized by Northern blot hybridization.

inoic acid, 13-*cis*-retinoic acid, and Ro15-0778 cause a rapid, dose-dependent decrease in ODC mRNA levels.

Furthermore, no decrease in ODC mRNA is seen in the absence of protein synthesis. However, since cycloheximide alone causes a twofold increase in ODC mRNA levels, the role of protein synthesis in the suppression of ODC mRNA levels by retinoids is not clear. For example, if cycloheximide suppresses the synthesis of a protein which mediates ODC mRNA turnover, the inhibition of the synthesis of this protein by cycloheximide would result in the stabilization of ODC mRNA even if retinoids had directly suppressed ODC mRNA synthesis.

Cycloheximide has been found to affect ODC mRNA levels in other cell types. For example, in BC_3Hl muscle cells, cycloheximide caused an increase in steady-state ODC mRNA levels and in ODC gene transcription. However, no changes in ODC mRNA half-life, which has been estimated to be 6–8 h [24], were detected [25]. Anisomycin also caused increases in ODC mRNA steady-state levels and in ODC gene transcription in Balb/c-3T3 cells and in the pheochromocytoma cell line PC12 [26], although these effects were small. These results, therefore, suggest that it is unlikely that either changes in mRNA half-life or gene transcription rates due to protein synthesis inhibition could account for the lack of response of ODC mRNA levels to retinoic acid in the presence of cycloheximide.

Recently, deThé et al [27] have reported that retinoic acid increases transcription of the β-retinoic acid receptor gene. This induction results in an accumulation of the β-retinoic acid receptor, a form of the receptor implicated in retinoic acid action in the skin [28,29]. If this amplification mechanism functions in human keratinocytes, then it is possible that increased retinoic acid receptor levels are mediating suppression of ODC gene expression. The suppression would therefore be dependent on new protein synthesis. We are currently exploring this possibility.

The data we have presented in this paper demonstrate that retinoic acid suppresses ODC activity in keratinocytes by decreasing ODC mRNA levels and thus the amount of enzyme synthesized. Since protein synthesis is required for this inhibition, these data are consistent with the hypothesis that retinoic acid suppresses ODC mRNA levels through an intermediate protein.

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