Regulation of the induction of ornithine decarboxylase in keratinocytes by retinoids

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Treatment of SV40-transformed keratinocytes (Z114) with epidermal growth factor (EGF) resulted in an increase in ornithine decarboxylase (ODC) activity and a dose-dependent increase in ODC mRNA levels. Pretreatment of keratinocytes with all-*trans*-retinoic acid inhibited the EGF induction of ODC activity. In both quiescent and EGF-stimulated cells, all-*trans*-retinoic acid inhibited ODC gene transcription and lowered ODC mRNA levels, whereas glyceraldehyde phosphate dehydrogenase expression remained unaffected. Treatment with all-*trans*-retinoic acid for 24 h resulted in a dose- and time-dependent decrease of up to 52% in EGF binding to EGF receptors and a 30–75% decrease in EGF-receptor quantity. In addition, when cells were treated with both UV radiation and all-*trans*-retinoic acid, their effects were additive in causing a decrease in EGF binding. Blocking of EGF receptors with a neutralizing antibody for EGF

receptors inhibited the induction of ODC activity by EGF. The effects of several other retinoids, including Ro15-0778, etretinate, Ro13-7410, etarotene, Ro40-8757, 13-*cis*-retinoic acid and acitretin, were also studied to determine their effects on EGF binding and ODC activity. Two of these other retinoids, 13-*cis*-retinoic acid and Ro13-7410, inhibited EGF binding the most (35–46%, $P < 0.001$); several others (etarotene, Ro40-8757 and etretinate) were less effective (7–16%), but significantly decreased EGF binding ($P < 0.05$), and two retinoids (Ro15-0778 and acitretin) showed no significant effect on EGF binding. In contrast, all of the retinoids tested inhibited the induction of ODC activity by EGF, although etretinate and Ro15-0778 were less effective. EGF signal transduction is important in ODC gene regulation, and retinoids are significant modulators of this pathway.

INTRODUCTION

Ornithine decarboxylase (ODC) is the rate-limiting enzyme for polyamine synthesis and is considered essential for cellular proliferation, because of the requirement for polyamines to interact with DNA [1]. ODC is a highly controlled enzyme that is induced by many trophic stimuli, including epidermal growth factor (EGF) [2]. Understanding the mechanisms whereby ODC is regulated may provide insight into approaches which one might consider in managing disorders of altered keratinocyte proliferation, e.g. keratinocyte hyperproliferation as seen in psoriasis and skin cancer.

Vitamin A is required for the normal growth, differentiation and development of epithelial tissues, and several retinoids have been found to exert valuable pharmacological effects in the skin. Thus, a greater understanding of their mechanism(s) of action may be valuable for designing better therapeutics. Retinoids have been known to be modulators of ODC activity for many years [2–4]. One of the most common screening assays for retinoids to predict their biological activity *in vivo* took advantage of the observation that active retinoids inhibit phorbol 12-myristate 13-acetate (PMA)-induced ODC activity in mouse epidermis. When topically applied, retinoic acid inhibits PMA-induced tumour promotion in mouse skin. In this regard, ODC induction is considered to be one of the mechanisms involved in tumour promotion [5]. We have been interested in the mechanism whereby retinoids modulate ODC activity and in developing assays *in vitro* for retinoids that might predict, with more convenience and possibly greater sensitivity, whether a retinoid has biological activity in human skin cells.

We report a cell culture system consisting of an immortalized

human foreskin keratinocyte cell line used to assess the influence of retinoids on the induction of ODC by EGF. We have used EGF as the inducing agent for ODC because of the consistency of this response and because it more closely represents a physiologically relevant stimulus for cell proliferation than utilizing phorbol esters. In addition, several hyperproliferative skin conditions, most notably psoriasis, appear to be stimulated by EGF-receptor signalling with transforming growth factor- α as the receptor ligand [6,7]. Previously we have reported that treatment of SV40-transformed keratinocytes with retinoic acid results in a dose-dependent blockage of the induction of ODC activity by EGF [2]. In addition, treatment of quiescent cultures with 10 μ M retinoic acid resulted in a decrease in ODC mRNA levels [2].

Although it has been suggested that retinoic acid may modulate the ODC gene through interaction with nuclear retinoic acid receptors (RARs), it has not been established that binding sites for RARs exist in the promoter region of the ODC gene. Our results reported here indicate that one mechanism whereby retinoids control the EGF induction of ODC is by blocking EGF signal transduction, and for several retinoids tested, e.g. retinoic acid, this interference probably occurs because of a decrease in EGF binding to the cell-surface EGF receptor.

To understand further the mechanism whereby retinoic acid interferes with the induction of ODC by EGF, we have characterized (1) the effect of retinoic acid on the levels of ODC mRNA in EGF-stimulated cultures, (2) the effect of retinoic acid on gene transcription by nuclear run-on assay, (3) the effect of retinoic acid on EGF binding and EGF-receptor amount, and (4) the effect of several other retinoids on EGF binding and ODC activity.

Abbreviations used: ODC, ornithine decarboxylase; EGF, epidermal growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; RAR, retinoic acid receptor.

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MATERIALS AND METHODS

Materials

Tissue culture media, plasticware and other reagents were obtained from vendors as previously described [2,8]. All-*trans*-retinoic acid, referred to as retinoic acid throughout, was obtained from Sigma (St. Louis, MO, U.S.A.). All other retinoid compounds were kindly provided by Stanley Shapiro, Hoffmann-LaRoche (Nutley, NJ, U.S.A.). Anti-(human EGF receptor) antibody was purchased from Sigma, and the neutralizing antibody for EGF receptor was obtained from UBI (Lake Placid, NY, U.S.A.).

Cell culture

Z114, a human foreskin keratinocyte cell line immortalized with SV40, was utilized in all experiments, the details of which have been previously described [2,8]. The cells were kindly provided to us by Vittorio Defendi, New York University [9]. Cells were grown in complete medium consisting of Dulbecco's modified Eagle medium supplemented with 10% (v/v) Nu-Serum IV, 0.5 µg/ml cortisol, 0.01 µg/ml cholera toxin and 10 ng/ml EGF. When indicated, cells were placed into basal medium consisting of Dulbecco's modified Eagle medium without the above additives. Cultures were grown in a humidified atmosphere of air/CO₂ (19:1) at 37 °C.

EGF binding

The details of ¹²⁵I-EGF binding experiments have been described previously [10,11]. Briefly, ¹²⁵I-EGF was added to basal medium. After incubation for the times described in the Figures, the dishes were washed three times with cold PBS containing 0.1% (w/v) BSA. Cells were solubilized in 0.1 M NaOH, and samples were counted for radioactivity in a Beckman 5500 γ-radiation counter. Non-specific binding represented 3–7% of the total binding, and was determined in parallel cultures which had a 200-fold excess of unlabelled EGF. Non-specific binding was subtracted from all points. Cell counts were determined with a haemocytometer. Scatchard analysis of the data was performed [12].

Northern-blot analysis

Northern-blot analysis was performed for ODC and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as previously described [2,8]. The cells were treated with retinoic acid, EGF, or ethanol vehicle as indicated in the individual experiments. Total cellular RNA was isolated by the guanidine thiocyanate/CsCl method and quantified by absorbance at 260 nm [13]. ODC cDNA was generously given by Dr. Olle Jänne, Rockefeller University [14]. GAPDH cDNA was kindly provided by Dr. M. B. Prystowsky (Albert Einstein–Montefiore Medical Center) [15]. ODC and GAPDH mRNAs were analysed by agarose/formaldehyde gel electrophoresis, followed by Northern blotting. Briefly, for each lane, 20 µg of total RNA was electrophoresed in an agarose/formaldehyde gel and blotted on to a nylon membrane. The membranes were hybridized with human ODC cDNA and murine GAPDH cDNA probes (sp. radioactivity 6 × 10⁸ c.p.m./µg of DNA) and exposed to X-ray film at –70 °C with an intensifying screen. The relative quantities of ODC and GAPDH mRNA were determined by laser densitometry.

ODC assay

ODC activity was determined as previously described [8]. Briefly, the release of ¹⁴CO₂ from DL-[1-¹⁴C]ornithine hydrochloride is

measured. The enzymically released ¹⁴CO₂ was captured on filter paper saturated with 1 M methylbenzathonium hydroxide in methanol. Enzyme activity is expressed as pmol of CO₂ released/h per mg of protein. Soluble protein extracted from the cultures was assayed for ODC activity and protein content by the Bradford method [16].

Nuclear run-on assay

Z114 cells were treated with retinoic acid, EGF, or ethanol vehicle as indicated in the experiments presented and were lysed in a Nonidet P-40 buffer [17]. For the transcription assay, nuclei (1 × 10⁷) were thawed and incubated in a reaction buffer containing 100 µCi of [³²P]UTP and unlabelled nucleotides [17]. Labelled nascent RNA transcripts were isolated. The ODC and GAPDH cDNAs were immobilized on to a nylon membrane and hybridized with the equal amount of labelled nascent RNA transcripts (8 × 10⁵ c.p.m.). The relative amounts of ODC and GAPDH mRNA from the cultures treated with retinoic acid, EGF, or control were determined by densitometry.

Immunoblot analysis

Solubilized extracts for immunoblotting were prepared from cell cultures pretreated with 10, 1 and 0.1 µM retinoic acid or vehicle (ethanol, 0.1%), for 24 h. The samples, containing 40 µg of cell extracts, were electrophoresed, transferred and incubated with anti-EGF-receptor antiserum for 60 min, followed by horseradish-peroxidase-conjugated second antibody. EGF receptor was immunodetected with enhanced chemiluminescence (ECL) reagents, and revealed by film exposure.

UV source and irradiation

Irradiation of monolayer cultures lightly moistened with PBS was performed with the lids of the culture dishes removed. Cells were exposed to UVB radiation, the details of which have been previously described [10]. After irradiation, cells were rinsed with PBS, fresh medium was added, and the cultures were returned to the incubator.

Statistics

Significance of differences between treated and untreated cultures was determined by Student's *t* test.

RESULTS

Retinoic acid suppresses ODC gene expression

Previously we have demonstrated that EGF maximally induced ODC mRNA in quiescent keratinocytes between 4 and 8 h upon activation with 50 ng/ml EGF. We have analysed the changes in the levels of ODC mRNA in quiescent keratinocytes upon activation with different doses of EGF (0–100 ng/ml). A dose-dependent relationship was observed between the amount of EGF and the amount of ODC mRNA in Z114 cells (Figure 1a). Although a 2-fold increase in ODC mRNA was observed in cells treated with EGF at 10 ng/ml, the usual concentration in complete medium, a 3.6-fold increase was observed when the concentration of EGF was increased to 100 ng/ml. Whereas we have previously described a maximum increase in ODC enzyme activity when cultures are treated with EGF at 50 ng/ml, these data show that mRNA levels continue to accumulate even when the enzyme activity has been maximized. In contrast, GAPDH showed no increase in mRNA levels at the different concentrations of EGF tested.

We show (Figure 1b) that retinoic acid decreased ODC mRNA

Figure 1 (a) EGF increases the level of ODC mRNA in a dose-dependent manner; retinoic acid (b) decreases ODC mRNA levels and (c) blocks ODC gene transcription in EGF-stimulated keratinocytes

(a) Total RNA was prepared from Z114 cells treated with EGF at concentrations from 0 to 100 ng/ml for 8 h. Samples of each RNA (20 μ g) were denatured by heating in sample buffer in a 60 °C water bath. The samples were electrophoresed on 1%-agarose gel, and transferred on to nylon membranes. The membrane was hybridized with 1×10^5 c.p.m./ml random-primer labelled ODC and GAPDH cDNA [(5–8) $\times 10^8$ c.p.m./ μ g] for 16 h at 42 °C. The X-ray film was exposed for 24 h. (b) Cells were grown in complete medium and placed in basal medium for 24 h, then treated with retinoic acid or its diluents for 24 h with or without EGF for 8 h as follows: (A) control, (B) ethanol (0.05%), (C) 5 μ M retinoic acid, (D) 50 ng/ml EGF + ethanol (0.05%), (E) 5 μ M retinoic acid + 50 ng/ml EGF. The amounts of ODC and GAPDH mRNA were determined by hybridizing the blots simultaneously with 32 P-labelled cDNA probes for each mRNA species. (c) Cells were pretreated with 5 μ M retinoic acid for a total of 24 h or combined with a 4 h incubation with 50 ng/ml EGF as follows: control (A), 5 μ M retinoic acid (B), 50 ng/ml EGF (C), and 5 μ M retinoic acid + 50 ng/ml EGF (D). The nuclear run-on transcripts were hybridized to ODC and GAPDH cDNA probes. The densities of ODC and GAPDH mRNA bands were determined by laser densitometry.

in quiescent keratinocytes and also blocked the EGF induction of ODC mRNA levels. Laser-densitometric analysis showed a 3.5-fold induction by EGF of ODC mRNA as compared with GAPDH mRNA (D); however, 5 μ M retinoic acid completely blocked the EGF induction of ODC mRNA (E). Retinoic acid decreased ODC mRNA in quiescent cells (C) by 30%.

To determine if the effect of retinoic acid on ODC mRNA

Figure 2 Retinoic acid decreases EGF binding to keratinocytes in a dose- and time-dependent manner

(a) Monolayers of sub-confluent Z114 cells were pretreated with 0–10 μ M retinoic acid or 0.1% ethanol for 24 h. The EGF binding was assayed by using 125 I-EGF added to basal medium at a dose of 5×10^5 c.p.m./ml at 4 °C for 3 h, as described in the Materials and methods section. (b) Monolayers of Z114 cells in 35 mm dishes were incubated in basal medium with 10 μ M retinoic acid or 0.1% ethanol at 37 °C. After the indicated times, cells were incubated with basal medium (1 ml) containing 5×10^5 c.p.m. of 125 I-EGF at 4 °C for 3 h. The EGF binding assay was performed as described in the Materials and methods section.

levels in EGF-stimulated or quiescent keratinocytes was exerted at the transcriptional level, the nuclear run-on assay was performed. We found that pretreatment of Z114 keratinocytes with 5 μ M retinoic acid resulted in blockage of the transcription of the ODC gene under EGF-stimulated conditions (Figure 1c). The ratios of the density of the ODC band to that of the GAPDH band in lanes A (control), B (5 μ M retinoic acid), C (50 ng/ml EGF) and D (50 ng/ml EGF and 5 μ M retinoic acid) were 0.6, 0.5, 2.0 and 0.6 respectively. Since ODC gene expression was at a low level under quiescent conditions (no added EGF), the decrease in ODC gene expression in cultures treated with 5 μ M

Figure 3 Retinoic acid treatment decreases the number of available EGF receptors

(a) Z114 cells were grown in 75 cm² flasks and subcultured at a density of 3×10^5 cells/well in 24-well trays. After 24 h of incubation in basal medium with 10 μM retinoic acid or vehicle as the control (0.1% ethanol), cells were incubated for 3 h at 4 $^{\circ}\text{C}$ in the presence of increasing concentrations of ^{125}I -EGF in a final volume of 0.25 ml of basal medium as described in the Materials and methods section. Non-specific binding was determined by using a 200-fold excess of unlabelled EGF, and was 3–7%. Wells contained approx. 4.2×10^5 cells in the vehicle control group and 3.9×10^5 cells in the cells treated with 10 μM retinoic acid. (b) Scatchard analysis indicated that retinoic acid decreased the number of EGF binding sites from 6.0×10^4 /cell to 2.9×10^4 /cell, and essentially did not change the EGF binding affinity ($K_d = 0.88$ nM in control cells and 0.82 nM in cells treated with 10 μM retinoic acid).

Figure 4 Retinoic acid results in a dose-dependent decrease in the quantity of EGF receptors

Cells were pretreated with 10 μM (B), 1 μM (C) or 0.1 μM (D) retinoic acid or ethanol (0.1%) (A) for 24 h. Portions of cell extracts (40 μg of protein) from each sample were electrophoresed, transferred and immunoblotted with anti-EGF-receptor antibody. EGF receptor (EGFR) bands were immunodetected by using ECL reagents and revealed by film expose.

retinoic acid was not dramatic. However, when we compared this ratio of nascent ODC mRNA transcripts to that of GAPDH mRNA transcripts with data from two additional experiments, retinoic acid reproducibly decreased ODC gene expression by 17–28% from control levels. Thus, under quiescent conditions retinoic acid modestly decreases ODC gene expression at the transcriptional level. In EGF-stimulated cultures, a greater decrease in ODC gene transcription by retinoic acid was observed, which may be related to interference with EGF signalling mechanisms.

Retinoic acid decreases the binding and amount of EGF receptor

The biological activity of EGF is mediated by binding to a specific cell-surface receptor. We extended our investigation to study the effect of retinoic acid on EGF binding to these cells, to determine whether this process might explain the inhibitory effect of retinoic acid on EGF induction of ODC expression. When Z114 keratinocytes were treated with 1 nM–10 μM retinoic acid for 24 h, a significant decrease in EGF binding was observed at ≥ 10 nM retinoic acid (Figure 2a). The decrease in EGF binding was dose-dependent.

When cells were treated with 10 μM retinoic acid for up to 24 h, a significant decrease in EGF binding was observed after 8 h (Figure 2b). At 8 h a 23% decrease in EGF binding was apparent; additional exposure to retinoic acid (up to 24 h) resulted in a significant further decrease in EGF binding (44%).

Saturation of ^{125}I -EGF binding to cells occurred at a lower EGF concentration when cells were pretreated with retinoic acid compared with control cultures (Figure 3a). Scatchard analysis revealed a 52% decrease in the number of available binding sites for EGF in the retinoic acid-treated cultures (Figure 3b). The treatment with retinoic acid, however, did not alter the rate of EGF internalization. With time, the decrease in cell-surface-bound EGF and the increase in cell-associated (internalized) EGF were parallel (results not shown). Since (1) the morphology of the cells treated with retinoic acid was unchanged, (2) the non-specific binding was in the same range (3–7%) in both retinoic acid-treated and control cells, and (3) the cell number was increased (from 3.0×10^5 /dish to 3.9×10^5 /dish) in cultures treated with retinoic acid for 24 h, which was similar to that in

Table 1 Blocking of EGF receptor with neutralizing antibody inhibits EGF-induced ODC activity

Cells were grown in complete medium and placed in basal medium for 24 h, then pretreated with retinoic acid, ethanol, neutralizing antibody for EGF receptor, or EGF, as follows: (A) ethanol (0.1%) for 24 h; (B) ethanol (0.1%) for 24 h with 50 ng/ml EGF for 4 h; (C) ethanol (0.1%) for 24 h, with incubation with neutralizing antibody (1:500) for 5 h and 50 ng/ml EGF for 4 h; (D) 1 μ M RA for 24 h. ODC activity was assayed as described in the Materials and methods section. Values are means $\pm$ S.D. ($n = 3$).

Sample	Treatment	ODC activity (pmol of CO ₂ /h per mg of protein)	t-Test result compared with (A)
A	Control	188 $\pm$ 46	
B	EGF (50 ng/ml)	1763 $\pm$ 306	$P < 0.001$
C	Antibody + EGF	196 $\pm$ 114	$P > 0.05$
D	1 μ M RA + EGF	492 $\pm$ 210	$P > 0.05$

Figure 5 Effects of retinoids on the induction of ODC activity by EGF

Cells were pretreated with different retinoids (5 μ M), including Ro15-0778, etretinate, etarotene, Ro13-7410, 13-*cis*-retinoic acid, Ro40-8757, acitretin and retinoic acid, or its vehicle, ethanol (0.05%), for 24 h, and harvested 7 h after addition of 50 ng/ml EGF. ODC activity was assayed as described in the Materials and methods section.

the control, we concluded that 10 μ M retinoic acid is not cytotoxic to Z114 cells.

We determined the effect of retinoic acid on the quantity of EGF-receptor protein to find an explanation for the observed decrease in EGF binding after retinoic acid treatment. Cells were pretreated with 10 μ M, 1 μ M or 0.1 μ M retinoic acid or ethanol (0.1%) for 24 h. The EGF receptor was quantitatively detected by Western blotting. Laser-densitometry scanning of the 170 kDa EGF receptor band showed a dose-dependent decrease in EGF-receptor amount caused by retinoic acid (Figure 4). A 75%, 54% and 30% decrease in the EGF-receptor amount was detected in cultures treated with 10 μ M, 1 μ M or 0.1 μ M retinoic acid respectively.

To confirm the essential role of EGF receptor in the EGF induction of ODC expression, cells were incubated with a neutralizing antibody to EGF receptor for 5 h (at 1:500). The induction of ODC activity by 50 ng/ml EGF was significantly blocked (Table 1).

Table 2 Effects of retinoids on EGF binding to Z114 cells

Cells were pretreated with ethanol (0.05%) or different retinoids (5 μ M) dissolved in ethanol, including Ro15-0778, etretinate, etarotene, Ro13-7410, 13-*cis*-retinoic acid, Ro40-8757, acitretin and retinoic acid, for 24 h. The cells were incubated with basal medium (1 ml) containing 5×10^5 c.p.m. of ¹²⁵I-EGF at 4 °C for 3 h. The EGF binding assay was performed as described in the Materials and methods section: values are means $\pm$ S.D. ($n = 3$).

Sample	Treatment	EGF binding (c.p.m./10 ⁵ cells)	t-Test result compared with (A)	Inhibition of binding (%)
A	Ethanol	1952 $\pm$ 45		
B	Ro13-7410	1265 $\pm$ 42	$P < 0.001$	35
C	Etarotene	1823 $\pm$ 33	$P < 0.05$	7
D	Ro40-8757	1640 $\pm$ 146	$P < 0.05$	16
E	Ro15-0778	1853 $\pm$ 55	$P > 0.05$	5
F	Etretinate	1660 $\pm$ 39	$P < 0.05$	15
G	13- <i>cis</i> -Retinoic acid	1172 $\pm$ 49	$P < 0.001$	40
H	Acitretin	1712 $\pm$ 199	$P > 0.05$	12
I	Retinoic acid	1055 $\pm$ 96	$P < 0.001$	46

Differential effects of various retinoids on ODC activity and EGF binding

All the retinoids that we tested at a concentration of 5 μ M significantly inhibited the EGF induction of ODC in our assay system (Figure 5). Etretinate and Ro15-0778 were much less effective at inhibiting ODC induction as compared with retinoic acid, 13-*cis*-retinoic acid, Ro40-8757, etarotene and Ro13-7410.

Retinoids show specificity in their effect on EGF binding to human keratinocytes. Two other retinoids, 13-*cis*-retinoic acid and Ro13-7410, were similar to retinoic acid in their ability to decrease EGF binding significantly ($P < 0.001$) to cultured human keratinocytes (Table 2). Of the other retinoids tested, etarotene, Ro40-8757 and etretinate showed a significant ($P < 0.05$) but less impressive decrease in EGF binding. Two retinoids, Ro 15-0778 and acitretin, did not significantly reduce EGF binding ($P > 0.05$). Thus, some retinoids interfere with EGF signal transduction through alternative mechanisms.

Retinoic acid and UVB radiation show additive effects in decreasing EGF binding to human keratinocytes

When cells were treated with 1 μ M and 10 μ M retinoic acid for 24 h, EGF binding was decreased by 25% and 55% respectively. Irradiation with 20 mJ of UVB/cm² resulted in a decrease of EGF binding by 64%. However, when treatment with 1 μ M or 10 μ M retinoic acid was used in combination with 20 mJ/cm² UVB, a greater decrease in EGF binding was observed, i.e. 85% or 93% respectively (Figure 6).

DISCUSSION

Retinoic acid treatment of Z114 cells clearly blocked the EGF induction of ODC mRNA and decreased ODC mRNA levels in quiescent cells. Similarly, retinoic acid caused a decrease in the ODC gene transcription in either the absence or presence of EGF, as measured by nuclear run-on assay. Interestingly, all of the retinoids tested were active in decreasing EGF induction of ODC enzyme activity. Etretinate and Ro15-0778 were much less effective at inhibiting ODC induction as compared with retinoic acid, 13-*cis*-retinoic acid, Ro40-8757, etarotene and Ro13-7410.

Three retinoids (all-*trans*-retinoic acid, 13-*cis*-retinoic acid and Ro13-7410) decreased the binding of EGF to the cell receptors by a substantial amount (35–46%). A significant, but modest,

Figure 6 Retinoic acid in combination with UVB causes additive inhibition of EGF binding

Monolayers of Z114 cells in 35 mm dishes were treated as follows: (A) ethanol (0.1%) for 24 h + sham-irradiation (control); (B) ethanol (0.1%) for 24 h + 20 mJ/cm² UVB; (C) 1 μM retinoic acid (RA) for 24 h + sham-irradiation; (D) 10 μM RA for 24 h + sham-irradiation; (E) 1 μM RA for 24 h + 20 mJ/cm² UVB; (F) 10 μM RA for 24 h + 20 mJ/cm² UVB. EGF binding was assayed as described in the Materials and methods section.

decrease (7–16%) in EGF binding was noted for etarotene, etretinate and Ro40-8757. Two other retinoids showed no significant effect on EGF binding. The decrease in EGF binding resulting from retinoic acid treatment is likely to be due to a decrease in the number of available receptors, because (1) the EGF-binding affinity and rate of receptor internalization were relatively unaffected, and (2) the immunoblotting data revealed a decrease in EGF-receptor amount.

The results of our EGF-binding experiments were compared with our data obtained from treating the cells with UVB (290–320 nm) radiation [10]. We previously reported that UVB treatment resulted in an immediate (apparent by 15 min after irradiation) decrease in EGF binding, whereas the onset of action after retinoic acid treatment reported here is delayed until 8–24 h after treatment. Unlike retinoic acid, UVB did not alter the quantity of EGF receptors. Following UVB treatment of Z114 cells, we have reported tyrosine phosphorylation of the EGF receptors coincident with the decrease in EGF binding, suggesting that these two events are related [10]. Preliminary data suggest that retinoic acid does not cause tyrosine phosphorylation of the EGF receptors. When we explored the combined effects of UVB and retinoic acid treatment, using conditions for each treatment that maximally inhibit EGF binding, we found their effects to be additive, consistent with the concept of two different mechanisms operative in down-regulation of EGF binding to keratinocytes. This may partially explain the basis for the observed benefit for treating psoriasis with combined retinoid and phototherapies [18].

We found no evidence of retinoid-induced cytotoxicity by morphological examination or by cell-viability assays, using

0.1–10 μM retinoic acid in our cultures. Although these concentrations are possibly higher than endogenous levels under normal circumstances, the data have possible relevance to the pharmacological effects of topically applied retinoids or systemically administered retinoids *in vivo*. Whether the cellular influences represent exaggerated normal responses to retinoids and/or pharmacological metabolic pathways remains to be determined.

When the cells used in our studies were immortalized, they were selectively grown in medium containing 10 ng/ml EGF, so that this concentration of EGF may be considered 'physiological' for these cells. Of note, information concerning the physiological levels of EGF in human skin is limited; another model *in vitro* reported in the literature shows that 2–200 ng/ml EGF induces a biological response, i.e. induction of transforming growth factor-α mRNA, in cultured keratinocytes [19]. We have previously demonstrated that 50–100 ng/ml is saturating for the induction of ODC enzyme activity [2].

The effects of retinoic acid on EGF-receptor number or EGF binding have been demonstrated by others to be different, depending on the cell type under study. For example, in SV40-transformed murine fibroblasts, retinoic acid treatment induced formation of EGF receptors [20]. Retinoic acid has been found to induce EGF receptors in other cell types, such as rabbit chondrocytes and fetal-rat lung cells [21]. Nevertheless, transcriptional activity of the EGF-receptor promoter has been shown to be inhibited by thyroid hormone and retinoic acid receptors in a ligand-dependent mechanism, using several diverse cell lines [22]. Ponc and colleagues [23] reported that there was very little effect of several retinoids on EGF binding, and a clear stimulation by Ro15-1570 of EGF binding to squamous-cell carcinoma cells (SCC-12F2). Their conditions were different from ours, in that they incubated their cells with the retinoids for 7 days, in contrast with 24 h in our experiments, and the concentration for EGF binding in their experiments was only 3 ng/ml, which may not have represented saturating conditions. We found a differential effect between retinoids in our experiments, which may reflect the conditions we used and/or the fact that we used an SV40-immortalized keratinocyte cell line instead of a squamous-cell carcinoma cell line. Results obtained from rabbit corneal epithelial cells were found to show a decrease in EGF receptor binding following retinoic acid treatment for 2–4 days; the number of receptors was decreased by approximately two-thirds [24]. Other cell lines have shown similar results, including human epidermoid carcinoma ME180 cells [11] and human glioma cells [25]. In the ME180 cells, the quantity of EGF-receptor protein was decreased by retinoic acid treatment, suggesting that retinoic acid decreases synthesis of the receptor [26]. The observation that some retinoids alter EGF binding (and possibly EGF-receptor gene regulation), whereas others do not, may help in characterizing the mechanisms whereby retinoids control ODC gene transcription.

Retinoids that affect EGF-receptor binding as well as inhibit ODC activity may prove to be more biologically effective for treatment of certain skin disorders, e.g. acne or photoaging, since both retinoic acid and 13-*cis*-retinoic acid are effective in these disorders. For acitretin and etretinate, agents active in the treatment of psoriasis, etretinate significantly blocked EGF binding (15%; $P < 0.05$) and acitretin did not, whereas acitretin was more effective at blocking ODC activity than etretinate. Although acitretin is considered to be the active metabolite of etretinate, the hydrolysis of the etretinate ester may not take place effectively in keratinocytes; *in vivo*, the conversion of etretinate into acitretin presumably takes place normally in other tissues, e.g. the liver. Therefore, the results shown here for etretinate may not reflect its metabolism in keratinocytes when

the drug is administered orally, but may help to explain its lack of efficacy when used topically. Whether the assays that we have described will have predictive value for new retinoid agents will be investigated in future studies.

The mechanism of modulation of ODC expression by retinoids is likely to be complex. In addition to the effects on EGF signal transduction outlined in this report, retinoids may act through other pathways known to decrease ODC expression, e.g. via transglutaminase, inactivating ODC by linking it to putrescine [27] or via increased antizyme production [28]. However, there may be additional retinoid-specific modulating pathways. For example, in HeLa cells, the α -RAR caused ligand-dependent suppression of the ODC gene at the transcriptional level [3]. Further studies into the influence of retinoids on EGF signalling pathways may provide valuable insight into the control of keratinocyte proliferation.

This research was supported by NIAMS Grant 1K08AR01838, a fellowship award from the Cosmetic, Fragrance and Toiletry Association through the Dermatology Foundation, and a Research Grant from Lever Brothers through the Dermatology Foundation. J.H.P. also received support for this research as the Irving Assistant Professor of Dermatology. The advice of Dr. I. B. Weinstein is gratefully acknowledged. Stanley Shapiro, Ph.D., Hoffmann-LaRoche, Nutley, NJ, is acknowledged for discussions relevant to the retinoid derivatives.

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