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Effects of Retinoids on Differentiation, Lipid Metabolism, Epidermal Growth Factor, and Low-Density Lipoprotein Binding in Squamous Carcinoma Cells

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The relationship among keratinocyte differentiation capacity, lipid synthesis, low-density lipoprotein (LDL) metabolism, plasma membrane composition, and epidermal growth factor (EGF) binding has been studied in SCC-12F2 cells. The differentiation capacity of the cells, i.e., ionophore-induced cornified envelope formation, was inhibited by various retinoids and stimulated by hydrocortisone. Retinoids that caused a significant reduction of cornified envelope formation, i.e., retinoic acid and 13-*cis*-retinoic acid, caused only minor changes in lipid synthesis and plasma membrane composition. Arotinoid ethylsulfone, having a minor effect on cornified envelope formation, caused a drastic inhibition of cholesterol synthesis, resulting in changes in the plasma membrane composition. Hydrocortisone stimulated cornified envelope formation but had only minor effects on lipid synthesis and plasma membrane composition. Of all retinoids tested, only arotinoid ethylsulfone caused a drastic increase in EGF binding, while hydrocortisone had no effect. Retinoic acid, arotinoid ethylsulfone, and hydrocortisone had no effects on LDL binding and only minor effects on LDL degradation. These results clearly demonstrate that the plasma membrane composition is not related to keratinocyte differentiation capacity, but most likely does determine EGF binding. Furthermore, EGF binding does not determine keratinocyte differentiation capacity.

Differentiation of keratinocytes has been demonstrated to be accompanied by a number of modulations in processes related to the plasma membrane. Thus it has been demonstrated that the ability of normal keratinocytes to form cornified envelopes is inversely related to the expression of low-density lipoprotein (LDL) receptors and LDL metabolism [1, 2]. Both processes, i.e., cornified envelope formation and LDL metabolism, were dependent upon the extracellular Ca^{2+} concentration [3], an established regulator of keratinocyte differentiation capacity [4–6]. Moreover, this correlation was extended to a variety of transformed keratinocytes, which exhibited a differential defect in differentiation capacity [1]. Closely coupled to the differentiation-related modulation of LDL metabolism were the modulations of cholesterol synthesis that were also observed [1, 3, 7].

In addition to the extracellular Ca^{2+} concentration, epidermal growth factor (EGF) has been indicated as a major factor in the regulation of skin development [8, 9]. Recently, we have demonstrated an inverse relationship between EGF receptor expression and cornified envelope formation in normal and transformed keratinocytes [6], comparable to LDL receptor expression. These findings have

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led to the hypothesis that the plasma membrane plays a central role in keratinocyte differentiation capacity, and changes in its properties lead to a modulation in differentiation capacity on the one hand and to differences in receptor expression (for example, of EGF and LDL) on the other. In this context, a number of studies in which the plasma membrane composition was modified experimentally by cholesterol depletion have recently been performed [10]. Using well-established inhibitors of cholesterol synthesis, such as 25-hydroxycholesterol and mevinolin, it was demonstrated that both the cholesterol synthesis and cholesterol–phospholipid ratio of the plasma membrane were significantly decreased. In addition, these inhibitors were demonstrated to inhibit cornified envelope formation and to stimulate EGF binding. These data were fully in agreement with previous findings and support the hypothesis described above.

In order to obtain more insight into the interrelationships among keratinocyte differentiation, plasma membrane composition, lipid synthesis, and EGF binding characteristics, we have approached the system from another direction. According to the hypothesis, inhibitors of keratinocyte differentiation should lead to a decreased cholesterol–phospholipid ratio of the plasma membrane and increased EGF binding, while inducers of keratinocyte differentiation should have the opposite effect. An attractive approach is the study of the effects of retinoids and hydrocortisone on these parameters. Retinoids (vitamin A analogs) have been demonstrated to affect significantly the differentiation of stratified squamous epithelia [11–19]. Furthermore, vitamin A has been demonstrated to influence the program of terminal differentiation of normal and transformed keratinocytes by affecting the level of mRNA, specific for particular keratins [20–22]. Finally, retinoids have been demonstrated to modulate EGF binding characteristics in a variety of cell lines [23–25]. On the other hand, hydrocortisone has been proven to act as an efficient inducer of keratinocyte differentiation [18, 19, 26, 27].

In this paper, we have studied the effects of various retinoids and hydrocortisone on cornified envelope formation, lipid synthesis, plasma membrane composition, and LDL and EGF binding characteristics in SCC-12F2 cells. SCC-12F2 is an established cell line of a squamous cell carcinoma [28, 29] which to a large extent retained the differentiation characteristics of normal keratinocytes [1, 6].

MATERIALS AND METHODS

Cell culture. The human squamous carcinoma cell line SCC-12F2, originating from facial epidermis, was kindly provided by Dr. J. Rheinwald [28, 29]. The cells were cultured using the Rheinwald–Green feeder-layer technique [30]. The culture medium was a mixture of Dulbecco–Vogt and Ham's F12 medium (3/1) supplemented with 5% fetal calf serum (FCS) and 0.4 μg/ml hydrocortisone.

For the experiments 10,000 SCC-12F2 cells/cm^2 together with 3000 3T3 cells/cm^2 were inoculated in culture medium supplemented with 1% FCS. The cells were refed on Days 4, 7, and 10 under yellow light with medium supplemented with 0.5 μl/ml stock solution of retinoids. The stock solutions of retinoids in absolute ethanol were freshly prepared. The controls received 0.5 μl/ml ethanol. All experiments were performed 11 days after the initiation of the culture. For the experiments the following retinoids were used: all-*trans*-retinoic acid (RA), 13-*cis*-retinoic acid (Ro 4-3780), etretinate (Ro 10-9359), etretin (Ro 10-1670), arotinoid acid (Ro 13-7410), and arotinoid ethylsulfone (Ro 15-1570).

Cornified envelope formation. The capacity of cornified envelope formation was determined according to Rice and Green [31].

Incorporation of [^{14}C]acetate into lipids. The cells were cultured for 6 days in media supplemented with 1% FCS supplemented with retinoids, at final concentrations of 0–3 μ*M* as indicated or 1 μ*M* hydrocortisone. Subsequently the cells were reincubated for 20 h in medium containing the same additions to which [^{14}C]acetate (2.5 μCi/ml, 59 mCi/mmol; The Radiochemical Center, Amersham) at a final concentration of 0.5 m*M* was added. All incubations were performed at 37°C in humidified atmosphere containing 10% CO_2. Next, the medium was removed and the cells were trypsinized, washed three times with PBS, and split into three fractions. Two of these were extracted with a chloroform/methanol (1/2, v/v) mixture according to Bligh and Dyer [32]. Organic phases were dried under a stream of N_2 gas at 30°C. The third fraction was lysed in 1 *N* NaOH, after which an aliquot was taken for protein determination [33]. The lipids of one portion of the cell extracts were separated into different neutral lipid classes by one-dimensional TLC on silica gel plates (Merck) using chloroform/methanol (98/2) as the first and hexane/chloroform (3/1) as the second developing system. Development was performed in the presence of authentic standards (Sigma). The second portion of the cell extract was used for phospholipid separation using HPTLC plates (Merck) and a developing system of chloroform/methanol/acetic acid/H_2O (100/60/6/4), in the presence of authentic standards (Sigma). Lipid fractions were visualized by iodine vapor and after destaining were scraped off and counted for radioactivity using Picofluor (Packard) as scintillation fluid.

Plasma membrane isolation. The plasma membranes of cultured SCC-12F2 cells were isolated according to the method described by Schmidt *et al.* [34], using cationic silica beads (kindly provided by Dr. B. J. Jacobson, Department of Biochemistry, University of Massachusetts, Amherst, MA).

Lipid extraction and determination. Lipids were extracted from membrane fractions using the method of Bligh and Dyer [32]. Organic phases were dried under a stream of nitrogen and the residues were dissolved in a suitable volume of chloroform/methanol (2/1). Sterol content in an aliquot of total lipid extracts was determined after evaporation of organic phases using a commercially available Monotest cholesterin kit (Merck).

Phospholipid determination was performed after evaporation of organic solvents and after destruction with perchloric acid at 180°C as phosphorus according to Rouser *et al.* [35].

The phospholipids were separated into different fractions by TLC on HPTLC plates (Merck) using chloroform/methanol/acetic acid/water (50/30/3/2) as the development system. Development was performed in the presence of authentic standards (Sigma). The individual phospholipid fractions were scraped off and inorganic phosphorus was determined.

Cell association and degradation of ^{125}I-labeled LDL. The cells were cultured for 6 days in media supplemented with 1% FCS and with 2 μ*M* RA, 2 μ*M* Ro 15-1570, or 1 μ*M* hydrocortisone. Subsequently the cells were reincubated for 24 h in media containing lipoprotein-deficient serum (LPDS [7]) (to induce the expression of LDL receptor [36]) and the same additions as before. ^{125}I-labeled LDL was then added (10 μg/ml), and the cells were reincubated for 3 h at 37°C. For the assessment of nonspecific binding, 300 μg/ml unlabeled LDL was added to the medium containing ^{125}I-labeled LDL. The cellular association and degradation of ^{125}I-labeled LDL were measured essentially according to Goldstein and Brown [36].

EGF binding. ^{125}I-labeled EGF binding was measured as described in detail previously [37]. Scatchard analysis was performed using the LIGAND program, written and described by Munson and Rodbard [38].

RESULTS

Effects of Retinoids on Cornified Envelope Formation

Retinoids have been demonstrated to modulate differentiation of epidermal cells. In order to establish the effects of retinoids on the differentiation capacity of SCC-12F2 cells, the ability of the cells to form a cornified envelope beneath the cell membrane was measured in the presence of various retinoids. The cells were incubated for 7 days in the presence of various concentrations of RA, Ro 4-3780, Ro 10-9359, Ro 10-1670, Ro 13-7410, and Ro 15-1570. In addition, the effects of hydrocortisone, a well-known inducer of keratinocyte differentiation, on cornified envelope formation have been determined. As shown in Fig. 1, all retinoids used are able to suppress cornified envelope formation in a concentration-

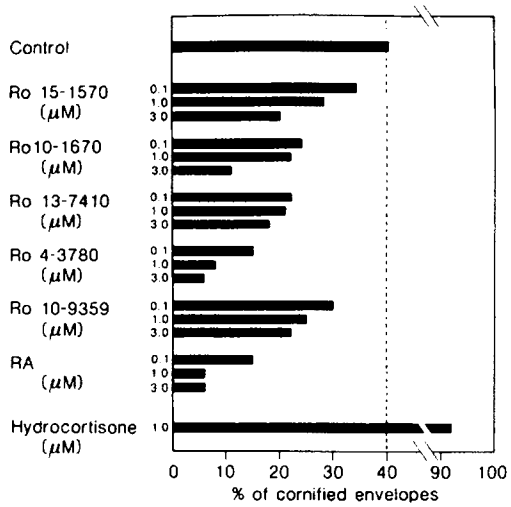

Fig. 1. Effect of retinoids and hydrocortisone on cornified envelope formation. The SCC-12F2 cells were incubated for 7 days in the presence of retinoids added at final concentrations of 0.1, 1.0, and 3.0 μ*M* or in the presence of 1 μ*M* hydrocortisone. Thereafter the cells were harvested by trypsinization and the competence of cells to form cornified envelopes was determined after a 4-h incubation of cells at 37°C in the presence of Ca^{2+} ionophore X-537A, as described in detail by Rice and Green [31].

dependent manner, while hydrocortisone causes an over twofold stimulation of cornified envelope formation. The ability of retinoids to suppress cornified envelopes follows the order RA >Ro 4-3780 >Ro 10-1670 >Ro 13-7410 >Ro 10-9359 >Ro 15-1570 (Fig. 1).

Effect of Retinoids on Lipid Synthesis

We have demonstrated recently that keratinocyte differentiation is accompanied by marked changes in lipid synthesis [M. Ponec *et al.*, in preparation] and that experimental modulations of the plasma membrane composition were accompanied by a modulated ability of the cells to form cornified envelopes [10]. Therefore we have studied here the effects of retinoids on lipid synthesis in SCC-12F2 cells. The retinoids tested were used at four different concentrations, i.e., 0.1, 0.5, 1.0 and 3.0 μ*M*, but for simplicity only the effects of the highest retinoid concentrations are presented in Table 1. At the lowest concentration (0.1 μ*M*) none of retinoids significantly influenced the rate of total lipid synthesis (data not shown). At a concentration of 3 μ*M* a high increase in the rate of total lipid synthesis is observed in the presence of Ro 10-9359 and a small stimulation in the presence of Ro 15-1570, Ro 10-1670, Ro 4-3780, and RA, while a small inhibition is observed in the presence of hydrocortisone (Table 1).

Analysis of [^{14}C]acetate incorporation into the various lipid classes demonstrates relatively small effects of the retinoids on the rate of phospholipid synthesis. A small stimulation is observed in the presence of Ro 15-1570 and Ro 10-9359 and a small inhibition in the presence of hydrocortisone.

The rate of cholesterol synthesis is significantly inhibited after exposure of the

TABLE 1

Effect of retinoids on lipid synthesis in SCC-12F2 cells

	[^{14}C]Acetate incorporation (dpm×10^{-3}/mg protein)				
	Total	Phospholipids	Cholesterol	Triglycerides	Cholesterol/ phospholipid
Control	980±27	390±18	75±2	480±4	0.19
Ro 15-1570	1161±20	587±3	20±2	539±16	0.03
Ro 10-1670	1189±11	439±2	73±1	668±8	0.17
Ro 13-7410	970±50	444±34	79±1	431±12	0.18
Ro 4-3780	1102±7	404±3	62±1	626±11	0.15
Ro 10-9359	1640±48	532±6	67±1	1026±39	0.13
RA	1047±18	396±1	56±2	588±17	0.14
Hydrocortisone	773±14	311±10	68±1	381±10	0.22

Note. SCC-12F2 cells were cultured to confluency and the rate of lipid synthesis was measured by [^{14}C]acetate incorporation into the various lipid fractions as described under Materials and Methods. The cells were exposed to the various retinoids for 7 days. The final concentration of all retinoids was 3 μ*M* and of hydrocortisone 1 μ*M*. Data are presented as means ±SE (N=6).

cells to Ro 15-1570, while only a small inhibitory effect or no effect at all is observed in the presence of the other retinoids or hydrocortisone (Table 1). The percentage of [^{14}C]acetate incorporated into free fatty acids and cholesterol esters was very low (about 2% of the total) and was only slightly affected by retinoids (data not shown). On the other hand, a high stimulation of triglyceride synthesis is measured after exposure of the cells to Ro 10-9359, and a relatively small stimulation in the presence of Ro 15-1570, Ro 10-1670, Ro 4-3780, and RA (Table 1). No effect on triglyceride syntesis is measured after exposure of the cells to Ro 13-7480, and a small inhibition is observed in the presence of hydrocortisone (Table 1).

The differential effects of the various retinoids on lipid synthesis result in a differential effect on the cholesterol–phospholipid ratio obtained from these data. As shown in Table 2 only Ro 15-1570 causes a large decrease in the value of the cholesterol–phospholipid ratio, while Ro 10-9359, RA, and Ro 4-3780 have only a small effect. Finally hydrocortisone causes a small increase in the cholesterol–phospholipid ratio (Table 2). These results clearly demonstrate that the inhibition of differentiation by some retinoids is accompanied by changes in lipid synthesis, which ultimately result in a decreased cholesterol-phospholipid ratio.

Effect of Retinoids on Cell Membrane Composition

As has been outlined in detail in previous studies [1, 2, 7] the rate of lipid synthesis cannot be translated simply to changes in the composition of the plasma membrane, since cholesterol synthesis in particular has been demonstrated to be dependent upon the presence of external lipoproteins and upon the expression of LDL receptors and the rate of LDL metabolism. In order to relate keratinocyte differentiation with the plasma membrane composition, plasma membranes from

TABLE 2

Effects of Ro 15-1570, RA, and hydrocortisone on phospholipid composition of purified plasma membranes of SCC-12F2 cells

	Percentage of total phospholipids			
	Control	Ro 15-1570	RA	Hydro-cortisone
Sphingomyelin	29	29	32	30
Phosphatidylcholine	21	23	20	22
Phosphatidylserine	15	10	12	14
Phosphatidylinositol	4	6	5	4
Phosphatidylethanolamine	29	30	30	29

Note. The cells were exposed to Ro 15-1570 (3 μ*M*), Ra (3 μ*M*), or hydrocortisone (1 μ*M*) for 7 days. Subsequently plasma membranes were purified and phospholipid composition was determined as described under Materials and Methods. Data are presented as percentages of total phospholipids.

SCC-12F2 cells exposed to RA, Ro 15-1570, and hydrocortisone were isolated, which caused a strong inhibition, a small inhibition, and a strong stimulation, respectively, in the ability of the cells to form cornified envelopes (Fig. 1). Subsequently the lipid composition of these isolated plasma membranes was determined and the cholesterol–phospholipid ratio calculated. As shown in Fig. 2, only Ro 15-1570 causes a reduction in the cholesterol–phospholipid ratio, while both RA and hydrocortisone have no effect whatsoever. Furthermore, analysis of the phospholipid composition of isolated membranes reveals that none of the compounds tested causes a significant modulation in phospholipid composition (Table 2).

Effects of Retinoids on EGF Binding

In previous studies, we have demonstrated a correlation between EGF binding and differentiation capacity in a variety of normal and transformed keratinocytes [6]. Furthermore, an experimental modification of the plasma membrane composition was demonstrated to result in a modulated EGF binding [10]. Therefore we have studied here the effects of the various retinoids on EGF binding in SCC-12F2 cells. The cells were treated for 7 days with various concentrations of retinoids and EGF binding was subsequently determined using an extracellular EGF concentration of 3 ng/ml. As shown in Fig. 3, of all the retinoids tested, only Ro 15-1570 has a clear stimulating effect on EGF binding, while only a small stimulation is found in the presence of high concentrations of Ro 10-1670, Ro 4-3780, and RA. Hydrocortisone does not affect EGF binding in SCC-12F2 cells (Fig. 3). Scatchard analysis of EGF binding revealed that the increase in binding, as shown in Fig. 3, is due to an increased number of EGF receptors.

Effect of Retinoids on LDL Binding and Metabolism

Since our previous studies revealed a similarity in the pattern of differentiation dependency of EGF and LDL receptor expression [3, 6], the effects of RA, Ro

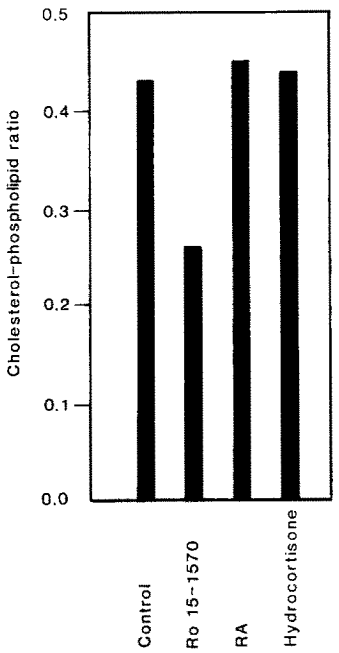

Fig. 2. Effect of Ro 15-1570, RA, and hydrocortisone on cell membrane cholesterol–phospholipid ratio. The cells were exposed to Ro 15-1570 (2 μ*M*), RA (2 μ*M*), and hydrocortisone (1 μ*M*) for 7 days. Subsequently the cholesterol and phospholipid contents of purified plasma membranes were measured, as described under Materials and Methods.

15-1570, and hydrocortisone have been studied on LDL binding and LDL degradation. As shown in Fig. 4, binding of LDL to SCC-12F2 cells is not affected by any of the compounds used. The degradation of LDL is slightly increased after exposure of the cells to RA and decreased in the presence of Ro 15-1570. In addition, hydrocortisone has no significant effect on LDL binding and degradation (Fig. 4).

DISCUSSION

Keratinocyte differentiation has been demonstrated to be accompanied by large modulations in cholesterol biosynthesis, LDL metabolism, as well as in expression of EGF receptors [1–3, 6]. These results suggested a strong relationship among differentiation, plasma membrane composition, and EGF receptor expression, comparable to other cell systems [39–41]. Support for this relationship in keratinocytes has been obtained recently in studies in which cholesterol synthesis was inhibited [10]. This treatment resulted in a decreased cholesterol–phospholipid ratio in plasma membranes, a decreased cornified envelope formation, and increased EGF binding.

In this paper we have measured the effects of established modulators of keratinocyte differentiation on plasma membrane composition, lipid synthesis,

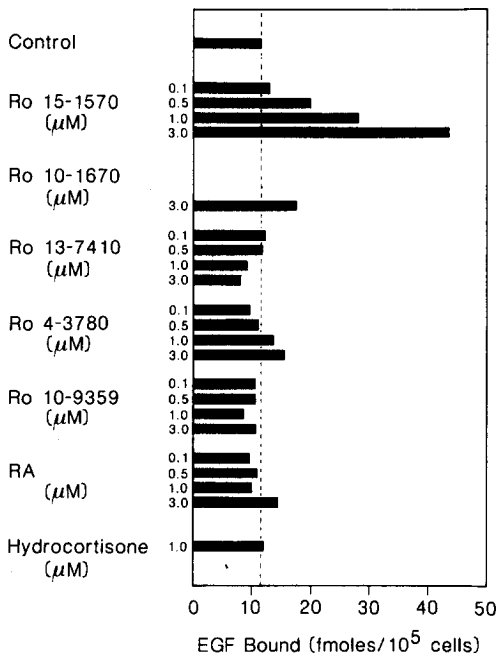

Fig. 3. Effect of retinoids and hydrocortisone on ^{125}I-labeled EGF binding. The cells were exposed for 7 days to various retinoids added at final concentrations of 0.1, 0.5, 1.0, and 3 μ*M* or to 1.0 μ*M* hydrocortisone. Subsequently the ^{125}I-labeled EGF binding at an extracellular concentration of 3 ng/ml was measured as described under Materials and Methods.

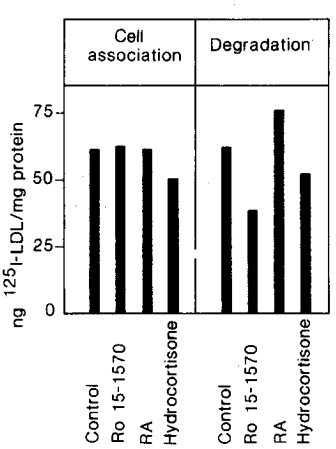

Fig. 4. Effect of Ro 15-1570, RA, and hydrocortisone on ^{125}I-labeled LDL binding. The cells were incubated for 6 days in the presence of RA (2 μ*M*), Ro 15-1570 (2 μ*M*), and hydrocortisone (1 μ*M*). Subsequently the medium was renewed by medium supplemented with 5% LPDS and the same additives as used before, and the cells were incubated for the next 24 h. The binding of ^{125}I-labeled LDL added at 10 μg/ml and in the presence or absence of the excess of nonlabeled LDL was measured, as described under Materials and Methods. In this figure the specific cell association and degradation of ^{125}I-labeled LDL are shown.

LDL metabolism, and EGF binding. These modulators include a variety of retinoids, which are able to inhibit differentiation [11–19], and hydrocortisone, which is known to stimulate differentiation [18, 19, 26, 27]. It is shown here that retinoic acid and 13-*cis*-retinoic acid are most effective in inhibition of the capacity of the cells to form cornified envelopes, while arotinoid ethylsulfone had only a small effect. Hydrocortisone caused a significant increase in the ability of SCC-12F2 cells to form cornified envelopes. These results are in accordance with recent observations [14, 42–44] demonstrating that the effects of retinoids and hydrocortisone on the capacity of keratinocytes to form cornified envelopes are due to a modulation of involucrin content and membrane-bound transglutaminase activity. According to the hypothesized relationship between the plasma membrane composition and the capacity of cells to form cornified envelopes, it was expected that RA and Ro 4-3780 would cause a significant reduction in the cholesterol–phospholipid ratio of purified plasma membranes, while hydrocortisone would cause a significant increase. However, it was found that Ro 15-1570, not RA and Ro 4-3780, caused a clear reduction of cholesterol synthesis and consequently a decrease in the cholesterol–phospholipid ratio of purified plasma membranes. Furthermore, hydrocortisone was shown not to affect lipid synthesis and plasma membrane composition. These results clearly demonstrate that the capacity of SCC-12F2 cells to form cornified envelopes is not related to the plasma membrane lipid composition.

In addition, the effects of retinoids and hydrocortisone on EGF binding were determined. These results demonstrated that only Ro 15-1570 had a clear stimulatory effect upon EGF binding, while almost no effect was observed in the presence of RA and hydrocortisone.

Our previous studies [1, 3, 6] have demonstrated a correlation between LDL and EGF receptor expression in relation to keratinocyte differentiation capacity. The results presented here show that in contrast to EGF the binding of LDL was not significantly affected by any of the compounds used. This suggests that the mechanisms involved in the regulation of LDL receptor expression are different from those involved in EGF receptor expression.

The results presented here suggest that retinoid-induced modulation of EGF binding is not related to the capacity of SCC-12F2 cells to form cornified envelopes, but is associated with the plasma membrane composition.

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