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Hydrocortisone is a potent antagonist of retinyl acetate but not retinoic acid

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Abstract. Growth of SCC-13 squamous carcinoma cultures in the presence of retinoids considerably reduced the expression of two differentiation markers, the cellular capability to form cross-linked envelopes, and the enzyme transglutaminase required for cross-linking. A limited survey of retinoids showed that all-trans retinoic acid, 13-cis retinoic acid, and arotenoid Ro 13-6298 were highly effective in the absence of hydrocortisone and were only slightly antagonized by its presence in the medium. In contrast, retinyl acetate, retinol, and retinol bound to its plasma binding protein were quite active in the absence of hydrocortisone but were essentially inactive in its presence. Dexamethasone was also highly effective in antagonizing the suppressive action of retinyl acetate on envelope formation, while the corticosteroid antagonists cortexolone and progesterone were inactive. These results suggest that there are separate pathways, which are differentially regulated by hydrocortisone, for either the metabolism or action of retinol and retinoic acid in SCC-13 cells.

Introduction

Vitamin A (retinol) and its analogs (retinoids) have significant effects on the differentiation of stratified squamous epithelia. Keratinization is arrested and mucous metaplasia occurs when chick embryonic skin is exposed to retinoids [9, 23]. Similarly, mammalian epithelia which have undergone squamous metaplasia during vitamin-A deficiency revert to a normal mucus-secreting or ciliated form during refeeding of the vitamin [17, 18]. Retinoids also alter the terminal differentiation of postnatal mammalian epidermis but do not fundamentally change its character: for example, the formation of human stratum corneum is disordered and desmosomes are reduced in number, but mucous metaplasia does not take place [7, 16, 33]. A coherent picture of vitamin-A action at the molecular level, however, is lacking in these systems.

In cell culture, vitamin A dramatically suppresses the formation of the cross-linked envelope, a specific feature of human keratinocyte terminal differentiation [10, 12]. The cross-linked envelope is a sodium dodecyl sulfate (SDS)-insoluble layer of protein formed beneath the cell membrane as a result of transglutaminase-catalyzed formation of ϵ-(γ-glutamyl)-lysine cross-links between glutamine and lysine residues of precursor proteins in the keratinocyte [27, 30]. Normal human keratinocytes, when cultured in medium containing fetal bovine serum depleted of vitamin A by solvent extraction, accumulate a substantial layer of superficial squames (containing cross-linked envelopes and abundant keratin) which comprise up to 40% of the total cells in highly confluent cultures. The addition of retinoids or the use of untreated serum in place of solvent-extracted serum reduces the number of spontaneously formed squames adhering to the surface of the culture by as much as 100-fold [10, 12, 20]. The extent of the effect depends on the epithelium of origin, with cultured conjunctival cells being the most sensitive to the retinoids tested, while epidermal cells are the least sensitive [10, 12].

Normal human keratinocytes in culture can be induced to form SDS-insoluble cross-linked envelopes by permeabilization to calcium ion, which is a required co-factor for cell transglutaminase [27]. This cross-linking is completed within a few hours and reflects the level to which the cells have already synthesized precursors required for envelope formation. Although retinoids reduce spontaneous envelope formation, they have little effect on envelope competence, i.e., the percentage of cells capable of forming envelopes following this treatment [4, 12]. In contrast, the squamous cell carcinoma line SCC-13, derived from human epidermis [26], forms spontaneous envelopes poorly in confluent culture but has a high degree of envelope competence, which is suppressed by growth in retinyl-acetate-containing medium [4, 28]. Hydrocortisone enhances envelope competence and also antagonizes the action of retinyl acetate [4]. In the present study, solvent extracted fetal bovine serum was used for cell culture so that the corticosteroid antagonism of vitamin A could be studied more carefully. Under these conditions, the suppressive effect of retinyl acetate on envelope competence is completely blocked by the inclusion of hydrocortisone [28]. We also show that retinoic acid greatly suppresses envelope competence, whereas hydrocortisone antagonizes this action to a far lesser extent. Moreover, the expression of transglutaminase, a key participant in envelope formation, is suppressed by the two different retinoids in parallel with envelope competence.

Methods

Materials. Retinyl acetate was obtained from Sigma (St. Louis, Mo), and all-trans retinoic acid was purchased from
Calbiochem (LaJolla, Calif); etretinate (Ro 10-9359), TMMP (the 4-methoxy-2,3,6-trimethylphenyl analog of retinoic acid), and the arylotin acid, were obtained by courtesy of Dr. W.E. Scott (Hoffman-LaRoche, Nutley, NJ). The nonionic detergent Emulgen 911 (Kao-Atlas, Tokyo) was a generous gift from Dr. John Dent (CIIT, Research Triangle Park, NC).

Serum retinol-binding protein (RBP) in a complex with transthretilin (TTR), formerly named prealbumin [11], was partially purified from human plasma using a modification of published methods [14], as kindly suggested by Dr. Paul Fitzgerald (Dept. of Anatomy, Harvard University). Briefly, the citrated platelet-poor plasma (0.5:1) was dialyzed for several days against 0.1 M sodium acetate (pH 5.6) and applied to diethylaminoethyl (DEAE)-Sephadex A-50 (Pharmacia, Piscataway, NJ), which was then rinsed with 0.3 M sodium acetate (pH 5.4) and eluted with 2.0 M sodium acetate buffer (pH 5.1). After concentration and dialysis, the eluate was chromatographed on DEAE-Sephadex A-50 using a NaCl gradient (0.2–0.4 M) in 20 mM Tris (pH 7.2). Concentrated peak fractions were pooled and applied to Sephadex G-100 in 20 mM Tris-Cl (pH 7.2) 0.15 M NaCl. Peak material with an optical density (OD) 280/330 ratio of 2.7 and an estimated 0.9 mg RBP was concentrated, dialyzed against isotonic phosphate-buffered saline, sterile filtered, and added directly to SCC-13 cultures.

Cell culture. Fetal bovine serum was depeopidized by solvent extraction [29] which entirely extracts retinol, as determined by reverse-phase HPLC of serum extracts on a C18Bondapak column (Waters Instruments, Milford, Mass [2]). SCC-13 cells grown with 3T3-feeder-layer support were inoculated at a density of approximately 2 × 10^4 cells per 60-mm dish in Dulbecco-Vogt Eagle’s medium supplemented with solvent-extracted serum (5%), and then fed every 2–3 days with medium containing this treated serum (2%). Corticosteroids and retinoids (solvents, ethanol and dimethylsulfoxide, respectively, at ≤0.1% in treated and control cultures) were added starting 3–5 days before the cells reached confluence. The total cell number, transglutaminase activity, and envelope competence were measured 5–10 days after confluence. A typical treatment protocol is shown below.

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>Confluence</th>
<th>Assay</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Retinoid/corticosteroid treatment</td>
<td></td>
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</tr>
</tbody>
</table>

The results obtained for competence were the same as when the retinoid/corticosteroid treatment was started 1 day after inoculation [28].

Envelope competence. To measure their capability of forming envelopes upon ionophore treatment, the cells were disaggregated with trypsin-EDTA, recovered by centrifugation, and resuspended at a density of 8 × 10^5 cells/ml in serum-free medium containing X537A (50 µg/ml), thereby permeabilizing them to calcium ion. After incubation for 2–3 h at 37°C, the medium was adjusted to 2% in SDS and 20 mM in dithioerythritol. Envelopes were scored by phase-contrast microscopy after at least 20 min at room temperature [4].

Transglutaminase assays. SCC-13 cultures were washed twice in 10 mM Na₂HPO₄ (pH 7.2) 0.15 M NaCl 0.5 mM EDTA before they were scraped from the dish into 2 mM Hepes (pH 7.4) 2 mM EDTA. Cells were broken open by either Dounce homogenization or bath sonication (E/MC-250 ultrasonic cleaner) in borosilicate tubes (12 × 75 mm); both methods of disruption gave equivalent results. The cell particulates were then separated from the supernatant by a 45-min centrifugation at 100,000 g. The total cell particulates from a single 6-cm dish were generally taken up in 2 ml 2 mM Hepes (pH 7.4) 2 mM EDTA 2 mM dithioerythritol 0.1% Emulgen 911 and then dispersed by sonication or homogenization prior to assay. In some experiments, the protease inhibitors antipain and leupeptin (Sigma) were included (10 µg/ml each), beginning at the cell-disruption step, without affecting transglutaminase activity.

Transglutaminase was assayed by the incorporation of ³H-purinose (40 µM, 50 Ci/mmol) into 2 mg/ml reductively methylated casein [19] in 100 mM Tris-Cl (pH 8.3) 2 mM CaCl₂ 2 mM dithioerythritol Emulgen 911 (0% to 1%). Reactions were incubated at 34.5°C for 20 min and quenched with 4 vol. 12.5% trichloroacetic acid (TCA). Assays were always checked for linearity with time and the amount of added cell homogenate. The acid precipitates were washed on Whatman GF/A glass-fiber filters in 5% TCA followed by 95% ethanol, dried, and scintillation counted. Enzyme activities (usually triplicate determinations) were normalized to protein content [3] in the particulate or soluble fractions assayed, and presented where appropriate as the mean ± standard error.

Results

Regulation of envelope competence

Initial experiments examined the envelope competence (percentage of cells forming cross-linked envelopes upon permeabilization to calcium ion) in SCC-13 cultures 5–10 days after confluence. When retinoids (without hydrocortisone) were added to the medium starting 3–5 days prior to confluence, the measured competence values were greatly suppressed. As shown in Fig. 1, envelope competence was progressively reduced from approximately 40% to 10% as the retinyl-acetate concentration was increased to 0.3 µM, above which the retinoids inhibited the cell growth rate. Retinoc acid was more powerful over this concentration range, reducing competence to 2% at 0.3 µM. When the medium contained hydrocortisone (1 µM) added simultaneously with these retinoids, the contrast in their activity was accentuated. Thus, retinyl acetate was totally ineffective, while the action of retinoic acid was only slightly antagonized, exhibiting a severalfold decrease in potency (Fig. 1).

In these experiments, the expanding colonies of treated and control cultures merged into confluent sheets within about 1 day of each other. In retinoic acid, where the cells appeared flatter than in controls, the density at the time of data collection was approximately 60% at 3 nM and 40% at 0.3 µM (or slightly higher in the presence of hydrocortisone) compared to that of controls. In contrast, cell densities appeared to be unaffected by retinyl acetate. This indicates that the measured effects of retinoic acid on competence were unlikely to be due to the decrease in cell density. Earlier work [4] has shown that when preconfluent SCC-13 cells (which have low competence) are growth-ar
Fig. 1. Effect of retinyl acetate and retinoic acid on envelope competence. Cells were grown in the indicated concentrations of retinyl acetate or retinoic acid with (●) or without (○) added hydrocortisone (10^{-6} M).

Fig. 2. Effect of various retinoids and the retinol-binding-protein transthyretin (RBP-TTR) complex on envelope competence in the presence (●) or absence (○) of hydrocortisone (10^{-6} M).

Fig. 3. Envelope competence in cultures grown in the presence of 3 × 10^{-7} M retinyl acetate and the indicated concentrations of dexamethasone (●), hydrocortisone (●), progesterone (○), or corticosterone (△). Measurements were made 5 days after the cultures had reached confluence. Values presented are averages of two independent experiments, except for 10^{-6} M hydrocortisone (six experiments).

RBP-TTR or retinyl acetate, i.e., from about 10 to 300 nM, could not cause a half-maximal reduction in envelope competence. The same increase was more than adequate to reverse the hydrocortisone effect in the case of all-trans and 13-cis retinoic acids and the arotinoid, although the concentration of retinoid required to reduce envelope competence by half appeared to be slightly greater in the presence of the steroid. This comparison clearly illustrates the dichotomy in the effectiveness of hydrocortisone toward the two groups of retinoids.

Figures 1 and 2 show that hydrocortisone increased envelope competence as much as twofold in the absence of added retinoid. These experiments were performed using a single lot of serum, from which several aliquots were solvent extracted. With two other lots of delipidized serum, the hydrocortisone stimulation of competence in the absence of added retinoid was similar in magnitude to that illustrated; however, hydrocortisone antagonism of retinoid action was not studied in detail. The development of defined (serum-free) growth media in which complete antagonism of the retinyl-acetate effect by hydrocortisone is observed could prove useful for further work.

Etretinate did not appear to be active in this system. Even at a concentration of 0.3 μM used in several experiments, the average decrease in competence from 55% to 50% or from 34% to 31% (in the presence or absence of hydrocortisone, respectively) was not statistically significant. In one experiment, the unesterified derivative of etretinate (TMMP) also showed little activity.

Figure 3 compares the relative ability of several steroids to prevent the suppression of envelope competence by retinyl acetate (0.3 μM). Dexamethasone was highly effective, producing, at 0.1–1.0 μM, a maximal response of approximately 80% competence compared to 10% in the absence of the steroid. The glucocorticoid antagonists corticosterone and progesterone, in contrast, were inactive at concentrations of up to 1 μM. At this elevated concentration, hydrocortisone was comparable to dexamethasone, but the latter...
Fig. 4. Particulate transglutaminase activity in cultures as a function of retinoid concentration in the presence (●) or absence (○) of hydrocortisone (10⁻⁶ M).

Fig. 5. Soluble (100,000-g supernatant) transglutaminase activities of SCC-13 cells treated as in Fig. 4. Hydrocortisone (10⁻⁶ M) or retinoids (3 x 10⁻⁷ M) were present during cell culture as indicated.

The specific activity of the particulate enzyme was reduced at least 20-fold in cultures treated with the highest concentration (0.3 μM) of either retinoid, although retinoic acid was slightly more potent than retinyl acetate. When hydrocortisone (1 μM) was in the growth medium, however, retinyl acetate was virtually without effect, while the potency of retinoic acid was reduced only slightly.

Transglutaminase activity in cell extracts was much greater in cultures after reaching confluence than during the rapid growth prior to confluence. As shown in Table 1, the specific activity of the particulate enzyme increased several fold during the transition of cultures from the preconfluent to the postconfluent state. This coupling of increased expression to confluence is in the same direction as that observed previously for envelope competence and involucrin content [4]. Hydrocortisone in the medium had a relatively small stimulatory effect on the high level of activity reached at confluence. When the growth medium was supplemented with charcoal-treated [1] instead of solvent-extracted (delipidized) fetal bovine serum, the effect of hydrocortisone was more striking. Transglutaminase activity was stimulated by a factor of 4.2 ± 0.6 (n = 4) in cultures grown with charcoal-treated serum, and by a factor of 1.4 ± 0.2 (n = 6) in those grown with solvent-extracted serum. In the presence of hydrocortisone, the high levels of enzyme expressed were essentially the same with either serum supplementation (Table 1). The present results are compatible with the differing influence of the two serum supplementations on envelope competence observed in a pilot study [28], presumably due to removal of retinol from the serum by solvent extraction but not by charcoal treatment [4]. Since human RBP, whose structure and function are highly conserved among mammals [25], suppresses envelope competence (Fig. 2), it is reasonable to assume that the RBP of fetal calf serum, which is apparently identical to adult bovine RBP [13], acts in the same way and can suppress transglutaminase activity. A stimulatory effect of hydrocortisone on the transglutaminase activity of cultured embryonic chick skin has also been described [22]; since a serum-free medium was used, however, it cannot be assumed that the hydrocortisone action was due to antagonism of vitamin A in this case [31].

The duration of retinoic acid exposure required for transglutaminase suppression is shown in Fig. 6. Parallel SCC-13 cultures were treated continuously with retinoic acid (0.06 μM), starting on different days before or after the cells reached confluence, and were then harvested simultaneously for assay of enzyme activity. Treatment of confluent cultures for up to 2 days had little effect on the enzyme activity. (The increase in activity after 1 day of

Table 1. Influence of confluence and hydrocortisone on transglutaminase activity.

<table>
<thead>
<tr>
<th>Hydrocortisone (1 μM)</th>
<th>Solvent-extracted serum</th>
<th>Charcoal-treated serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preconfluent</td>
<td>Postconfluent</td>
<td>Preconfluent</td>
</tr>
<tr>
<td>-</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>+</td>
<td>6</td>
<td>27</td>
</tr>
</tbody>
</table>

*Units of nanomoles putrescine per milligram per hour. A representative experiment using parallel cultures; the pre- and postconfluent cultures were harvested 14 days apart.

Regulation of transglutaminase

SCC-13 cells contain two forms of transglutaminase, one extractable by detergent from cell particulates, and the other cytosolic. According to immunochemical and physical criteria [32], the two are closely related. The addition of all-trans retinoic acid or retinyl acetate to the growth medium in the absence of hydrocortisone greatly suppressed the levels of both transglutaminases in confluent cultures. Figure 4 illustrates the effect on the particulate form of the enzyme, which accounts for approximately 90% of the total activity in SCC-13 cells [32], and Fig. 5 shows phenomena of similar magnitude for the cytosolic form of the enzyme.
A central finding is that hydrocortisone is a far more potent antagonist of retinyl acetate than of retinoic acid. Earlier workers who studied embryonic chick skin mucous metaplasia by vitamin A and its antagonism by hydrocortisone [9] did not look for or observe this disparity. Retinyl acetate is very likely hydrolyzed to retinol in cell culture. That retinyl acetate and retinol have similar effects on SCC-13 cell envelope competence rules out the possibility that hydrocortisone specifically blocks retinyl-acetate action by blocking its conversion to retinol. Hydrocortisone may affect a pathway which is required for retinol action but is not essential for retinoic acid action under the conditions employed here. It is apparent that hydrocortisone can act in the absence of retinoids to increase envelope competence, consistent with its action to increase transglutaminase activity.

The relationship between retinol and retinoic acid action is unclear. For example, it is not certain whether retinol itself has a special function in the reversal of squamous metaplasia, or whether it must first be metabolized to retinoic acid. Dietary retinol is absolutely required for the visual and reproductive function in rats, and vitamin-A-deprived rats fed retinoic acid are both blind and sterile, but grow normally and are otherwise quite healthy [11]. This has led to the hypothesis that retinoic acid is the biologically active form of vitamin A required for reversal of squamous metaplasia and maintenance of normal mucus-secreting epithelia. Future experiments should address the question of whether hydrocortisone blocks the conversion of retinol to retinoic acid (or some other common active form), or whether it blocks a separate pathway available to retinol but not retinoic acid. Hydrocortisone-induced specific destruction of retinol but not retinoic acid has not been excluded by these experiments, however, nor has an effect of retinoic acid on hydrocortisone utilization specifically been ruled out.

The present study also demonstrates a novel retinoid-dependent suppression of keratinocyte transglutaminase. In contrast, retinoid exposure of skin or cultured keratinocytes of the mouse induces transglutaminase activity [5, 34], and retinoic acid induces tissue transglutaminase in mouse peritoneal macrophages [21]. These findings could reflect differences in species, in the type of cell used (malignant or normal), or in the type of transglutaminase studied. Both the particulate and cytosolic enzymes of SCC-13 cultures occur in normal human epidermal cells and appear to be keratinocyte specific. A set of three monoclonal antibodies which immunoprecipitates the two forms has also been used to demonstrate immunoreactivity in the most differentiated strata of human epidermis but not in the basal or lower spinous layers or the dermis [32]. In addition to these two enzymes, normal human keratinocytes in culture contain an additional, soluble transglutaminase with distinct immunologic and physical properties which could account for the retinoid-stimulated activity observed in other systems [32].

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Fig. 6. Particulate transglutaminase activity in confluent SCC-13 cultures as a function of days of exposure to retinoic acid. Retinoic acid (6 × 10⁻⁸ M) was added to the medium of parallel cultures starting 1 to 9 days prior to harvest and at each feeding thereafter (day 0 cells were not exposed to retinoic acid). The first group treated with retinoic acid (9 days prior to harvest) was in a preconfluent state. Cultures were harvested simultaneously about 5 days after reaching confluence.

Exposure was not statistically significant.) Treatment starting when the cells were in a preconfluent state (9 days prior to harvest) prevented the large increase in activity normally occurring in postconfluent cultures. Comparison with the data in Table 1, which show that preconfluent cells have low transglutaminase activity, suggests that the primary effect of retinoic acid is to inhibit transglutaminase synthesis, and that, once synthesized, the enzyme is relatively stable and insensitive to the action of the retinoid.

Discussion

The present results provide further insight into retinoid suppression of differentiation in SCC-13 cells and suggest new avenues for research into the mechanisms of retinoid action. Simultaneous reduction in the levels of both involucrin [4] and transglutaminase readily accounts for the observed lowering of envelope competence by retinoids, although reduction of other envelope components [30] may occur as well. Purification of these participants in keratinocyte envelope formation [27, 30, 32] now makes possible the investigation of vitamin-A action on their synthesis or breakdown, as in current studies of vitamin-A-dependent expression of keratins in this cell type [6, 15].

Normal keratinocytes cultured from several human epithelia, including epidermis, show little or no effect of retinoids on envelope competence [12], but significant suppression of spontaneous envelope formation can occur, depending upon the tissue of origin. The conjunctival keratinocyte, which is sensitive to retinyl acetate even in the presence of hydrocortisone, provides a promising bioassay for retinoid efficacy [10]. The relative activity of various retinoids in the conjunctival cell is rather similar to that in SCC-13 cultures, but in the latter, these agents directly affect envelope competence. This curious dichotomy, a reflection of regulatory aberrations that can occur in neoplastic cells, may prove useful in studying elements of the keratinocyte differentiation program as well as mechanisms of retinoid action.
References

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Note added in proof
Recent results with mouse epidermal cell cultures [Lichti U, Ben T, Yuspa SH (1985) J Biol Chem 260:1422–1426] and several other human squamous carcinoma cell lines [Rubin AL, Rice RH, in preparation] demonstrate that, indeed, the form of transglutaminase whose expression is stimulated by retinoids is distinct from the form suppressed by retinoids in SCC-13 cells in the present study.