Modulation of Involucrin and Envelope Competence in Human Keratinocytes by Hydrocortisone, Retinyl Acetate, and Growth Arrest

Polly R. Cline and Robert H. Rice

ABSTRACT

Involucrin accumulation and ionophore-assisted envelope formation, markers of keratinocyte differentiation, were found to be highly dependent on culture conditions in the malignant epidermal keratinocyte line, SCC-13, derived from a human squamous cell carcinoma. In confluent cultures, approximately one-half of the cells were competent to form envelopes when grown in medium without hydrocortisone or retinyl acetate supplementation. Addition of hydrocortisone to the medium during growth resulted in up to 90% competence, while addition of retinyl acetate instead resulted in as low as 10% competence. Hydrocortisone partially antagonized the effect of retinyl acetate when both agents were added together. Involucrin levels, measured by radioimmunoassay, were modulated essentially in parallel with envelope competence under the various conditions tested. When the cells were grown in medium supplemented with hydrocortisone, the levels shortly after confluence were over 50-fold higher than in sparse cultures. Regardless of hydrocortisone or retinyl acetate addition, less than 1% of the cells were competent in sparse cultures of growing cells, but up to 90% exhibited this property after growth arrest in serum-free medium containing hydrocortisone. High levels of competence were correlated with cessation of cell division but not with loss of colony-forming efficiency; under optimal conditions, two-thirds of the cells were capable of both envelope formation and colony initiation. Normal human epidermal cells showed a 4- to 5-fold increase in envelope competence from sparse to confluent culture but were insensitive to the suppressive effect of retinyl acetate. The results suggest that some potential differentiated character of malignant keratinocytes may be suppressed in vivo by physiological agents such as vitamin A.

INTRODUCTION

During their normal program of terminal differentiation, epidermal keratinocytes synthesize beneath the plasma membrane, in vivo (13) and in culture (29), a protein envelope stabilized by \( \epsilon \)-(\( \gamma \)-glutamyl)lysine isopeptide bonding (23). Identical to the marginal band visible in electron micrographs (cf. Refs. 9 and 17), this structure is inducible in a majority of cultured epidermal cells by suspension in semisolid medium, which permits the final stages of terminal differentiation (9). Envelope formation in suspension is not diminished in extent by protein synthesis inhibition (24) and is greatly accelerated by ionophores and other agents permitting influx of calcium (25). The envelopes, containing an envelope precursor protein (25) called involucrin (31), then become stabilized by calcium-dependent transglutaminase cross-linking. Thus, envelope formation as a consequence of ionophore treatment provides a simple, although artificial, test for this distinctive aspect of keratinocyte differentiation.

Keratinocyte lines derived from human squamous cell carcinomas can now be established in culture on a fairly routine basis (21) and serve as suitable models for aberrant terminal differentiation (20). The original characterization of several lines revealed a reduced commitment to spontaneous envelope formation in suspension, compared to normal cells, but included the important observation that suspension itself elicited some "envelope competence," visible upon subsequent ionophore treatment (20). The present work demonstrates this phenomenon in surface culture and explores hormonal and physiological factors influencing involucrin expression and envelope competence.

Hydrocortisone (2) and vitamin A (5) have long been known to affect the differentiation of numerous epithelia including epidermis. Hydrocortisone accelerates stratified squamous differentiation in culture of chick (7, 27) and mouse epidermis (33), while vitamin A inhibits such differentiation of chick skin in culture (7) and appears necessary in vivo to prevent squamous metaplasia of many epithelia (34). More recent reports, that hydrocortisone in chick skin (14, 28) and vitamin A in mouse (18) and human epidermal cells (8) influence the expression of specific proteins (keratins and transglutaminase), suggested that these agents may also modulate other aspects of keratinocyte differentiation including envelope formation.

MATERIALS AND METHODS

Culture Conditions. Human keratinocytes were cultivated in the presence of a feeder layer of lethally irradiated 3T3 cells according to standard methods (19). SCC-13 cells were grown in Dulbecco-Vogt Eagle's medium supplemented with fetal bovine serum (5%), hydrocortisone (0.4 \( \mu \)g/ml), and retinyl acetate as specified. For these experiments, the fetal bovine serum was depleted of steroids by treatment with 0.1 volume of 0.9% NaCl solution containing 10% (w/v) charcoal (Amend Drug and Chemical Co., Irvington, N. J.) and 1% (w/v) dextran T-70 (Pharmacia, Uppsala, Sweden) at 55° for 30 min with subsequent removal of the charcoal by centrifugation and sterile filtration (1). Measured concentrations of retinol and retinyl palmitate in the medium were 2.8 and 0.2 \( \times 10^{-10} \) M, respectively. In experiments using retinyl acetate, the medium was changed at 2-day intervals, and dimethyl sulfoxide, solvent for retinyl acetate, was held to a 0.02% or lower final concentration. Normal foreskin epidermal cells (strain N, 40 to 50 generations) were grown as above with added epidermal growth factor (10 ng/ml), cholera toxin (5 ng/ml), insulin (5 \( \mu \)g/ml), transferrin (5 \( \mu \)g/ml), and triiodothyronine (2 \( \times 10^{-11} \) M).

Cross-Linked Envelope Formation. 3T3 feeder cells were sprayed from surface cultures with EDTA. The keratinocytes, adherent to the dishes, were disaggregated with trypsin and EDTA and suspended at 8 \( \times 10^5 \) cells/ml in serum-free medium containing ionophore X537A at a concentration of 50 \( \mu \)g/ml (Hoffman-LaRoche Inc., Nutley, N. J.). The samples were incubated for 2 hr at 37°, treated with sodium dodecyl sulfate

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PENDENCE OF COMPETENCE ON CULTURE DENSITY.

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experiments, envelope competence became appreciable at con-
additions, few cells were able to form envelopes at early times
in culture upon treatment with the ionophore X537A. In these
mous cell carcinoma (21), competence to form cross-linked
experiment illustrated was performed at least twice.

RESULTS

In the keratinocyte line, SCC-13, derived from a human squa-
mous cell carcinoma (21), competence to form cross-linked
velopes depended markedly upon culture conditions. Chart 1
shows that, regardless of hydrocortisone and retinyl acetate
additions, few cells were able to form envelopes at early times
in culture upon treatment with the ionophore X537A. In these
periments, envelope competence became appreciable at con-
fluence, when the many small colonies began to coalesce, and
reached a maximum after several more days. In other experi-
ments at lower inoculation densities, the proportion of cells
able of forming envelopes became appreciable as the isolated
colonies became larger, but not to as great an extent as in the
post-confluent state. In parallel work, the 2 keratinocyte lines,
SCC-9 and SCC-12F, also exhibited essentially the same de-
pendence of competence on culture density.

When SCC-13 cells were cultivated without added hydrocor-
tisone or retinyl acetate, 40 to 50% became competent to form
velopes by several days after confluence (Chart 1). Cells grown
in the presence of hydrocortisone exhibited increasing compet-
ence during the week preceding confluence and reached final
levels of 70 to 90% in postconfluent cultures. Competence was
considerably reduced in cultures treated with retinyl acetate in
the medium, as shown. Maximal effects of 5- to 10-fold were
obtained at a retinyl acetate concentration of approximately 0.3
µM, as illustrated in Chart 1a, which had little, if any, effect on
cell growth. Chart 1b illustrates the 2- to 3-fold decrease in
velope competence observed in medium with retinyl acetate
added to 0.1 µM. This effect was of similar magnitude in the
presence or absence of added hydrocortisone. When the cul-
tures were held at confluence for extended periods of up to 2
weeks, after which time they began to deteriorate, envelope
competence did not increase further and even declined slightly.

The involucrin content of SCC-13 cultures grown under condi-
tions modulating envelope competence has been measured by
radioimmunoassay. As illustrated in the standard curves pre-
ented in Chart 2, this assay is suitable for involucrin amounts
of 10 to 100 ng, providing adequate sensitivity for the present

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1 The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesul-
conic acid.
Modulation of Involucrin and Envelope Competence

work. Serial dilutions of extracts of both SCC-13 and normal epidermal cells gave curves parallel to the standard curve. When known amounts of involucrin were diluted serially in such extracts, and the measured amount of extract involucrin was taken into account, the curves were coincident with the standard curves, as shown in Chart 2. Confirming that the involucrin did not appear to be subject to proteolytic degradation in these assays, simultaneous inclusion of the inhibitors, benzamidine (10 mM), phenylmethylsulfonyl fluoride (0.1 mM), and Trasylol (200 kallikrein inhibitor units), had no effect on the curves obtained. Extracts of human fibroblasts contained no detectable involucrin in the assay, while the contents of normal epidermal cells at confluence under the growth conditions used were measured as 0.5 to 1.2% of soluble cell protein.

As shown in Chart 3, radioimmunoassay of cell extracts indicated that sparse cultures of growing SCC-13 cells had very low levels of involucrin (less than 0.01% of soluble cell protein) regardless of hydrocortisone or retinyl acetate addition to the medium. As the cells reached confluence, involucrin levels rose substantially, a change evident qualitatively by gel electrophoresis. When cells were grown in the presence of hydrocortisone, involucrin rose over 50-fold to 0.5% of soluble cell protein, comparable to the levels measured in normal epidermal cells. Without hydrocortisone in the medium, this rise was less pronounced, but still over 10-fold, to about 0.1% of soluble cell protein. Cells grown in the presence of added retinyl acetate (0.3 μM) exhibited less marked but still substantial increases in involucrin content. As illustrated in Chart 3, retinyl acetate reduced the content by about 5-fold in cells grown with added hydrocortisone and about 2-fold in cells without the corticosteroid. Thus, involucrin levels were modulated in the same direction as envelope competence by culture conditions.

Since a majority of the cells in these experiments exhibited competence in confluent culture under optimal conditions, suspension in semisolid medium, originally observed to elicit tissuc competence (20), is unnecessary. To test whether cessation of DNA synthesis (which occurs in suspension) elicits competence, cultures of SCC-13 at a preconfluent state were incubated in serum-free medium, gently arresting growth in G1 or G0 (15). For this experiment, cells were cultured in medium containing serum until reaching 20 to 30% of the confluent density. After rinsing and changing the culture to serum-free medium, the cell number nearly doubled and remained constant thereafter with little if any microscopic change in appearance such as stratification. After 15 days in this condition, as illustrated in Chart 4, nearly all of the cells incubated with 1 μM hydrocortisone were competent, while about one-half without hydrocortisone were competent. Hydrocortisone added at 0.02 μM, equal to the dissociation constant of the glucocorticoid receptor for this ligand in human keratinocytes (16), was similarly effective. Except for the slower approach to maximal competence, the final result was comparable to that for growth in the presence of serum (Chart 1). In both cases, the extent of spontaneous envelope formation (no added ionophore) was approximately 10% of the induced value, and retinyl acetate was at least as effective in suppressing envelope competence as in growing cultures.

To test whether envelope competence indicated a commitment to terminal differentiation, resulting in irreversible loss of the capacity for cell division, cells grown to confluence were tested for colony-forming efficiency. Inspection of the results of a representative experiment in Table 1 reveals that envelope competence and colony initiation are readily compatible. In cultures grown without added hydrocortisone or retinyl acetate, one-third of the cells had all of the components necessary to form envelopes and were capable of growth and colony initiation, while two-thirds of the cells grown in the presence of hydrocortisone exhibited these properties. This finding has been confirmed with cultures incubated in serum-free medium as shown in Chart 4; even when competence was 90% in cells grown with hydrocortisone, colony-forming efficiency remained appreciable (52%). As seen in Table 1, cells in the cultures treated with retinyl acetate had reduced envelope competence and slightly lower colony-forming efficiency, also inconsistent with the notion that induction of competence represents a terminal state.

The dependence of envelope competence on culture density is shown in Chart 5 for normal human epidermal cells. While not so striking as in SCC-13, an increase of approximately 4-fold is evident from the time of earliest measurement (Day 2) to confluence. This increase was faster in cultures grown in the presence of hydrocortisone, due at least in part to more rapid colony expansion (22), but the cultures grown without added steroid eventually became equally competent. In contrast to the squamous cell carcinoma keratinocytes, however, the normal epider-
Table 1  
Colony-forming efficiency and envelope competence in confluent SCC-13 cultures

<table>
<thead>
<tr>
<th>Additives</th>
<th>Cross-linked envelopes (%)</th>
<th>Colony-forming efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>37</td>
<td>98</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>Retinyl acetate</td>
<td>18</td>
<td>48</td>
</tr>
<tr>
<td>Retinyl acetate + hydrocortisone</td>
<td>28</td>
<td>81</td>
</tr>
</tbody>
</table>

DISCUSSION

Spare cultures of growing SCC-13 keratinocytes were virtually incapable of envelope formation and accumulated little involucrin, but competence and involucrin were both elicited by cessation of growth. Thus, the growing cells appear not to express envelope competence, because they do not regulate their removal from the proliferative compartment and thus stop cycling. In contrast, the commitment to terminal differentiation is evident in cultures of normal keratinocytes by their stratification, where the noncyering superficial cells accumulate involucrin, not detected in basal cells in culture (31) or in vivo (3, 25). Stratification of normal cells is not required for involucrin accumulation, however, and appears to be a consequence rather than a cause of terminal differentiation (32). Similarly, expression of involucrin and envelope competence in the present work occurs with little if any stratification and is not indicative of a terminal differentiation process. Keratinocytes that are envelope-competent and capable of colony initiation must be quite rare in normal epidermal cultures, but can constitute a large fraction of SCC-13 populations.

The present experiments show clear effects of hydrocortisone and retinyl acetate on envelope competence and involucrin accumulation in SCC-13 keratinocytes. Enhancement of competence by hydrocortisone and suppression by vitamin A are consistent with the known effects of these agents on the histological appearance of skin explants in culture (27, 33). Each agent is effective in the absence of the other, with its action presumably mediated by a distinct cytoplasmic binding protein (16, 18), but the effect of retinyl acetate at high concentration is dominant, as is vitamin A in embryonic chick skin explants (6). The present culture system may prove to be advantageous for elucidating the biochemical mechanisms by which these agents affect structural features of keratinocytes. If involucrin accumulation in SCC-13 occurs analogously to that in normal epidermal cells, it presumably reflects increased levels of specific mRNA (31). While hydrocortisone is known to increase transcription of message for certain proteins such as growth hormone (4), the action of retinoids on transcription of specific structural or regulatory genes remains to be characterized.

Retinyl acetate has been shown to reduce considerably spontaneous envelope cross-linking in human keratinocytes cultured from esophagus and vagina without affecting involucrin levels (10). Similarly, retinoic acid has been reported to reduce spontaneous envelope cross-linking in suspended epidermal cells derived from guinea pig skin (35). In both cases, ionophore-inducible envelope formation was essentially unaffected, indicating that the retinoids reduced cell permeabilization during terminal differentiation but not enzyme and structural protein components below required levels. These findings, including the insensitivity exhibited by normal epidermal cells (10), stand in marked contrast to retinyl acetate suppression of involucrin content and envelope competence in SCC-13. In the latter cells, the influence of retinoids on envelope structural proteins other than involucrin has not been investigated. However, recent experiments in the laboratory have shown that transglutaminase activity is also suppressed, as distinguished from the paradoxical stimulation of the mouse keratinocyte enzyme by retinoic acid at high concentration (36).

Despite exhibiting conspicuously low envelope competence under routine culture conditions (20), SCC-13 cells were capable of expressing levels of competence and involucrin characteristic of normal epidermal cells under optimal conditions. The mechanism is unclear by which malignant keratinocytes, such as SCC-13, acquire increased sensitivity to physiological agents to which the normal cells have only limited response. The great dependence of differentiation features on culture conditions in these experiments raises the possibility that reduced differentiation in vivo may be at least partly a consequence of this sensitivity. Vitamin A levels in human plasma (26), for example, range above concentrations effective on SCC-13 in culture. Squamous dysplasias of the uterine cervix, which show markedly reduced involucrin levels compared to those in surrounding regions of normal stratified squamous epithelium (30), may represent a striking example of this effect.

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REFERENCES


18. Rask, L., Anundi, H., Bohme, J., Eriksson, U., Ronne, H., Sege, K., and
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