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High-Cell-Density Phorbol Ester and Retinoic Acid Upregulate Involucrin and Downregulate Suprabasal Keratin 10 in Autocrine Cultures of Human Epidermal Keratinocytes

Yves Poumay,* Françoise Herphelin,* Patrick Smits,† Isabelle Y. De Potter,* and Mark R. Pittelkow‡

*Département Histologie-Embryologie, Facultés Universitaires Notre-Dame de la Paix, B-5000 Namur, Belgium;

†Laboratory of Molecular Biotechnology, Department of Biochemistry, UIA, B-2610 Wilrijk, Belgium; and

‡Department of Dermatology, Mayo Clinic, Rochester, Minnesota 55905

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Autocrine growth of human epidermal keratinocytes is initiated in subconfluent cell cultures in the absence of exogenous growth factors, at low calcium concentration of the medium and with sufficient cell density. Culture confluence inhibits keratinocyte proliferation and upregulates expression of early, keratin 10 (K10), and late, involucrin, markers of differentiation. In this report, the phenotype of autocrine keratinocytes was studied at high cell density (post-confluence), specifically after treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), or all-*trans* retinoic acid (RA). At postconfluence, K10 is decreased but not involucrin. TPA upregulates involucrin expression, but not K10 in subconfluent keratinocytes. Treatment of confluent keratinocytes with RA downregulates K10, but upregulates involucrin. This *in vitro* culture model, unlike others, simulates for the first time the *in vivo* effects of RA, a member of the retinoid family which potently modulates keratinocyte differentiation and the expression of selected gene products. It thus can be developed to further examine epidermal differentiation. © 1999 Academic Press

Stratification and differentiation of keratinocytes in the human epidermis is a process that generates and maintains the stratum corneum, an impermeable barrier made up of keratinized anucleate cells and isolates the organism from its environment, including the ability to protect against chemicals and pathogens that come into contact with the skin surface (1). The continuous renewal and progressive maturation of keratinocytes during their upward migration through the epidermis creates the distinct basal, spinous, granular and cornified layers constitutive of this tissue, and

abnormalities in epidermal differentiation can directly be linked to dermatological diseases (1, 2). The availability of *in vitro* models of human and rodent keratinocyte culture during the last two decades has allowed investigation of the cellular and molecular mechanisms regulating differentiation (1–4). However, each model has been recognized as having specific advantages as well as disadvantages such as the presence of serum, feeder layers or comparison of human and mouse keratinocytes (2, 4). Consequently, when initiating *in vitro* investigations of the epidermal differentiation process in keratinocyte, one model must be selected, ideally taking into account the particular process or environmental conditions the study will be dealing with.

Autocrine cultures of human epidermal keratinocytes were recently described, and can be established in a defined culture medium at low calcium concentration, when cell density is sufficient to provide factors that sustain cell proliferation (5, 6). Autonomously growing autocrine keratinocyte cultures reach confluence where upon terminal differentiation is induced within the cell population. We have previously shown that growth-arrest induced by confluence of the culture is associated with strong induction of the early differentiation markers, suprabasal keratins 1 (K1) and 10 (K10), and occurs independently of the medium calcium concentration (7). Thus, these specific autocrine conditions are particularly suited for studies of the effects of individual growth factors on the initiation of epidermal differentiation (7, 8).

Among the genes expressed by keratinocytes during the epidermal differentiation program *in vivo* are those expressed early in the process, such as K1 and K10, and others that are expressed later (1, 3). A representative later marker is involucrin, a component of the

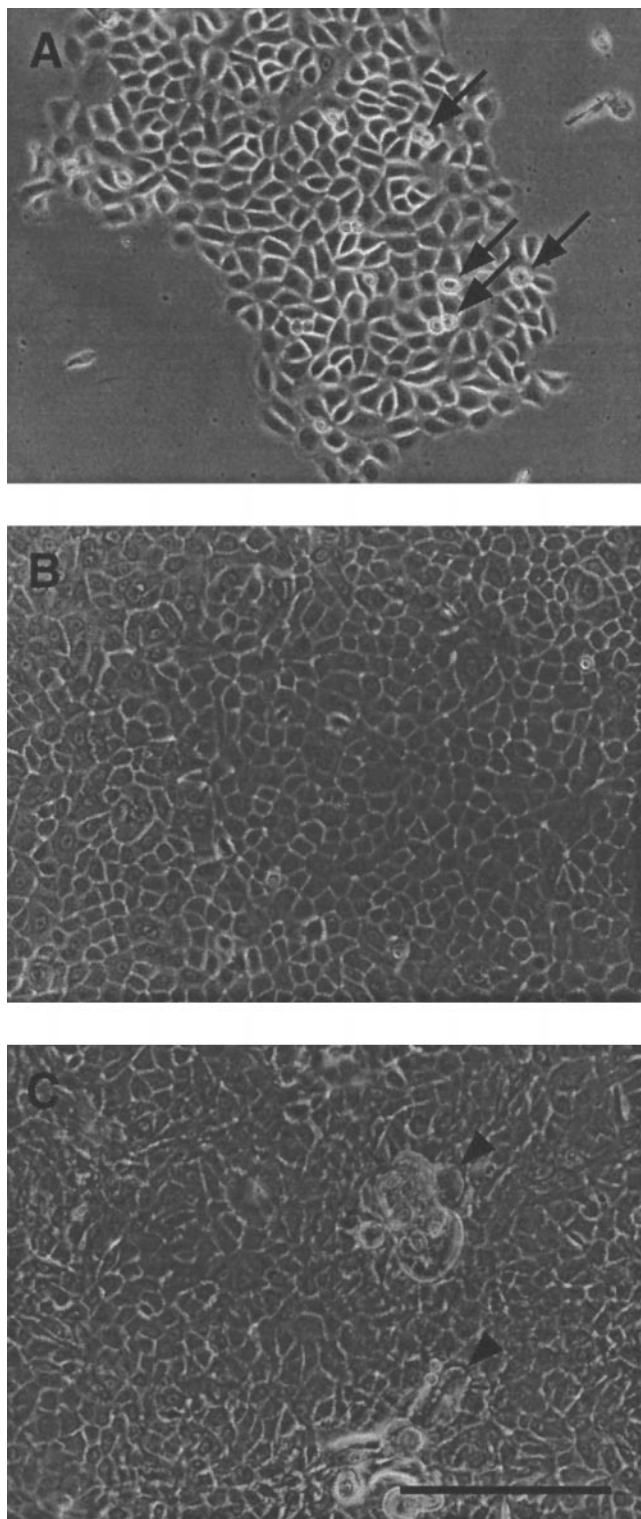


FIG. 1. Morphology of autocrine cultures of human epidermal keratinocytes at various cell densities. Keratinocytes were plated in secondary cultures in KGM-2 medium, then switched to basal medium when cells were covering approximately 40% of the substratum. Autocrine keratinocytes were photographed using inverted phase contrast microscopy at subconfluence after one day of autocrine growth (A), on the first day of complete confluence of the culture (B), and four days after confluence at postconfluence (C). Arrows indicate

cornified envelope, that characterizes keratinized cells of the stratum corneum and is expressed late in differentiation program *in vivo*, in the upper spinous and granular epidermal layers (1, 3, 4). Interestingly, whereas the early markers K1 and K10 are induced at confluence of autocrine keratinocyte cultures (7), recent studies have further shown that postconfluent cultures (i.e., cultures maintained under autocrine growth conditions for several days after confluence) continue to follow the program of epidermal differentiation and trigger expression of later markers (9) suggesting that not only the onset of epidermal differentiation, but also the regulation of later phenomena, can be studied in this model.

In the present work, we verify that the expression of early and late differentiation markers are differentially regulated by cell density in autocrine keratinocyte cultures (9), and we demonstrate that autocrine cultures are sensitive to compounds known as modulators of epidermal differentiation. Most interesting, we find that all-*trans* retinoic acid (RA) upregulates involucrin in normal keratinocytes, providing *in vitro* data that parallel for the first time *in vivo* effects of RA (10).

MATERIALS AND METHODS

Cell Culture

Human adult normal skin samples were obtained at plastic surgery (Dr. B. Bienfait, Clinique St. Luc, Namur-Bouge). Keratinocytes were isolated by the trypsin float technique as described (11), and primary cultures were initiated in complete, 0.15 mM (low) calcium growth medium (KGM-2, BioWhittaker) containing growth factors and hormone supplements. The low calcium concentration was maintained throughout all the experiments reported here. Culture medium was renewed every two days. Keratinocytes harvested by trypsinization of primary proliferating subconfluent cultures were cryopreserved. After thawing, cells were plated into secondary cultures at 5×10^3 cells/cm² in complete medium, then, once approximately 40% of the culture substratum was covered by keratinocytes, cultures were washed repeatedly with KBM-2 medium prepared by excluding bovine pituitary extract, insulin, transferrin, epinephrine and EGF from complete culture medium. The KBM-2 medium is designated hereafter as basal medium. Washed keratinocytes were then incubated in the basal medium to initiate their autocrine growth at subconfluence (5) and terminal differentiation at confluence (7). Secondary autocrine cultures were also refed every other day with

cells undergoing mitosis and arrowheads indicate cellular stratification. Bar: 250 μ m.

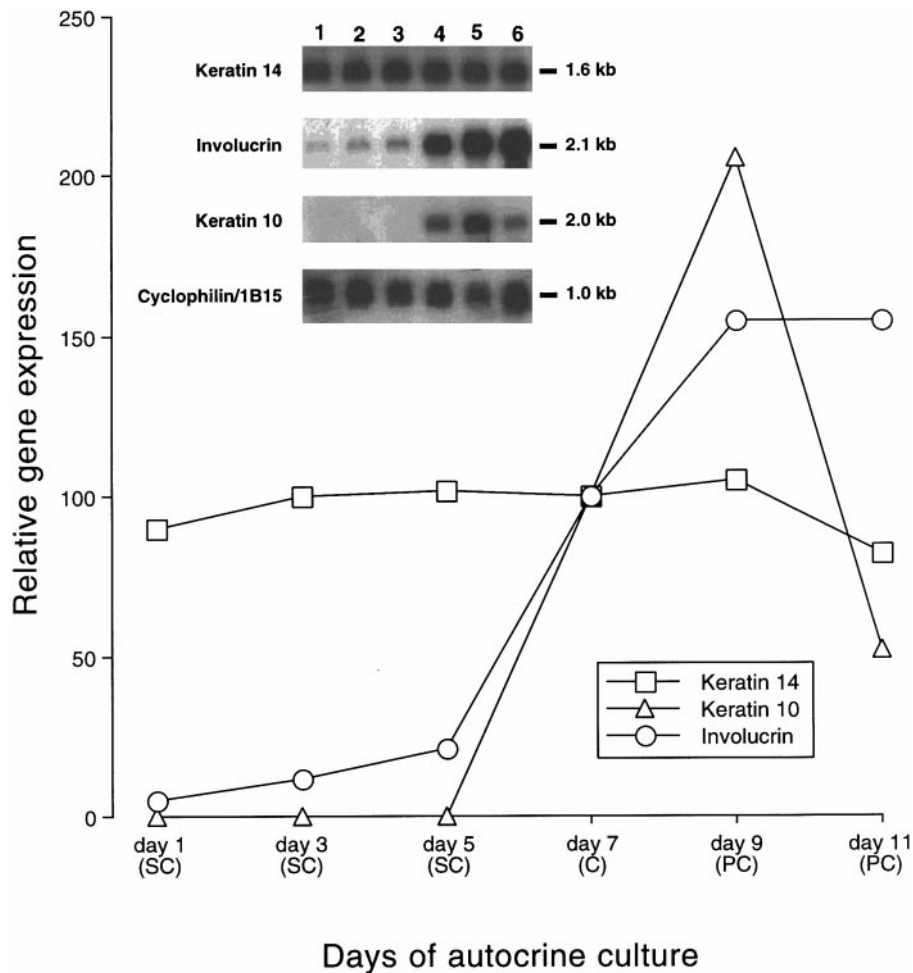


FIG. 2. Modulation of epidermal differentiation-related RNA expression by cell density in autocrine cultures of human keratinocytes. Poly(A)RNA was extracted from keratinocytes cultured in autocrine conditions for 1 (lane 1), 3 (lane 2), 5 (lane 3), 7 (lane 4), 9 (lane 5), and 11 (lane 6) days. Subconfluence (SC), confluence (C), and postconfluence (PC) of the culture were determined by direct phase contrast microscopic observation. Relative gene expression was calculated as the ratio of the keratin 10, keratin 14, or involucrin gene expression to the expression of the cyclophilin/1B15 gene, using densitometric measurements of the respective Northern blot hybridizations. The results are expressed as percentages of the relative gene expression calculated on the first day of culture confluence (day 7).

basal medium. Autocrine subconfluent keratinocytes grown in basal medium were treated with 1, 10, or 100 ng/ml of 12-*O*-tetradecanoylphorbol 13-acetate (TPA, Sigma) for 18 h. Treatment of autocrine keratinocytes cultured in basal medium with 10^{-8} - 10^{-6} M all-*trans* retinoic acid (RA, Sigma) was performed for 18 h at subconfluence or confluence. Stock solutions of TPA and RA were prepared in dimethyl sulfoxide (DMSO). DMSO 0.01% was also included in control cultures. Representative experiments are shown on the figures, each experiment being reproduced at least three times with similar results.

Poly(A)RNA Isolation and Northern Blot Analysis

Poly(A)RNA from autocrine cultures was isolated and analyzed by Northern blotting as described previously (7). The cDNAs specific for the human basal

keratin 14 (K14), suprabasal keratin 10 (K10) (12), or cDNA specific for human involucrin (13) were used to detect RNA expression of epidermal differentiation markers. The membrane was also hybridized with the housekeeping gene cyclophilin/1B15 cDNA to verify equivalent loading and transfer of RNA (14). The relative gene expression was analyzed using densitometric measurements performed by the NIH Image Analysis software package based on comparison of each gene product with expression of cyclophilin/1B15.

Western Blot Analysis

Cell lysates were analyzed by Western blotting as described (7). Involucrin protein was detected by incubation for 2 h with 1:250 dilution of a rabbit polyclonal antibody (Harbor) and K10 protein by incubation with 1:250 dilution of the DEK10 mouse monoclonal anti-

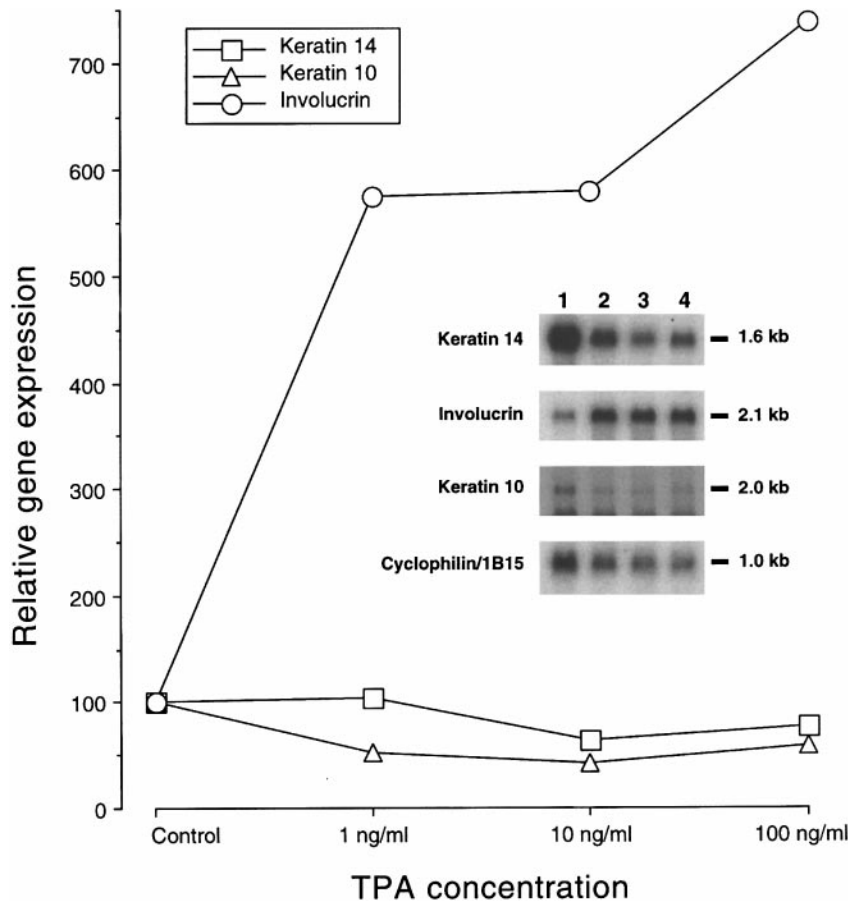


FIG. 3. Modulation of epidermal differentiation-related RNA expression by TPA in subconfluent autocrine keratinocyte cultures. Poly(A)RNA was extracted from keratinocytes cultured in autocrine conditions at subconfluence after treatment for 18 h with DMSO 0.01% (Control, lane 1), or with 1 ng/ml (lane 2), 10 ng/ml (lane 3), or 100 ng/ml (lane 4) of TPA. Relative gene expression was calculated as in Fig. 2. Results are expressed as percentages of the relative gene expression calculated in the control culture.

body (Cappel). Immunoreactivity was detected with 1:1000 dilutions of horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (Dako), and visualized using the Supersignal West Pico chemiluminescent substrate (Pierce).

RESULTS

Cell Density Regulates Expression of K10 and Involucrin in Autocrine Culture Conditions

Subconfluent keratinocytes grown under autocrine culture conditions demonstrated mitoses in each replicating colony (Fig. 1A). At culture confluence, keratinocyte growth-arrest, as reported previously (7, 11), is documented by the absence of mitotic figures and the typical quiescent appearance (Fig. 1B). Four days after culture confluence, at postconfluence, cellular stratification is clearly evident in many areas of the culture (Fig. 1C). Northern blot analysis of epidermal gene expression at increasing cell densities shows that, following the onset of terminal differentiation at culture

confluence (7), K10 and involucrin are differentially controlled during the postconfluent period of culture (Fig. 2). Indeed, whereas K10 is strongly induced by confluence, we demonstrate that K10 is downregulated during postconfluent culture. Similar results are observed for K1 mRNA expression (data not shown). However, downregulation of the K1 and K10 transcripts is accompanied by cellular accumulation of both cytoskeletal keratin proteins (7, data not shown). Unlike suprabasal keratins, involucrin is faintly expressed by subconfluent keratinocytes. However, involucrin expression is enhanced respectively 8.3 and 12.8 fold at confluence (day 7) and postconfluence (day 11), in comparison with expression of subconfluent cultures analyzed on the third day (day 3) of autocrine conditions (Fig. 2). This observation is in accordance with late expression of involucrin in the upper layers of normal epidermis. On the other hand, basal K14 gene expression is not markedly regulated by cell density of autocrine cultures, suggesting that keratinocytes exhibiting the basal phenotype persist under these dif-

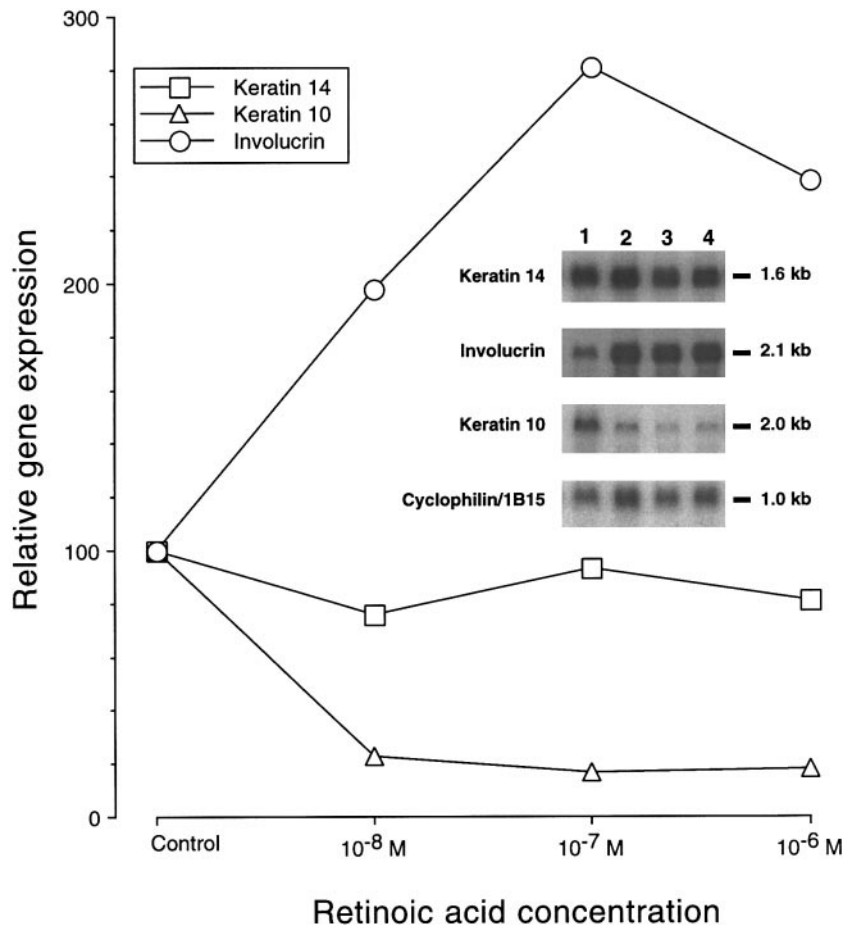


FIG. 4. Modulation of epidermal differentiation-related RNA expression by RA in confluent autocrine keratinocyte cultures. Poly(A)RNA was extracted from keratinocytes cultured in autocrine conditions at confluence after treatment for 18 h with DMSO 0.01% (Control, lane 1), or with 10^{-8} M (lane 2), 10^{-7} M (lane 3), or 10^{-6} M (lane 4) all-*trans* retinoic acid. Relative gene expression was calculated as in Fig. 2, and expressed as in Fig. 3.

differentiating culture conditions and are in homeostatic balance with terminal differentiating cells.

TPA Regulates Epidermal Differentiation under Autocrine Culture Conditions

TPA induces terminal differentiation of keratinocytes *in vivo* (15). Subconfluent cultures were treated with TPA to assess whether this active phorbol ester is able to trigger differentiation in epidermal keratinocytes cultured in autocrine conditions. Involucrin mRNA expression is clearly induced by TPA (Fig. 3) similarly to previous reports on keratinocytes cultured in different models (16, 17). However, no induction of the suprabasal K10 expression could be found. On the contrary, the weak K10 expression seen by prolonged exposure of the Northern blot was inhibited by TPA (Fig. 3), an effect already reported for K1 and K10 expression in mouse cells (18). Interestingly, the coordinate inhibition of K14 mRNA expression by TPA in human keratinocytes has not been observed for mouse

cells, further suggesting distinctive regulation of epidermal keratin expression between the two species (7, 19). Diminution of K14 mRNA expression by exposure of human keratinocyte to TPA indicates progressive loss of the basal cell phenotype that parallels the loss of clonogenic, proliferative potential of TPA-treated human keratinocytes (20).

Retinoic Acid Differentially Regulates K10 and Involucrin Expression in Differentiating Autocrine Cultures

RA is known to exert a wide range of effects on keratinocytes during epidermal differentiation. In these sets of experiments, all-*trans* retinoic acid was added to the medium of autocrine keratinocyte cultures to determine whether this retinoid also regulates differentiation under these conditions. When 10^{-8} - 10^{-6} M RA were added to subconfluent, growing autocrine cultures of keratinocytes, no effect on K14, K10 or involucrin mRNA expression was found (data not

shown). However, addition of 10^{-8} - 10^{-6} M RA to the medium of confluent differentiating cultures resulted in the inhibition of K10 mRNA expression (Fig. 4), similar to previously reported results (21, 22). K14 expression was unaffected by the range of RA concentrations tested, but by contrast, for each RA concentration tested the relative gene expression of involucrin increased at least two fold for cultured keratinocytes grown in autocrine conditions at confluence (Fig. 4) and postconfluence (data not shown). We believe this is the first report of a differential *in vitro* pro-differentiating effect of RA. To confirm these results, we used western blot analysis to determine whether the upregulation of involucrin mRNA expression by RA is paralleled by enhanced involucrin protein expression (Fig. 5). Indeed, when identical confluent autocrine keratinocyte cultures were treated for 18 h with 10^{-8} - 10^{-6} M RA, an increase in involucrin level was seen compared to control cultures (Fig. 5B). Conversely, increasing RA concentrations resulted in decreasing K10 protein level (Fig. 5C) (21, 22).

DISCUSSION

Application of the autocrine keratinocyte culture system to investigate the regulation of epidermal differentiation and effects of pharmacologic and other chemical agents provides the potential to totally define the culture environment of the keratinocyte. To date, these defined culture conditions have been used to examine early events of keratinocyte commitment to differentiation (7), but they are also useful for studies of later events in the keratinocyte terminal differentiation process. Indeed, we demonstrate here that continued culture of keratinocytes after confluence is attained not only maintains a subpopulation of basal cells expressing K14 but also induces later differentiation markers. Subsequently the mRNA encoding the early K10 marker is downregulated but involucrin is further upregulated. These findings correlate well with the inherent steps of differentiation seen in epidermis and suggest that the autocrine keratinocyte culture system after confluence is a robust model for investigating molecular regulators of epidermal growth and differentiation (9).

The regulation of differentiation at postconfluence has been reported to be mediated by activation of protein kinase C (PKC) in human keratinocytes (9). The tumor-promoter and PKC-activator TPA is well-known as a strong inducer of terminal differentiation in keratinocytes (1, 15, 16, 20). Indeed, we found in autocrine cultures of keratinocytes that TPA strongly upregulates expression of involucrin, but downregulates expression of specific keratin genes. These results are in accordance with the AP-1 activating effects of TPA and its enhancer activity on the involucrin promoter (17, 23). These observations in human cells also correlate

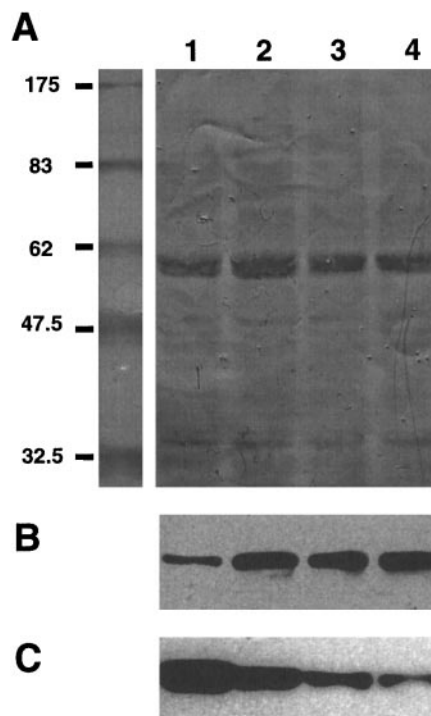


FIG. 5. Western blot analysis of involucrin and K10 protein expression in confluent autocrine keratinocyte cultures treated with RA. Cellular protein extracts were prepared from keratinocytes cultured in autocrine conditions at confluence after treatment for 18 h with DMSO 0.01% (lane 1), or with 10^{-8} M (lane 2), 10^{-7} M (lane 3), or 10^{-6} M (lane 4) all-*trans* retinoic acid. Amido black total protein staining of the membrane (A). Immunodetection of involucrin (B) or K10 (C) was performed on the blot shown on A. Position of molecular weight markers expressed in kDa are shown on the left of A.

with inhibitory effect of TPA on the suprabasal K1/K10 expression in mouse cells (18, 24). Finally, we show that the phenotypic effects induced in low density autocrine cultures by the phorbol ester mimic the regulation of the epidermal differentiation marker expression observed in postconfluent high density cultures.

Treatment of differentiating autocrine cultures with RA produced unexpected observations related to involucrin expression. It is well established that RA regulates epidermal differentiation. However, the main disparity concerning the keratinocyte response to RA is the effect of the compound *in vitro* or *in vivo*. Basically, RA has consistently inhibited epidermal differentiation in previous studies performed on cultured keratinocytes (1, 21, 25-31), but with *in vivo* application of the compound, no significant alteration of K1 and K10 has been found, and an increased number of cell layers expressing filaggrin and involucrin are routinely detected (10). Also, loricrin expression was reduced *in vivo* only after acute treatment with RA, whereas an increased number of cell layers expressing loricrin is observed after chronic treatment (10). In confluent autocrine keratinocyte cultures, we find here inhibition of K10 expression at both mRNA and protein levels, con-

firming previous *in vitro* studies (21, 22, 28), but, of significance, we observe that involucrin is upregulated by RA in autocrine culture conditions. This result is surprising initially, because several previous reports have demonstrated inhibition of involucrin expression by RA in cultured keratinocytes (32, 33). However, these results were observed in malignant keratinocytes isolated from human squamous cell carcinomas (SCC). Further scrutiny of investigations performed on normal human cultured keratinocytes (22, 26) raises doubt as to whether RA has a clear suppressing effect on involucrin expression. Together, these previous results suggest that the regulation of involucrin expression by RA might differ between normal and SCC keratinocytes. The difference may be explained by altered RA receptor (RAR) expression in SCC (34). However, by analyzing the 5' regulatory sequences of the human involucrin gene using the transcription element search software (TESS) package (URL: www.cbil.upenn.edu, May 20, 1999) to localize potential RA responsive elements, several imperfect (20% of mismatch allowed) sequences are identified. These sequences may potentially bind the predominant RA receptors in human skin, RAR- γ and RAR- α (35).

In conclusion, the present study demonstrates that autocrine culture conditions for keratinocytes which we have previously developed provide an adequate model for future investigations, in a defined environment, of epidermal differentiation. We suggest that this model should be utilized when discrepancies exist with other *in vitro* models and the *in vivo* state. In particular, using this autocrine *in vitro* model, we have identified for the first time that involucrin expression is upregulated as a pro-differentiating effect of RA in epidermal keratinocytes similar to its effect *in vivo*.

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