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Suppression of keratinocyte differentiation in SSC-9 human squamous carcinoma cells by benzo[a]pyrene, 12-*O*-tetradecanoylphorbol-13-acetate and hydroxyurea

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In the human squamous carcinoma cell line SCC-9, the expression of two markers of keratinocyte differentiation, involucrin and transglutaminase, was greatly stimulated when growing cultures reached confluence. However, the two markers differed temporally in their induction, with transglutaminase reaching maximal levels shortly after confluence and involucrin a week later. If replication was arrested with hydroxyurea prior to confluence, transglutaminase induction occurred within several days but involucrin levels were completely suppressed. Such a striking degree of uncoupling also resulted when the cells were treated with polycyclic aromatic hydrocarbons such as benzo[a]pyrene but not with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a potent inducer of aryl hydrocarbon hydroxylase, or with pyrene. Chronic treatment with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate suppressed expression of both transglutaminase and involucrin. However, suppression of the latter (evident in greatly reduced mRNA levels) was considerably more potent and powerful. These findings demonstrate uncoupling of keratinocyte differentiation, potentially useful in analysis of its multiple regulatory influences. They also emphasize the utility of sensitive keratinocyte targets for studying the mechanisms by which model carcinogens disturb the orderly progression of events in their differentiation program.

Introduction

Generation of malignancy in normal cells is a multistep process that can be set in motion by genetic damage. Alteration of developmental or other processes not clearly involving DNA damage can also be important (1), evident particularly in keratinocytes during promotion by such potent compounds as 12-*O*-tetradecanoylphorbol-13-acetate (TPA*) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (2,3). Uncommon genetic events often appear to be rate limiting in producing malignant change. However, recent evidence points to enhancement of its incidence or detection by heritable perturbation of intracellular processes that occurs throughout carcinogen-exposed populations (4,5). Thus, study of the modulation of target cell differentiation by carcinogen treatment may further our understanding of neoplastic progression. Moreover, analysis of the perturbation of differentiation is likely to offer insight into the mechanisms stabilizing normal programming.

For these purposes, keratinocytes derived from human tumors appear highly appropriate. It is now a routine matter to establish continuous epithelial cell lines from squamous carcinomas of the

skin and oral cavity (6), the esophagus (7) and other sites using 3T3 feeder layer support. These cells have been shown to lack certain differentiation features characteristic of normal keratinocytes, such as rapid attenuation of colony forming ability when held in suspension (8). While the growing cultures are commonly deficient in certain differentiation features, exhibiting low levels of involucrin and particulate transglutaminase, for example, they can express substantial levels after reaching confluence (9–11). Inasmuch as the carcinoma cells exhibit marked sensitivity to important physiological effectors (calcium, retinoids, glucocorticoids) and carcinogens [TCDD, polycyclic aromatic hydrocarbons (PAHs)], they promise to be valuable subjects for studying the mechanisms by which such agents act (12,13). Moreover, the divergence of these cells from normal properties does present opportunities for resolving the regulation of normal keratinocyte differentiation into constituent elements and for examining the molecular basis of contrasting responses to model carcinogens.

Materials and methods

Cell culture

SSC-9 malignant lingual keratinocytes, generously provided by Dr J.G. Rheinwald (6), were cultivated (14) with support from a feeder layer of lethally irradiated 3T3 cells using a mixture of Dulbecco–Vogt Eagle's and Ham's F-12 media (3:1) supplemented with fetal bovine serum (2%), hydrocortisone (0.4 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), T₃ (20 pM) and adenine (0.18 mM). The cells were inoculated at a density of 1–2 × 10⁴/6-cm dish and reached confluence in 11–14 days. Cultures were treated with TPA (Sigma Chemical Co., St Louis, MO) or TCDD (kindly provided by Dr H. Green) at each medium change (performed at 3-day intervals) starting 2 days after inoculation. Cells were treated similarly with hydroxyurea or PAHs (Sigma) starting several days prior to confluence. Controls contained the same solvent concentrations (generally 0.1% dimethylsulfoxide) as treated cultures. After a total of 30–40 days, the cultures were rinsed twice with 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄ (pH 7.2), scraped into plastic microfuge tubes with approximately 1 ml of ice-cold buffer (10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1% Emulgen 911 nonionic detergent) and stored frozen until assay.

Assays

Samples were disrupted by sonication immediately upon thawing. Aliquots of each crude homogenate were assayed in duplicate for transglutaminase activity by incorporation of [³H]putrescine into reductively methylated casein (11). After a 30-min incubation at 37°C, protein-bound radioactivity was recovered by precipitation with trichloroacetic acid, rinsed onto glass fiber filters and scintillation counted. SCC-9 lacks expression of the soluble tissue transglutaminase even when treated with high retinoic acid concentrations (12). Occasional checks confirmed that the various treatment conditions did not fortuitously induce this enzyme.

After removal of aliquots for transglutaminase assay, the remaining crude homogenate was subjected to microfugation for 3 min. Involucrin in the supernatant was measured by a sensitive enzyme-linked immunoassay (15). Triplicate aliquots of appropriate dilution (1:10–1:2000) in 0.1 ml were incubated overnight with rabbit anti-involucrin antiserum, transferred to a 96-well plate containing adsorbed partially purified involucrin, incubated 30 min and washed. Antibodies remaining bound to the wells were quantitated by treatment with protein A–alkaline phosphatase conjugate and subsequent incubation with *p*-nitrophenylphosphate. Absorbance values at 405 nm were measured using a Titertek Multiskan and involucrin values calculated from standard curves generated in parallel with samples of chromatographically purified involucrin (16).

Values of transglutaminase activity and involucrin content were normalized to total cell protein determined using bicinchoninic acid (17) from Pierce Chemical Co. (Rockford, IL).

*Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenzo[*a*]anthracene; B[a]P, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAH, polycyclic aromatic hydrocarbon.

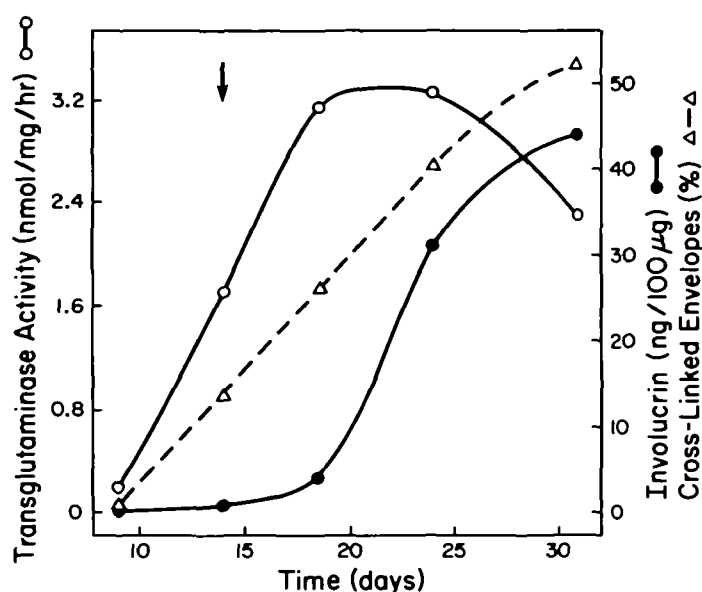


Fig. 1. Expression of transglutaminase, involucrin and envelope forming ability as a function of time from inoculation. Duplicate cultures at the times indicated were assayed for each marker. The vertical arrow gives the time at which the cultures reached confluence.

Cross-linked envelope forming ability was measured by phase-contrast microscopy. The cells were disaggregated with trypsin, suspended in serum-free medium containing 100 μ M X537A ionophore and treated with 2% sodium dodecyl sulfate, 20 mM dithioerythritol several hours later (9). Values reported are the means of two independent experiments.

Involucrin mRNA level

RNA was isolated from treated and control cultures dissolved in 6 M guanidine thiocyanate by centrifugation through CsCl (18). Total cellular RNA (15 μ g) was electrophoresed by standard methods in 1% agarose gels containing 0.66 M formaldehyde (19) and transferred to Gene Screen Plus. Fluorescent visualization of the samples in the gels and after transfer as recommended (19) confirmed that the same amounts of RNA were compared. The membrane was prehybridized in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate for 1 h at 42°C. Denatured salmon sperm DNA (0.1 mg/ml) and a nick-translated 32 P-labeled probe (2×10^6 c.p.m./ml) (a 4.5-kb *Xba*I–*Bam*HI restriction fragment of pAI-3H6B (20) kindly provided by Dr M.A. Phillips) were added and the hybridization continued for 36 h. Prior to autoradiography, the membrane was washed twice each with $2 \times$ SSC at room temperature (5 min), $2 \times$ SSC, 1% SDS at 60°C (30 min) and $0.1 \times$ SSC at room temperature (30 min). In parallel to experimental samples, involucrin mRNA was readily detected from cultured normal human epidermal cells (positive control) and was not observed from cultured human dermal fibroblasts (negative control).

Results

Growth arrest

Measurements of transglutaminase activity and involucrin content in SCC-9 cells showed that these differentiation markers greatly increased as rapidly growing cultures became confluent. As shown in Figure 1, the transglutaminase specific activity rose over 10-fold, while that of involucrin increased at least 50-fold. The rise in enzymatic activity coincided with the merging of colonies and formation of a continuous monolayer. While the actual assignment of confluence is subjective (uncertain by ± 1 day), it was clear in each of five experiments that the rise in involucrin occurred 7–10 days later than that of transglutaminase. These experiments also demonstrated a dramatic increase in cross-linked envelope structures inducible by ionophore treatment of the cells after confluence as illustrated. These observations have also been made in SSC-12F2 cells (21), an independently derived human epidermal line (22), and thus may be characteristic of

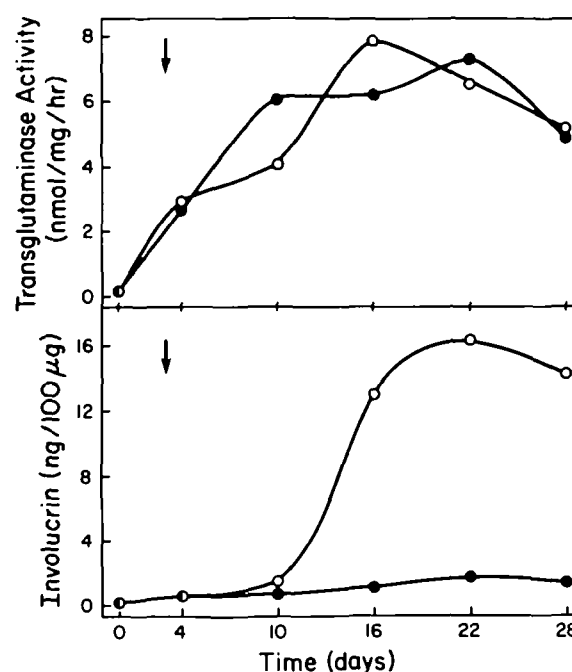


Fig. 2. Uncoupling of transglutaminase and involucrin expression by arrest of DNA synthesis. At time 0 (1 week after inoculation), parallel pre-confluent cultures were grown in medium without (○) or with (●) 0.3 mM hydroxyurea supplementation. At the times indicated, cultures were harvested in duplicate and assayed for transglutaminase activity and involucrin content. The vertical arrow at day 4 indicates when the control cultures reached confluence. This experiment has been carried out three times with essentially identical results, of which a representative example is illustrated.

neoplastic keratinocytes. Succeeding work has concentrated on expression of the specific markers transglutaminase and involucrin since ionophore-inducible envelope formation, involving the cross-linking of a number of proteins in human (23,24) and mouse epidermal cells (25), is not as well characterized and hence more difficult to interpret.

The SSC-9 cells stratify little, but their number keeps increasing slowly after confluence as cell division proceeds at a decreasing rate for a week or more (A.L. Rubin and R.H. Rice, unpublished observation). Thus, the above difference in time of appearance could logically reflect a greater sensitivity of involucrin expression to residual growth than that of transglutaminase. To test this possibility, the time course was measured after treating pre-confluent cultures with hydroxyurea, an inhibitor of ribonucleotide reductase (26). In preliminary trials, a concentration of 0.3 mM was found just sufficient to stop cell growth. While the protein content of cultures increased nearly 40%, due to an increase in cell size, the cell numbers remained static at approximately 20% of the number attained at confluence in untreated controls. In the treated cultures (Figure 2, top panel), transglutaminase increased in specific activity with essentially the same time course as in the control cultures. However, involucrin levels remained very low for the duration of the treatments, up to 4 weeks after confluence. Virtually identical results (27) were obtained using 1 mM α -difluoromethylornithine, a concentration of this potent inhibitor of ornithine decarboxylase (28) just sufficient to stop cell growth. In other experiments without growth inhibitors, qualitatively similar results were obtained when the cells were switched to serum-free medium at zero time. However, the uncoupling was not so dramatic, since cell growth continued for several generations approximately to confluence.

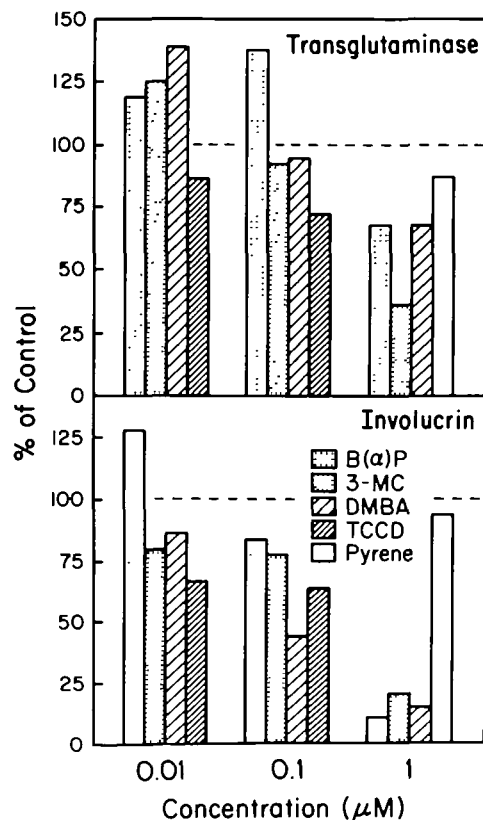


Fig. 3. Concentration dependence of polycyclic aromatic hydrocarbon and TCDD effects on transglutaminase and involucrin expression. Starting several days before confluence, cultures were exposed in duplicate for approximately 3 weeks to agents at the concentrations indicated and then assayed. The values illustrated give the means of two experiments in which control values (100%) for transglutaminase and involucrin were 4.1 nmol/mg/h and 11 ng/100 μg respectively.

PAH compounds

When growing SCC-9 cultures were treated with PAH compounds, initial experiments indicated that expression of involucrin was suppressed considerably more than that of transglutaminase. Figure 3 gives the results of treating the cultures with one of several such agents over a range of concentrations with subsequent assay of differentiation markers 2 weeks after confluence. As shown in the bottom panel, B[a]P, 3-methylcholanthrene (3-MC) or 7,12-dimethylbenz[a]anthracene (DBMA) at the highest concentration tested (1 μM) all reduced involucrin content to ~10% of the values in control cultures. By contrast, the structurally similar compound pyrene was ineffective. To determine whether this action was mediated primarily by the Ah receptor, for which the first three compounds but not pyrene are ligands, TCDD was also employed. TCDD generally produces its maximal effects in the concentration range of 10 nM, compatible with its affinity for the receptor, and is metabolized poorly if at all by the aryl hydrocarbon hydroxylase which it induces (3). The data show little effect of TCDD at 10 or 100 nM on involucrin accumulation. Thus, under these conditions the suppressive action of the PAHs is likely to be due to their metabolites and not simply from induction of the battery of genes controlled by the Ah receptor. As seen in Figure 3 (top panel), the level of transglutaminase in the cells was altered only to a modest extent at the high concentrations of the agents, where the activities were 40–70% of those in untreated controls. The

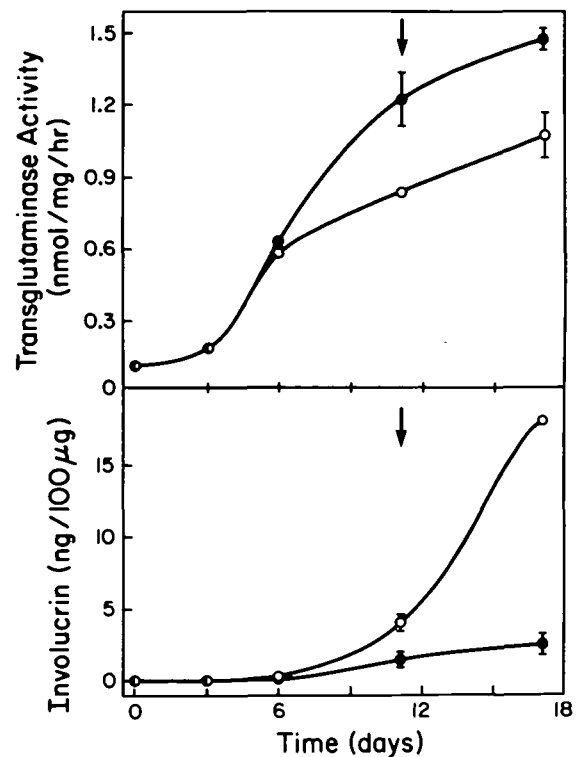


Fig. 4. B[a]P suppression of involucrin expression. Starting at day 0, 11 days before treated and control cultures reached confluence (vertical arrows), the medium was supplemented with 0.3 μM B[a]P (●) or solvent alone (○). This result is representative of three experiments. The involucrin content in the treated cultures did not increase with culture for an additional 2 weeks. The error bars show the range of values obtained for duplicate cultures, typical for all the experiments illustrated.

small effect on the transglutaminase level noted with TCDD in the growth medium was only marginally less than that obtained with B[a]P and DMBA, suggesting that metabolites of the latter were of minimal efficacy, in contrast to their influence on involucrin levels.

In the initial experiments illustrated in Figure 3, it was clear that 1 μM concentrations of the carcinogenic PHAs reduced the cell growth rate while TCDD did not. [The culture medium contained 1 μM hydrocortisone, which prevents inhibition of SCC-9 growth by TCDD (29).] To clarify whether the observed action of the PAHs could reflect a nonspecific toxicity, the time course of transglutaminase and involucrin expression was examined in cultures treated with 0.3 μM B[a]P, where growth inhibition was not apparent. The treated and control cultures reached confluence simultaneously. Involucrin was almost completely suppressed as seen in Figure 4 and for the duration of the analysis up to several weeks after confluence, while the transglutaminase was if anything slightly higher than in the untreated control cultures.

TPA

As in the case of most tumorigenic keratinocyte lines (30,31), the tumor promoter TPA had no inhibitory effect on growth of SCC-9 cells. Cultures were treated with various concentrations of this agent and after several weeks at confluence were assayed for transglutaminase activity and involucrin content. As shown in Figure 5, the expression of involucrin was suppressed half maximally at ≤1 nM and nearly completely (98%) at high concentration. Transglutaminase was also suppressed, although not as completely (85% at 1 μM) and with approximately 10-fold

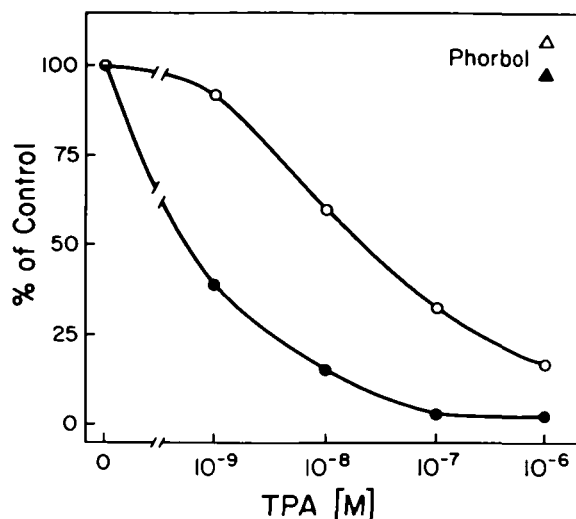


Fig. 5. Suppression of involucrin and transglutaminase by TPA. Duplicate cultures treated continuously for 26 days were harvested 19 days after confluence and assayed for transglutaminase (○) and involucrin (●). Specific activities obtained are presented as percentages of those for transglutaminase (3.8 nmol/mg/h) and involucrin (25 ng/100 μg) in untreated control cultures. Phorbol (1 μM) had no effect on levels of transglutaminase (Δ) or involucrin (▲).

higher EC₅₀. Upon acute treatment of confluent cultures with 0.3 μM TPA, the levels of neither involucrin nor transglutaminase changed significantly for at least a day afterwards. This finding indicates that changes were not occurring rapidly due to, for example, direct phosphorylation of either marker. In parallel, B[a]P also had little acute effect on confluent cultures (including morphology), although a modest reduction in marker levels occurred after several days.

To determine whether the observed virtually complete suppression of involucrin reflected a reduction in the level of involucrin message, RNA samples from cultures treated for several weeks at confluence were examined using an involucrin cDNA probe (20). When equal amounts of RNA were electrophoresed in agarose gels and hybridized with the probe, as illustrated in Figure 6, it was clear that the mRNA levels in cultures treated with TPA (lane 1) were dramatically less (not detectable) than those in the control untreated cultures (lane 2).

Discussion

The present results reveal distinct regulation of keratinocyte transglutaminase and involucrin during differentiation. Ordinarily, cells of the basal and immediately suprabasal layers of normal stratified squamous epithelia contain little if any of either protein. The marked increase in their expression at essentially the same point in the upper spinous layer of epidermis (10) could reflect quite similar or identical regulatory signals. However, the temporal difference in their appearance upon reaching confluence in SCC-9 cultures indicates that this coincidence *in vivo* cannot be interpreted so simply. Thus, involucrin expression in culture was considerably more sensitive than that of transglutaminase to suppression by the agents under study. Indeed, it is clear that treatment of SCC-9 cells with certain agents can completely uncouple expression of the markers, creating a state of intermediate differentiation in which signals necessary to proceed in the program are not generated. Although neoplastic cells usually exhibit peculiarities in their regulation of differentiation,

1 2



Fig. 6. Suppression of involucrin mRNA by TPA. Cultures treated continuously for 26 days with 0.3 μM TPA (lane 1) or solvent alone (lane 2) were harvested 19 days after confluence and the extracted RNA was probed with ³²P-labeled involucrin cDNA. The result is representative of two experiments.

and hence identification of such a state in normal cells is uncertain, this finding is likely to be of assistance in resolving elements co-ordinating the normal program.

The differentiation of many cell lines, including malignant keratinocytes (9), has been found to occur at the end of log phase growth as cells linger in the G₀ or G₁ parts of the cell cycle. The effect of antimetabolites such as hydroxyurea or α-difluoromethylornithine in suppressing involucrin could be attributed to arrest in the S phase instead. PAHs may well have a tendency to stop the cells outside of G₀ or G₁; genotoxic alkylating agents including aflatoxin B₁ (32) interfere with transit through S phase. In any case, it appears that expression of transglutaminase is not so strictly limited during the cell cycle since it is expressed by cells arrested in S phase.

In human and mouse epidermal cells, sensitivity to phorbol ester tumor promoters is hypothesized to require some differentiated function (33). Thus, these agents reduce colony formation and induce cross-linked envelopes in the majority of keratinocytes cultivated from normal human epidermis in low- or high-calcium medium (31), but a resistant population (presumably basal cells) is evident (30). Although these responses are not observed in keratinocyte carcinoma lines such as SCC-9 and SCC-25 (30,31), the present work shows that SCC-9 cells are indeed responsive to TPA, but not in growth inhibition. In contrast to mouse epidermal cells, which in low-calcium medium cease growing and exhibit increased transglutaminase activity upon TPA treatment (33,34), SCC-9 cells display a suppression of transglutaminase as well as involucrin. This action also occurs in the nontumorigenic keratinocyte line SCC-12F2 (21), which is known to be substantially reduced in colony forming ability by TPA (30,35).

Chronic treatment with TPA leads to dramatic depletion of protein kinase C in many cell types, including A431 (36) and SCC-12F neoplastic human keratinocytes (E.Choi and W.A. Toscano, Jr, personal communication). This phenomenon, which

may be due to enhanced proteolysis (37), has also been reported in mouse skin (38). It is plausible that the effect of this depletion is to prevent protein kinase C-dependent action in treated cells. If certain aspects of keratinocyte differentiation are stimulated by acute TPA treatment, then chronic treatment paradoxically may appear to suppress the differentiation. It could even be imagined that reactive B[a]P metabolites could have some affinity for and damage protein kinase C, thereby reducing the effective concentration of this enzyme and leading to the observed suppression of involucrin synthesis.

TPA has been found to alter the activity of DNA-binding proteins which serve as transcription regulatory factors (39,40), providing a possible mechanism by which the programming in treated cells could be affected. Although involucrin is quite sensitive to proteolytic degradation and could be subject to rapid depletion as a consequence of protease activation, it is clear that suppression of this marker occurs at the level of mRNA, due to alteration of its transcription, degradation or processing. The present results (differences in EC₅₀ and extent of suppression) suggest distinctions in the coupling of protein kinase C to the two differentiation markers studied. This finding could reflect either a divergence in the pathways by which each marker is indirectly affected or perhaps the separate influence of different protein kinase C isozymes having distinct enzymatic properties (41,42). Our recent observation of TPA-stimulated serine phosphorylation of the transglutaminase near its membrane anchorage region (R.Chakravarty and R.H.Rice, in preparation) raises the possibility of altered activity due to this (or other) post-translational modification.

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