Modulation of Tissue and Epidermal Transglutaminases in Mouse Epidermal Cells after Treatment with 12-O-Tetradecanoylphorbol-13-acetate and/or Retinoic Acid in Vivo and in Culture
Ulrike Lichti and Stuart H. Yuspa

ABSTRACT
Retinoic acid (RA) induces tissue transglutaminase (TGASE) and inhibits terminal differentiation induced either by calcium ion or by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in primary mouse epidermal cells in culture. The relevance of these effects on cultured cells to the antipromoting action of RA was investigated in female BALB/c and CD-1 mice in vivo. Tissue TGASE was distinguished from epidermal TGASE on the basis of different thermolability at pH 9 or elution from the anion exchanger Mono Q. After topical application of 3 to 5 μg (10 to 17 nmol) of RA to the shaved back skin, the specific activity of tissue TGASE increased up to 30-fold primarily in the basal cell fraction of Percoll-separated epidermal cells. Enzyme activity returned to basal levels by 7 days. Treatment with TPA (10 μg or 17 nmol/mouse) induced an increase in epidermal TGASE which reached a maximum at 12 h after application, primarily in suprabasal cells. RA applied 1 h before TPA caused no reduction of TPA-induced epidermal TGASE, but the increase in tissue TGASE due to RA was markedly inhibited by TPA. The effects of TPA and RA on TGASE activities in primary epidermal cells in culture were similar to those in vivo except that RA reduced the induction of epidermal TGASE by TPA. In culture the induction of epidermal TGASE by TPA was independent of Ca2+ concentration in the medium above 0.03 mM, but cornified envelope formation was markedly enhanced by Ca2+ above the level required for maintaining a basal cell population (0.03 to 0.05 mM). The TPA-induced formation of cornified envelope in the presence of elevated Ca2+ was completely inhibited by RA if cells were pretreated with RA for 24 h. Our results are consistent with RA causing a reprogramming of epidermal cells that alters their response to differentiation stimuli.

INTRODUCTION
Phorbol ester tumor promoter action in mouse epidermal cells appears to involve both acceleration of differentiation of keratinocytes which have made the commitment to differentiation as well as enhanced proliferation of basal keratinocytes (1). Carcinogen-altered cells (2) and papilloma-derived cells (3) have been found to be uniformly resistant to the differentiation-inducing influence of TPA.2 The dual action of the tumor promoter, which depends on the state of the target cell, should favor the clonal expansion of premalignant cells and may be critical for the process of papilloma development (4, 5).

The mechanism by which retinoids inhibit skin tumor promotion by phorbol esters (6) is unclear. There is evidence to suggest that suppression of polyamine metabolism by retinoids is a contributing factor (7, 8). However, the expected inhibition by retinoids of phorbol ester-stimulated epidermal DNA synthesis resulting from decreased polyamine synthesis has not been observed either in vivo (9) or in vitro (10). Nevertheless, selective suppression of proliferation of the carcinogen-altered cells by retinoids could account for their antipromoting activity. In fact, the growth in culture of a series of papilloma-derived cell lines was found to be markedly inhibited by RA (11).

Retinoids could also inhibit phorbol ester-mediated tumor promotion by interfering with the acceleration of differentiation of normal cells surrounding initiated cells. Retinoids are known to modify epidermal differentiation as determined by a reduction in differentiation-associated proteins and structures, namely certain keratins (12, 13), filaggrin,3 involucrin (14), epidermal TGASE (15–17), and CEs (15, 18–20).

In studies on the induction of epidermal TGASE during terminal differentiation of keratinocytes, it was discovered that RA induces tissue TGASE in cultured mouse epidermal cells (18, 21). Tissue TGASE can be distinguished from the epidermis-specific enzyme by its greater affinity for anion exchange resins, by being entirely cytosolic, and by its greater thermal sensitivity at pH 9 (21). The induction of tissue TGASE by retinoids has also been observed in human keratinocytes (17), in mouse macrophages (22), and in human myelocytic leukemia cells (23). This enzyme appears to be constitutively present in many types of cells, but its function is unknown (24). The conditions which favor tissue TGASE induction in cultured mouse keratinocytes are those that select for basal cells, suggesting that these cells are the source of the tissue enzyme in epidermis (21). These cells are also believed to be principal targets for phorbol esters in tumor promotion (25).

The current study was designed to analyze the kinetics and target cell specificity for the induction of both tissue and epidermal TGASEs by RA and TPA in vivo. Combined effects of the two agents were also evaluated under treatment conditions in vivo which are relevant to phorbol ester tumor promotion and its inhibition by retinoids. Finally the effects of these agents on TGASEs in epidermal cells in vivo and in vitro were compared to validate the in vitro model and to obtain clues to the underlying mechanisms of action of these agents.

MATERIALS AND METHODS
Treatment of Animals. The choice of the two mouse strains, BALB/c and CD-1, was based on the large body of information obtained in this laboratory on cultured epidermal cells from BALB/c mice and on the observations of the antipromoting effects of retinoids in CD-1 mice (6). No differences were found in the response of these two mouse strains to RA with respect to TGASE inductions. Female mice, 7 to 9 wk old, were obtained from the Frederick Cancer Research Facility, Frederick, MD (BALB/c) or from Charles River Breeding Laboratories, Portage, MI (Charles River CD-1). The back skins of mice were shaved with hair clippers, and 3 days later animals in the resting phase of their hair cycle were treated by applying 200 μl of acetone alone or containing RA or TPA to the shaved area. RA (all-trans; Hoffmann La Roche, Nutley, NJ) was dissolved in acetone at 5 mg/ml, stored at 4°C, and used within 1 wk. Dilutions in acetone were made immediately prior to application to the animals. TPA (LC Services, Woburn, MA) was dissolved in acetone at 30 mg/ml, stored at 4°C, and used within 1 wk. Dilutions in acetone were made immediately prior to application to the animals. TPA (LC Services, Woburn, MA) was dissolved in acetone at 30 mg/ml, stored at 4°C, and used within 1 wk. Dilutions in acetone were made immediately prior to application to the animals.

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1 To whom requests for reprints should be addressed, at NIH, Bldg. 37, Rm. 3B25, Bethesda, MD 20892.2 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; DTT, dithiothreitol; CE, cornified envelope; PBS, Ca2+- and Mg2+-free Dulbecco’s phosphate-buffered saline; RA, all-trans-retinoic acid; TGASE, transglutaminase; FPLC, fast protein liquid chromatography.

3 S. Nagae and U. Lichti, unpublished observations.
dissolved in acetone at 0.05 mg/ml just before use. Usually 5 animals were used per treatment group. Animals were sacrificed by cervical dislocation, and remaining hair was removed with treatment with Nair followed by washing with water, Betadine, water, and 70% ethanol. Depliation with Nair reduced the cell yield from TPA-treated epidermis and was omitted for all animals in experiments involving TPA treatments. Nair did not affect cell yield or TGASE activity from control or RA-treated epidermis.

Outline of Analysis of TGASEs in Epidermis. Epidermis was separated from dermis either by heat treatment (26) or by trypsin treatment (27). Epidermis obtained after treatment with trypsin was further separated into cornified material and dissociable cells. For some experiments, these cells were fractionated on Percoll (Pharmacia, Piscataway, NJ) density gradients (28) into basal and suprabasal cells. Extracts from cells were assayed for epidermal and tissue TGASEs either by the more rapid differential heat stability assay (see below) or by separation on the anion exchanger Mono Q (Pharmacia, Piscataway, NJ) using the Pharmacia FPLC system (21). The two methods gave comparable results. Extracts from whole epidermis or stratum corneum could only be monitored by Mono Q-FPLC, because the differential heat stability assay grossly underestimated the content of tissue TGASE in these extracts.

Separation of Epidermis by Heat Treatment. Back skin was removed and stretched dermis side up on the rough side of a porcelain spot plate. Subdermal fat, fascia, and muscle (hypoderms) were removed by scraping with a razor blade while holding the skin in place with a scoop-type spatula. The skin was pick up with filter paper applied to the dermal side, immersed in PBS (Ca2+- and Mg2+-free Dulbecco's phos- phate-buffered saline; Gibco, Grand Island, NY) at 37°C for 30 s, quickly cooled in ice-cold PBS, and returned to the porcelain block. More hypodermal material was scraped off, followed by removal of the epidermis from the opposite side of the dermis by scraping. Tissue fractions were chopped with scissors as necessary, quickly frozen on dry ice, pulverized in liquid nitrogen with mortar and pestle, and stored at −20°C.

Preparation of Epidermal Cells from Trypsin-treated Skin and Fractions on Percoll Gradients. Skin was prepared and stretched on filter paper as for the separation of epidermis by heat treatment. The skin was cut into three pieces and first floated on PBS (5 skins/150-mm culture dish). PBS was removed by suction, 10 to 20 ml of 1% trypsin (T-1005; Sigma, St. Louis, MO) in PBS were added, and the skins were incubated at 37°C for 45 min. Trypsin was removed by suction, the epidermis peeled from the dermis, and either the epidermis alone or in some cases both tissues were transferred to separate tubes containing 20 ml of high calcium medium (see “Other Methods” below) containing 0.2 μg/ml DNase (Type I; Coopertiomedical, Malvern, PA) and 8 μg/ml soybean trypsin inhibitor (Sigma, St. Louis, MO). Tissues were agitated on a rotary platform (100 rpm) with two or three brief vortexing periods during 10 to 15 min at room temperature. Tissues and sus- pended cells were filtered through 100-mesh Nytex (Martin Supply Co., Baltimore, MD), and tissues were rinsed with more medium. In cases where the stratum corneum was to be analyzed, it was washed with PBS by centrifugation, frozen on dry ice, and pulverized in liquid nitrogen. Cells in the combined filtrates were collected by centrifugation at 800 rpm for 3 min. Cells that were to be assayed without fractionation on Percoll were washed 3 times with PBS, counted, and frozen on dry ice. The yield of cells dissociated by this procedure was 10 to 13 million cells for epidermis and 1 to 3 million for the dermis. Cells to be fractionated on Percoll (28) were suspended after the first centrifugation in cold 50% Percoll (Percoll/high-calcium medium/10×PBS, 1/0.9/ 0.1, v/v/v) at a cell density of 1 million cells or less per ml. Density marker beads (Pharmacia) were added, and the tubes were centrifuged in the Sorvall SS34 rotor at 10,000 rpm for 40 min. Morphology confirmed that cells lighter than the 1.062 g/cm2 red marker beads were primarily suprabasal and that those heavier were basal cells. Percoll fractions were diluted at least 5-fold with PBS, and cells were recovered by centrifugation at 200,000 for 5 min, washed 2 more times with PBS (800 rpm, 3 min), counted, and frozen.

Preparation of Cell Extracts. Frozen cells and pulverized tissues were suspended in Buffer A [0.02 M sodium phosphate (pH 7.2)/0.5 mM EDTA/10 mM DTT/50 μg/ml phenylmethylsulfonyl fluoride] at a concentration of approximately 5 to 10 million cells/ml, one epidermis/ ml, one dermis/ml, and one hypoderms/5 ml. Cells were sonicated with five 10-s bursts, and tissues, with ten 10-s bursts of the sonicator (Heat Systems Model W185; Heat Systems-Ultrasonics, Inc., Plain- view, NY; microprobe, setting 4) with 10-s cooling periods between sonication periods. For chromatography on Mono Q, Triton X-100 (Research Products International Corp., Elk Grove, IL) was added to the resulting lysate (L1) to a final concentration of 1%, and the lysate was incubated at 37°C for 5 min yielding lysate L2. This was centrifuged at 45,000 rpm for 60 min to yield the Triton-soluble extract S2, which was filtered through a 0.2-μm filter before chromatography.

Differential Heat Stability Assay for TGASE Activity in Epidermal Cells. Cell lysates in Buffer A (L1) were centrifuged at top speed (approximately 130,000 × g) in the Beckman Airfuge for 15 min to yield S1, the buffer-soluble enzyme fraction containing all of the tissue and some of the epidermal TGASEs. S2, obtained by centrifuging the Triton-containing lysate (see above), contains all of the tissue and most of the epidermal TGASEs. Preincubating S1 or S2 with all of the assay components except putrescine (i.e., at pH 9) at 37°C for 10 min leads to loss of 90 to 100% of tissue TGASE with recovery of virtually all epidermal TGASE activity (21). Measuring TGASE activity by the standard assay at 28°C in lysates, supernatants, and preincubated supernatants [e.g., S1 (37°C)] allows calculation of the fraction of epidermal TGASE in the original lysates: [L2 − S2 + S2 (37°C)/L2] for L2 and [L1 − S1 + S1 (37°C)/L1] for LI, assuming 100% destruction of tissue TGASE and 100% survival of epidermal TGASE during the preincubation. In experiments where these values were compared, the same fractions of epidermal TGASE activity were obtained, suggesting that cellular components solubilized by Triton X-100 do not influence the heat stability of the enzymes. In most experiments the specific activity of S2 was determined, and the fraction of epidermal TGASE in S2 was calculated from the ratio of the activities in S2 (37°C) and S2. Relative specific activities of epidermal and tissue TGASE deter- mined by this procedure agreed closely with results from FPLC separations performed on the same S2 fractions when epidermal cells were analyzed (correlation coefficient = 0.99 for epidermal TGASE and 0.98 for tissue TGASE). The absolute values for both enzyme activities were consistently higher in the thermal stability assay relative to the FPLC assay by a factor close to that expected from the recovery of total activity from the column (e.g., a factor of 1.7 for 60% recovery). Thus this difference is probably due to the decreased stability of the enzymes after partial purification on Mono Q.

Other Methods. Assay for TGASE at pH 9 and chromatography of extracts on Mono Q were performed as previously described (21). Enzyme activity elution profiles from Mono Q columns are given as a ratio, expressed as the percent activity of 52 was determined, and the fraction of epidermal TGASE in S2 was calculated from the ratio of the activities in S2 (37°C) and S2. Relative specific activities of epidermal and tissue TGASE deter- mined by this procedure agreed closely with results from FPLC separations performed on the same S2 fractions when epidermal cells were analyzed (correlation coefficient = 0.99 for epidermal TGASE and 0.98 for tissue TGASE). The absolute values for both enzyme activities were consistently higher in the thermal stability assay relative to the FPLC assay by a factor close to that expected from the recovery of total activity from the column (e.g., a factor of 1.7 for 60% recovery). Thus this difference is probably due to the decreased stability of the enzymes after partial purification on Mono Q.

RESULTS

TGASE Activity in Various Layers of Skin from RA-treated Mice. At the start of these investigations, separation of epider-
TGASEs. Note different scales for the three ordinales. TGASE activities in C
the column. Epidermis was separated from dermis by the heat treatment method.
sacrifice. Enzyme activity values were normalized to 1 mg of protein applied to
of epidermis (A), dermis (B), and hypodermis (C) of BALB/c mice treated with
contamination of epidermal preparations with hypodermal ma-
terial could significantly alter the measured content of tissue
not substantially affected by the RA treatment. Therefore, slight
TGASE (Fig. 1C). The enzyme appears to be constitutive and
and RA-treated animals contained very high levels of tissue
corresponds to that of the major activity in epidermis, while
identity of these activities was not further investigated.
In contrast to other layers of skin, hypodermis of both control
and RA-treated animals contained very high levels of tissue
TGASE (Fig. 1C). The enzyme appears to be constitutive and
of control mice (Fig. IA) may be largely of hypodermal origin.
us to suspect that the enzyme found in heat-separated epidermis
fractions of control, trypsin-separated epidermis (Fig. 2) lead
altered elution profile which suggests differentiation-associated
stratum corneum was virtually devoid of tissue TGASE activity.
other fractions by basal cells; however, the results suggest
induction of tissue TGASE in the lower spinous cells as well as
in basal cells. The highest specific activity of epidermal TGASE
was found in the fraction enriched in suprabasal cells. The
stratum corneum was virtually devoid of tissue TGASE activity.
The epidermal TGASE activity in this fraction showed an
altered elution profile which suggests differentiation-associated
processing of this enzyme. The low tissue TGASE levels in all
fractions of control, trypsin-separated epidermis (Fig. 2) lead
us to suspect that the enzyme found in heat-separated epidermis
are considered to be more reliable and form the basis of
the conclusions drawn from our studies. The specific activity of
total, epidermal, and tissue TGASE was similar for trypsin-
separated and heat-separated portions of epidermis from the
same group of animals, suggesting that destruction of TGASEs
by the heat treatment did not occur.

RA Dose-Response of Tissue TGASE Induction. The RA
dose-response for tissue TGASE induction was measured in

![Fig. 1. Separation on Mono Q of TGASE activities in Triton X-100 extracts
of epidermis (A), dermis (B), and hypodermis (C) of BALB/c mice treated with
10 µg of RA in 200 µl of acetone/mouse (+) or with acetone alone (--) 24 h
before sacrifice. Enzyme activity values were normalized to 1 mg of protein
applied to the column. Epidermis was separated from dermis by the heat
treatment method. The arrows indicate elution positions of epidermal (Peak I)
and tissue (Peak II) TGASEs. Note different scales for the three ordinales.
TGASE activities in C superimposed at almost all points for the two treatment
groups.](cancerres.aacjrnl.org)

![Fig. 2. Epidermal and tissue TGASE activities in basal (B), suprabasal (SB),
and cornified (C) layers of epidermis from BALB/c mice treated as in Fig. 1
with RA or acetone (Ac). Epidermis was obtained by trypsin treatment of skin,
and cells from the epidermis were separated by Percoll gradient centrifugation
into basal and suprabasal cells. A, TGASE activity profiles normalized to 1 mg
of protein of Triton X-100 extracts separated on Mono Q; B, summation of
peak area activities: Fractions 8 to 16 for epidermal (solid bars) and Fractions
21 to 24 for tissue (hatched bars) TGASE, activity corresponds to that in 1 mg
of protein.](cancerres.aacjrnl.org)
Percoll-fractionated cells from trypsin-separated epidermis by the differential heat stability assay. Histological examination showed that the trypsin treatment allowed some epidermal cells to remain associated with the dermis; therefore cells that could be released from the dermis by agitation in culture medium were also assayed but not fractionated on Percoll. Mice received a single application of RA and were sacrificed 22 h later. Fig. 3 shows that maximum induction of tissue TGASE was produced by 3 to 10 μg of RA per mouse.

Fig. 3A shows calculated values of epidermal and tissue TGASE activities for all epidermis-derived cells combined including epidermal cells that were loosely associated with the dermis. Fig. 3, B and C, shows tissue and epidermal TGASE activities, respectively, assayed in basal, suprabasal, and dermis-associated epidermal cells. The combined values in Fig. 3A were calculated on the basis of the fraction of cells obtained from each source, which ranged from 9 to 22% (12.6 ± 5.1) for dermis-associated, 15 to 26% (19.7 ± 4.2) for basal, and 63 to 74% (67.9 ± 6.6) for suprabasal epidermal cells for the various groups of animals treated with the different amounts of RA. The variation in cell recoveries did not correlate with the concentration of RA used in the treatment. The trypsinization procedure appears to leave a substantial number of basal (and suprabasal) epidermal cells attached to the dermis which show a similar response to RA treatment as basal cells. Their higher specific activity for both epidermal and tissue TGASEs compared to the basal cell enzyme levels suggests that some enzyme activity is lost during the Percoll fractionation procedure to which the epidermis-derived cells were subjected. In order to obtain a more complete representation of basal cells, it may be necessary to combine the loosely attached cells from the dermis with those obtained from the epidermis before Percoll fractionation.

Induction of Epidermal TGASE by TPA in Basal and Suprabasal Epidermal Cells. The time course for changes in TGASE activities after treatment of mice with 10 μg of TPA is shown in Fig. 4. Epidermal cells obtained from trypsin-separated epidermis were fractionated on Percoll and assayed by the differential heat sensitivity assay. Epidermal TGASE increased primarily in the suprabasal cell-enriched populations with a time course similar to that observed in vitro (32). Enzyme induction is thought to be accelerated in those cells that have made the commitment to terminal differentiation, which includes some cells in the basal cell layer as well as suprabasal cells. The slight increase in tissue TGASE is a consistent finding (cf. Fig. 5A); the significance of this is not understood. It may be due to inflammatory cells infiltrating from the dermis.

Time Course for Tissue TGASE Induction by RA and Effect of TPA. The effect of combined treatments with TPA and RA on TGASEs in epidermis was examined both in heat-separated epidermis and in cells from trypsin-separated epidermis. The

![Graph](https://example.com/graph.png)
results were found to be similar for the two separation methods. The more extensive study involving FPLC analysis of extracts from unfractionated epidermal cells is shown in Fig. 5. In the combined treatments, the RA treatment preceded the TPA application by 1 h. The area under the activity peaks of the FPLC elution profiles normalized to 1 mg of protein is plotted as a function of time after treatment for tissue (Fig. 5A) and epidermal (Fig. 5B) TGASEs.

The results in Fig. 5A show that the early increase in tissue TGASE induced by RA is followed by a slow decline to basal levels by Day 7 after treatment. In comparison the decline in epidermal TGASE, induced by TPA, was more rapid (Fig. 5B). RA did not suppress the endogenous level nor the TPA-induced increase in epidermal TGASE. TPA had only a slight effect on the basal level of tissue TGASE, but markedly suppressed the RA-induced increase of this enzyme (Fig. 5A). The expected suppression by RA of TPA-induced epidermal TGASE observed in vitro (see below) did not appear to occur in vivo. It is reasonable to expect various subpopulations of epidermal cells to respond differently to RA. Selective suppression of epidermal TGASE by RA in basal cells was not observed, however. Specific activities (pmol/mg ± SD, n = 3 to 5) of epidermal TGASE in basal cells assayed 18 to 22 h after treatment by the differential heat stability assay were 95.3 ± 24.0 for TPA, 80.9 ± 15.1 for RA, 140.3 ± 61.3 for RA + TPA, and 27.0 ± 16.6 for acetone.

Characteristics of Differentiation of Epidermal Cells in Culture Induced by Ca²⁺ and TPA. Primary mouse epidermal cells in culture can be made to mimic the behavior of cells in vivo by controlling the calcium concentration in the medium (31). The induction of epidermal TGASE by TPA is most easily demonstrated in cells cultured in low-calcium medium in which the endogenous level of epidermal TGASE is low (32). Raising the calcium concentration of the medium at the time of treatment with TPA did not affect the increase in TPA-induced epidermal TGASE activity 8 h later as seen in Fig. 6A. This suggests that calcium from the medium is not required for the induction of the enzyme. On the other hand, the formation of cornified envelopes within 48 h after treatment with TPA was markedly dependent on the calcium concentration in the medium (Fig. 6B). The requirement for elevated calcium concentration for cornified envelope formation presumably reflects the calcium dependence of TGASE activity necessary for cross-linking to occur, but may also be necessary for the synthesis of precursor proteins for the cornified envelope. The cross-linking process occurs in vivo in cells of the upper granular layer (33), and in vitro in the absence of TPA in a small fraction of the cells maintained in high-calcium medium (Fig. 6B).

Inhibition of Cornified Envelope Formation by RA in Culture. Previously the inhibition by RA of cornified envelope formation in mouse epidermal cells, induced to differentiate in high-calcium medium, was demonstrated by measuring the reduction in the number of ϵ-(γ-glutamyl)lysine cross-links (18). The inhibition by RA of TPA-induced cornified envelope formation in the presence of high-calcium medium is shown in Fig. 7. Pretreatment of cells with RA before exposure to TPA gave the greatest inhibition of cornified envelope formation. A similar requirement for pretreatment of cells with RA was also observed for inhibition of calcium-induced cornified envelope formation in a papilloma-derived cell line in which a large number of cornified envelopes can be induced by calcium alone (20).

Antagonism between RA and TPA or Ca²⁺ in the Induction of TGASEs in Cultured Epidermal Cells. The requirement for a pretreatment with RA for complete inhibition of cornified envelope formation suggested that the cells become altered in their ability to respond to TPA or calcium during exposure to the retinoid. Therefore in the examination of the antagonism between RA, TPA, and calcium in the induction of TGASEs in cultured cells, the order in which cells were exposed to these agents was varied. Treatment times were chosen for maximum induction of TGASEs by the various agents, i.e., 8 h for TPA-induced epidermal TGASE and 48 h for calcium-induced epidermal or RA-induced tissue TGASEs. Triton X-100 extracts of cells were examined by the FPLC method for the contents of the two TGASEs. The results in Table 1, Experiment 1, show that the induction of epidermal TGASE by TPA is diminished by RA, particularly if the cells were first pretreated with RA. RA also reduced epidermal TGASE induction by calcium (Table 1, Experiment 2), and pretreatment of cells with the retinoid enhanced the effect. As in vivo, TPA essentially prevented the induction of tissue TGASE by RA (Experiment 2), but could not reverse the induction which resulted from the
Antagonism between retinoic acid and TPA or Ca\(^{2+}\) in the induction of transglutaminases in cultured primary mouse keratinocytes

Primary mouse epidermal cells were grown in low-calcium medium for 6 days. Pretreatment and treatments were in low-calcium (0.05 mm Ca\(^{2+}\)) medium unless indicated by “Ca\(^{2+}\)” (high-calcium medium, 1.2 mm). Concentrations of agents were 3 \(\mu\)M RA and 0.17 \(\mu\)M TPA. Triton X-100 extracts of cells were chromatographed on Mono Q, and peak area activities were normalized to 1 mg of protein applied to the column.

<table>
<thead>
<tr>
<th>Pretreatment (24 h)</th>
<th>Treatment (8 or 48 h)*</th>
<th>Epidermal Peak I</th>
<th>Tissue Peak II</th>
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</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>79.3</td>
<td>16.2</td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>227.7</td>
<td>20.6</td>
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<td>TPA + RA</td>
<td>149.8</td>
<td>10.3</td>
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<tr>
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</tr>
<tr>
<td>RA</td>
<td>95.5</td>
<td>210.1</td>
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</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
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<tr>
<td>RA</td>
<td>401.0</td>
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<tr>
<td>Ca(^{2+}) + RA</td>
<td>336.4</td>
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<td></td>
</tr>
<tr>
<td>RA + Ca(^{2+})</td>
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<tr>
<td><strong>Effect of TPA and Ca(^{2+}) on RA-induced tissue transglutaminase</strong></td>
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<tr>
<td>RA</td>
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<td>2062.6</td>
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</tr>
<tr>
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<td>114.6</td>
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<td>336.4</td>
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<tr>
<td>Ca(^{2+}) + RA</td>
<td>953.5</td>
<td>382.0</td>
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</table>

* Cells were analyzed for constituent TGASEs by the FPLC method 8 h after treatment in Experiment 1 and 48 h after treatment in Experiment 2. One entry appears twice in the table for ease in comparisons.

Discussion

In experiments demonstrating the inhibition of mouse skin papilloma formation by RA, the retinoid is effective at 1.7 to 17 nmol/mouse (0.5 to 5 \(\mu\)g/mouse) applied 1 h before each treatment with the promoter, typically 10 \(\mu\)g of 17 nmol of TPA 2 times per wk (6). The retinoid is required throughout the entire promotion phase or at least during the second stage of a two-stage promotion protocol (34). Furthermore, RA enhances rather than inhibits complete carcinogenesis by 7,12-dimethylbenz[a]anthracene (35), and RA itself has promoting activity when applied repeatedly to carcinogen-initiated skin (36, 37). RA does not inhibit tumor promotion by some agents chemically unrelated to phorbol esters (38). These findings suggest that the retinoid, when applied with TPA, counteracts the phorbol ester action specifically.

If both the induction of terminal differentiation and the stimulation of proliferation are required for phorbol ester-mediated tumor promotion, an analysis of the influence of retinoids on these pathways is important for an understanding of their antipromoting activity. We have focused our studies on the differentiation pathway, since it has been shown that retinoids do not reduce phorbol ester-mediated hyperplasia (9, 10). Our studies demonstrate that the response of epidermal cells to TPA and RA with respect to TGASE induction is remarkably similar in vivo and in vitro. The induction of epidermal TGASE by TPA in vivo parallels previously reported data on the induction of epidermal differentiation in vitro (1). Furthermore RA reduced tissue TGASE primarily in basal cells in vivo and in vitro. In combined treatments with TPA and RA, RA was found to suppress the RA-induced increase in tissue TGASE both in vivo and in vitro. However, although retinoic acid markedly reduced the induction of epidermal TGASE and associated formation of cornified envelopes in vitro, retinoic acid did not inhibit, but slightly enhanced, the induction of epidermal TGASE in vivo under the single treatment conditions studied. The observed enhancement could be the result of a decreased rate of cell death in the upper layers of the epidermis accompanied by a reduced turnover of preexisting epidermal TGASE. Suppression of TPA-induced increase in extractable epidermal TGASE activity is therefore not a mechanism by which RA counteracts the tumor-promoting activity of TPA. It must be emphasized, however, that our data give information on total enzyme activity that can be extracted from cells and tissue but not on intracellular activity. Whether the intracellular activity of epidermal TGASE in vivo is modulated by RA remains to be determined.

Two major differences between the in vivo and in vitro experiments may explain the differing responses of the induction of epidermal TGASE by TPA to RA: (a) cells at risk and (b) the relative concentrations of RA and TPA. Many of the cells in which TPA induces epidermal TGASE in vivo are suprabasal (Fig. 4) and may have no counterpart in low-calcium cultures. The induction of TGASE by TPA in these cells may not be affected by RA. In the experiment shown in Fig. 5 total, rather than separated, basal and suprabasal cells were assayed. Hence the fraction of cells in which epidermal TGASE induction by TPA can be inhibited by RA may be small in vivo and large in vitro. Analysis of basal cells from animals treated with both TPA and RA did not reveal selective suppression of epidermal TGASE in this population, but the results are complicated by an increase of this enzyme activity after treatment with RA alone. The ratio of the concentrations of TPA and RA in vivo is close to 1, while the concentration of RA was almost 18 times higher than that of TPA in the culture medium. The choice of doses of TPA and RA used in vivo was guided by those used for tumor promotion experiments in BALB/c and CD-1 mice. The doses of TPA and RA used in vitro were those giving maximal induction of epidermal (39) and tissue (18) TGASE, respectively. A high dose of RA is required in vitro probably because the half-life of RA in culture is about 10 h due to rapid metabolism by the cells. RA at 3 \(\mu\)M did not appear to be toxic to confluent epidermal cells as used in these experiments, judging by cell count.

Whether the induction of tissue TGASE by RA plays a role in the inhibition of tumor promotion is still unclear. The optimal dose for induction of tissue TGASE in vivo is of the order of magnitude found effective for the inhibition of tumor promotion (6). Hence the induction of tissue TGASE could play a significant role in the subsequent behavior of the cells in which it occurs. Under the single-exposure conditions of our experiments, TPA treatment of mice markedly reduced the induction of tissue TGASE by RA, suggesting that this manifestation of RA action may be unrelated to its antipromoting action. To entirely rule out a role for tissue TGASE in antipromotion, however, the effect of repeated applications of RA and

* L. M. De Luca and U. Lichti, unpublished observations.

79
TPA on TGASEs in the epidermis will have to be examined. As the time course of TGASE induction (Fig. 5) indicates, the next application of RA and TPA in a promotion/antipromotion protocol would occur at a time when epidermal and possibly tissue TGASEs are still elevated (72 to 96 h) relative to levels in untreated skin. Hence the consequences of a second and subsequent treatments may very well be different from those of the first treatment.

Epidermal cells transformed in culture or in vivo generally are more sensitive to RA with respect to induction of tissue TGASE. Some of these cell lines have high endogenous levels of TGASE activity (40, 41), later demonstrated to be of the tissue type (21). This could be merely a reflection of a more homogeneous cell population in the cell lines in which a greater percentage of the cells respond. Alternatively, it could represent a significant differential response pattern of normal and neoplastic cells to retinoids. Nevertheless, in two cell lines tested so far, TPA also reduced the induction of tissue TGASE by RA.5

The designation of Peak I activity as epidermal TGASE was made on the basis of this being the predominant form in untreated epidermis as well as in TPA- or Ca2+-treated epidermal cells (21). Peak II activity from RA-treated cultures grown in low-calcium medium was identified as tissue TGASE on the basis of cross-reactivity with anti-guinea pig liver TGASE antiserum (42). The RA-induced Peak II activity from mice in vivo behaves like the corresponding activity from cultures not only on Mono Q but also with respect to solubility and heat sensitivity. Heterogeneity of Peak I activity has been observed frequently (cf. Figs. 1 and 2). It is not clear whether this is due to different enzymes, to differentiation-associated processing of the epidermal enzyme, or to enzymatic modification during the extraction procedure. Several reports published recently suggest the existence of several types of cellular TGASEs as well as different forms of a given type of TGASE (43–46).

The large number of cornified cells in intact skin makes it difficult to measure an increase in these structures due to TPA treatment in vivo. In vitro the increase is rapid and substantial. The inhibition of formation of cornified envelopes by RA was virtually complete in parallel with the effect on epidermal TGASE if cells were pretreated with RA before exposure to TPA. This could mean that fewer cells entered the stage of differentiation in which cells become responsive to TPA by an increase in epidermal TGASE and cornified envelope formation. In a papilla-derived cell line (PE), which responds to calcium, but not to TPA, by a substantial increase in epidermal TGASE and cornified envelopes, RA was found to inhibit TGASE induction only 50% under conditions where cornified envelope formation was completely blocked (20). This suggests that RA interferes at a point in differentiation beyond TGASE induction, e.g., at formation of cornified envelope precursor protein synthesis or envelope assembly (activation of TGASE). This question can be approached when markers for cornified envelope precursor proteins become available for mouse epidermis.

Our evidence is consistent with RA altering the response of epidermal cells to the differentiation-inducing effect of TPA. In vivo several applications of the retinoid may be required to demonstrate a difference in response to TPA. At this point one can only speculate about the nature of the alteration in RA-treated cells, whether the change is at the level of protein kinase C or a consequence of the interaction of TPA with this or other receptors. The involvement of protein kinase C in the induction of epidermal TGASE by TPA is indicated by the ability of phospholipase C or diacylgycerols to induce this enzyme (47). The altered response to phorbol esters is likely to be the result of a significant reprogramming of basal keratinocytes under the influence of RA (42), possibly involving tissue TGASE-mediated modification of regulatory molecules.

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Modulation of Tissue and Epidermal Transglutaminases in Mouse Epidermal Cells after Treatment with 12-\(\alpha\)-Tetradecanoylphorbol-13-acetate and/or Retinoic Acid \textit{in Vivo} and in Culture

Ulrike Lichti and Stuart H. Yuspa


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