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PHORBOL ESTER TUMOR PROMOTERS INDUCE EPIDERMAL TRANSGLUTAMINASE ACTIVITY

Stuart H. Yuspa, Theresa Ben, Henry Hennings and Ulrike Lichti

In Vitro Pathogenesis Section, Laboratory of Experimental Pathology, National Cancer Institute, Bethesda, MD 20205

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ABSTRACT

Epidermal basal cells in culture have low levels of epidermal transglutaminase, the enzyme responsible for the formation of the cross-linked envelope in differentiated cells. The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and other active (but not inactive) phorbol ester skin tumor promoters induce transglutaminase activity. Sloughing of differentiated cells accompanies the rise in transglutaminase activity. Phorbol esters do not affect transglutaminase activity when added directly to cell lysates. Corticosteroids have little influence on transglutaminase induction by phorbol esters. Retinoic acid induces transglutaminase activity, but activity does not further increase when basal cells are treated with both retinoic acid and 12-O-tetradecanoylphorbol-13-acetate.

INTRODUCTION

Phorbol ester tumor promoters produce a variety of biological, physiological and biochemical changes in vivo and in vitro. Those effects which are relevant to tumor promotion have been difficult to assign because of the large number of cellular responses and the often disparate nature of responses in different cell types. Since phorbol esters are promoting agents primarily in mouse skin and other squamous epithelia in vivo, the availability of a mouse epidermal cell culture model (1) has been useful to define and study responses in the primary target tissue for tumor promotion. Recently this approach has been enhanced by the development of techniques which selectively cultivate the epidermal basal cell, the putative target cell for epidermal carcinogenesis (2).

Abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; TG, transglutaminase.

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Among the many biological effects of phorbol esters, alterations in differentiation have received wide attention (3). Both induction and inhibition phenomena have been observed. In general little is known of the mechanism involved in any of the observed alterations in differentiation. Major advances in epidermal biology in recent years have led to a much greater understanding of the events involved in epidermal differentiation (4,5). In particular the enzyme epidermal transglutaminase, a calcium-dependent soluble protein, appears to play a central role in the formation of the cornified envelope (6-9). This study was designed to investigate the effect of phorbol esters on the activity of epidermal transglutaminase in an effort to define and explain the phorbol ester induced alterations in epidermal differentiation.

MATERIALS AND METHODS

Source of Chemicals:

Phorbol esters were obtained from Chemical Carcinogenesis, Eden Prairie, MN. [2,3-^{3}H(N)]-putrescine (25-40 Ci/mmole) was from New England Nuclear. Mezerine and fluocinolone acetonide were generously supplied by Dr. Thomas Slaga, Oak Ridge National Laboratory. Retinoic acid was from Hoffman-La Roche. Putrescine dihydrochoride and Trizma Base were from Sigma (St. Louis, MO). Casein (Hammerstem) was from Schwartz Mann (Orangeburg, NY) ethylene diamine tetracetic acid (EDTA) and $CaCl_2$ were from Fisher (Fairlawn, NJ), DMSO (silylation grade) was from Pierce Chemical Co (Rockford, IL) and ethylene bis (oxyethylenenitrilo)-tetracetic acid (EGTA) was from J.T. Baker (Phillipsburg, NJ).

Cell Culture:

Epidermal cells were prepared from Balb/c newborn mice as previously described (10) and plated at $8x10^6$ cells per 100 mm culture dishes. Cultures were grown under low calcium conditions (0.07mM) in Medium 199 and 2% chelex treated fetal calf serum as reported elsewhere (2). Six days after plating, cells were exposed to phorbol esters, other test agents or solvent (0.1% DMSO). At appropriate times after exposure, medium was removed, the cells washed 3 times with assay buffer (see below) and frozen.

Transglutaminase Assay:

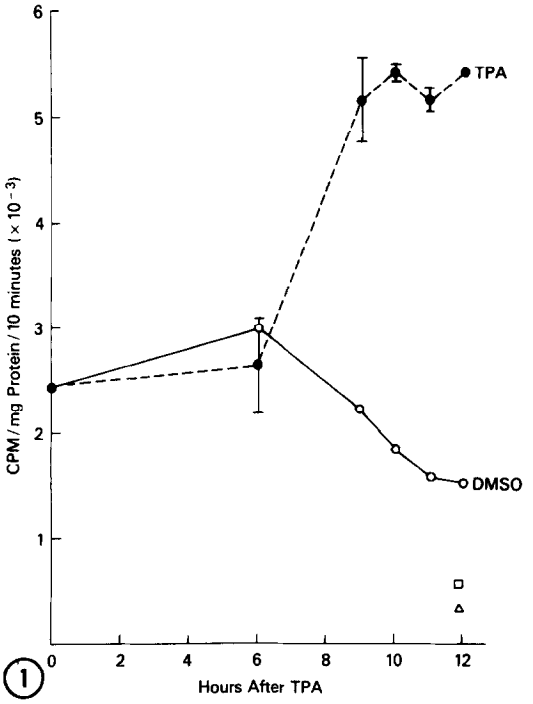
Epidermal transglutaminase was assayed by an adaptation of the method of Ogawa and Goldsmith (7) which measures the enzyme catalyzed formation of ε amino-γ-glutamyl bonds between ^{3}H-putrescine and casein. Approximately $6x10^6$ epidermal cells were lysed by freeze-thawing in 300 µl of buffer mixture composed of 50 mM Tris (pH 7.5), 2.5 mM dithiothreitol, 0.13 M NaCl, 0.83 mM EDTA and 8.3 mM $CaCl_2$. The reaction mixture consisted of a total of 200 µl as follows: 100 µl cell lysate, 20 µl casein (20 µg/ml), and 30 µl ^{3}H-putrescine (5 mM final concentration) and the additional 50 µl as buffer or EGTA (100 mM). After 10 minutes at 37°, 50 µl of reaction mixture was spotted on Whatman 3MM filter paper strips (previously washed with 50 µl of 100 mM EGTA and dried) and immediately immersed

in ice cold 10% TCA containing 1.0% putrescine. Filter papers were gently agitated through 3 TCA washes for 20 minutes each, rinsed with ice cold absolute ethanol and dried. Radioactivity bound to casein which precipitated on filter paper was counted in Instagel (Packard, Downers Grove, IL) in a Beckman LS 300 scintillation counter. Background radioactivity from parallel assays lacking cell lysate was substracted from all samples. Protein concentration for each lysate was determined by the method of Lowry as detailed by Lane (11). The Ca^{++} dependence of enzymatic activity was confirmed by the addition of a final concentration of 25 mM EGTA to the assay mixture. Preliminary experiments indicated linear enzyme kinetics at 37°C during 20 minutes of assay, therefore, assays were terminated after 10 minutes of incubation. All assays were performed in duplicate on replicate samples from each experiment and all experiments were performed at least twice.

RESULTS

Previous studies had indicated that cultivation of mouse keratinocytes in medium with a reduced calcium concentration resulted in selective monolayer growth of cells with characteristics of basal cells (2,12). One such characteristic was a relatively low level of transglutaminase activity (13) since that enzyme is associated with the differentiated cell layer (6). Upon elevation of extracellular calcium concentration, attached cells undergo a programmed differentiation which is associated with an elevation in transglutaminase activity and cornified envelope production (2,13,14 and unpublished results). When cells grown under low calcium conditions are exposed to TPA (Fig 1), transglutaminase activity increases after a lag of 6-8 hours. In multiple experiments, peak activity increases of 3-5 fold occurred generally 12-14 hours after TPA exposure commenced. Activity returned toward basal levels by 24-48 hours. Figure 1 also indicates that the transglutaminase activity increase is due to induction of new protein synthesis rather than a direct TPA activation of the enzyme since the activity increase is inhibited by either actinomycin D or cycloheximide at doses which inhibit RNA or protein synthesis by >95%. Further evidence against direct activation of transglutaminase activity is seen in Fig. 2. Addition of a series of TPA doses directly to the assay system had no significant effect on activity.

The induction of transglutaminase activity occurs at TPA doses as low as 10 ng/ml (1.6×10^{-8}M) and is optimal at doses around 100 ng/ml



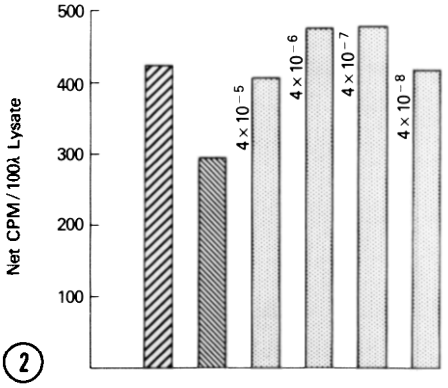


Figure 1. Epidermal transglutaminase activity in mouse epidermal basal cells exposed to TPA. Epidermal cells were grown for 6 days prior to treatment in 0.02 mM Ca^{++} medium. TPA (1.6×10^{-8}M) or DMSO (0.1%) exposure was begun at 0 time and samples were removed at intervals for transglutaminase assays. Each point represents duplicate assays performed on duplicate samples. ●-----● TPA; O-----O DMSO. Other plates were treated in an identical fashion except that 20 μg/ml cycloheximide □ or 0.1 μg/ml actinomycin D △ were added along with TPA and samples were removed at 12 hours.

Figure 2. Effect of adding TPA to transglutaminase assay. Replicate epidermal cells lysates were assayed for transglutaminase activity with addition of 5μl buffer ▨, 5μl DMSO ▨ or 5μl TPA solution in DMSO ▨ to give a final concentration of TPA indicated on figure in molarity.

(Fig 3). At higher doses (1000 ng/ml) the level of induction decreases dramatically. This decrease at higher dose is not due to a change in the kinetics of induction since activity is lower at all time points studied over a 24 hour interval. Figure 2 had indicated that even high concentrations of TPA did not directly inhibit enzyme activity. Therefore the decreased induction at higher TPA concentrations must reflect either a physiological change in the cell or a non-specific toxic effect.

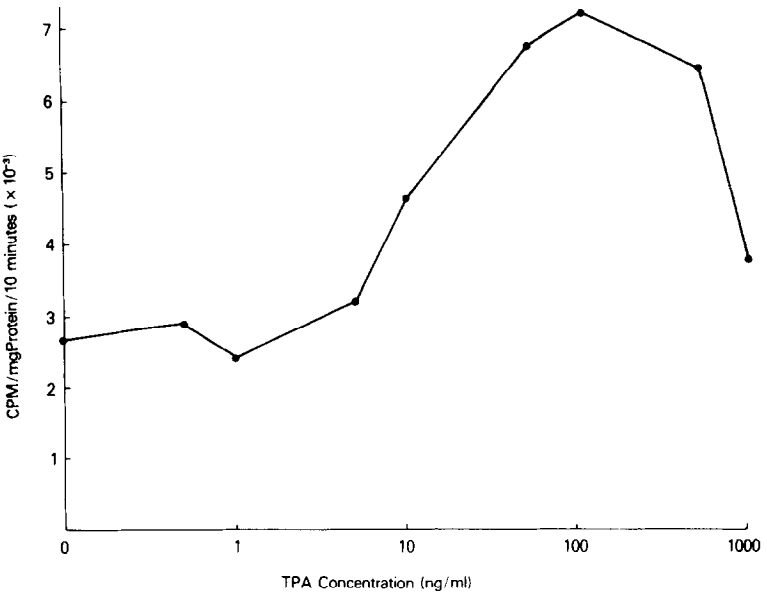


Figure 3. Effect of TPA concentration on induction of epidermal transglutaminase. The protocol was identical to that of figure 1. Samples were removed for assay 14 hours after exposure to various concentrations of TPA.

To determine if the induction of transglutaminase was a property shared by other biologically active phorbol esters and analogues, epidermal basal cells were exposed to a number of these compounds and transglutaminase activity assayed after 14 hours. Figure 4 demonstrates that TPA, phorbol dibenzoate and mezerine all were active for this property. Mezerine was approximately equipotent to TPA as has been previously reported for many other properties of mezerine except complete tumor promotion (15). Phorbol dibenzoate was slightly less active than TPA which is in agreement with its promoting potency (16). The non-promoting analogues, 4-O-methyl TPA, phorbol-13-acetate and phorbol (not shown) were without effect on TG activity.

Antipromoters are known to modify a number of specific effects of phorbol esters (1,17), but their role in differentiative responses has not been well characterized. Corticosteroids and retinoids are of particular interest since they are potent inhibitors of skin tumor promotion. Fluocinolone acetonide, a potent steroidal antipromoter, does not

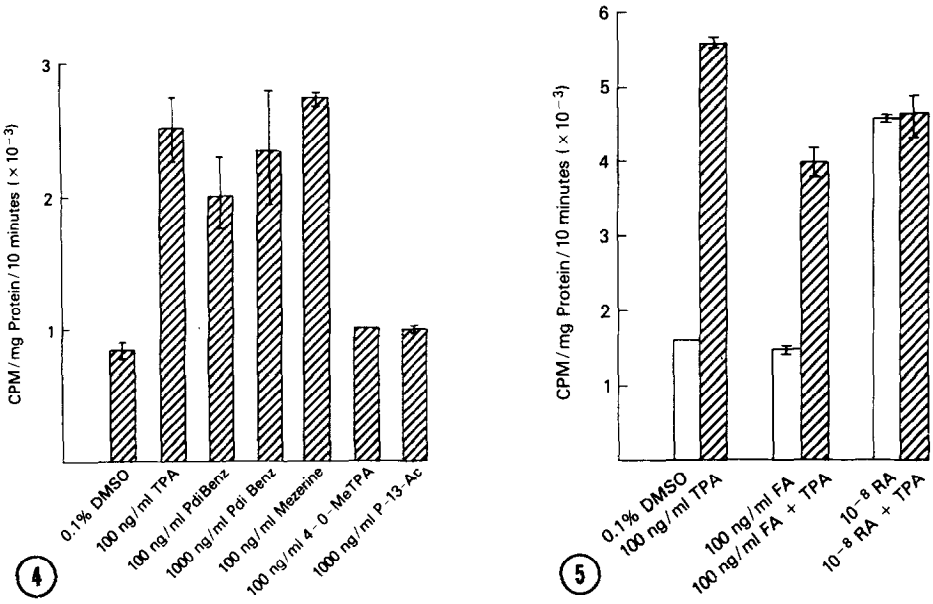


Figure 4. Epidermal transglutaminase activity in mouse epidermal basal cells exposed to various phorbol esters and mezerine. The protocol was identical to that of figure 1 and all samples were assayed at 14 hours after exposure. PdiBenz, phorbol dibenzoate; 4-O-Me TPA, 4-O-methyl TPA; P-13-Ac, phorbol-13-acetate.

Figure 5. Effect of antipromoters on the induction of epidermal transglutaminase by TPA. The protocol was identical to that of figure 1 and antipromoters and TPA were added simultaneously at 14 hours before freezing samples for assay. FA, fluocinolone acetonide; RA, retinoic acid.

itself alter transglutaminase activity and has only a slight modifying effect on TPA induced activity (Fig 5). Retinoic acid is a potent inducer of transglutaminase as we have reported (13). TPA, in concert with retinoic acid, produces no additional increase in TG activity above the retinoid alone suggesting TG activity is maximally induced by either retinoid or TPA. Alternatively the retinoid may be capable of blocking a further induction by TPA.

DISCUSSION

TPA and other active phorbol esters are capable of increasing epidermal transglutaminase activity in a population of basal cells in vitro. Morphologic changes (see below) which accompany TG increase suggest that terminal

differentiation is induced. Studies utilizing inhibitors of protein or RNA synthesis indicate that the increase requires new protein synthesis but cannot distinguish between synthesis of TG itself or of an activator molecule. Interestingly, in our studies, cycloheximide alone failed to increase TG activity, whereas cycloheximide at very high doses was reported to accelerate cross-linking in human keratinocytes (9). Direct activation of TG activity by TPA is unlikely since TPA was without effect when added directly to the cell lysate and since there is a lag of 8 hours between TPA exposure and enzyme activity increase. Direct activation of TG enzyme activity has been reported in erythrocytes treated with calcium ionophores (18) and in lymphocytes exposed to supraoptimal concentrations of plant lectins (19).

A previous report suggested that hydrocortisone could induce epidermal transglutaminase in embryonic chick skin explants and this was associated with accelerated differentiation (20). In our studies, the steroid fluocinolone acetonide did not induce activity and in concert with TPA caused a slight inhibition of the induction. Retinoic acid, a potent antipromoter, induced TG activity without producing a morphological change in the cells. We have reported that retinoic acid inhibits morphological differentiation in our culture model (13) when differentiation is induced by increasing extracellular Ca^{++}. Thus, elevated TG activity is not necessarily associated with induction of morphological differentiation. A striking morphological change occurs in a subpopulation of basal cell cultures exposed to TPA. These changes (decreased adhesiveness, distinct cell outlines, rounded shape and sloughing) are consistent with induced differentiation and correspond temporally to the transglutaminase increase. Thus the TPA altered cellular environment must be permissive for differentiation to occur in association with elevated transglutaminase activity while the retinoic acid treated cell cannot differentiate in association with elevated TG activity.

The results obtained in this study are most readily interpreted to indicate that TPA and other active phorbol esters induce differentiation in a

portion of the epidermal basal cell population. One mechanism whereby such an induction could occur would be via altered Ca^{++} flux or redistribution of intracellular Ca^{++} produced by TPA (21). Changes in environmental calcium are known to regulate epidermal differentiation in vitro (2,12,14). Other data support the conclusion that TPA induces epidermal differentiation. There is an accelerated appearance of the "differentiated pattern" of epidermal keratin proteins after TPA treatment in vitro (22). TPA produces accelerated sloughing of keratinized material into the culture medium and rapid maturation of ^{3}H-thymidine prelabeled cells (unpublished data). In vivo TPA increased the synthesis of differentiation associated proteins (23,24). In contrast, evidence for a dedifferentiation or blocked differentiation of epidermis produced by promoters has also been presented (25,26). Taken together these results indicate that subpopulations of basal cells may respond differently to the same exposure, perhaps related to their degree of commitment to differentiate at the time of treatment.

How can the induction of epidermal differentiation reported here and elsewhere relate to the promoting properties of phorbol esters? Recently we have reported that treatment of keratinocyte cultures with chemical carcinogens yield cell foci which fail to terminally differentiate when challenged to do so by elevation in medium Ca^{++} (1,27). If such cells are initiated cells, they would have a distinct growth advantage if surrounding normal cells were induced to differentiate in some way, for example, by promoter treatment. Ultimately, such foci could expand into a tumor. Such a possibility is testable with in vitro model systems and is currently under study.

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