Divergent Responses in Epidermal Basal Cells Exposed to the Tumor Promoter 12-O-Tetradecanoylphorbol-13-acetate

Stuart H. Yuspa,1 Theresa Ben, Henry Hennings, and Ulrike Lichti

In Vitro Pathogenesis Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Maryland 20205

ABSTRACT

Mouse epidermal basal cells can be selectively cultivated in medium with 0.02 to 0.09 mM Ca2+ and can be induced to differentiate by medium containing 1.2 mM Ca2+. Basal cell cultures were studied to determine if all cells in this population responded identically to the skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Studies on the induction of the enzyme epidermal transglutaminase by TPA demonstrated a 2- to 4-fold increase in activity within 12 hr of exposure. This activity increase paralleled morphological differentiation in approximately 50% of the basal cell population, and differentiating cells sloughed from the culture dish within 24 to 48 hr as transglutaminase activity returned to basal levels. The cells which remained were resistant to induced differentiation by 1.2 mM Ca2+ medium, in that they failed to demonstrate increased transglutaminase activity or decreased thymidine incorporation, both characteristics of control basal cells induced to differentiate by 1.2 mM Ca2+. Cells remaining after a single exposure to TPA did not respond to a second exposure with an induction of transglutaminase if the interval between exposures was 4 days. TPA-pretreated cells did not undergo a transient decrease in thymidine incorporation (characteristic of control cells) when exposed to TPA a second time but instead were directly stimulated to proliferate by the phorbol ester, indicating that such cells were not refractory to the promoter. When the treatment-free interval after TPA was extended from 4 to 10 days, transglutaminase inducibility was restored in basal cells to either TPA or 1.2 mM Ca2+ as inducers. These results indicate that heterogeneity exists within the epidermal cell population and that exposure to phorbol esters induces differentiation in some cells, while stimulating proliferation in others. Such heterogeneous responses would cause a selective redistribution of the epidermal cell population and could lead to clonal expansion of initiated cells.

INTRODUCTION

Phorbol esters are potent tumor promoters for initiated mouse skin and induce a variety of biological and biochemical changes in skin and in cultured cells. In cell culture, changes produced by phorbol esters, as reported from various laboratories, have often appeared contradictory and vary depending on the species or cell type studied, culture conditions, or phorbol ester concentration. Phorbol ester-induction changes in mouse skin have been more consistent from laboratory to laboratory. Nevertheless, the responses in skin seem paradoxical, since both differentiative and proliferative processes (generally considered mutually exclusive in the skin) are stimulated with about the same temporal sequence (27). For example, the induction of polyamine biosynthesis (19) and the decrease in histidase activity (5), considered as proliferative functions in skin, coincide with the induction of transglutaminase activity (28) and stimulation of keratin protein synthesis (25), which are differentiation-related activities. Likewise, the peaks of stimulated DNA synthesis and synthesis of the differentiation-specific histidine-rich protein coincide in TPA-treated mouse skin (13).

An unusual and poorly studied aspect of tumor promotion on mouse skin is the change in tissue responses which occur with multiple exposures to phorbol esters when exposures occur with the frequency required for tumor promotion. For example, the induction of ODC2 is more rapid and of greater magnitude with multiple exposures (16, 18), and the stimulation of DNA synthesis occurs earlier, reaches a higher peak value, and lacks the initial inhibitory phase (23). Likewise, multiple TPA exposures greatly amplify the elevation of epidermal cyclic guanosine 3':5'-monophosphate, which is barely measurable after a single exposure (7). These enhanced responses are not maintained if intervals between promoter exposures are prolonged, and prolonged intervals also eliminate the promoting action of these agents. Sensitization of the tissue toward enhancement of certain (proliferative) responses suggests that cells particularly sensitive for this response program to phorbol esters may be selected during the promotion process. Alternatively, cells could be transiently altered by a single exposure to respond differently to subsequent treatments.

Recently, this laboratory has developed techniques to selectively culture mouse epidermal basal cells (10, 29). Epidermal basal cells can be selectively cultivated by growth in medium with reduced ionic calcium concentration (0.02 to 0.09 mM) (10). These cells have morphological, cell kinetic, and marker protein characteristics of basal cells (30) and grow as a monolayer with a high proliferation rate. Basal cells can be subcultured and cloned (32). When the Ca2+ concentration of culture medium is elevated to levels found in most commercial preparations (1.2 to 1.4 mM), proliferation ceases, and terminal differentiation rapidly ensues with squamous differentiation and sloughing of cells occurring by 72 to 96 hr (10). Using this methodology, we have reported that basal cells are the principle target for the induction of ODC by phorbol esters and that the ability to respond to phorbol esters is lost rapidly after the cells become committed to differentiate (15). It is not known if committed cells are capable of responding to phorbol esters by parameters other than ODC induction. If only basal cells are responsive, considerable heterogeneity must exist among basal cells because of the divergence of the program of induced responses toward both differentiation and proliferation.

1 To whom requests for reprints should be addressed.
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This report presents evidence for the existence of heterogeneity among basal cells with regard to pharmacological responses to TPA. Since the divergent responses would result in the loss of one cell compartment and the expansion of the other, we propose that these programmed responses are crucial for tumor promotion.

**MATERIALS AND METHODS**

**Cell Culture.** Epidermal cells were isolated from newborn BALB/c mice by methods described previously (28). Medium 199 without Ca²⁺ was obtained from the NIH Media Unit and supplemented with 2% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.), which had been passed over a column of Chelex resin to remove calcium (10). Calcium chloride was then added to a final concentration of 0.07 mM (low Ca²⁺) or 1.2 mM (high Ca²⁺). Isolated cells were plated in 0.07 mM Ca²⁺ medium on plastic culture dishes and maintained in this medium for 4 to 7 days prior to first exposure to phorbol esters. For experiments in which several treatments were examined with a 10-day interval, cells were grown in Ca²⁺-free Eagle's minimal essential medium (M. A. Bioproducts, Walkersville, Md.) plus 10% Chelex-treated fetal bovine serum (0.02 mM Ca²⁺) (10). Both minimal essential medium and Medium 199 also contained penicillin (100 units/ml), streptomycin (100 µg/ml), and Fungizone (0.25 µg/ml) (Grand Island Biological Co., Grand Island, N. Y.). Because of the differentiation-inducing effects of TPA, treated cultures were plated at 10 x 10⁶ cells/100-mm dish, while controls were plated at 5 x 10⁵ cells/100-mm dish, so that approximately equal cell numbers were available in posttreatment studies.

**Chemicals.** TPA was obtained from Chemicals for Carcinogenesis Research, Eden Prairie, Minn., and dissolved in dimethyl sulfoxide for use in cell culture (29). [methyl-³H]Thymidine (6 Ci/mmol) was from New England Nuclear, and the chemicals used in biochemical assays were from sources reported previously.

**Biochemical Assays.** Assays for the induction of DNA synthesis (33) and epidermal transglutaminase activity (28) have been described previously. All experiments were performed at least twice with a minimum of duplicate samples.

**RESULTS**

When cultured basal cells (0.07 mM Ca²⁺) are exposed to TPA, there is a striking morphological change in some cells. While the untreated population is relatively homogeneous, certain cells in the treated cultures could be observed to undergo a morphological change, consisting of a transient dendritic shape, cell rounding, and detachment from the monolayer over a 24-hr period (Fig. 1). Examination of detaching cells indicates a 7-fold increase in cornified envelopes, determined by their resistance to detergents (8) when compared to controls. Morphological changes parallel a substantial increase in epidermal transglutaminase activity (28), the enzyme responsible for formation of the cornified envelope. As the rounded cells detach, transglutaminase activity decreases. This response to TPA appears to be a programmed induction of terminal differentiation in some epidermal cells, while other cells in the same culture are not induced to differentiate by TPA (Fig. 1).

To determine if cells resistant to the differentiation-inducing effect of TPA were nevertheless altered by the promoter, cultures containing basal cells which survived the treatment with TPA were washed free of cornified cells within 24 to 48 hr of TPA exposure and studied further. They were compared to previously untreated epidermal cells with regard to their re-

**Fig. 1.** Effect of TPA treatment on morphology of epidermal basal cells. Mouse epidermal cells were plated at 10 x 10⁶ (TPA group) or 5 x 10⁵ (dimethyl sulfoxide control) per 100-mm plate in 0.07 mM Ca²⁺ medium and treated with 0.1% dimethyl sulfoxide (A), or TPA (50 ng/ml) (B) on Day 5 in culture. Phase-contrast photomicrograph was taken 12 hr later when many cells were rounded and forming cornified envelopes in the TPA-exposed group. ×100.
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response to a signal to differentiate (high Ca²⁺ medium which induces differentiation in untreated basal cell cultures) and in their response to a second TPA exposure. Chart 1 represents activity of epidermal transglutaminase at various times following the transfer of TPA-pretreated or control basal cells to medium containing 1.2 mM Ca²⁺. Control cultures show a rapid increase in transglutaminase activity within 24 hr which is sustained as the cells undergo terminal differentiation. This is associated with the formation of cornified squamae (10, 11). In contrast, epidermal transglutaminase activity did not immediately rise in response to Ca²⁺ in TPA-pretreated cells, suggesting a block in response to a signal for differentiation in these cells.

Characteristically, basal cell cultures have a high proliferation rate when measured by thymidine incorporation into DNA (10). When extracellular Ca²⁺ is increased to induce terminal differentiation, the rate of DNA synthesis drops rapidly (10). This is seen in control cultures depicted in Chart 2, where thymidine incorporation decreases by 60 to 70% within 48 hr after basal cells are exposed to 1.2 mM Ca²⁺ medium. We have shown previously that TPA exposure to cultured epidermal cells causes a significant decrease in thymidine incorporation for 24 hr, which is followed by a significant increase in DNA synthesis (33). If basal cell cultures (0.07 mM Ca²⁺) treated with TPA are switched to 1.2 mM Ca²⁺ at the time of maximum decrease in DNA synthesis (24 hr, zero time in Chart 2), they are insensitive to the effect of Ca²⁺ on proliferation and demonstrate a steep increase in [methyl-³H]thymidine incorporation over the next 48 hr before declining to the lower rates of control cells at later times. Thus, basal cells selected by a TPA treatment (which are resistant to TPA-induced differentiation) do not respond to the Ca²⁺ signal by rapidly turning off proliferation and commencing differentiation as in control cells. The insensitivity of these TPA-pretreated cells to the Ca²⁺ signal, both for increased transglutaminase activity and DNA synthesis depression, persists for some time without further TPA exposure, suggesting that this subpopulation of TPA-treated basal cells is in a proliferative mode and is incapable of undergoing terminal differentiation.

In vivo data (7, 16, 18, 23) have indicated that a single TPA treatment of mouse skin alters the response to subsequent treatments if the interval is sufficiently short, and the foregoing studies suggest that this may be due to selection of cells with a particular response pattern. Several characteristic TPA responses were studied in basal cells treated previously with TPA in vitro. Chart 3A indicates that basal cells, pretreated previously with a single TPA exposure in vitro and cultured in the absence of TPA for 4 days, show no depression of DNA synthesis upon retreatment but are stimulated to proliferate directly, just as mouse skin in vivo (13, 23). This is in contrast to control basal cells treated primarily with TPA in which a steep depression of thymidine incorporation occurs followed by a recovery. Similar differences are observed when epidermal transglutaminase is studied (Chart 3B). TPA-pretreated basal cells fail to demonstrate a rise in transglutaminase activity (and also do not produce cornified cells) when treated a second time with an interval of 4 days. Control cells demonstrate the expected 2- to 3-fold increase. These studies indicate that basal cells remaining several days after TPA exposure are resistant to the differentiative effects of phorbol esters but are responsible to the proliferative effects.

The altered response pattern to a second TPA exposure or to 1.2 mM Ca²⁺ is not a permanent characteristic of the basal cells persisting after a single exposure to TPA. Chart 4 indicates that TPA-pretreated basal cells cultured for 10 days in

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Chart 1. Effect of TPA pretreatment on Ca²⁺-induced epidermal transglutaminase activity. Mouse epidermal cells were plated at 10 × 10⁶ (TPA group) or 5 × 10⁶ (dimethyl sulfoxide control) per 100-mm plate in 0.07 mM Ca²⁺ medium and treated with TPA (100 ng/ml) or dimethyl sulfoxide (0.1%) on Day 5 in culture. After a 24-hr exposure (zero time), cultures were washed and refed in 1.2 mM Ca²⁺ medium, and duplicate plates were frozen at indicated times. Transglutaminase activity was assayed on frozen-thawed cell lysates as described previously (28), except that activity was based on DNA rather than on protein as the denominator to exclude protein contributed by dead squamae which develop in 1.2 mM Ca²⁺ medium.

Chart 2. Effect of TPA pretreatment on the inhibition of proliferation associated with Ca²⁺-induced differentiation. The culture and treatment conditions were the same as described in Chart 1. At each time point studied, duplicate dishes were pulsed for 1 hr with 1 μCi [methyl-³H]thymidine per ml prior to harvest. DNA-specific activity was assayed as described previously (17).
The absence of phorbol esters have regained the capacity to differentiate in response to 1.2 mM Ca\(^{2+}\). Similarly, these cultures also demonstrate a transglutaminase induction in response to another TPA exposure (data not shown). Morphological studies confirm that heterogeneity in response to TPA has been reestablished during a 10-day interval and is similar to that shown in Fig. 1, although it is uncertain whether the same proportion of cells is induced to differentiate.

**DISCUSSION**

Earlier studies on the incorporation of [methyl-\(^3\)H]thymidine into DNA and on the induction of ODC activity by phorbol esters, using a culture model containing cells of all epidermal strata, had led us to propose that several classes of responding cells with differing sensitivities to TPA exist in the epidermis (15, 16, 33). This conclusion was supported by studies of the kinetics of ODC induction in cultures containing only basal cells exposed to TPA, which indicated that response kinetics fell into 2 patterns, either a 3- or 9-hr peak, with TPA concentrations below or above 10 ng/ml, respectively (15). Other data utilizing polyacrylamide gel electrophoresis indicated that TPA accelerated the shift from a basal cell keratin protein pattern to a stratum corneum pattern when epidermal cells were exposed in vitro (25). In contrast, morphological data indicated that TPA was also capable of inhibiting epidermal differentiation in vitro under conditions where control cells were highly differentiated (33). Thus, it appeared from *in vitro* studies that TPA had both accelerating and inhibitory effects on keratinocyte differentiation.

The capability to selectively cultivate epidermal basal cells in medium with a low Ca\(^{2+}\) concentration has provided a unique opportunity to study the interaction of phorbol esters with their putative target cells in skin. It should be emphasized that reduced Ca\(^{2+}\) growth conditions appear to select for normal basal cells. These cells have a basal cell ultrastructure, have a high growth fraction and growth rate, and synthesize basal cell-specific proteins (29). The possibility that Ca\(^{2+}\) may be a physiological regulator of epidermal differentiation in vivo has been discussed previously (9, 10). Recently, a unique Ca\(^{2+}\) binding protein for skin has been described and localized solely to the basal layer (24). Its function is currently unknown. In our *in vitro* model, basal cells cultured in <0.1 mM Ca\(^{2+}\) medium appear to respond to phorbol esters as do basal cells in vivo; at least for all parameters studied so far. We have already demonstrated that basal cells appear to be the target cell for ODC induction (15), that TPA induces basal cells to mature (28), and that specific new epidermal proteins are synthesized in response to phorbol esters (3).

These current results could explain a puzzling aspect of the biology and pharmacology of phorbol esters in skin. We have now demonstrated divergent responses among subpopulations of epidermal cells to the same TPA exposure with the induction of differentiation and physical loss of one subpopulation and stimulated growth of another population. The stimulated cells predominated *in vitro* and are transiently resistant to differentiation signals (1.2 mM Ca\(^{2+}\) or a second TPA exposure), presumably because they are in a proliferative program. They respond to retreatment with TPA by a direct stimulation of DNA synthesis (without an initial inhibition). These cells are also more sensitive to ODC induction by phorbol esters\(^3\) and thus are not refractory to TPA. It appears that this population, either constitutively or because of an effect of TPA, has only a single (proliferative) response pattern. Given enough time between TPA exposures,

\(^3\) U. Lichti and S. H. Yuspa, unpublished data.
heterogeneity of responsiveness is restored. These results mimic the response patterns for multiple TPA exposures in vivo (7, 16, 18, 23).

Several other aspects of these in vitro observations are consistent with in vivo data. Careful morphological and cell kinetic analyses of mouse epidermis treated with TPA have indicated that a rapid migration of cells out of the basal layer occurs shortly after exposure (1, 6). There is a large increase in the number of nucleated suprabasal cells prior to the first mitotic peak (1, 21). This latter observation had been attributed previously to reactivation of nuclear and cytoplasmic organization in superficial cells which were otherwise not seen in control skin (21). A more plausible explanation is that basal cells, induced to differentiate by TPA, migrate into the upper layers during the first 12 to 24 hr after exposure. This same reasoning would explain the initial inhibition of DNA synthesis which is observed after TPA exposure, since the proliferative pool would be transiently decreased of basal cells induced to mature prior to the activation of the proliferative subpopulation at 18 to 24 hr.

The cellular or molecular basis for the heterogeneity of epidermal basal cells remains to be determined. Previously morphological and cell kinetic data have suggested that basal cells in the interfollicular epidermis are heterogeneous (12, 20). Basal cells with differing cell cycle characteristics have been described. It has also been proposed that maturation begins while cells are still present in the basal layer. Our data are the first indication that basal cells may differ in their pharmacological responsiveness, although morphologically they appear homogeneous in vitro. TPA exposure results in the death and loss of one population via terminal differentiation and stimulates the growth of another. Further studies of the apparently homogeneous stimulated cells may yield information concerning the molecular or cellular basis for their response patterns. The finding that cultures return to divergent response patterns after a prolonged TPA-free interval suggests that TPA is not causing cells to change behavior to a uniform response through genetic alterations in some cells. It seems more likely that basal cells differ in their maturation potential in vitro as in vivo. TPA may accelerate differentiation in more mature cells and prevent differentiation in less mature cells by stimulating proliferation. In the absence of TPA, a maturing population reaccumulates, and this population is then again stimulated to terminal differentiation by TPA. With frequent TPA exposure, the cells would remain in a less mature state (21). Studies to confirm this mechanism for heterogeneity are currently in progress.

The studies reported here demonstrate the ability of phorbol esters to produce a balanced and programmed heterogeneous response in mouse epidermal cells with regard to differentiation and proliferation. These programmed responses would result in a significant growth advantage to the growth-stimulated population at the same time it provided space for cell proliferation along the basement membrane because of the loss of other basal cells by induced differentiation and migration out of the basal layer. In other studies, we have developed evidence that initiated cells demonstrate an altered program of differentiation in that they are less likely to respond to a signal to differentiate (14, 34). Such cells might be expected to respond to TPA in the proliferative mode, find room to expand due to loss of surrounding cells, and clonally expand. As suggested previously, the clonal expansion of differentiation-altered cells could yield a benign tumor (30, 31, 34), which is the major lesion in 2-stage skin tumorigenesis. Our model requires that a subsequent change in a papilloma cell is necessary for carcinoma development (31). In 2-stage protocols in mouse skin, carcinomas appear to develop from papillomas independently of continued promoter exposure; that is, the same number of carcinomas develop relative to papillomas if TPA is continued or discontinued after papilloma formation (2, 26). Thus, the conversion step(s) from benign to malignant is likely to be independent of phorbol esters (30, 31). This model of phorbol ester action in vivo differs from reports on the ability of these agents to promote malignant transformation in certain cell lines in vitro (4, 17). This paradox suggests that phorbol esters may have additional biological activities when used under some conditions in cell culture, although their effects on cultured mouse epidermal cells appear to correlate closely to their activity on mouse skin.

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