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Initiator and Promoter Induced Specific Changes in Epidermal Function and Biological Potential

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Mouse epidermal basal cells can be selectively cultivated in medium with a calcium concentration of 0.02–0.09 mM. Terminal differentiation and sloughing of mature keratinocytes occur when the calcium concentration is increased to 1.2–1.4 mM. When basal cell cultures are exposed to chemical initiators of carcinogenesis, colonies of cells that resist calcium-induced differentiation evolve. Likewise, basal cells derived from mouse skin initiated in vivo yield foci that resist terminal differentiation. This defect in the commitment to terminal differentiation appears to be an essential change in initiated cells in skin and is also characteristic of malignant epidermal cells. This model system has also provided a means to determine if basal cells are more responsive to phorbol esters than other cells in epidermis and to explore the possibility that heterogeneity of response exists within subpopulations of basal cells. The induction of the enzyme ornithine decarboxylase (ODC) was used as a marker for responsiveness to phorbol esters. ODC induction after exposure to 12-0-tetradecanoylphorbol-13-acetate (TPA) in basal cells is enhanced 20-fold over the response of a culture population containing both differentiating and basal cells. When basal cells are induced to differentiate by increased calcium, responsiveness to TPA is lost within several hours. In basal cell cultures, two ODC responses can be distinguished. After exposure to low concentrations of TPA or to weak promoters of the phorbol ester series, ODC activity is maximal at 3 hr. With higher concentrations of TPA, the ODC maximum is at 9 hr. These results are consistent with the presence of subpopulations of basal cells with differing sensitivities to TPA. Other studies that use the enzyme epidermal transglutaminase as a marker for differentiation support this conclusion. In basal cell culture TPA exposure rapidly increases transglutaminase activity and cornified envelope development, reflecting induced differentiation in some cells. As differentiated cells are sloughed from the dish, the remaining basal cells proliferate and become resitant to induced differentiation by 1.2 mM calcium. These data provide additional evidence of basal cell heterogeneity in which TPA induces one subpopulation to differentiate while another is stimulated to proliferate and resists a differentiation signal. Tumor promoters, by their ability to produce heterogeneous responses with regard to terminal differentiation and proliferation, would cause redistribution of subpopulations of epidermal cells in skin. Cells that resist signals for terminal differentiation, such as initiated cells,

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would be expected to increase in number during remodeling. Clonal expansion of the intitiated population could result in a benign tumor with an altered program of differentiation. In skin, benign tumors are the principal product of 2-stage carcinogenesis. Subsequent progression to malignancy may involve an additional step, probably a genetic alteration, that is independent of the tumor promoter.

Key words: tumor promotion, carcinogenesis, epidermal cells, 12-0-tetradecanoylphorbol-13-acetate (TPA), terminal differentiation, initiation, transglutaminase, ornithine decarboxylase

Studies performed in mouse skin have provided the basis for subdividing chemical carcinogenesis into 2 stages, initiation and promotion [1, 2]. Important concepts in carcinogenesis first noted in skin studies were the covalent binding of initiators to cellular macromolecules, the irreversibility of initiation, and the reversibility of tumor promotion. More recently these concepts have been confirmed in a variety of in vivo and in vitro models. During the last several years our laboratory and others [3–6] have used cultures of mouse epidermal cells to study chemical carcinogenesis. The use of such a specialized cell culture model has the distinct advantage of a large in vivo data base with which to validate results obtained under the artificial conditions of in vitro growth. In virtually every instance where in vivo and in vitro comparisons have been possible, results in vitro have reproduced or predicted the data available from studies on mouse skin in vivo [5]. Epidermal cell cultures also provide a model to study the relationship between normal differentiation and malignant transformation and to identify specialized markers associated with the transformed state. The parallels defined for the in vivo and in vitro skin models predicted that in vitro epidermal carcinogenesis would be subdivided into initiation and promotion phases to allow independent study of each stage. In addition it was expected that carcinogen treatment would yield cells with preneoplastic characteristics, as well as fully malignant cells, thus providing a variety of cell types for further study. These predictions have been confirmed recently [5, 7, 8].

We have tried to use the biology of skin carcinogenesis in vivo as a guide to design in vitro experiments that would yield relevant data at the cellular level. Little is known of the biology of initiation. The change occurs rapidly, after a single exposure to initiator and in only a few cells, as best as can be determined. Initiation is heritable, appears to be associated with carcinogen-induced DNA damage, and occurs with greater frequency when the target tissue is rapidly proliferating. Initiation produces no recognizable biochemical or biological change in the cell in the absence of promotion. Much evidence suggests that initiation is a genetic event, but the nature of the change has not been defined.

Promotion requires repeated exposures and considerable time to produce tumors. The biological changes produced by promoters have been well characterized [9, 10]. If one lists the carcinogenesis model systems where promotion has been demonstrated (skin, liver, esophagus, colon, bladder, breast, stomach, respiratory tract) [11], it is clear that the phenomenon occurs in the epithelium of complex tissues. Most commonly, target epithelial cells are organized in a stratified or maturing arrangement, usually in a terminally differentiating lining epithelium. It is not clear whether tissue complexity is a fundamental requirement for a multistage mechanism or if other, less complex, organ sites have simply not been adequately explored. In mouse skin, the most widely studied model for tumor pro-

motion, basic biological observations have indicated that tissue complexity may be associated with a requirement for a multistage mechanism of carcinogenesis (see below). The most potent promoters in mouse skin are esters of the diterpene alcohol phorbol isolated from Croton tiglium plants. The most active of the phorbol esters is 12-0-tetradecanoylphorbol-13-acetate (TPA), which has been extensively studied in skin and cell culture and has provided many clues to the mechanism of promotion. Table I summarizes some biological observations from the promotion phase of skin carcinogenesis in which phorbol esters were used as promoters. While there is a requirement for prior initiation for tumor production, promoters seem to induce biochemical changes in many or all epidermal cells, not just initiated cells. Repeated promoter exposures sensitize the tissue for certain responses to subsequent exposure such as stimulation of DNA synthesis [12], stimulation of ornithine decarboxylate induction [13, 14], and elevation of cyclic guanosine monophosphate levels [15]. This suggests a selection of sensitive cells during promotion, a change more likely to occur in complex tissues where a variety of target cells exist. Initiation-promotion protocols in skin are most efficient at producing premalignant lesions (papillomas) and very inefficient at producing malignant tumors, although carcinomas ultimately arise from some papillomas even long after promotion has been terminated [16–18]. The tumors produced by 2-stage protocols in skin are monoclonal [19], indicating an origin from a single (presumably) initiated cell, without recruitment of normal cells into the tumor mass. The monoclonal origin of tumors, the suggestion of tissue remodeling with repetitive promoter exposure, and the requirement for repeated promoting stimuli prior to the development of tumors strongly suggest that cell selection is involved in promotion.

Our studies have been directed toward defining the cellular changes involved in multistage carcinogenesis. Using the mouse epidermal cell culture model, we have attempted to define the biological nature of the initiated cell that would allow it to express its altered phenotype in response to repeated promoter exposures. Studies have also been designed to determine which cells in the complex structure of the epidermis are responsive to tumor promoters and to determine if all responsive cells respond in a like manner. Heterogeneity in response might be expected to lead to cell selection. These studies are summarized in the following sections.

MATERIALS AND METHODS

Cell Culture

Epidermal cells were isolated from Balb/c or SENCAR newborn and adult mice by methods previously described [4, 5]. Recent studies in our laboratory have shown that epidermal basal cells can be selectively cultivated by growth in medium

TABLE I. Characteristics of Mouse Skin Tumor Promotion

Tumors are produced only in initiated skin.
Biochemical changes appear to occur in many cells, not just initiated cells.
Cell selection or tissue sensitization leads to enhanced responses with multiple applications.
Benign tumors (papillomas), which commonly regress, are the major neoplasm produced.
Malignant tumors are rare, arise from papillomas, occur late, and may occur independently of continued promoter exposure.
Tumors are monoclonal.

with reduced ionic calcium concentration (0.02–0.09 mM) [20]. These cells have morphological, cell kinetic, and marker protein characteristics of basal cells [21] and grow as a monolayer with a high proliferation rate. Basal cells can be subcultured and cloned [22]. When the calcium concentration of culture medium is elevated to levels found in most commercial preparations (1.2–1.4 mM), proliferation ceases and terminal differentiation rapidly ensues, with squamous differentiation and sloughing of cells occurring by 72–96 hr [20]. This simple physiological manipulation has been useful in approaching questions regarding initiation and promotion. Medium used for short-term experiments was Medium 199 containing 2% fetal bovine serum (Reheis Chemical Co., Kankakee IL) and a calcium concentration of 0.07 mM. Medium for long-term experiments was Eagles minimal essential medium containing 8% fetal bovine serum, nonessential amino acids, and a calcium concentration of 0.02 mM. Both media contained penicillin 100 units/ml, streptomycin 100 μg/ml, and fungizone 0.25 μg/ml. The preparation of each medium has been described previously [7, 20].

Biological and Biochemical Assays

Assay procedures rely on the modulation of terminal differentiation in epidermal cells via medium calcium concentrations and alterations in biological or biochemical functions produced by the tumor promoter TPA. To monitor induced epidermal differentiation, the striking change in morphology, the rapid decrease in DNA synthesis, and the increase in activity of epidermal transglutaminase, the enzyme responsible for formation of the cornified envelope [20, 23], were assayed. Techniques for isolation and characterization of differentiation-resistant cells have been previously published [5, 7]. To monitor responses to tumor promoters, exposed cultures were assayed to measure the stimulation of DNA synthesis [24], the induction of ornithine decarboxylase activity [25], and the increase in epidermal transglutaminase [23].

RESULTS

Biological Changes in Initiation

In vivo, only a small number of epidermal basal cells proliferate at a particular time, and those that leave the basement membrane are obligated to terminally differentiate. In epidermal tumors (both papillomas and carcinomas) the proliferating population increases 10-fold and proliferating cells are observed away from the basement membrane area [17]. Other characteristics of epidermal differentiation are maintained in tumors, particularly the papillomas, which are the principal product of 2-stage skin carcinogenesis [26]. This suggests that, during the process of carcinogenesis, an alteration occurs in initiated cells that is expressed as the ability to proliferate under conditions where normal cells cannot or where normal cells are obligated to differentiate. This alteration is likely to be an early event in the transformation process and would be a premalignant trait, perhaps a key change in initiation. The responses of cultured basal cells to increases in extracellular calcium resemble the events associated with the commitment to differentiate and migrate away from the basement membrane in vivo. It might be expected, in analogy with in vivo data, that carcinogens could alter the basal cell response to calcium-induced terminal differentiation. Support for this idea was provided by the observation that cultured malignant epidermal cells continued to

proliferate when switched from 0.7 mM Ca^{++} medium [27] and could be selected from a large excess of normal cells that did not survive induced differentiation [7].

Primary epidermal cells from newborn mice were plated in 0.02 mM Ca^{++} medium, treated on day 3 with a chemical carcinogen, maintained for 3–9 weeks in low calcium, and then switched to 1.4 mM Ca^{++} medium for an additional 4 weeks. Surviving epithelial colonies were fixed, stained with rhodamine B, and counted. Results of these experiments have been recently published [5, 7]. Treatment of cultures with 7,12-dimethylbenz[a]-anthracene (DMBA) or N-methyl-N′-nitro-N-nitrosoguanidine yielded 4–20-fold more colonies resistant to calcium-induced differentiation than solvent controls. Colony number was proportional to carcinogen dose for both agents. Cells obtained from colonies in carcinogen-treated cultures demonstrated characteristic epidermal traits, including the ability to vertically stratify and produce squames in 1.4 mM Ca^{++}. They could be subcultured but did not grow in agar or produce tumors in syngeneic mice. In prolonged subculture these cells did become tumorigenic and produced squamous cell carcinomas (unpublished). Cells selected in this assay differ from normal basal cells in that they fail to demonstrate the proliferative block that accompanies calcium-dependent terminal differentiation, and they continue to proliferate under conditions where normal cells are obligated to terminally differentiate. This change is analogous to changes described for basal cells in mouse skin tumors in vivo and is likely to be fundamental to the development of neoplasia in a terminally differentiating tissue.

Current studies indicate that the alteration in differentiation outlined above is associated with the initiation step in skin carcinogenesis. Adult mice were initiated with 25 μg of DMBA given once topically, and after 6 weeks with no further treatment the initiated skin was removed, and the epidermal cells were isolated and cultured in 0.02 mM Ca^{++} medium. Keratinocytes for both initiated and control skin grew well under these conditions. Cultures were switched to medium containing 1.2 mM Ca^{++} after 11–17 weeks and after 4 weeks in 1.2 mM Ca^{++} were fixed and stained with rhodamine. Table II indicates that in cultures derived from Balb/c mouse epidermis, colonies survived Ca^{++}-induced differentia-

TABLE II. Differentiation-Resistant (Rhodamine +) Colonies From Mouse Epidermis Treated In Vivo With Initiating Dose of 7,12-Dimethylbenz[a]anthracene [DMBA]

Experiment	In vivo treatment	Time in vitro (wks)		Plates +/ total plates
		0.02 mM Ca^{++}	1.4 mM Ca^{++}	
308 PB	DMBA (25 μg × 1)	11-17	4	5/8
(Balb/c)	Acetone (200 μl × 1)	11-17	4	0/7
308 PS	DMBA (25 μg × 1)	11-17	4	7/7
(SENCAR)	Acetone (200 μl × 1)	11-17	4	4/5

Epidermal cells were prepared from skins of 4–6 adult mice per group treated in vivo as shown in the second column. Pooled cell preparations were plated at 2×10^6 viable cells per 35 mm culture dish in 0.02 mM Ca^{++} Eagle's minimal essential medium containing 8% chelex-treated fetal calf serum (Reheis Chemical Company, Kankakee, IL). During weeks 11–17 in culture, 1 or 2 dishes from each group were switched to 1.4 mM Ca^{++} medium and maintained for 4 additional weeks while terminal differentiation led to detachment and sloughing of many cells. Plates were then fixed in 10% formalin and stained with 1% rhodamine B, which stains stratifying epidermal colonies. Plates were scored as positive or negative depending on whether there were red colonies > 5 mm in diameter.

tion only from initiated skin, whereas all control keratinocytes terminally differentiated in 1.2 mM Ca^{++}. The striking difference in colony formation between cultures of keratinocytes from initiated and control epidermis is shown in Figure 1. As in the model described in the previous paragraph, cells with characteristic epidermal features form the colonies in this assay. These cells can be subcultured in 1.2 mM Ca^{++} medium and are being tested for tumorigenicity. Two additional experiments with a similar protocol have confirmed these results for Balb/c mice. Table II also shows that cultures derived from SENCAR mouse skin demonstrate differentiation-resistant colonies in both control and initiated groups. This has been confirmed in a second experiment (not shown). SENCAR mice are extremely sensitive to skin carcinogenesis by initiation and promotion protocols and develop a significant number of benign tumors with TPA promotion alone [28]. We have previously suggested that SENCAR mouse skin is constitutive for initiation [5, 28], and these in vitro results are in agreement with that conclusion. Thus from cultured keratinocytes of skin initiated in vivo and from basal cells exposed to initiators in vitro, foci can be derived that resist the signal to cease proliferation in association with terminal differentiation. It seems reasonable that this trait, which is also characteristic of cultured malignant epidermal cells, is a fundamental and early change in carcinogenesis.

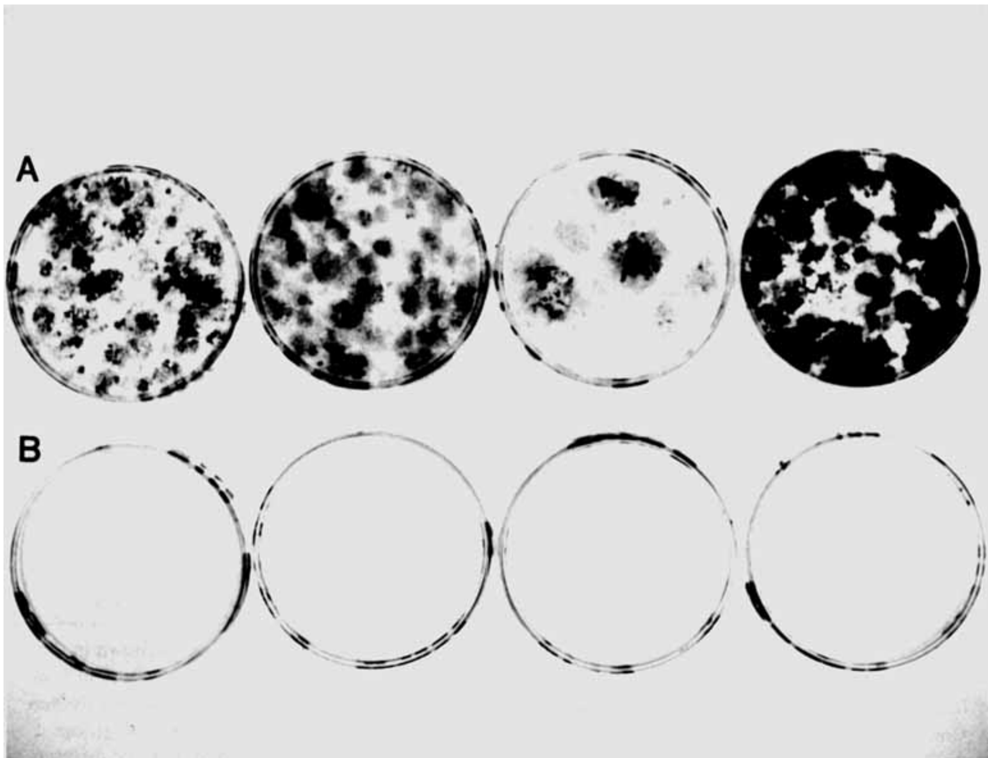

Fig. 1. Rhodamine-stained plates representative of Experiment 308PB. In vivo exposure to: A, DMBA 25 $\mu g \times 1$; B, acetone only. Cultures were grown in 0.02 mM Ca^{++} medium for 11–17 weeks and 1.2 mM Ca^{++} medium for 4 weeks prior to fixation and staining.

The Target Cell for Tumor Promoters

We have emphasized that tumor promotion is a process that is characteristic of carcinogenesis in complex tissues. Complex tissues such as the epidermis are composed of more than one cell type or of cells in differing biological states. We used our culture model to investigate whether both basal and differentiating cells were equally sensitive to the tumor promoter TPA. The induction of the enzyme ornithine decarboxylase (ODC) was used as an index of responsiveness. Primary epidermal cells cultured in low-calcium media were up to 20-fold more responsive to TPA than cells grown in medium with 1.2 mM Ca^{++} [29]. Low-calcium conditions also permitted cells to remain responsive to TPA throughout their culture lifetime, whereas cells plated in 1.2 mM Ca^{++} became unresponsive to TPA within 42 hr in culture [29]. When cells grown in 0.07 mM Ca^{++} medium for 3 days were switched to 1.2 mM Ca^{++} at various times prior to exposure to TPA (Fig. 2),

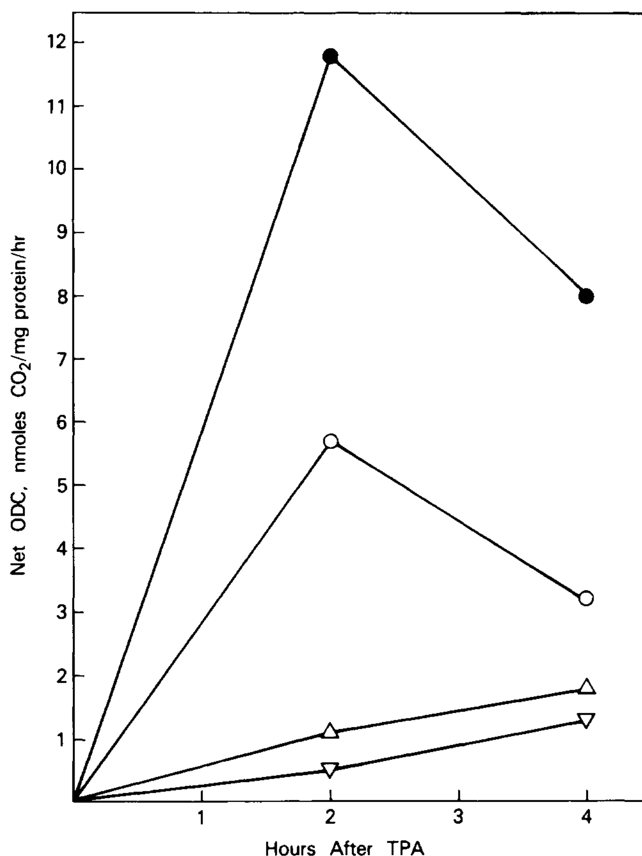

Fig. 2. Loss of ODC inducibility by TPA as a function of time after calcium induced differentiation. Primary epidermal cells were cultured in 0.07 mM Ca^{++} medium for 3 days. Cultures were then switched to 1.2 mM Ca^{++} medium and exposed to TPA (0.01 μg/ml) or DMSO (0.1%) at various times. ODC activity was assayed at 2 and 4 h after TPA exposure, times previously determined to be maximal for ODC induction under these conditions. Results represent net ODC (TPA-induced ODC minus ODC in DMSO control) on duplicate dishes assayed in duplicate (variation $< 10\%$). ●—● 0.07 mM Ca^{++} and TPA; ○—○ simultaneous 1.2 mM Ca^{++} and TPA; △—△ 1.2 mM Ca^{++} 2 h prior to TPA; ▽—▽ 1.2 mM Ca^{++} 4 h prior to TPA.

responsiveness was rapidly lost. Within 4 hr, ODC induction ws less than 10% that observed in cells maintained in 0.07 mM Ca^{++}. Even when TPA was added simultaneously with the increase in calcium, a significant decrease in induction was observed. These changes in ODC inducibility associated with modulation of extracellular calcium are not directly related to Ca^{++} concentrations or to the inability of cells to elevate ODC activity, since ultraviolet light is capable of inducing ODC equally at all calcium concentrations [29]. These results suggest that only epidermal basal cells are the target cell for TPA action (at least with regard to ODC induction) and that basal cells become refractory to TPA action early after commitment to terminal differentiation. The molecular basis for this lost responsiveness is under study.

Heterogeneity in Basal Cell Responsiveness to Tumor Promoters

The indication that primarily the epidermal basal cells are responsive to TPA was surprising in view of the large number of changes observed in mouse skin or epidermal cell cultures exposed to phorbol ester tumor promoters. These responses, listed in Table III, demonstrate that a variety of skin functions are altered, many of which occur with the same temporal pattern. When divided as in Table III, both differentiative and proliferative events occur simultaneously. Since these processes are generally thought to be mutually exclusive in epidermis, if basal cells are the only responsive population, considerable heterogeneity must exist among basal cells in the program of induced responses. Experimental data from this laboratory on the induction of ODC had supported that idea. When epidermal cells were exposed to weakly promoting phorbol esters, ODC activity peaked at 3 hr after exposure, whereas peak activity after exposure to strong promoters occurred at 9 hr [14]. Similarly, basal cells exposed to very low concentrations (1 ng/ml) of TPA demonstrated the 3 hr ODC peak, whereas higher concentrations (100 ng/ml) resulted in a maximum at 9 hr and very low activity at 3 hr [29]. These results were consistent with the presence of at least 2 populations of cells, a particularly sensitive population with peak ODC activity at 3 hr and a population of less sensitive cells with later peak ODC activity.

Other data support the idea of divergence of responses to TPA among the cells of the basal cell population. For example, TPA accelerated the shift from a basal cell keratin protein pattern on polyacrylamide gels to a stratum corneum

TABLE III. Effects of Phorbol Esters on Mouse Epidermis In Vivo and In Vitro

Differentiative Functions	Unassigned function	Proliferative functions
↓ DNA synthesis [12]	Cyclic nucleotide changes [15]	↑ DNA synthesis [12]
↑ RNA and protein synthesis [30]	↑ 13.5K, 25K, 35K, 50 K proteins [34]	↑ RNA and protein synthesis [30]
↑ Synthesis of histidine-rich protein [31]	↑ Phospholipid synthesis [35]	↑ Polyamine biosynthesis [25]
↑ Epidermal transglutaminase [23]	↑ Histone phosphorylation [36]	↑Prostaglandin synthesis [37]
↑ 68K keratin [32]	Dark cells [26]	↓ Histidase activity [38]
↑ Suprabasal cells [33]		↓ Chalone responsiveness [39]
↑ Cornified cells [23]		

pattern when epidermal cells were exposed in vitro [32]. This suggested that differentiation was induced. In contrast, morphological data indicated that TPA was also capable of inhibiting epidermal differentiation in vitro [24, 40]. Thus is appeared that TPA had both accelerating and inhibitory effects on keratinocyte differentiation. TPA was also reported to preferentially stimulate the proliferation and migration of a particular epidermal cell type, the dark basal cell [26, 41].

Direct evidence for heterogeneity of basal cell responses to TPA has been obtained by recent in vitro studies. Basal cell cultures exposed to TPA and maintained in 0.07 mM Ca^{++} rapidly undergo a morphological change in which a portion of the cell population becomes rounded and eventually detaches from the monolayer. Detaching cells are cornified, and the process is associated with a substantial increase in epidermal transglutaminase activity [23]. This effect does not appear to be the result of cytotoxicity but rather a programmed response to TPA [23] in which terminal differentiation is induced in a subclass of basal cells. Other basal cells in the same culture look relatively unaffected by TPA. Thus, within the same total population, variability is easily documented.

To determine if basal cells resistant to the differentiation-inducing effect of TPA were nevertheless altered by the promoter, cells surviving the treatment with TPA in 0.07 mM Ca^{++} were washed free of cornified cells after 24 hr and studied further. If the remaining basal cells were unaffected by TPA, they would be induced to differentiate by 1.2 mM Ca^{++}. If, however, the cells had been changed by the promoter in some other way, they might be expected to exhibit an altered response to the calcium signal. Such a finding could explain the inhibition of epidermal differentiation observed after TPA exposure. Table IV shows results of these experiments. In control cultures (no TPA exposure) elevation of Ca^{++} results in a rapid increase in transglutaminase activity and a substantial decrease in thymidine incorporation into DNA, events associated with terminal differentiation. However, in TPA-treated cultures, the transglutaminase activity did not increase in response to calcium for at least 48 hr. Similarly, the high rate of DNA

TABLE IV. Effect of TPA Treatment on Basal Cell Response to Calcium-Induced Differentiation

Parameter of Ca^{++} induced differentiation	Time (h) after culture in 1.2 mM Ca^{++} (% of zero time control in 0.07 mM Ca^{++})		
	24	48	72
Transglutaminase[a]			
TPA Pretreated	79	81	251
Control	330	378	495
DNA Synthesis[b]			
TPA Pretreated	111	88	62
Control	65	33	40

[a]Primary epidermal cells were cultured in 0.07 mM Ca^{++} medium for 5 days, exposed to 0.1 μg/ml TPA or 0.1% DMSO for 20 h, washed, and cultured in 1.2 mM Ca^{++} medium. Duplicate dishes were assayed for transglutaminase activity.

[b]Primary epidermal cells were cultured in 0.07 mM Ca^{++} medium for 4 days, exposed to 0.1 μg/ml TPA or 0.1% DMSO for 20 h, washed and cultured in 0.07 mM Ca^{++} medium for an additional 24 h and then switched to 1.2 mM Ca^{++} medium. Duplicate dishes were pulsed with ^{3}H-TdR (1 μ Ci/ml) for 1 h at 24-h intervals and assayed for thymidine incorporation into DNA.

synthesis characteristic of basal cells continued even when TPA-exposed basal cells were switched to 1.2 mM calcium conditions. Identical results were obtained when TPA exposure was continued in 1.2 mM calcium medium (the assay period) or when TPA exposure was for only 24 hr in 0.07 mM Ca^{++} prior to the assay period. These results suggest that the population of basal cells resistant to TPA-induced differentiation was transiently resistant to a physiological differentiation signal. This heterogeneity in response to phorbol esters among target cells within the same tissue could form the basis for the cell selection observed during tumor promotion in skin, and it may be critical for the promoting activity of these agents.

DISCUSSION

In normal epidermis (as in other tissues) coordinated growth and differentiation appear to occur in discrete units or columns composed of a small number of cells of which approximately 10 are basal cells [42]. The regulation of maturation may be relatively autonomous within an epidermal unit, but inter-unit regulation of growth and differentiation seems certain, as evidenced by the overall uniformity of skin thickness and the overlapping cellular arrangement of cornified cells in adjacent units [43]. Within a unit, individual basal cells lose mitotic capacity as they begin maturation while still on the basement membrane, then they migrate into the suprabasal region, where they ultimately become cornified and desquamate. Under steady-state conditions, cell loss equals cell production, and adjacent units (proposed as groups of 6 to account for hexagonal overlapping of cornified cells [43]) mature at similar rates, further suggesting inter-unit regulation. If this regulatory structure remained intact, the skin would be protected from deviant behavior of an individual cell by restricting altered cells in a single epidermal unit to the division and maturation rate of surrounding units.

Our studies indicate that initiation of carcinogenesis results in an alteration in the regulation of maturation of a basal cell and its progeny such that initiated cells proliferate under conditions where normal cells are obligated to cease proliferation. In vitro this is expressed as an alteration in the response of epidermal cells to elevation of extracellular calcium to 1.4 mM. This change in calcium concentration induces terminal differentiation in normal (unexposed) cells. In contrast, exposure to carcinogens in culture or in situ prior to culture results in colonies of altered epidermal cells capable of continued proliferation in 1.4 mM calcium. These cells nevertheless retain their differentiative capability in vitro as they migrate away from the plastic substrate and cornify, producing mature squames, which stain red with rhodamine. An analogous change in vivo would yield cells that retain growth capacity along the basement membrane and even in the nucleated suprabasal region of an epidermal unit but that differentiate in the upper layers, producing cornified cells which interact with adjacent units. The intact unit structure would restrict the expansion of initiated cells to the area of a single unit. Assuming that initiation is an infrequent event, this change in proliferative capacity in only a small number of units would be nearly impossible to detect experimentally in vivo in the absence of tumor promotion, but it could be expressed during the calcium selection exerted by our in vitro assay.

Both our in vitro studies and previous studies in vivo indicate that exposure to phorbol ester tumor promoters induces differentiation in some basal cells and disrupts normal epidermal structure and communication [26]. Basement mem-

brane area (in many epidermal units) is vacated as basal cells, induced to differentiate, migrate to upper layers [33]. Because of the heterogeneous response to phorbol esters among basal cell subpopulations, other basal cells (including initiated cells) are stimulated to proliferate and are transiently blocked from terminal differentiation. The net effect is a selective regenerative hyperplasia in which initiated cells (among others) clonally expand to repopulate the basal cell area and occupy several epidermal units. Repetition of this sequence of events after each exposure to the promoter amplifies these changes, resulting in a miniclone of initiated cells [26], occupying many units, in which proliferative functions are maintained in many cells and a high labeling index is noted experimentally [17]. Under these conditions the constraints on cell production of surrounding adjacent epidermal units no longer apply to most of the cells in the initiated clone, and excess production leads to localized areas with multiple layers of initiated cells and their more differentiated progeny [26]. Ultimately, with continued promoter exposure, a benign tumor, with multiple proliferative cells and multiple differentiative layers, will result. The rapid rate of proliferation in the papilloma predisposes these clones of initiated cells to a subsequent genetic change. Thus the promoter-independent conversion from papilloma to carcinoma [18], which occurs with low frequency in 2-stage mouse skin carcinogenesis protocols, may result from a spontaneous random genetic event in an individual papilloma cell. Alternatively, some initiated clones could be programmed to become carcinomas at the time of the initiating event, but require a papilloma stage to progress to the malignant phenotype.

This hypothesis regarding the biological mechanisms of initiation and promotion is consistent with experimental observations in skin carcinogenesis in vivo and in vitro and addresses the biological activity reported for phorbol esters in skin. Such a scheme provides rational approaches to molecular studies of initiation and promotion and allows certain predictions relating to skin carcinogenesis. Since benign tumors would be clonal expansions of initiated cells, such tumors must be monoclonal. Studies of 2-stage carcinogenesis in chimeric mice have indicated this to be the case [19]. Preferential growth and migration of specific epidermal cell types should be apparent after promoter exposure; dark cells represent one such cell type [26, 41]. Specific promoter-induced loss of basal cells in vivo through induced differentiation is also predicted by this scheme, and a concomitant migration of cells out of the basal layer as they differentiate would be expected. Both phenomena are reported as early changes in mouse skin treated with TPA [33]. The phenotype of the initiated cells, of early benign growths (miniclones), and of the final papillomas should be the same. Unfortunately this is not known for skin. This model suggests that population redistribution is essential to promotion and predicts that other techniques to produce such a change will act as promoting stimuli. In this regard, wounding [30] and skin abrasion [44] are promoting influences in skin. In contrast, a purely hyperplastic stimulus that does not involve regeneration, such as skin massage,is not a promoting influence [45]. This model also predicts that agents that interfere with either the differentiative or proliferative effects of phorbol esters would inhibit promotion. Antipromoting steroids are potent inhibitors of TPA-induced proliferation [46], and retinoids are known to alter epidermal differentiation [47]. One might also expect that a critical element in promotion would be the early escape of initiated cells from the confines of neighboring normal cells to form

a miniclone less subject to the growth regulation of its surroundings. This would require balanced heterogeneous responses, and TPA may be particularly effective in this regard, while other agents such as mezerein are less effective or are particularly damaging to initiated cells. Once a miniclone is established, other agents can accelerate the clonal expansion through a proliferative stimulus. This would explain the apparent multistage nature of promotion [48].

We have restricted our interpretations of current data from this laboratory to the formulation of a scheme for tumor development in mouse skin. However, the observation of an alteration in the proliferative block that is normally associated with terminal differentiation is not unique to skin carcinogenesis. Carcinogen-induced changes have been reported in several epithelial models in which cells proliferate in tissue regions normally occupied only by nonproliferating maturing cells [49, 50]. Intestinal biopsies from cancer-prone patients with familial polyposis have shown that crypt cells in nonadenomatous areas proliferate in the luminal portion of the mucosa, a region without proliferative activity in normal intestinal epithelium [51]. Cultured cells from polyposis patients behave as if they are constitutively initiated [52]. Likewise, stimuli that produce a regenerative hyperplasia or heterogeneous responses in target cells have promoting activity in models other than skin. Wounding and irritation are such stimuli and act as promoters in the colon and respiratory tract [53, 54]; cigarette smoke and hormones may be other examples. Thus, these concepts evolving from studies with cultures of mouse keratinocytes may yield information applicable to a number of models for epithelial carcinogenesis and may reflect processes important in human carcinogenesis.

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