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Action of Phorbol Esters in Cell Culture: Mimicry of Transformation, Altered Differentiation, and Effects on Cell Membranes

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The carcinogenic process is usually multifactor in its causation and multistep in its evolution. It is likely that entirely different molecular mechanisms underlie the many steps in this process. In contrast to initiating carcinogens, the action of the tumor-promoting phorbol esters does not appear to involve covalent binding to cellular DNA and they are not mutagenic. Recent studies in cell culture have revealed two interesting biologic effects of the phorbol esters and related macrocyclic plant diterpenes. The first is that at nanomolar concentrations they induce several changes that resemble those seen in cells transformed by chemical carcinogens or tumor viruses. These include altered morphology and increased saturation density, altered cell surface fucose-glycopeptides, decrease in the LETS protein, increased transport of deoxyglucose, and increased levels of plasminogen activator and ornithine decarboxylase. In transformed cells exposed to phorbol esters the expression of these features is further accentuated. Phorbol esters do not induce normal cells to grow in agar but they do enhance the growth in agar of certain transformed cells. The second effect of the phorbol esters is inhibition of terminal differentiation. This effect extends to a variety of programs of differentiation and is reversible when the agent is removed. With certain cell culture systems induction of differentiation, rather than inhibition, is observed. Both the transformation mimetic and the differentiation effects are exerted by plant diterpenes that have tumor-promoting activity but not by congeners that lack such activity. The primary target of phorbol esters appears to be the cell membrane. Early membrane-related effects include enhanced uptake of 2-deoxyglucose and other nutrients, altered cell adhesion, induction of arachidonic acid release and prostaglandin synthesis, inhibition of the binding of epidermal growth factor to cell surface receptors, altered lipid metabolism, and modifications in the activities of other cell surface receptors. A model of "two stage" carcinogenesis encompassing the known molecular

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and cellular effects of initiating carcinogens and tumor promoters is presented. According to this model, initiating carcinogens induce stable alterations in the cellular genome but these are not manifested until tumor promoters modulate programs of gene expression and induce the clonal outgrowth of the initiated cell.

Key words: tumor promoters, phorbol esters, plasminogen activator, epidermal growth factor, carcinogenesis

It is likely that most human cancers do not result from simple exposure to a single exogenous agent but rather a complex interaction between multiple environmental (exogenous) and host (endogenous) factors. The carcinogenic process is often a multistep one occurring over an appreciable fraction of the lifespan of the host. It is probable that each of these steps reflects qualitatively different biologic and biochemical events and that they may be mediated by different types of agents. In other words, the simple exposure of an individual to a DNA-damaging agent may not be sufficient to induce cancer if the subsequent steps in a multistep process are rate-limiting. This has obvious implications for cancer prevention. It may, for example be possible to reduce the incidence of certain human cancers by preventing (or reversing), not the initial step, but the later steps in a multistep carcinogenic process.

The existence of at least two distinct phases in chemical carcinogenesis, termed "initiation" and "promotion," is well illustrated in studies on mouse skin. There is also increasing evidence that a process similar to promotion occurs during tumor induction in certain other tissues and in other species (for review, see refs. 1–6). It is of interest that the most potent promoters on mouse skin, the phorbol esters and related diterpenes, are naturally occurring substances. Although there has been the impression that the action of the phorbol esters as tumor promoters is confined to mouse skin, recent studies indicate that this is not the case (Table I).

One wonders to what extent other promoters and carcinogenic cofactors occur in our diet, in other aspects of the natural environment, or in industrial products, and to what extent they are limiting determinants in the causation of specific human cancers.

The identification of such factors requires better knowledge of their mechanisms of action as well as simple in vitro assays that can be used for their detection. Table II contrasts the biologic properties of carcinogens and tumor promoters on mouse skin.

It should be stressed that although many carcinogens undergo metabolic activation and bind covalently to DNA (and are therefore mutagenic) this is not true for the tumor promoters. A few years ago we became interested in developing cell culture systems which could be used to study the mechanism of action of tumor promoters and which might

TABLE I. Examples in Which Phorbol Esters Enhance Carcinogenesis in Tissues Other Than Mouse Skin

Type of tumor	Species	Agents	Reference
Ovary, intestine	Mouse	DMBA or urethane (diaplacental) + TPA (postnatal)	2
Forestomach	Mouse	DMBA + TPA	3
Stomach	Rat	MNNG + croton oil	4
Esophagous[a]	Human	? + diterpene from croton flavens	6

[a] Suggestive evidence.

TABLE II. Comparison of Biologic Properties of Initiating Agents and Promoting Agents

Initiating agents	Promoting agents
1. Carcinogenic by themselves – "solitary carcinogens"	1. Not carcinogenic alone
2. Must be given *before* promoting agent	2. Must be given *after* the initiating agent
3. Single exposure is sufficient	3. Require prolonged exposure
4. Action is irreversible and additive	4. Action is reversible (at early stage) and not additive
5. No apparent threshold	5. Probable threshold
6. Yield electrophiles that bind covalently to cell macromolecules	6. No evidence of covalent binding
7. Mutagenic	7. Not mutagenic

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serve as rapid assays for screening environmental substances for promoting activity. This article will summarize studies from our own laboratory and from other laboratories on the biologic and biochemical effects of the phorbol esters in various cell culture systems. Aspects of this subject have recently been reviewed elsewhere [7].

MIMICRY OF TRANSFORMATION

One of the first effects we observed was that 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and several related macrocylic plant diterpenes, are extremely potent inducers of plasminogen activator (PA) synthesis in both chick embryo fibroblasts (CEF) and HeLa cultures [8–11]. We have previously presented evidence that this effect is highly specific, that it involves de novo macromolecular synthesis, and that it correlates with the tumor-promoting potency of a series of phorbol ester analogs [7–11].

In view of the results obtained with PA, it was natural to ask whether TPA and related compounds also enhance the expression of other biologic markers frequently associated with transformation and tumorigenicity. Elsewhere we have summarized data from several laboratories indicating that TPA does induce several properties in normal cells that mimic those often seen in transformed cells [7–10]. This mimicry includes changes in cell morphology, growth properties, cell surfaces properties, and specific enzymes. We must stress, however, three aspects of the effects obtained when normal cells not previously exposed to an initiating carcinogen are incubated with TPA. 1) Not all cells display the full set of phenotypic changes of mimicry. 2) TPA-treated normal cells do not mimic all of the properties of fully transformed tumorigenic cells. It is of particular importance that they do not acquire the capacity for growth in agar. The stimulatory effects of TPA on the growth in agar of adenovirus-transformed cells are described below. 3) In contrast to fully malignant cells, the maintenance of transformation properties in normal cells is dependent upon the continuous presence of the promoting agent, and the cells revert to normal when the agent is removed from the medium. In mouse skin previously exposed to a carcinogen the repeated application of TPA, however, can lead to "autonomous" malignant tumors. TPA can enhance the stable transformation of fibroblast cultures previously exposed to a chemical carcinogen, UV, or x-irradiation [12–14] or an adenovirus [15, 16]. The latter results indicate that "initiated" cells have a qualitatively different response to TPA than completely normal cells.

PHENOTYPIC ENHANCEMENT AND EFFECTS ON ADENOVIRUS TRANSFORMATION

An intriguing aspect was our finding that TPA causes a further increase in PA synthesis in transformed cells that are already synthesizing high levels of PA [8–10]. We refer to this phenomenon as "enhancement." Studies with chick embryo fibroblasts transformed by a temperature-sensitive mutant of Rous sarcoma virus (RSV) showed that enhancement of TPA-induced PA synthesis required continuous expression of the sarc gene of RSV [8–10]. This finding has been confirmed and extended by other investigators [17, 35]. The results suggest that there is an interaction between products of the sarc gene and cellular events triggered by TPA. We are currently studying the nature of this interaction. Other examples of an enhanced response to TPA by transformed cells have now been seen in terms of morphologic changes [8–10, 17], ornithine decarboxylase (ODC) induction [18], and prostaglandin synthesis [19]. The phenomenon of enhancement may be a useful model for understanding tumor promotion and progression, since it provides examples in which previous changes in phenotype alter a cell's subsequent response (both quantitatively and qualitatively) to a tumor-promoting agent.

The results obtained with RSV-transformed cells suggested that tumor promoters could interact with oncogenic viruses in the transformation process. There are several examples in which chemical and physical agents interact synergistically with viruses in the carcinogenic process both in vitro and in vivo (for review see refs. 15, 20, and 21). Indeed, it seems likely that certain human cancers may be due to interactions between chemical agents and types of viruses which alone would have little or no oncogenic potential. This is important to keep in mind in the search for viruses that might play a role in human cancer causation. These agents may not have all of the properties of oncogenic viruses seen in experimental animal systems and, when assayed alone, they may not be capable of cancer induction in the absence of chemical cofactors.

To explore these aspects of chemical-viral interactions we have recently developed an in vitro system in which the transformation of rat embryo (RE) cells is markedly enhanced when, after infection with a mutant (ts125) of adenovirus type 5, the cells are grown in the presence of TPA [15, 16]. The presence of TPA caused an increased number of foci of transformation. Foci also appeared earlier and were larger than those obtained with adenovirus in the absence of TPA. Phorbol, 4αPDD, and 4-0-MeTPA were inactive in this system. The addition of TPA could be delayed until after viral uptake and integration of adenovirus sequences into the host genome had occurred, thus indicating that the enhancement by TPA was not exerted on these steps. This is in contrast to the ability of certain initiating carcinogens to enhance DNA virus transformation [15, 16, 21, 22].

One of the best in vitro markers for the tumorigenicity of transformed fibroblast or epithelial rodent cells is anchorage independence, ie, ability to grow in agar or agarose suspension, although there are a few exceptions (for review see Fisher et al [16]). It was of interest, therefore, to examine the effects of TPA on this property in adenovirus-transformed cells. In recent studies, we have found that although TPA does not enhance the growth in agar of normal RE cells, it does induce the growth in agar of morphologically transformed adenovirus-infected RE cells [16] (Table III).

This effect appears to be inductive and not due to simple cell selection [81]. Yet it is irreversible, since when the TPA is removed the cells now grow in agar with a higher efficiency than prior to exposure to TPA. Colburn et al [23] have found that certain

TABLE III. Effect of TPA on Growth in Agar of Normal and Adenovirus-Transformed Rat Embryo Cells

Cell type	Agar-cloning efficiency (%)		Ratio
	−TPA	+TPA	(+TPA/−TPA)
Normal			
Secondary rat embryo	< 0.001	< 0.001	–
Adenovirus-transformed			
Ad-A18-E	< 0.001	0.1	> 100
Ad-A18-L	0.1	0.5	5.0
Ad-E7-E	< 0.001	0.2	>200
Ad-E7-L	1.2	2.7	2.3

Morphologically transformed clones were isolated from cultures of secondary rat embryo cultures previously infected with a mutant of human adenovirus type 5 (H5ts125) and tested for growth in agar in the presence and absence of 100 ng/ml TPA. E, early passages (< 10) of these clones; L, later passages (> 25). For additional details see Fisher et al [16].

serially passaged mouse epidermal cell cultures also undergo an irreversible increase in anchorage-independent growth when exposed to TPA. This phenomenon may represent a useful in vitro model system for studying the process of tumor progression.

EFFECTS ON DIFFERENTIATION

Since it is likely that carcinogenesis involves major disturbances in differentiation, it was of interest to determine whether TPA would affect the differentiation of certain well-defined tissue culture systems. Table IV summarizes examples from our own laboratory and from the literature indicating that TPA is a highly potent inhibitor of terminal differentiation in a variety of cell systems.

This effect on differentiation is not simply a consequence of toxicity or growth inhibition, and, in certain cases, is reversed when TPA is removed from the culture. Nor is the effect limited to a specific program of differentiation or species (Table IV). Evidence has also been obtained that, as with the phenomenon of mimicry of transformation, the relative potencies of a series of phorbol ester analogs as inhibitors of differentiation correlates with their potencies as promoters on mouse skin [24].

Recently, cell systems have been found in which TPA induces rather than inhibits differentiation. This has now been seen with a certain clone of murine erythroleukemia cells [34], murine and human myeloid leukemia cells [36, 37, 79, 80], and a human melanoma cell culture [38]. Reciprocal effects of the same agent on differentiation, depending on the type of cell culture, have been seen with other agents, including glucocorticoid hormones, cyclic AMP, and BUdR. It is possible that the ability of TPA either to induce or inhibit differentiation depends on the nature of the membrane, and/or membrane constituents of the target cell. The late Dr. Morris Kupchan and his colleagues found that certain macrocyclic plant diterpenes inhibited the growth of a transplantable mouse lymphoma [39]. We wonder whether this was due to an inductive effect on differentiation and whether this approach can be further exploited in the therapy of certain neoplasms which retain the capacity for differentiation [40].

TABLE IV. Examples of TPA Inhibition of Differentiation

Cell system	Type of differentiation	References
Murine erythroleukemia	Erythroid	24, 25, 72
Chicken embryo myoblasts	Myogenesis	26
Chicken embryo chondroblasts	Chondrogenesis	27, 49
Murine 3T3	Lipocytes	28
Murine neuroblastoma	Neurite	29, 71
Murine melanoma	Melanogenesis	30
Sea urchin	Embryogenesis	31, 32, 33

EFFECTS ON MEMBRANES AND THE CELL SURFACE

Early studies on the effects of TPA in cell culture suggested that the cell surface membrane may be the major target of TPA action [75]. More recent studies have reinforced this hypothesis. Table V is a list of effects of TPA on cell surfaces and membranes.

We have studied the uptake of ^{3}H-TPA by cells in culture and found that it is linear across a wide range of TPA concentration and does not appear to be saturable [55]. Thus far, we have been unable to demonstrate a specific high-affinity saturable receptor, although we are still pursuing this aspect. Cell fractionation studies indicated that the uptake was almost entirely into the membranous fractions of the cell and appeared to be a simple partitioning of the highly hydrophobic compound into the lipid phase of the membrane. Uptake by the nucleus was extremely low. Cellular uptake was not inhibited by a large excess of nonradioactive TPA, inhibitors of energy metabolism, inhibitors of macromolecular synthesis, or cytochalasin. There was no evidence of covalent binding to cellular macromolecules and almost all of the cell-associated ^{3}H-TPA was released when the cells were placed in serum containing medium lacking TPA or when cells were extracted with lipid solvents [55]. Thus the detection of a specific TPA receptor is complicated by the extensive non-specific binding.

In view of the evidence that TPA is concentrated largely in the lipid phase of cell membranes, in collaboration with D. Schachter's laboratory at Columbia University, we have looked for evidence of a change in the physical properties of cell membranes by studying the fluorescence polarization of an asymmetric chromophore, 1,6-diphenyl-1,3,5-hexatriene (DPH) [45]. Concentrations of TPA as low as 0.1 ng/ml (10^{-10}M) produced a reproducible decrease in fluorescence polarization of DPH. The change was detected within less than 1 h and was not blocked by cycloheximide or actinomycin D, suggesting that it occurs directly at the membrane level. Other phorbol esters having tumor-promoting activity (PDD and PDB) also exerted this effect, whereas the compounds phorbol and 4αPDD, which lack tumor-promoting activity, were inactive. These results suggest that TPA produces a generalized change in the physical properties of the lipid phase of cellular membranes and that this effect appears to be a direct one. A similar effect of TPA on fluorescence polarization of DPH has been found in human lymphoblastoid cells [46]. One interpretation is that TPA results in an increase in membrane fluidity, but other interpretations have not been excluded [45].

Additional evidence that an early site of action of TPA is the cell membrane comes from studies with murine erythroleukemia cells. We have found that, whereas these cells usually grew in suspension, within 30–120 min after exposure to TPA they become adherent to tissue culture plates and take on an epithelioid or fibroblastic appearance [48].

TABLE V. Effects of TPA on Cell Surfaces and Membranes in Cell Culture

	References
Altered Na/K ATPase	41
Increased uptake 2-DG, ^{32}P, ^{86}RB	42, 43, 57
Increased phospholipid synthesis	44, 74
Increased membrane lipid "fluidity"	45, 46
Increased release arachidonic acid, prostaglandins	19, 47
Altered morphology and cell-cell orientation	8, 17, 41, 42
Altered cell adhesion	48, 49, 73
Altered fucose-glycopeptides	9, 10
Decreased LETS protein	50
"Uncoupling" of β-adrenergic receptors	51, 52, 53
Inhibition of binding of EGF to receptors	54, 60

The induction of adhesion is not blocked by inhibitors of RNA or protein synthesis, although it is temperature-dependent. Studies in progress indicate that when a series of diterpenes are assayed for induction of adhesion, in a sensitive clone of murine erythroleukemia cells, their relative potency generally correlates with their activity as promoters on mouse skin. Thus, this assay may provide a simple rapid screening test for this class of tumor promoters. The fact that this process also does not appear to require macromolecular synthesis suggests that it is due to a primary effect of TPA on cell membranes. Effects on cell adhesion have also been seen with chondroblasts and lymphoblastoid cultures [49, 73].

Another early response to TPA is the release from membrane phospholipids of arachidonic acid, which is associated with a stimulation of prostaglandin synthesis. This effect was recently described by Levine and Hassid [19] and we have extended this finding to CEF and 10T½ cell cultures [47]. The response in CEF cultures is shown in Figure 1. It occurs rapidly, is not seen with nonpromoting diterpenes, and is inhibited by transretinoic acid. The latter compound is an inhibitor of tumor promotion on mouse skin [56]. A curious aspect is that although the TPA-induced release of arachidonic acid and prostaglandins is not inhibited by actinomycin D it is inhibited by cycloheximide or puromycin [47]. The significance of TPA-induced membrane phospholipid deacylation in terms of the other effects of TPA on cell function are not clear at the present time. The fact that it is inhibited by inhibitors of protein synthesis, whereas certain other early responses to TPA such as increased 2-deoxyglucose uptake [57] and altered membrane "fluidity" [45] are not, suggests that it is not the initial or primary effect of TPA on cells.

We have previously postulated that the phorbol ester tumor promoters may act by usurping the function of a cell surface receptor whose normal function is to mediate the action of a growth regulator or homone yet to be identified [9, 54]. Consistent with this hypothesis are (i) the low concentrations at which TPA acts in cell culture (approximately 10^{-8} to 10^{-10}M); ii) the remarkable similarity in structural requirements seen when a variety of phorbol esters and related macrocyclic diterpenes are tested in diverse systems; and iii) the highly pleiotropic and reversible effects of these compounds. Since the earliest effects of TPA appear to occur at the cell membrane, we further postulated that the putative receptors are on the cell surface and the endogenous growth regulator may be a polypeptide hormone.

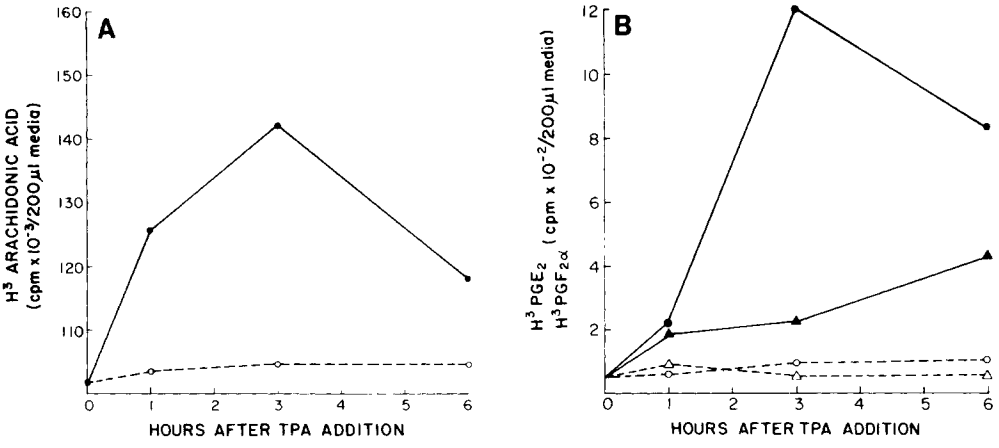

Fig. 1. The accumulation of arachidonic acid and prostaglandins E_2 and $F_{2\alpha}$ in culture medium from CEF. TPA (8×10^{-8}M) or 0.1% DMSO was added in 2 ml of serum-containing medium to cultures prelabeled with [^{3}H]-arachidonic acid and aliquots of the media were collected at the specified times. Control cultures received 0.1% DMSO as vehicle control. Radioactivity was extracted and analyzed by thin-layer chromatography for arachidonic acid and prostaglandins E_2 and F_2. Results are the means from two different cultures. A) Arachidonic acid released, plus TPA (•—•) or 0.1% DMSO (○– – –○). B) Prostaglandin E_2 released, plus TPA (•—•) or 0.1% DMSO (○– – –○); prostaglandin F_2 released, plus TPA (▲———▲) or 0.1% DMSO (△– – –△). For additional details see Mufson et al [47].

A possible candidate for the polypeptide hormone is epidermal (EGF), since it shares a number of biologic effects with TPA [54]. We have shown that EGF, like TPA, is a potent inducer of plasminogen activator in HeLa cell cultures [58]. In addition, we found that TPA and related tumor promoters are extremely potent inhibitors of the binding of ^{125}I-EGF to its cell surface receptors [54]. This effect is seen with a variety of human, rat, and murine cell cultures and is preferential for the EGF receptor [59]. For example, TPA does not inhibit the binding of insulin to its receptors [59]. These findings have been confirmed and extended by other investigators [60, 77].

There is evidence that the concentration of EGF required for maximum biological effect is considerably lower than that required to saturate receptor binding [70, 78]. Therefore, in most of our studies we have used a low concentration of ^{125}I-EGF (approximately 0.04 nM) to provide assurance that we were dealing with physiologic concentrations and to maximize sensitivity to the TPA effect. Under these conditions with HeLa cells the inhibitory effect of TPA is noncompetitive with EGF [54]. This is also true with the macrocyclic diterpene mezerein (Lee and Weinstein, unpublished studies). On the other hand, recent studies by other investigators [60, 77] using 3T3 cells suggest that the TPA inhibition of EGF-receptor binding is competitive with EGF. This apparent discrepancy may relate to differences between HeLa and 3T3 cells or to the much higher concentrations of EGF used in the latter studies. The finding that cells transformed by murine sarcoma viruses have a decrease in EGF receptors and actually synthesize a polypeptide growth factor [61] suggests that changes in the EGF effector system may play an important role not only in the carcinogenic process but also in maintenance of the transformed state.

Figure 2 shows that when ^{125}I-EGF was added to HeLa cultures there was rapid binding which was linear for about 30 min. The amount of bound material declined after 90 min [59]. When TPA (30 ng/ml) was added simultaneously with the EGF, there was rapid

inhibition of ^{125}I-EGF binding which was apparent within 15 min and persisted for at least 150 min. The preincubation of cells with TPA 10 min prior to the addition of EGF gave results similar to those obtained when the two were added simultaneously.

The effects of delayed addition of TPA are also shown in Figure 2. The addition of TPA either during the phase of linear binding of ^{125}I-EGF, or the plateau phase, resulted in a rapid decline of ^{125}I-EGF binding. This effect was apparent within 5–10 min of addition of the TPA. In a separate experiment cells were incubated with ^{125}I-EGF for 50 min to achieve plateau binding. The medium was removed and either TPA (33 ng/ml) or DMSO was added and the rate of loss of radioactivity was measured during a subsequent 90-min period. In the control culture there was a gradual decline, whereas in the TPA-treated culture there was a rapid decline, in cell associated radioactivity [59]. Thus TPA is capable of reversing the initial binding of ^{125}I-EGF to cells and also enhances the loss of cell-associated EGF that normally occurs at later time points.

Additional studies indicated that the radioactivity released from the cells by TPA was largely intact ^{125}I-EGF rather than degraded material, as judged by chromatography on Biogel P-6 columns [59]. These results provided evidence that TPA does not exert its effect on cellular binding of EGF by enhancing the degradation of EGF either via direct proteolysis or enhanced cellular internalization and proteolysis.

The influence of temperature on the ability of TPA to affect EGF binding is summarized in Table VI. Although at 37°C TPA caused approximately a 77% inhibition of ^{125}I-EGF binding, at 22°C the inhibition by TPA was 60% and at 4° it was only 26%, compared to the control values at the same temperatures. Additional studies indicated that when ^{125}I-EGF binding was studied over a period of several hours at either 22° or 4°C in the absence or presence of TPA, the percentage inhibition by TPA was greater at all times at 22° than at 4°C. Although TPA had only a small effect on EGF binding when added to cells at 4°C, if cells were preincubated with TPA at 37° (in the absence of EGF) there was a marked inhibition in their capacity to subsequently bind ^{125}I-EGF at 4°C compared to the binding obtained at 4°C with cells not previously exposed to TPA at 37°.

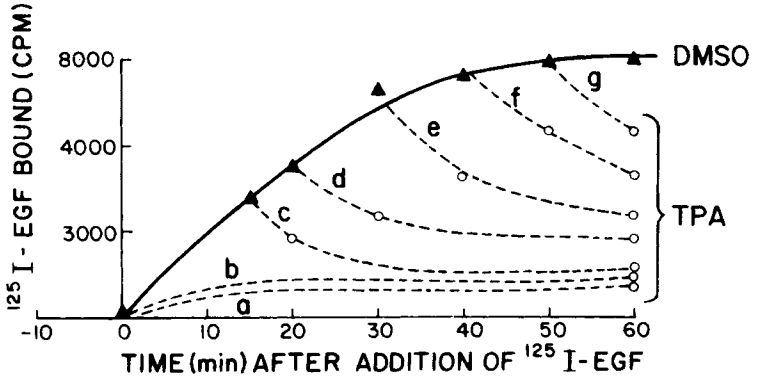

Fig. 2. Time course of binding of ^{125}I-EGF to HeLa cells at 37°C and the effects of addition of TPA at various times. ^{125}I-EGF was added at time zero and cell-associated material was determined at various time intervals in the absence (▲——▲) or presence (○– – –○) of 33 ng/ml TPA. The TPA was added at different times as indicated: a) –10 min; b) time 0; c) time 15 min; d) time 20 min; e) time 30 min; f) time 40 min; g) time 50 min. All plates received 11,488 cpm ^{125}I-EGF (specific activity 77 μCi/μg). For additional details see Lee and Weinstein [59].

TABLE VI. Effect of Temperature on TPA Inhibition of EGF Binding

Temperature	^{125}I-EGF bound (cpm)		% of Control
	Control	+TPA	
4°	3,110	2,295	73.8
22°	6,634	2,612	39.4
37°	7,499	1,687	22.5

HeLa cells were incubated with ^{125}I-EGF (22,727 cpm, specific activity 57 μCi/μg) plus or minus TPA (22 ng/ml) for 60 min at the indicated temperature and the amoung of cell-bound material was measured as described in Lee and Weinstein [54].

To determine the effect of temperature on the ability of TPA to enhance the loss of previously bound EGF from cells, we first allowed cells to bine ^{125}I-EGF at 37° for 50 min, shifted them to either 4° or 22°, added either DMSO or TPA, and then measured the rate of loss of cell-associated ^{125}I-EGF. We found that at 22°C TPA induced a rapid loss of cell-associated ^{125}I-EGF, whereas at 4°C it did not [59].

Taken together, the above results suggest that TPA inhibits EGF binding not by binding directly to the "active site" of the EGF receptor but by indirectly altering the conformation or inducing the clustering of EGF receptors. This could reflect the binding of TPA to sites on the EGF receptor which have an allosteric effect, or TPA-induced changes in the lipid microenvironment in which the EGF receptors are embedded. Conformational changes and temperature-dependent receptor clustering are known to markedly affect the function of various hormones receptors [62, 69, 70, 76].

MODELS OF TPA ACTION AND THEIR RELEVANCE TO TUMOR PROMOTION

Figure 3 integrates the various cellular effects of TPA into a comprehensive model. The primary action of TPA appears to be at the cell surface membrane. Changes in cell surface morphology and cellular adhesion and an apparent increase in membrane lipid fluidity provide evidence that TPA produces a generalized change in cell membrane structure. These early effects do not appear to require de novo RNA and protein synthesis, but their physical and biochemical basis remains to be elucidated. These structural changes presumably account for several effects of TPA on membrane function including an alteration in membrane-associated Na/K ATPase, increased transport of ^{86}Rb, ^{32}P, and 2-deoxyglucose, enhanced phospholipid synthesis, and phospholipid deacylation (Table V). The functions of β-adrenergic receptors [51–53], the receptor for epithelial growth factor [54, 60], and perhaps other receptors involved in growth control are also altered. There are also alterations in cell surface glycoproteins [9, 10, 50], although these appear to occur later than the changes in membrane phospholipids. The ability of retinoids to antagonize certain actions of TPA [47, 74], to inhibit tumor promotion on mouse skin [56], and to inhibit certain other forms of carcinogenesis [63], may be due to reciprocal effects of the retinoids at the membrane level. Since a number of the effects of TPA resemble those of hormonal agents, it is possible that TPA acts by usurping, or disturbing, the function of a cellular receptor-response pathway normally used by an endogenous growth regulatory substance. Studies with TPA may therefore provide clues to the general phenomenon of hormonal carcinogenesis. In addition, the compound provides a useful probe for studies on membrane structure and function.

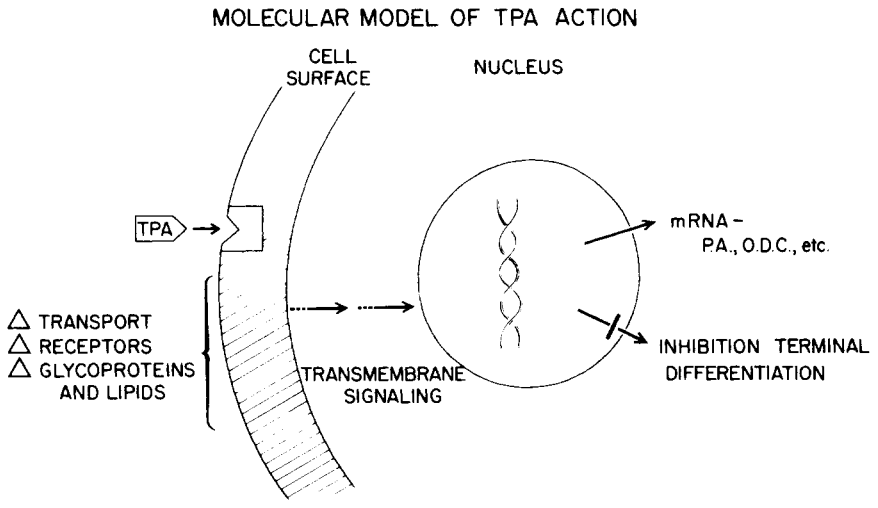

Fig. 3. Schematic model of the primary action of TPA on the cell surface with secondary effects on nuclear function. Reproduced from Weinstein et al [7].

Following the above early effects of TPA on cell membranes, there are a series of later or secondary cellular responses which require RNA and protein synthesis and therefore may reflect the action of "transmembrane signals" on nuclear and cytoplasmic functions. As in the case of certain polypeptide hormones and mitogens that exert their primary effects at the cell surface, the nature of these transmembrane signals is not well understood at the present time. These later responses to TPA include induction of plasminogen activator and ornithine decarboxylase synthesis, inhibition or stimulation of DNA synthesis, altered cell surface glycoproteins and effects on the expression of pre-existent programs of terminal differentiation (Table IV).

We must emphasize that cell types differ considerably in terms of their responses to TPA. Differences in response to the same hormone by different target cells are well known in endocrinology. Clonal variants of murine erythroleukemia cells that are resistant to TPA inhibition of terminal differentiation have recently been isolated [64] and these may prove useful in dissecting out the diverse actions of TPA. Since the transformation process itself leads to changes in cell surface structure and function, one might anticipate that cells previously altered by exposure to a chemical or viral carcinogen would have quantitatively and/or qualitatively different responses to TPA compared to completely normal cells. This aspect could in part explain the phenomenon of "enhancement" observed when transformed cells are exposed to TPA. Mechanisms involving sequential alterations in the response of the same cell type to tumor promoters and growth-controlling substances may underlie the stepwise process of tumor promotion and progression.

A number of years ago, Berenblum [65] postulated that tumor promoters act by inducing disturbances in differentiation and several observations in mouse skin provided indirect support for this hypothesis [66, 67, 68]. The results in cell culture systems provide direct evidence that the phorbol esters can be potent modifiers of terminal differentiation (Table IV). This effect may be an important clue to their ability to act as tumor promoters on mouse skin. A possible model is illustrated schematically in Figure 4. The stem cells in the epidermis are continually dividing; yet the tissue as a whole is in a stage of balanced

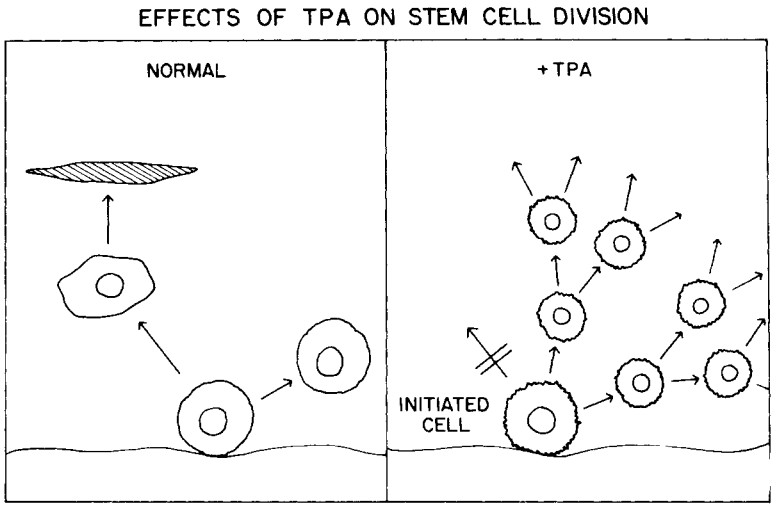

Fig. 4. Schematic representation of the normal mode of asymmetric stem cell division in epidermis and of the hypothesis that TPA induces exponential growth of an initiated stem cell, thus yielding a clone of such cells from which tumors can arise. Reproduced from Weinstein et al [7].

growth and a stable stem cell pool size is maintained. This is probably achieved by a regular asymmetric division of the stem cell. One daughter cell becomes a stem cell and the other daughter cell is committed to keratinize and terminally differentiate, thus irreversibly losing its growth potential. If an "initiated" stem cell were restrained to the stem cell mode of division, it could not increase its proportion in the stem cell pool. If, however, the stem cell division mode were interrupted by the action of a promoting agent, the initiated cell could undergo exponential division, thus yielding a clone of similar cells. Since TPA can also induce phenotypic changes in cells that mimic those of transformed cells, the micro-environment of a clone of such cells might itself enhance their further outgrowth and development into a tumor.

The fact that TPA can induce rather than inhibit differentiation in certain cell systems may, in part, explain its tissue specificity as a tumor promoter. In addition, this action might be exploited as a novel approach to cancer therapy in those tumors in which this type of compound induces terminal differentiation.

Although the above the speculations provide plausible models for thinking about mechanisms by which the phorbol esters enhance the induction of papillomas on mouse skin previously exposed to an initiating agent, they do not readily explain why repeated applications of these agents to mouse skin eventually result in the formation of malignant tumors that do not regress even after application of TPA has been stopped. Stated in other terms, the question is: How is a cellular response mechanism that is normally inductive converted to one which is constitutive or autonomous? This remains one of the major dilemmas in carcinogenesis. It seems likely that the answer to this question relates to the nature of the irreversible change in cells produced by the initiating carcinogens. Elsewhere, we have raised the question of whether the latter lesion is a simple random point mutation or a more complex change in genome structure related to normal mechanisms of cellular differentiation [7]. One hypothesis of two-stage carcinogenesis is that the initiating agent results in the acquisition of an aberrant program of differentiation, but this program remains dormant until expression of the related genes is induced by the promoting agent. With

repeated induction the expression of this program becomes "locked in" by a mechanism (yet to be discovered) similar to those that provide stability to normal states of differentiation [7].

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