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Chemically Unrelated Tumor Promoters Induce Identical Morphological Changes in Cultured Rat Oral Epithelium

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Abstract—*By use of phase contrast microscopy, transmission and scanning electron microscopy the cytomorphological effects of different known tumor promoters (TPA, teleocidin, mezerein and anthralin) were studied and compared to the cytomorphological effects of a variety of non- or weak promoting irritants (ethylphenylpropiolate (EPP), phorbol, acetone, ethanol and dimethyl sulfoxide (DMSO)). The studies were conducted in cultures of stratifying rat tongue epithelial cells. It was demonstrated that the tumor promoters induce characteristic cytomorphological alterations, the most striking changes being elongation of the cells and formation of long cytoplasmic extensions together with induction of so-called "dark cells". The non-promoting irritants exerted well-known cytotoxic reactions like cell rounding and cell sloughing. It is suggested that the characteristic tumor promotor induced cytomorphological effects partly reflects a block of the intercellular communication and thus should be paid more attention as an important characteristic event among the pleiotropic effects exerted by tumor promoters.*

INTRODUCTION

SINGLE application of the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) is known to induce marked hyperplasia and morphological changes in the epithelium of mouse back skin [1–3]. The most striking effect on the epidermis seems to be the induction of "dark" keratinocytes [2, 4]. Morphological changes induced by TPA have also been reported in a variety of *in vitro* systems including human fibroblasts [5], chick embryo fibroblasts [6], mouse fibroblasts [7, 8], mouse epithelial cells [9], canine kidney cells [10], monkey kidney cells [11], hamster oral epithelial cells [12], and human epithelial cells [13–15]. In these studies, the most frequent observation has been the alteration of the phenotype of TPA-treated cells toward a pronounced elongation and formation of long cytoplasmic extensions.

The present paper describes that not only TPA but also different classes of tumor promoters such as teleocidin, anthralin and mezerein induce identical characteristic morphological changes in cultured rat oral squamous epithelial cells.

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MATERIALS AND METHODS

Tissue culture

Subcultures of rat tongue squamous epithelial cells were prepared as previously described [16]. Briefly, the cells were plated in plastic culture flasks (Falcon 3013) as droplets of cell suspension containing 2×10^5 cells per 20 μl and were supplied with complete culture medium consisting of minimum essential medium with Earle's salt solution (MEM), 5% calf serum and 40 μg/ml gentamycin sulfate. The incubation temperature was 30° C and the gaseous environment consisted of 95% air and 5% CO_2.

Preparation and application of test chemicals

TPA and phorbol were purchased from P-L Biochemicals, GmbH, W. Germany, ethylphenylpropiolate (EPP) from EGA-Chemie, W. Germany, and mezerein from LC Service Corp., U.S.A. Anthralin was commercially available (Trøjborg Apotek, Aarhus, Denmark), whereas teleocidin was kindly donated by Dr. Fujiki, National Cancer Center Research Institute, Tokyo, Japan. Reagent grade acetone and ethanol were used.

All chemicals were dissolved in dimethyl sulfoxide (DMSO), purchased from Pierce Chemical, U.S.A., to a final concentration in the culture

medium of 0.2% DMSO. The influence of a wide range of concentrations of each chemical on cell proliferation was prior to the performance of morphological studies assessed by computerized measurements of the outgrowth areas during a 14-day period (Table 1). The test chemicals were added to confluent cell cultures day 4 after subcultivation.

For each dose level of all chemicals microscopy examinations were made in duplicate flasks. Observations were conducted in two identical experiments performed with a 4-week interval.

Phase contrast microscopy

The cultures were examined in a Leitz Diavert phase contrast microscope and photographed every 10 min during the first 4 hr following test chemical application. The cultures were subsequently examined during 1 week with increasing intervals from 20 min to 6 hr.

Scanning electron microscopy

Two hours following exposure to test chemicals, when morphological cell changes were fully developed, the medium was removed and cultures were processed for scanning electron microscopy. They were rinsed in MEM (30° C) followed by fixation for 2 hr at 4° C in a 1 : 1 mixture of 2.5% glutaraldehyde and 2% aqueous OsO_4 (1 : 1) in 0.2 M sodium cacodylate (pH 7.4). After rinsing in the same buffer, specimens (16–20 sq mm) were cut out from the bottom of the culture flasks with a warm scalpel and were critical point dried with Polaron E3000 CPD. Specimens were mounted on metal stubs using a double-coated tape (Scotch 665), supplemented with Silverpaint and coated with gold-platinum with Polaron E5000 Diode Sputtering System. Examination was made in a Philips 505 scanning electron microscope.

Transmission electron microscopy

Two hours after exposure to test chemicals the cultures were processed for TEM study. They were fixed in 2.5% glutaraldehyde-2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 hr at room temperature, rinsed in the same buffer overnight and then post-fixed in cacodylate buffered OsO_4 for 1 hr at 4° C. Following staining *en bloc* with 1% aqueous uranyl acetate and dehydration in ascending concentrations of ethanol (50–100%), the cultures were infiltrated with a mixture of ethanol and hydroxypropyl methacrylate (HPMA) for 30 min each in ratios of 2 : 1, 1 : 2 and finally in pure HPMA. This was followed by an infiltration with HPMA and Epon in ratios of 2 :1, 1 : 2 each for 2–3 hr and then embedded *in situ* with pure Epon. After polymerization, the epon blocks were peeled from the flasks. Thin sections were cut parallel or vertical to the substratum with a diamond knife, double stained with uranyl acetate and lead citrate, and examined in a Philips 301 transmission electron microscope at 60 kV. Semithin sections were stained with toluidine blue for light microscopic observation.

Table 1. *Dose range of testchemicals per* ml *medium. Toxicity was assessed by computerized measurements of the outgrowth areas during a* 14 days *period. Non-toxic doses did not affect the growth rate*

	Toxic doses	Non-toxic doses
TPA	1 µg	
	250 ng	
	100 ng	
		10 ng
		1 ng
Teleocidin	1 µg	
	100 ng	
		10 ng
		1 ng
Mezerein	500 ng	
	100 ng	
		10 ng
		1 ng
Anthralin	100 µmol	
		10 µmol
		1 µmol
EPP	5 µg	
	1 µg	
		500 ng
Phorbol	500 ng	
	100 ng	
		10 ng
Acetone	1% v/v	
	0,1% v/v	
		0,01% v/v
Ethanol	10% v/v	
	1% v/v	
		0,01% v/v
DMSO	10% v/v	
	5% v/v	
		1% v/v

RESULTS

As a general observation no significant differences either between flasks having received identical treatment or inter-experimentally were recognized.

Findings with phase contrast microscopy

Within 10 min after exposure to TPA, teleocidin, and mezerein in non-toxic doses (Table 1), morphologic changes of the cultured rat epithelial cells became evident and reached a maximum after 2–3 hr. These changes consisted of marked widening of the intercellular spaces and transformation of the polygonal epithelial cells to elongated, triangular or spindleshaped, fibroblastoid cells (Figs. 1, 3, 5 and 6). Many of these morphologically

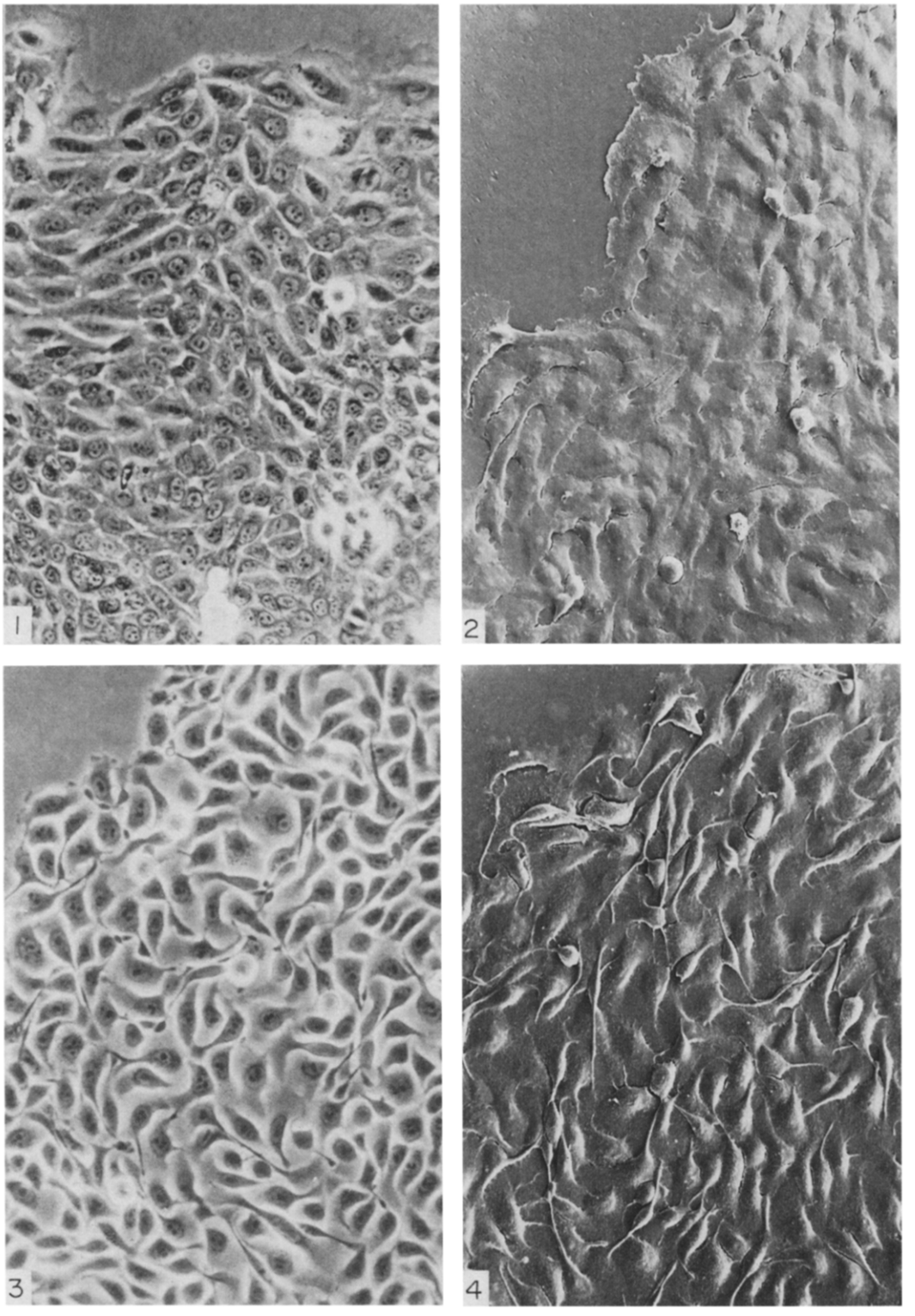


Fig. 1. *Culture of rat tongue squamous epithelial cells. Untreated control culture day* 4 *following subcultivation. Phase contrast* × 240.

Fig. 2. *As Fig.* 1. *SEM* × 240.

Fig. 3. *Culture exposed to* 100 ng *TPA per* ml *medium day* 4 *following subcultivation. Phase contrast* × 240.

Fig. 4. *As Fig.* 3. *SEM* × 240.

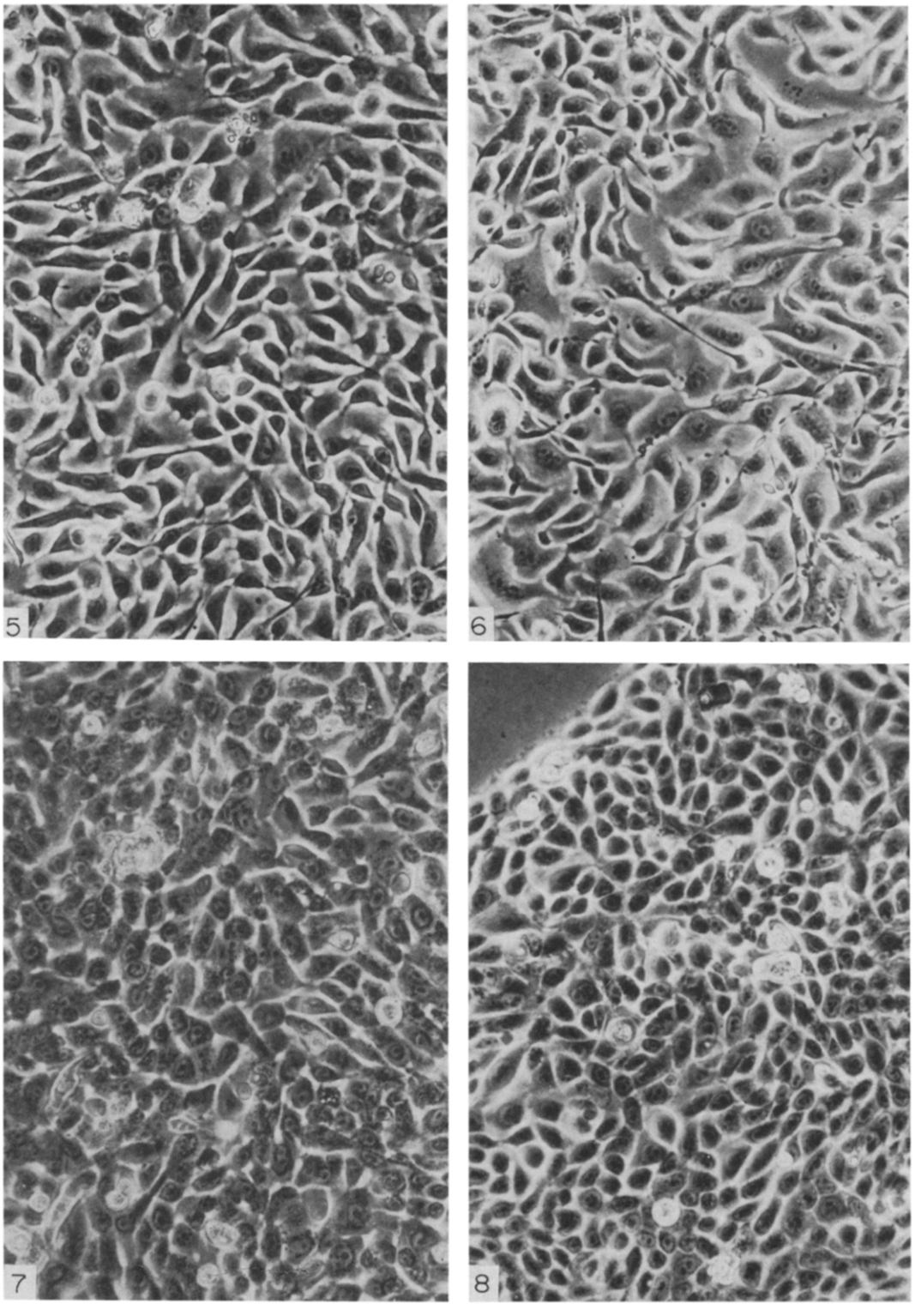


Fig. 5. *Culture exposed to* 100 ng *teleocidin per* ml *medium day* 4 *following subcultivation. Phase contrast* × 240.
Fig. 6. *Culture exposed to* 100 ng *mezerein per* ml *medium day* 4 *following subcultivation. Phase contrast* × 240.
Fig. 7. *Culture exposed to* 100 ng *phorbol per* ml *medium day* 4 *following subcultivation. Phase contrast* × 240.
Fig. 8. *Culture exposed to* 1 μg *EPP per* ml *medium day* 4 *following subcultivation. Phase contrast* × 240.

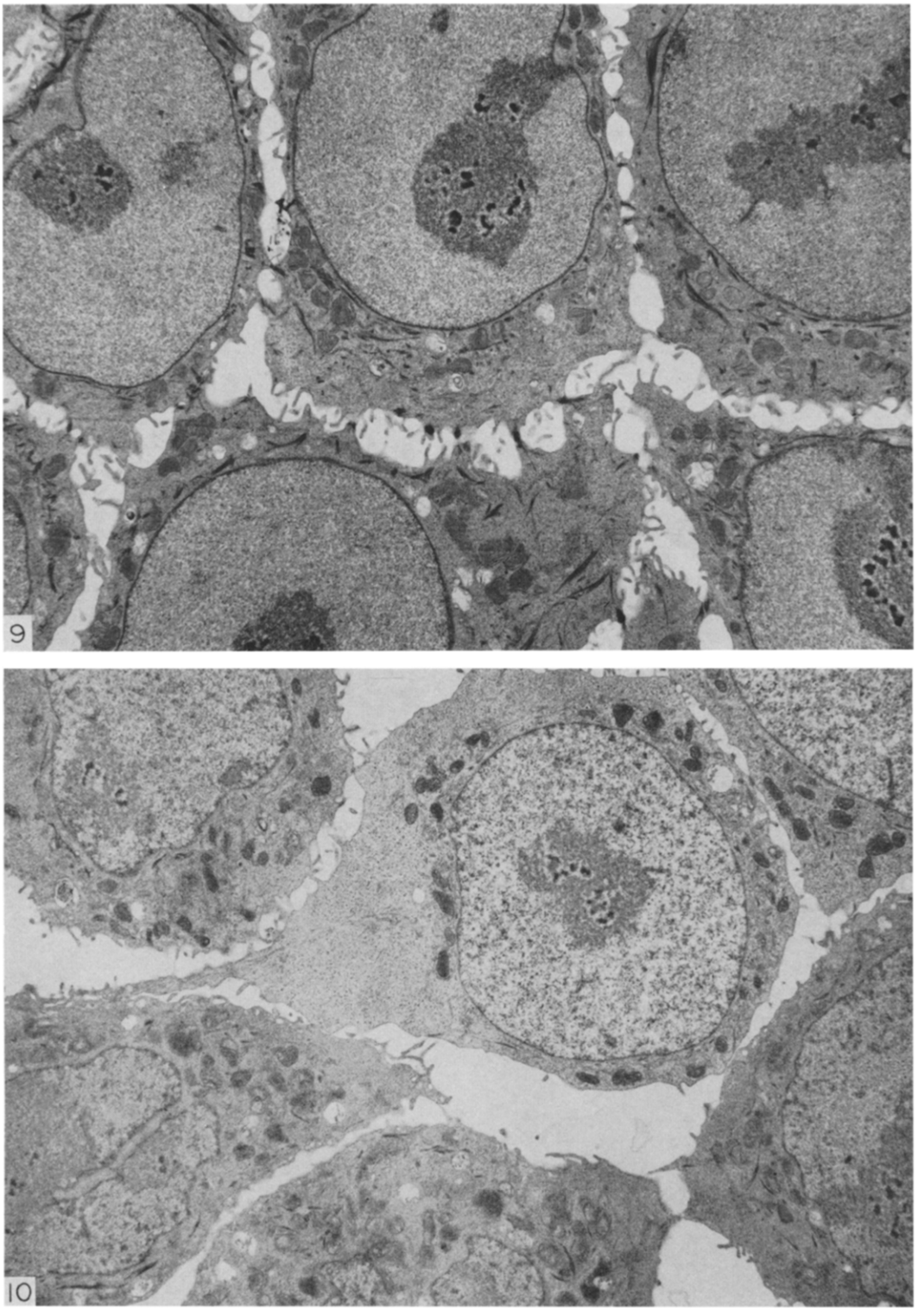


Fig. 9. *Control culture. Polygonal cell shape, several intercellular junctions. TEM* × 5000.

Fig. 10. *Culture exposed to TPA,* 100 ng *per* ml *medium during* 1 hr. *Elongated cell shape. Organelles concentrated in the perinuclear region. Very few intercellular junctions. TEM* × 5000.

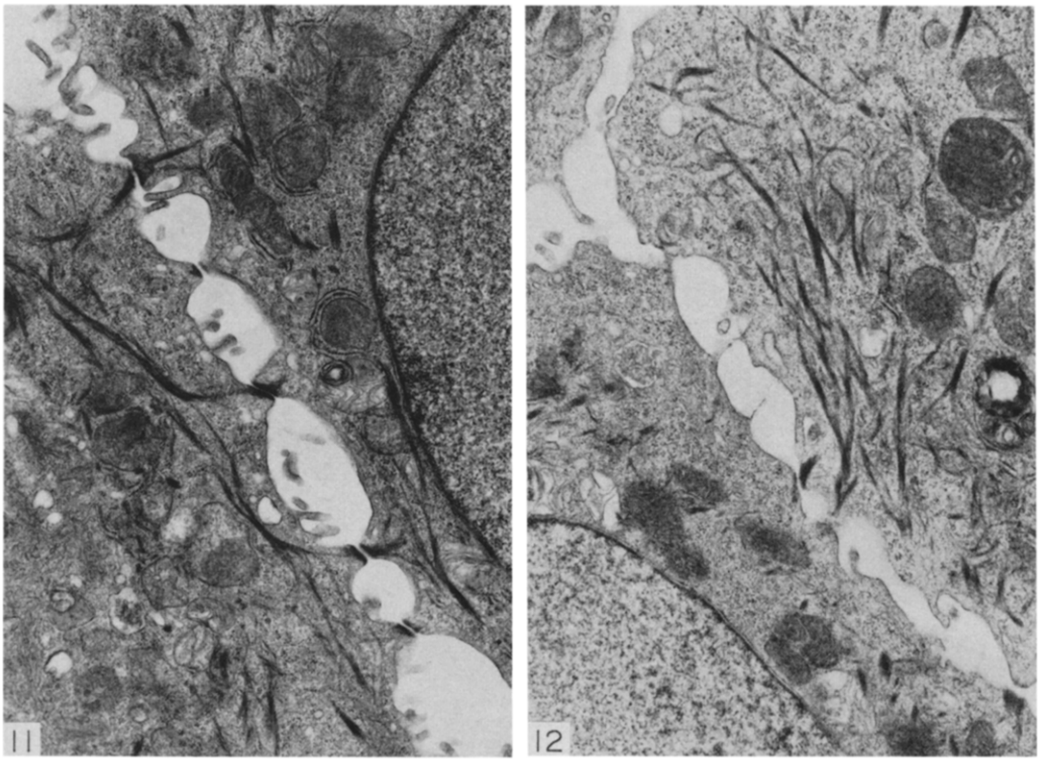
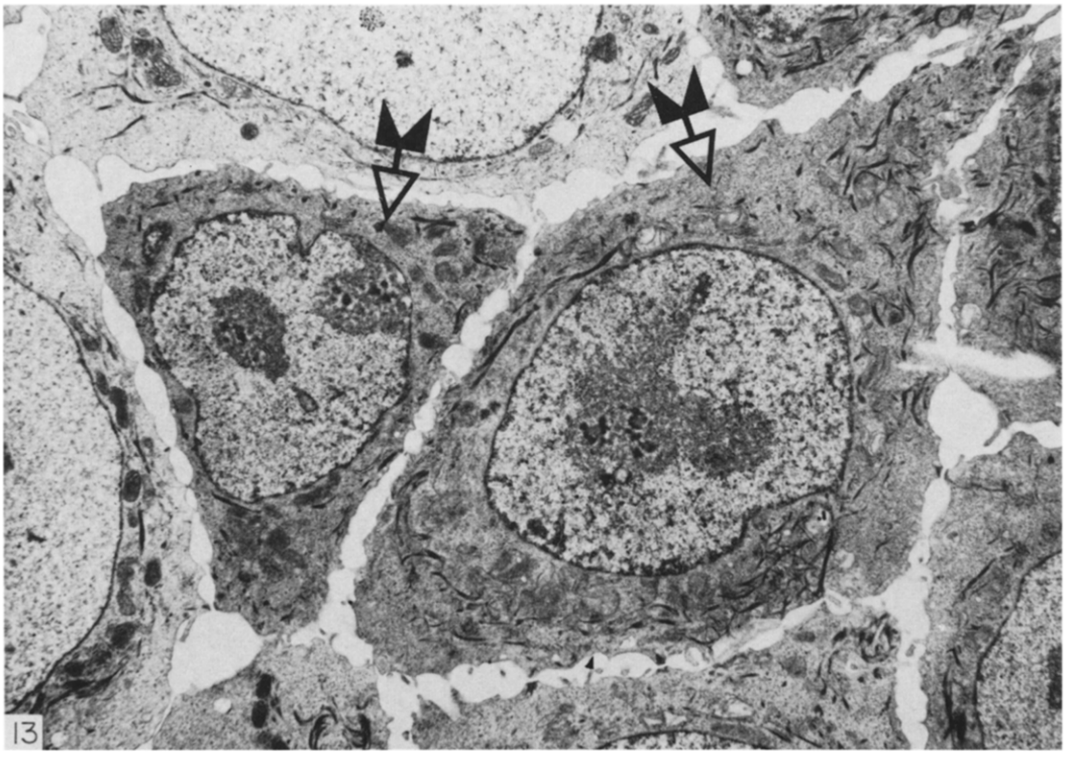


Fig. 11. *Control culture. Adjacent cells connected with numerous symmetric desmosomes. TEM* × 15000.

Fig. 12. *Culture exposed to TPA*, 100 ng *per* ml *medium during* 1 hr. *Asymmetric or disorganized desmosomes. TEM* × 15000.



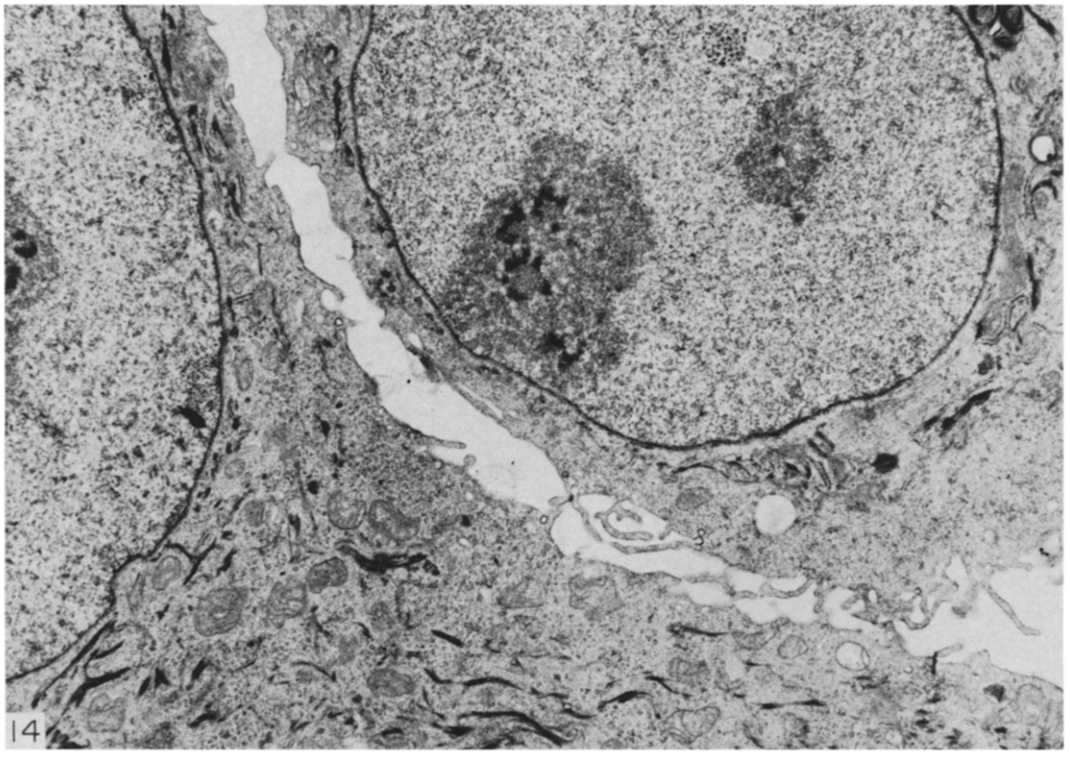


Fig. 13. *Survey of culture exposed to teleocidin,* 100 ng *per* ml *medium during* 1 hr. *Spindle shaped cells and virtually no intercellular junctions. Light cells and dark cells (arrows). TEM* × 5000.

Fig. 14. *Treatment as in* Fig. 13. *No intercellular junctions between closely apposed cells. TEM* × 15000.

modified cells displayed long thin cytoplasmic extensions up to 50 μm in length. The cellular response to anthralin developed more gradually than to the other tumor promoters used, but was morphologically identical to the other tumor promoter treated cultures after 8–10 hr. The morphological changes induced by the tumor promoters were reversible, in that the fibroblastoid cells slowly regained their original polygonal shape during a period of 24–36 hr. A change to medium not containing tumor promoter, accelerated at any time the normalization of cell morphology.

Application of tumor promoters in toxic doses (Table 1) resulted in granulation and vacuolization of the cytoplasm and formation of rounded cells.

Treatment of the cultures with non-toxic doses of the non-promoting irritants EPP, phorbol, acetone, ethanol and DMSO did not provoke morphological changes like those seen in the tumor promoter treated cultures. Cytotoxic reactions, i.e. granulation and vacuolization of the cytoplasm and increasing cell rounding occurred in the cells treated with toxic concentrations of these non-tumorpromoting chemicals, but cellular shape and intercellular relations generally stayed normal (Figs. 7 and 8).

Findings with scanning electron microscopy

The control cultures consisted of polygonal epithelial cells whose surfaces were covered with small villi or ridges. The cells were closely apposed with numerous tight interdigitations (Fig. 2). In sharp contrast, the promoter treated cultures showed a marked variation in cell size, shape and surface morphology. A dominating feature was the presence of elongated, fibroblastoid cells with long slender cytoplasmic extensions traversing great distances to interconnect with other cell bodies or processes several cell lengths away (Fig. 4). Cells with more pronounced microvillous surfaces were also an occasional finding. Although some other cells still retained their polygonal shape and relatively close intercellular relations, the intercellular spaces were generally dilated and tight intercellular interdigitations were not observed. Several mitotic cells could be identified even in areas where intercellular regulation mechanisms between the majority of the cells by morphological criterias were shown to be out of control.

The rounded cells seen at the LM level after exposure to strongly toxic doses of promoting and non-promoting test chemicals could easily be identified in SEM, the cell membranes showing numerous blebs. Neither fibroblastoid cells nor cells with long slender cytoplasmic extensions were observed in these cultures.

Findings with transmission electron microscopy

Figure 10 illustrates a typical survey electron micrograph of cells treated with TPA. Compared with control cultures, the cells were apparently devoid of their cell-to-cell contacts (Fig. 9). The tonofilaments were decreased in number and showed fragmentation and formation of irregular aggregates and whorls. Tonofilaments often showed detachment from desmosomes, dispersed distribution and much less prominent bundle formation than in control cells. Microfilaments frequently seen at the periphery of untreated cells were virtually absent in the TPA-treated cells. Desmosomes became hardly visible and, if recognizable, displayed separation between the apposing attachment plaques, asymmetry in width, disappearance of an intercellular contact layer and loss of insertion of tonofilaments. Gap function-like structures frequently seen between the control cells were extremely rare between the promoter-treated cells (Figs. 11–14). Furthermore, the cell organelles were often concentrated in the perinuclear region leaving a broad organelle-free zone at the cell periphery.

Long slender cytoplasmic extensions, the most characteristic finding in the tumor promoter treated cells, were revealed to contain tonofilaments and microtubules running parallel to their long axis, free ribosomes, numerous pinocytotic vesicles, some coated vesicles and vesicular or tubular profiles of smooth membrane bound structures. Vesicles and smooth membranous structures were concentrated particularly at the tip of cell extensions, which were either attached to other cell bodies and processes or freely protruded into the intercellular spaces often with bulging ends. Lysosomal-like bodies such as multivesicular bodies were also present in the cell processes.

The so-called "dark cells", clearly demonstrated in toluidine blue stained semi-thin sections, could easily be recognized in promoter-treated cultures in TEM. They appeared in groups or singly throughout the cultures. Both cytoplasmic and nuclear matrices showed high electron density, and densely packed ribosomes further contributed to the dark appearance of these cells. Relatively large nuclei with coarse nucleoplasm, prominent nucleoli and abundant cytofilaments were additional characteristics of the dark cells.

DISCUSSION

The present study has demonstrated that not only TPA, as earlier demonstrated, but also other known tumor promoters are capable of inducing characteristic morphological changes in exposed cells. The observations made by phase contrast microscopy are in full accordance with previously described findings in TPA-treated cells *in vitro*, either epithelial cells or fibroblasts of different origin [5–14]. The occurrence of elongated cells, long cytoplasmic extensions and rounded cells has also been reported in the epidermis of mouse back

skin following TPA treatment [1–4]. The present ultrastructural observations of numerous microtubules, pinocytotic vesicles and multivesicular bodies in the tumorpromoter treated cells also correspond well with previous *in vivo* observations [2].

Based on the ultrastructural features, the electron dense cells frequently observed in promoter treated cultures were identified as "dark cells" which have been described in some *in vivo* studies as a remarkable tumor promoter effect [1–4]. The widened intercellular spaces and decreased intercellular connections shown by SEM are well in accordance with the TEM findings exhibiting a dramatic decrease of the amount of intercellular junctions.

One study [8] has reported that a variety of potent tumor promoters cause a rapid but reversible disorganization of microfilaments in mouse BALB/3T3 fibroblasts. The present study supports these findings by the demonstration of disorganized microfilaments in the tumor promoter treated epithelial cells.

In non-toxic as well as toxic doses the non-tumor-promoting chemicals were not able to provoke morphological changes similar to those induced by tumor promoters. Thus the characteristic phenotypical changes induced by tumor promoters cannot be interpreted as purely toxic effects. Further, the changes are transient and of relatively short duration, which is in agreement with a few reports stating that changes are initiated within 2–120 min [8, 11, 21] and almost completely reversed within 7–10 hr [11].

Different classes of tumor promoting chemicals possess the ability to inhibit the metabolic intercellular communication [16]. It is known that the metabolic cooperation between the cells is mediated by their gap junctions [17] and recent studies have demonstrated a decreased number of gap junctions in TPA-treated Chinese hamster V-79 cells [18] and in mouse epidermis *in vivo* [19, 20]. The present study has clearly demonstrated that tumor promoter treatment decreases the number of intercellular junctions between epithelial cells in culture. The observed morphological changes may well indicate that the tumor promoters exert influence on the metabolic cooperation between cultured epithelial cells, as previously shown in fibroblastic cultures [16, 22, 23].

Tumor promoting phorbol esters have been shown to induce increased membrane fluidity of cells [25, 26] and because of this membrane-mediated effect some morphological changes might be expected. Although it has been demonstrated that the morphological changes can be induced by TPA even in enucleated cells [24], any relationship between the morphological configuration of cells and the changes at the molecular level still remain uncertain.

Epithelial cells when seeded *in vitro* are known to exhibit elongated, fibroblastoid morphology and formation of long cytoplasmic extensions (attachment bridges) during the initial stages of cellular adherence [27]. Individual epithelial cells seeded on plastic will go through a stage of reassociation during which the attachment bridges are numerous [27]. Once contacts to neighboring cells have been established, the cell layer attain normal pavement-like morphology. Similar observations have been made in subcultures in the present *in vitro* system. The morphological changes of tumor promoter treated cells thus seem to mimic those of newly seeded, untreated cells establishing intercellular contacts. It is therefore suggested that the tumor-promoting chemicals transiently make the cells morphologically react as individual cells through interference with the intercellular communications and possibly by membranous alterations at the molecular level.

The frequent observation of mitotic cells in the tumor promoter treated cultures strongly indicate that the cells do not suffer from toxic injuries. It further demonstrates that tumor promoter treated cells do undergo division, even while the general intercellular communication is inhibited and the metabolic regulation of the cells is out of normal control. The observation supports the theories of Trosko *et al.* [28] that tumor promoters by blocking the intercellular communication inhibit the repressor effects of normal cells on adjacent initiated cells thus allowing these to expand into miniclones. Further repeated exposure to a tumor promoter or a hyperplasiogenic agent could result in the expansion of these clones into a tumor mass [28].

To our knowledge chemically induced tumor promotion in rat epithelium has not yet been performed. Still the accordance between our observations and observations made in a variety of animal and cell culture studies representing sensitive targets for tumor promotion make us put forward the tentative suggestion that the present findings are related to the tumor promoting effect of the compounds tested.

Since the modifying effect on cell morphology seems to be a common characteristic of tumor promoting chemicals in spite of their different chemical structure, it should be elucidated whether this effect reflects common critical events concerning intercellular communication between epithelial cells.

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REFERENCES

1. Raick AN, Burdzy K. Ultrastructural and biochemical changes induced in mouse epi-

dermis by a hyperplastic agent, ethylphenylpropriolate. *Cancer Res* 1973, **33,** 2221–2230.
2. Raick AN. Ultrastructural, histological, and biochemical alterations produced by 12-*O*-tetradecanoyl-phorbol-13-acetate on mouse epidermis and their relevance to skin tumor promotion. *Cancer Res* 1973, **33,** 269–286.
3. Raick AN. Late ultrastructural changes induced by 12-*O*-tetradecanoyl-phorbol-13-acetate in mouse epidermis and their reversal. *Cancer Res* 1973, **33,** 1096–1103.
4. Klein-Szanto A, Major S, Slaga T. Quantitative evaluation of dark keratinocytes induced by several promoting and hyperplastic agents: Their use as an early morphological indicator of tumor-promoting action. In: Hecker E *et al.*, eds. *Carcinogenesis—A Comprehensive Survey*. New York, Raven Press, Vol 7, 305–309.
5. Diamond L. O'Brien S, Donaldson C, Shimuza Y. Growth stimulation of human diploid fibroblasts by the tumor-promoter, 12-*O*-tetradecanoyl-phorbol-13-acetate. *Int J Cancer* 1974, **13,** 721–730.
6. Driedger P, Blumberg P. The effect of phorbol diesters on chicken embryo fibroblasts. *Cancer Res* 1977, **37,** 3257–3265.
7. Sivak A. Induction of cell division in BALB/c-3T3 cells by phorbol myristate acetate or bovine serum: Effects of inhibitors of cyclic AMP phosphodiesterase and NA^+-KA^+-ATPase. *In Vitro* 1977, **13,** 337–343.
8. Sakiyama S, Hiwasa T. Disorganization of microfilaments caused by tumor promoters in mouse fibroblasts. *Gann* 1984, **75,** 4–7.
9. Yuspa S, Ben T, Patterson E, Michael D, Elgjo K, Hennings H. Stimulated DNA synthesis in mouse epidermal cell cultures treated with 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer Res* 1976, **36,** 4062–4068.
10. Ojakian GK. Tumor promoter-induced changes in the permeability of epithelial cell tight junctions. *Cell* 1981, **23,** 95–103.
11. Schliwa M, Nakamura T, Porter K, Euteneuer U. A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. *J Cell Biol* 1984, **99,** 1045–1059.
12. Schuster GS, Singh BB, Erbland JF, Ringler MS. Effects of 12-*O*-tetradecanoylphorbol-13-acetate on oral epithelium in vitro. *J Oral Path* 1984, **13,** 382–389.
13. Parkinson JK, Emmerson A. The effects of tumor promoters on the multiplication and morphology of cultured human epidermal keratinocytes. *Carcinogenesis* 1982, **3,** 525–531.
14. Hawley-Nelson P, Stanley J, Schmidt J, Gulling M, Yuspa S. The tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate, accelerates keratinocyte differentiation and stimulates growth of an unidentified cell type in cultured human epidermis. *Exp Cell Res* 1982, **137,** 155–167.
15. Willey JC, Saladino AJ, Ozanne C, Lechner JF, Harris CC. Acute effects of 12-*O*-tetradecanoylphorbol-13-acetate, teleocidin B, or 2,3,7,8- tetrachlorodibenzo-p-dioxin on cultured normal human bronchial epithelial cells. *Carcinogenesis* 1984, **5,** 209–215.
16. Jepsen A, MacCallum D, Lillie J. Fine structure of subcultivated stratified squamous epithelium. *Exp Cell Res* 1980, **125,** 141–152.
17. Trosko JE, Yotti LP, Warren ST, Tsushimoto G, Chang E. Inhibition of cell–cell communication by tumor promotors. In: Hecker E *et al.* eds. *Carcinogenesis—A Comprehensive Survey*. New York, Raven Press, 1982, Vol. 7, 565–585.
18. Loewenstein W. Junctional intercellular communication and the control of growth. *Biochim Biophys Acta* 1979, **560,** 1–65.
19. Yancey S, Edens J, Trosko J, Chang C, Revel J. Decreased incidence of gap junctions between chinese hamster V-79 cells upon exposure to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. *Exp Cell Res* 1982, **139,** 329–340.
20. Kalimi GH, Sirsat SM. Phorbol ester tumor promoter affects the mouse epidermal gap junctions. *Cancer Lett* 1984, **22,** 343–350.
21. Kalimi GH, Sirsat SM. The relevance of gap junctions to stage I tumor promotion in mouse epidermis. *Carcinogenesis* 1984, **5,** 1671–1677.
22. Fitzgerald D, Murray A. A new intercellular communication assay: Its use in studies on the mechanism of tumor promotion. *Cell Biol Int Rep* 1982, **6,** 235–242.
23. Enomoto T, Sasaki Y, Shiba Y, Kanno Y, Yamasaki H. Tumor promoters cause a rapid and reversible inhibition of the formation and maintenance of electrical cell coupling in culture. *Proc Natl Acad Sci US-Biol Sci* 1981, **78**(a), 5628–33.
24. Nagle DS, Blumberg PM. Activity of phorbol ester tumor promoters on enucleated swiss 3T3 cells. *Cancer Res* 1980, **40,** 1066–1072.
25. Fisher PB, Falmm M, Schachter D, Weinstein IB. Tumor promoters induce membrane changes detected by fluorescence polarization. *Biochem Biophys Res Commun* 1979, **86,** 1063–1068.
26. Castagna MC, Rochette-Egly C, Rosenfield C, Mishal Z. Altered lipid microviscosity in lymphoblastoid cells treated with 12-O-tetradecanoyl phorbol-13-acetate, a tumor promoter. *FEBS Lett* 1979, **100,** 62-66.
27. Karasek M, Liu S. Keratinization of epidermal cells in cell culture. In: Seiji M, Bernstein J, eds. *Biochemistry of Cutaneous Epidermal Differentiation*. Tokyo, University of Tokyo Press, 1977, 336–358.
28. Trosko JE, Chang C, Mecalf A. Mechanism of tumor promotion: potential role of intercellular communication. *Cancer Invest* 1983, **1,** 511–526.