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TUMOR PROMOTERS INHIBIT METABOLIC COOPERATION IN COCULTURES OF EPIDERMAL AND 3T3 CELLS

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Summary: Cultures of mouse epidermal cells (HEL/37) were prelabelled by incubation for 2-3 h with ^{3}H-uridine. The cells were washed and cocultured for 4 h with unlabelled 3T3 cells. Communication between 3T3 and HEL/37 cells, as evidenced by the transfer of label from HEL/37 cells to contacting 3T3 cells, was inhibited by inclusion of the tumor promoting agents, 12-O-tetradecanoylphorbol-13-acetate (10^{-7}-10^{-9} M) and phorbol-12,13-didecanoate (10^{-7} M) during coculture. Non-promoting derivatives of these phorbol esters did not inhibit transfer of label.

Tumor promoters are non-carcinogenic compounds which greatly enhance tumorigenesis in cells previously initiated by exposure to a low dose of a carcinogen (1). Initiated cells will not develop into tumors unless repeatedly exposed to the promoting stimulus. Despite intensive investigation, the mechanism of promotion is unknown (see Refs. 2 and 3 for discussion of models). Much of the information on promoter action has come from studies on 2-stage carcinogenesis in mouse skin, and from more recent work on the effects of promoters on cultured mammalian cells. Tumor promoters induce many biochemical and morphological changes in cells (some of which are characteristic of the transformed phenotype), and have recently been shown to have profound effects on the terminal differentiation of several cultured cell types (see 3, 4 for references). It is not known which of these changes are causally related to promotion.

One of the requirements for successful promotion is the ability of initiated cells to escape local tissue discipline and to selectively pro-

Abbreviations: DMSO, dimethyl sulfoxide; PDD, phorbol-12,13-didecanoate; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor.

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liferate into clonal outgrowths of transformed cells. It seemed to us possible that initiated cells may be limited in their ability to develop into tumors by metabolic interaction with neighboring normal cells, and that promoters may directly interfere with such communication between cells.

In the present communication, we show that tumor promoters can inhibit the establishment of metabolic cooperation between cocultured mammalian cells. The existence of junctions which permit direct communication between cells has been repeatedly demonstrated (5). Such junctions have been detected by measurement of electrical coupling between cells, by micro-injection of tracer molecules and by metabolic cooperation experiments (5). We have used a variant of the latter technique in which radioactive nucleotides in prelabelled donor cells are transferred to initially unlabelled cells maintained in coculture (6). The transfer of label between cells in contact is detected by autoradiography. It has not been unequivocally established that metabolic cooperation involves transfer of molecules through structures physically characterized as gap junctions, but this is likely (7, 8).

Cell lines and culture conditions. The mouse epidermal cell line (HEL/37; used at passages 160-174) was obtained from Dr. N.E. Fusenig, Institute for Biochemistry, German Cancer Research Centre, Heidelberg. The line developed spontaneously from untreated primary cultures, has been characterized as epidermal by morphological and immunological methods and expressed tumorigenic behavior after transplantation *in vivo* ((9); personal communication from Dr. Fusenig). Swiss albino 3T3 cells (used at passages 30-33) were obtained from Dr. M. Stanley, Department of Pathology, University of Adelaide. Both cell lines were maintained in Eagle's minimum essential medium supplemented with 10% foetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), and containing antibiotics (penicillin, 100 I.U./ml; streptomycin, 100 µg/ml). All incubations were conducted at 37° in a humidified atmosphere of 5% CO_2. HEL/37 cells were subcultured by removing confluent cells from the dishes by incubation in a solution containing 5.6 mM glucose, 0.15 M NaCl, 2.7 mM EDTA, pH 7.4 and 0.1% trypsin (Flow Labs, Rockville, U.S.A.) at 37° for 10 min. For removal of the 3T3 cells, the glucose-NaCl solution contained 5.4 mM EDTA and no trypsin.

Coculture experiments. HEL/37 cells were plated onto glass cover slips in 35 mm plastic dishes (Lux) at a density of 1×10^5 cells per dish. The cells were used after 2 days of culture (3.7×10^5 cells per dish), a time of exponential growth. The medium was removed and replaced with 1 ml of medium containing 5 µCi [5-^{3}H] uridine (5 Ci/mmole). After incubation for 2 h or 3 h at 37°, the medium was removed and the cells washed 4 times with complete medium containing 0.1 mM unlabelled uridine.

The dishes were then cocultured with 5.0 x 10^4 3T3 cells in 2 ml of medium containing either appropriate concentrations of the phorbol esters dissolved in 2 µl DMSO or DMSO alone. The addition of phorbol esters did not affect the plating efficiency of 3T3 cells during coculture. After incubation for 4 h at 37°, the cocultures were washed in 1 ml of medium containing 0.1 mM uridine. The cells were washed with 1 ml of fixer solution (17.3 M methanol-0.87 M acetic acid), incubated with a further 1 ml of fixer for 10 min at room temperature, and washed in running water for 2 h. The cover slips were mounted on glass slides and processed for autoradiography using Ilford K-2 emulsion. The slides were developed (4 min; 20°; Kodak D-19 developer) after 6 days of exposure, stained with hematoxylin (Harris) and mounted.

3T3 cells in contact with HEL/37 cells were scored as labelled or unlabelled by comparison with randomly chosen areas visually free of cells. Slides were examined under coded, blind conditions by two investigators independently (A.W.M. and D.J.F.), and at least 250 cells were scored on each slide. Both sets of data showed the same differences between test and control groups, and the results presented are those determined by one investigator (A.W.M.).

Incorporation of [5-^{3}H] uridine into acid-soluble and acid-insoluble material. Epidermal cells were cultured and labelled with ^{3}H-uridine for 3 h as described above. The dishes were washed with 1 ml medium containing 0.1 mM uridine, the cover slips transferred to clean dishes and washed a further five times. The cells were incubated in 2 ml medium containing phorbol esters or DMSO. After 4 h of incubation at 37° the medium was removed and the cells extracted in 2 ml of cold 5% (w/v) trichloroacetic acid (30 min; 2°). The cells were washed with a further 2 ml of cold 5% trichloroacetic acid and the original extract and washings combined. The acid insoluble residue was dissolved in 2 ml of 0.1 N NaOH (1 h; 20°) and the solution acidified by addition of 0.3 ml 1 N HCl. Aliquots of both the acid-soluble and the acidified NaOH extracts were added to 10 ml of a Triton X 114-Xylene scintillation fluid and radioactivity measured with a Packard Tri-Carb scintillation spectrometer.

Other chemicals. PDD, 4α-PDD and 4-O-methyl TPA were obtained from PL-Biochemicals, Milwaukee, Wisconsin, U.S.A.; TPA was obtained from Cambrian Chemicals, Croydon, England. [5-^{3}H] Uridine (sp. act. 5 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, England. EGF was purified to homogeneity from the submaxillary glands of male Swiss albino mice (10).

RESULTS AND DISCUSSION

The tumor promoters TPA and PDD strongly inhibited the transfer of radioactivity from prelabelled HEL/37 mouse epidermal cells to 3T3 cells (Table 1). No inhibition was observed with either 4-O-methyl TPA or 4α-PDD, which are non-promoting derivatives of the active phorbol esters (11), or with EGF. Both cell types used in this study have specific surface receptors for EGF (12, unpublished). In control cocultures or cocultures treated with inactive phorbol derivatives, about 50% (experiment

Table 1. Effect of tumor promoters on metabolic cooperation in cocultures of HEL/37 and 3T3 cells

Treatment	Proportion labelled cells	Total cells scored	Range of proportion labelled cells on individual slides	Chi^2 (vs control)	P value
Experiment 1					
DMSO control (5)	0.46	1477	0.440-0.510		
10^{-7} M TPA (5)	0.096	1441	0.064-0.177*	484	<0.001
10^{-8} M TPA (3)	0.084	879	0.078-0.097	362	<0.001
10^{-7} M 4-O-methyl TPA (3)	0.45	979	0.402-0.545*	0.50	>0.30
10^{-7} M PDD (3)	0.114	850	0.086-0.156*	294	<0.001
10^{-7} M 4α-PDD (3)	0.47	883	0.466-0.473	0.04	>0.95
Experiment 2					
DMSO control (4)	0.350	1217	0.346-0.357		
10^{-7} M TPA (3)	0.060	918	0.051-0.073	251	<0.001
10^{-9} M TPA (5)	0.064	1527	0.046-0.093	359	<0.001
10^{-9} M EGF (5)	0.335	1536	0.318-0.360	0.59	>0.30

The transfer of label from HEL/37 donor cells to 3T3 recipient cells was determined by autoradiography as described in the Materials and Methods section. In experiments 1 and 2, the labelling times with [^{3}H] uridine were 3 and 2 h respectively. Recipient cells in contact with donor cells were scored under coded, blind conditions as labelled or unlabelled. Numbers in parentheses refer to the number of replicate slides examined for each treatment. The Chi^2 analysis was carried out on the pooled data for each treatment group.

*Chi^2 analysis indicated heterogeneity between the replicate slides in these treatment groups.

1) and 30% (experiment 2) of the 3T3 cells in contact with HEL/37 cells were labelled over the 4 h period. In all coculture experiments only 3T3 cells which were in visible contact with epidermal cells were labelled. Thus the transfer of label is unlikely to be due to the release of [^{3}H] uridine from the prelabelled HEL/37 cells and its subsequent uptake by the 3T3 cells. Although the nature of the material transferred is not known, previous experiments with different cell types have indicated that nucleotides but not nucleic acids can pass between cells (6). The inhibition

Table 2. Effect of TPA and PDD on the distribution of label between the acid-soluble and acid-insoluble pools of HEL/37 cells prelabelled with [^{3}H] uridine.

Treatment	Total d.p.m. per 10^6 cells (X 10^{-4}) Acid-soluble	Acid-insoluble
DMSO	160± 6	772±25
10^{-7} M TPA	147±12	727±59
10^{-7} M PDD	173± 7	843±37

HEL/37 cells were prelabelled with [^{3}H] uridine, washed and then incubated for a further 4 h with DMSO (control), 10^{-7} M TPA or 10^{-7} M PDD as described in the Materials and Methods section. Each figure is the mean ± S.E. of determinations carried out on 5 separate dishes.

of label transfer by the active tumor promoters was dramatic. Although detailed concentration curves have not yet been done, TPA was effective at the lowest concentration tested (10^{-9} M). One trivial explanation of this data could be that TPA and PDD rapidly decrease the amount of radioactivity present in the uridine nucleotide pool and therefore available for transfer to recipient 3T3 cells. However, as shown in Table 2, neither TPA nor PDD decreased the amount of radioactivity remaining in acid-soluble material extractable from HEL/37 cells after 4 h in culture.

The scoring of the cells was done under coded, blind conditions. Consequently, experimental artifact is likely only if promoters caused some morphological change in the cells which generated a systematic change in scoring patterns. The major effect observed was a marked promoter-induced decrease in the proportion of 3T3 cells, in contact with HEL/37 cells, which were labelled after coculture. We do not feel that cellular identification was a problem as the recipient 3T3 cells were, in general, less densely labelled than the donor HEL/37 cells, and because of the morphological differences between the two cell lines which were not obviously changed by either TPA or PDD. Nevertheless, it will be necessary

to confirm the inhibitory effects with a variety of cell systems and using different techniques to detect communication between cells.

Little is known about the assembly of gap junctions, but it is probable that it requires the lateral movement of intra-membrane proteins (13). If so, modulation of assembly by tumor promoters would not be unexpected as these compounds are known to interact with the surface of cells (3, 14, 15) and to cause changes in cell surface fluidity (16). It is also possible that promoters inhibit molecular transfer without affecting junction assembly (17). In either event, it will be important to determine whether promoters inhibit communication between cells with already established gap junction links. In the present experiments TPA and PDD were added as coculture commenced and therefore prevented the *establishment* of effective transfer channels.

While caution should be exercised in extrapolating too widely from the present results, we feel that a more detailed examination of the effects of tumor promoters on intercellular communication is now warranted.

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