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AN ORGAN CULTURE OF ADULT MOUSE SKIN: AN IN VITRO MODEL FOR STUDYING THE MOLECULAR MECHANISM OF SKIN TUMOR PROMOTION[1]

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SUMMARY: A simple method to culture explants of adult mouse skin in a modified Eagle's HeLa cell medium was developed in order to further study the biochemical responses to the tumor promoting phorbol esters. The skin explants remained viable for at least 48 hr, as determined by their ability to incorporate ^{3}H-thymidine into DNA as well as to induce epidermal ornithine decarboxylase (EC 4.1.1.17) activity following 12-0-tetradecanoylphorbol-13-acetate addition. The time course of induction of ornithine decarboxylase activity by the tumor promoter was similar to that observed with intact mice. Furthermore, the addition of retinoic acid and indomethacin, the agents that are known to inhibit the induction of ornithine decarboxylase activity by topically applied TPA, also inhibited the induction of ornithine decarboxylase activity by TPA in skin explants.

Topical application of the potent tumor-promoting agent 12-0-tetradecanoylphorbol-13-acetate (TPA) to mouse skin results in numerous biochemical changes that lead to enhanced cell proliferation. Examples include enhanced incorporation of ^{32}P into phosphatidylcholine, increased prostaglandin synthesis, sequential activation of RNA, protein, and DNA synthesis, increased phosphorylation of histones, decreased histidase activity, altered cyclic nucleotide metabolism, and induction of ornithine decarboxylase (ODC) activity (see reviews 1,2,3,4). The relevance of these TPA-induced alterations to the mechanism of skin tumor promotion is not clearly understood. Recently, it has been proposed that the induction of ODC activity and DNA synthesis play a role in skin tumor promotion by TPA (5-10). Thus, prior treatment

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of mouse skin with indomethacin or retinoic acid inhibited the induction of ODC activity, the increase in DNA synthesis, and the formation of skin tumors by TPA.

In order to facilitate further the elucidation of the molecular mechanism of skin tumor promotion by TPA and other agents that modify tumor promotion, a simple, easily manipulable short-term organ culture of adult mouse skin explants responsive to TPA was developed, and the results are presented in this communication.

MATERIALS AND METHODS

Female Charles River CD-1 mice, 7-9 weeks of age, were housed and treated as described previously (5). The backs of the mice were shaved 3 to 4 days before experimentation, and only those mice not exhibiting hair regrowth during this period were used. TPA was obtained from Dr. Peter Borchert, Eden Prairie, MN. Indomethacin and β-retinoic acid were obtained from Sigma Chemical Company, St. Louis, MO. Dimethylsulfoxide (Fisher Scientific Company, Fair Lawn, NJ) was used as a vehicle for TPA, indomethacin and retinoic acid; its final concentration in the medium never exceeded 0.4%. DL-[1-^{14}C]Ornithine hydrochloride (specific activity, 49.9 mCi/mmol) and [methyl-^{3}H]thymidine (specific activity, 52.4 Ci/mmol) were purchased from New England Nuclear, Boston, MA.

Skin was excised from the shaved backs of mice after cervical dislocation. The subcutaneous fat and muscle were scraped off with a razor blade. Skin explants (approximately 2 x 1 cm) from the back of each mouse were transferred to a flask containing modified Eagle's HeLa cell medium (25 ml per 8 skin pieces) with or without 10% bovine serum at 37°C. The medium was gassed with 95% oxygen and 5% CO_2 for 5 min, the appropriate additions were made, and the flasks were sealed and incubated in a shaking water bath at 37°C. At appropriate times after incubation, skin pieces were removed to ice-cold distilled water, the epidermis was separated by a brief heat-treatment (55° for 20 sec.), and biochemical determinations were then made.

ODC activity in the soluble epidermal extracts was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine hydrochloride (11).

Incorporation of [^{3}H]thymidine into DNA was determined as described previously (11). DNA content was determined by the method of Burton (12), and protein content was determined by the procedure of Lowry *et al.* (13).

RESULTS

Viability of skin explants. Various criteria can be used to test the viability of mouse skin explants in culture. Examples include the determination of DNA synthesis as observed by the rate of thymidine uptake as well as by autoradiography, the mitotic index and rate, the rate of oxygen uptake, and the response to various stimuli such as increased ODC

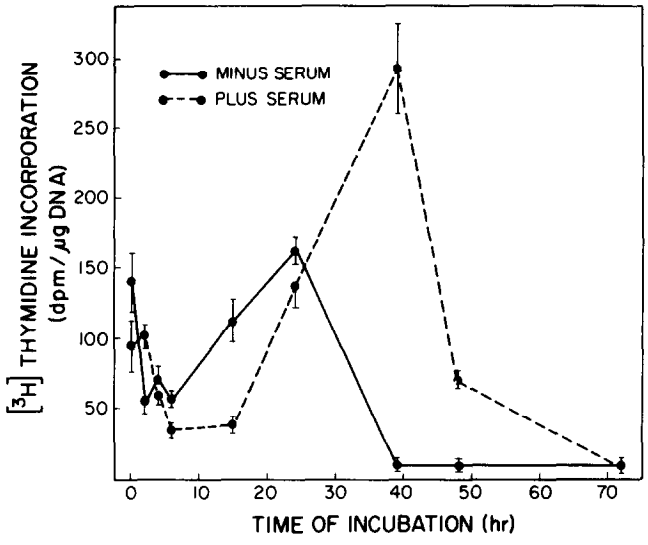

Figure 1. Incorporation of tritiated thymidine into epidermal DNA of mouse skin explants incubated in vitro.

Skin explants were incubated in Eagle's HeLa cell medium with or without 10% bovine serum. At indicated times after incubations, cultures were pulsed with tritiated thymidine (1 μCi/ml medium) for 30 min, and radioactivity associated with hot acid-soluble DNA was determined. Each point represents the mean ± S.E. of 4 determinations carried out on 8 skin pieces prepared from 4 mice.

activity following addition of TPA to medium containing skin explants. We determined the rate of thymidine incorporation into DNA and the induction of ODC activity by TPA as the measure of viability of skin explants during culturing time. The rate of incorporation of tritiated thymidine into DNA is shown in Figure 1. In this experiment, skin explants were incubated in flasks containing Eagle's HeLa cell medium with or without 10% bovine serum; medium was changed every 24 hr after incubation. At various times after incubation, cultures were pulsed with tritiated thymidine (1 μCi/ml) for 30 min, and DNA synthesis was measured as tritiated thymidine incorporation into hot acid-soluble material. Skin explants incubated in the medium either with or without serum exhibited an initial drop in the rate of thymidine incorporation. A peak of DNA synthesis was observed at 24 and 39 hr in the absence and presence of serum respectively. Incorporation of thymidine

Table 1. The induction of epidermal ODC activity by TPA in incubated mouse skin explants.

Mouse skin explants were prepared and incubated in medium with or without 10% bovine serum for the indicated times. Epidermal ODC activity was determined at 4 hr after addition of DMSO or 1μM TPA in DMSO following the indicated times of incubation. Each value is the mean ± S.E. of determinations carried out on 8 skin pieces from 4 mice.

Time of incubation (days)	ODC activity (nmol CO_2/30 min/mg protein)			
	- Serum		+ Serum	
	- TPA	+ TPA	- TPA	+ TPA
0	0.29 ± 0.05	3.27 ± 0.4	0.18 ± 0.03	3.26 ± 0.08
1	0.67 ± 0.05	4.18 ± 0.29	0.53 ± 0.05	4.49 ± 0.51
2	0.12 ± 0.05	1.15 ± 0.32	0.13 ± 0.01	4.24 ± 0.31
3	0.02	0.02	0.02	0.04

declined at 39 hr after incubation in serum-free medium. However, inclusion of 10% bovine serum in the medium shifted the decline in thymidine incorporation to 48 hr after incubation.

The induction of epidermal ODC activity by TPA at various times after incubation of skin explants is shown in Table 1. ODC activity was determined 4 hr after the addition of 1 μM TPA into the medium. A dramatic increase in ODC activity by TPA was observed up to 24 hr in serum-free cultures and up to 48 hr in cultures containing 10% bovine serum.

Histological examination of the skin sections before and after culture did not reveal gross changes in the architecture of epidermis for 24 hr in serum-free and for 48 hr in serum-containing medium.

Effect of TPA on the induction of ODC activity in incubated skin explants. In order to elucidate the use of this *in vitro* system for studying the mechanism of action of TPA, a time and dose effect of TPA addition in incubated skin explants on the induction of ODC activity was determined. A time course of induction of epidermal ODC activity by 0.16 μM TPA in skin explants incubated in serum-free medium is shown in Figure 2. TPA addition resulted in a dramatic increase in ODC activity with peak activity at about 8 hr, and enzyme activity declined to the original level at 22 hr. The time

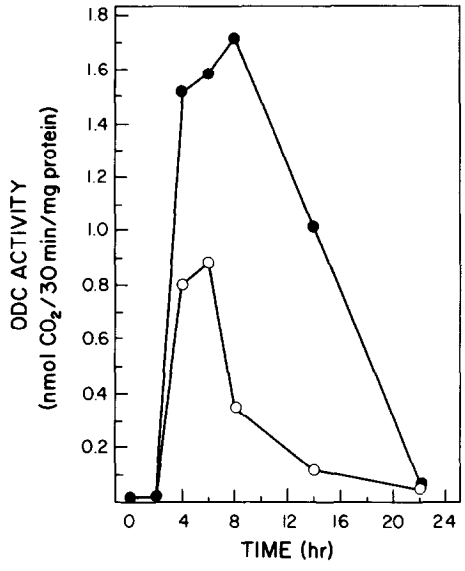

Figure 2. Effect of TPA on induction of epidermal ODC activity in incubated skin explants.

Skin explants were incubated in Eagle's HeLa cell medium without serum in the presence of 0.16 μM TPA in DMSO (●) or DMSO alone (O). Epidermal ODC activity was determined at various times following incubation. Each value is the mean of 2 determinations carried out on 6 skin pieces prepared from 3 mice.

course of ODC induction by TPA is similar to that seen in vivo with intact mice (5,9); however, a large increase in epidermal ODC activity was observed in incubated skin pieces without the addition of TPA (Figure 2).

Induction of ODC activity was dependent on the concentration of TPA in the medium (Figure 3). A significant increase in ODC activity was observed 6 hr following the addition of 0.01 μM TPA concentration, and about a 25-fold increase in ODC activity was observed with 10 μM TPA. Furthermore, inclusion of 10% bovine serum in the medium did not alter the degree of induction of ODC activity by TPA (Figure 3).

Inhibition by indomethacin and retinoic acid of the induction of ODC activity caused by TPA. Topical application of indomethacin, a prostaglandin synthesis inhibitor, inhibits the induction of ODC activity by TPA (8). As shown in Figure 4, indomethacin also inhibited the induction of ODC activity by TPA in incubated skin explants. Addition of 0.3 mM indomethacin

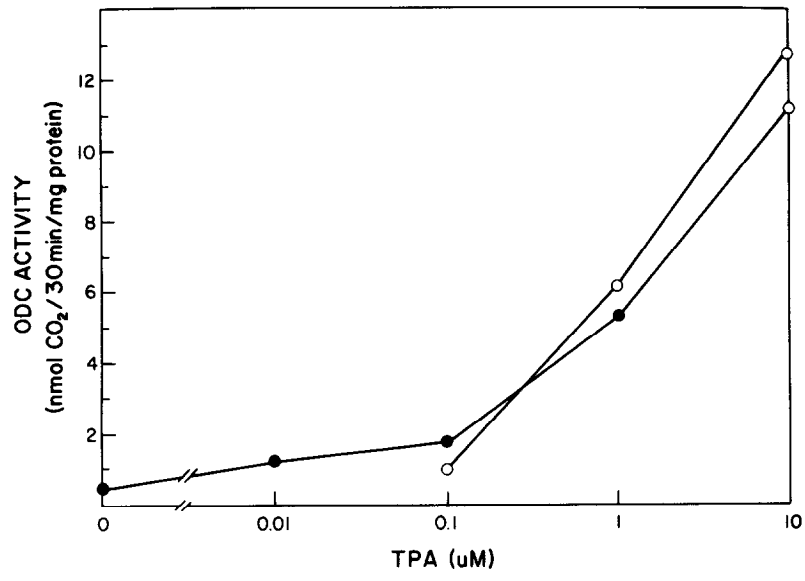

Figure 3. Effect of TPA concentration on epidermal ODC activity in incubated skin explants.

Skin explants were incubated in medium with (○) or without (●) 10% bovine serum in the presence of various concentrations of TPA; ODC activity was determined 4 hr after TPA addition. Each value is the mean of 2 determinations carried out on 6 skin pieces prepared from 3 mice.

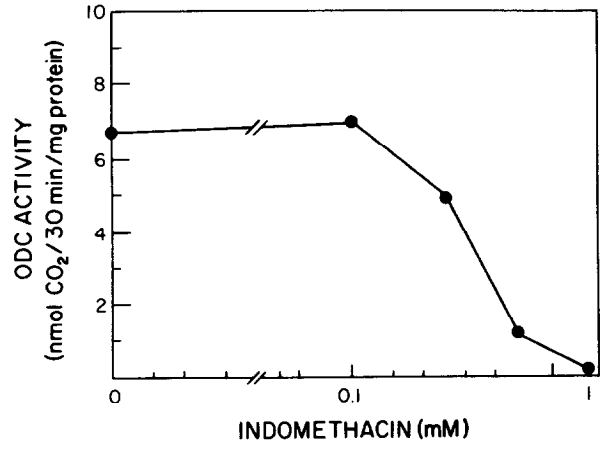

Figure 4. Inhibition by indomethacin of the induction of ODC activity by TPA.

Skin explants were incubated in serum-free medium in the presence of 1 μM TPA with various concentrations of indomethacin. Epidermal ODC activity was determined 4 hr after TPA addition. Each value is the mean of 4 determinations carried out on 8 skin pieces obtained from 4 mice.

inhibited by 27% the induction of ODC activity; an 84% inhibition was observed after the addition of 0.5 mM indomethacin. Furthermore, retinoic acid, a potent *in vivo* inhibitor of the induction of mouse epidermal ODC activity by TPA, also inhibited the enzyme induction in skin explants; addition of 2 μM retinoic acid inhibited by 79% the induction of ODC activity (ODC activity was 7.76 and 1.65 nmol CO_2/30 min/mg protein in the presence of 1 μM TPA and 1 μM TPA plus 2 μM retinoic acid respectively).

DISCUSSION

The need for *in vitro* experimental models, which have *in vivo* relevance, to elucidate the molecular mechanism of action of carcinogens, tumor promoters, and pharmacological agents has been well recognized. *In vitro* cultivation of mammalian skin, a model for studying epidermal growth and differentiation, has been described by several investigators (14-22). In this context, the work of Reaven and Cox (17) on adult human skin in organ culture and of Halprin et al (21) on postembryonic mouse skin in explant culture is noteworthy. In their studies (17,21), skin remained viable and grew for several days. We now describe a simple method to culture explants of adult mouse skin in a modified Eagle's HeLa cell medium. Under these conditions, skin explants remained viable and responsive to tumor promoter action for about 48 hr and thus may provide a useful model system to elucidate the biochemical mechanism of action of the tumor-promoting agent TPA.

It appears from the results presented (Figure 1 and Table 1) that skin explants maintained metabolic potential for at least 48 hr after incubation. Clearly, skin explants exhibited enhanced DNA synthesis. However, a drop in thymidine uptake was observed for the initial several hours. A similar decline in thymidine uptake has been shown in organ culture of other mammalian skins (15,17,22). The possible cause of this drop in DNA synthesis remains speculative.

The time course of induction of ODC activity by TPA in incubated skin explants was similar to that observed following topical application of TPA to mouse skin. Furthermore, the addition of indomethacin or retinoic acid, the potent in vivo inhibitors of ODC induction, also inhibited the induction of ODC activity by TPA in skin explants. These results indicate that there is a good correlation between the effects of TPA in in vitro and in in vivo mouse skin systems. The effect of TPA on several other biochemical events in this in vitro system remains to be determined.

In conclusion, the short-term organ culture of adult mouse skin described in this paper provides a simple, easily manipulable, in vitro system that mimics the adult mouse skin in vivo. In this system, as in epidermal cells in culture, the mechanism of action of the skin tumor promoter TPA, and of various agents such as retinoids and steroids, can be studied under precisely defined conditions.

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