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Tumor Promotion and the Induction of Epidermal Ornithine Decarboxylase Activity in Mechanically Stimulated Mouse Skin[1]

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ABSTRACT

Wounding by incision was a promoting stimulus in mouse skin previously initiated with 7,12-dimethylbenz-(a)anthracene. Skin massage elicited a marked proliferative response in skin but was not a promoting stimulus.

Wounding mouse skin, either by multiple scalpel incisions or by stripping with silicon carbide paper, led to a marked induction of ornithine decarboxylase activity. In both instances activity was maximal between 20 and 26 hr after wounding, with a secondary rise at 72 hr. Skin massage did not lead to a detectable increase in ornithine decarboxylase activity over the same time period.

INTRODUCTION

The role of cell proliferation in the promotion of tumors in mouse skin initiated with carcinogens has been debated for some years (8, 9, 13, 14, 20, 23). Although cell proliferation and hyperplasia are correlated with the promoting activity of some phorbol esters, an increased rate of cell proliferation does not necessarily lead to the promotion of initiated cells either *in vivo* (8, 9, 19, 20) or *in vitro* (14). Thus, although enhanced proliferative activity may be necessary for tumor promotion, it is likely that other specific biochemical changes are also required. It has recently been claimed that the induction of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) activity is a specific early change associated with tumor promotion (15). The decarboxylase is thought to be the rate-limiting step in the biosynthesis of polyamines in mammalian tissues (21). Enzymic activity is enhanced after the treatment of mouse skin with a range of promoting agents, but not when treated with nonpromoting but hyperplastic agents (16).

The contention that the induction of ornithine decarboxylase activity represents a biochemical change specific for promotion, which is separable from events leading to cell proliferation and hyperplasia, is of considerable importance. In these studies, this proposal has been examined further by measuring ornithine decarboxylase activity in skin after the induction of proliferative activity by physical manipulation. The treatments used were skin massage, which elicits a proliferative response (1, 2) but is not promoting, and skin wounding which induces hyperplasia (2) and is also a promoting stimulus (8).

MATERIALS AND METHODS

Materials. Croton oil, colchicine, DMBA,[3] and L-ornithine were obtained from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol and pyridoxal 5'-phosphate were from Calbiochem Ltd., Melbourne, Australia. DL-[1-^{14}C]Ornithine (specific activity, 59 mCi/mmole) was obtained from The Radiochemical Centre, Amersham, England. All other chemicals were of analytical reagent grade.

Treatment of Animals. Animals used were Swiss albino mice approximately 3 months of age and were maintained as described before (15). Female mice were used unless otherwise stated. The dorsal skin was shaved 7 days prior to use and only those mice not showing hair regrowth were used for experimentation. Skin massage was carried out by gently rubbing the shaved area with the forefinger for 30 sec. Mice were wounded, under light ether anesthesia, by a simple scalpel incision (4 cm long) through the dorsal skin, or by removal of the keratinized layer by gently rubbing with fine silicon carbide paper (Grade 800-AP). Mice wounded by the latter procedure were treated with a commercial depilatory agent (Dorothy Gray, Sydney, Australia) 5 days prior to use. In initiation-promotion experiments mice were wounded with a single incision, and in experiments to measure ornithine decarboxylase activity mice were wounded 4 to 6 times with approximately 3 mm between each incision.

Ornithine Decarboxylase Assay. Soluble epidermal extracts were prepared as described (17) but in 50 mM Tris-chloride (pH 7.4). Extracts were prepared from the skins of individual mice.

Ornithine decarboxylase assay mixtures contained 0.4 μmole of pyridoxal 5'-phosphate, 1 μmole of dithiothreitol, 0.2 μmole of L-ornithine, 100 μmoles of Tris-chloride (pH 7.4), 0.02 μmole of sodium bicarbonate, 0.5 μCi of DL-[1-^{14}C]ornithine, and 0.5 ml of epidermal extract (0.4 to 1.5 mg of protein) in a final volume of 2 ml. Sodium bicarbonate was added to reduce possible binding of [^{14}C]carbon dioxide by proteins in the cell-free extracts. Assays were carried out for 60 min at 37° as described (17); after termination of the reactions, flasks were left for 3 hr to ensure complete collection of the labeled carbon dioxide.

Protein was determined according to the method of Lowry *et al.* (10).

Initiation-Promotion Experiments. Groups of 30 mice were initiated by treating the dorsal skin with 25 μg of DMBA in 0.2 ml of acetone. Control groups were treated with 0.2 ml of acetone. Promotion was begun after another

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[3] The abbreviation used is: DMBA, 7,12-dimethylbenz(a)anthracene.

14 days of treatment. The treatments tested for promoting activity were biweekly applications of croton oil (0.2 ml of a 0.5% solution in acetone), biweekly skin massage, and wounding with a single scalpel incision. The healed wounds were reopened every 2 weeks and were treated with antibiotic powder (Tricin; V. R. Laboratories Pty. Ltd., Australia) on each occasion.

One control group was initiated with DMBA and "sham"-wounded by drawing the back of a scalpel blade along the dorsal skins at 2-week intervals. After each sham wounding, these animals were also treated with antibiotic powder.

Mice were examined weekly for papilloma formation.

Mitotic Index Determination. Mice were given i.p. injections of 0.1 mg of colchicine in 0.2 ml of 0.9% chloride solution 4 hrs prior to sacrifice. Histology was carried out as described before (22). The mitotic index is expressed as the number of metaphase cells per 100 nucleated, interfollicular epidermal cells.

RESULTS

It is clear from the results shown in Chart 1 that skin wounding elicits a marked promotion of tumors in skin initiated with DMBA. As reported previously (8), the tumors developed along the margins of the wounds. The total tumor yield was much less than that observed after croton oil promotion, presumably indicative of the smaller skin area exposed to the promoting stimulus. No papillomas were observed in groups that had not been initiated with DMBA.

In the experiments described in Chart 1, no tumors were observed in skin that was massaged twice a week over an 18-week period. As observed previously (1, 2), skin massage induced a marked proliferative response in the basal epidermal cells. These results are summarized in Table 1. Experiments were carried out to determine the effect of both single and multiple skin massage treatments on the mitotic index. The basal mitotic index was significantly higher in mice massaged twice weekly for 16 weeks and then left for 96 hr after the final massage, than in mice that had received no massage treatment ($p < 0.01$). However, an increase in the epidermal mitotic index was observed 24 hr after massage in both groups of animals. These data suggest that each massage treatment used in the initiation-promotion experiment would elicit a proliferative response in the basal cell layer of epidermis.

Chart 2 shows the activity of epidermal ornithine decarboxylase after skin wounding and massage. A substantial increase was observed after wounding by either the infliction of multiple cuts or by rubbing with fine silicon carbide paper. Although the magnitude of the increase varied, the general shape of the response curve was similar after both forms of wounding. No increases in ornithine decarboxylase activity were detectable after skin massage. In a separate experiment, it was established that no increases in decarboxylase activity occurred at 6, 8, or 12 hr after either wounding or massaging the skin (data not shown). In the experiments described in Chart 2, animals were sacrificed at varying times of the day. Separate experiments established that there were no detectable changes in basal ornithine decarboxylase activity at the various times used.

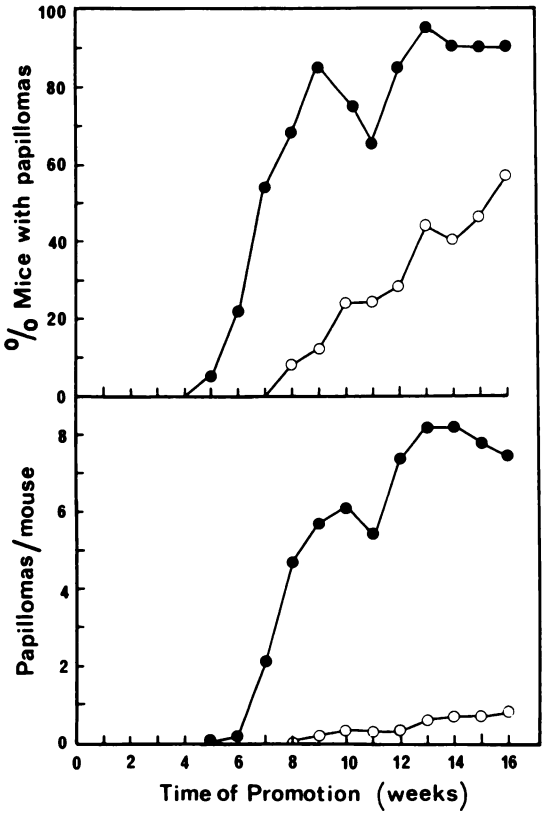

Chart 1. The promoting effect of wounding in mouse skin. Mouse skin was initiated by treatment with 25 μg of DMBA (male mice were used in these experiments). After 2 weeks mice were treated with 0.2 ml of 0.5% croton oil in acetone (●) or were wounded by a single cut with a scalpel blade (○). Croton oil treatment was continued twice weekly, and the skin wounds were reopened every 2 weeks (see "Materials and Methods").

Table 1

Effect of skin massage on the mitotic index of mouse epidermal cells

Male mice (7 months old) taken from the initiation-promotion experiment described in Chart 1 were used; animals had been initiated with DMBA 18 weeks before the experiment. Control mice that had not previously been massaged were given a single massage treatment 24 hr prior to sacrifice. Mice that had received biweekly massage for 16 weeks were left for 96 hr after the last treatment and then massaged 24 hr prior to sacrifice. All mice were given i.p. injections of 0.1 mg of colchicine in 0.2 ml of 0.9% NaCl solution 4 hr before sacrifice.

Each value represents the mean ± S.E. of determinations carried out on 5 animals (see "Materials and Methods").

Treatment	Mitotic index
No massage	0.78 ± 0.19
Single massage (24 hr)[a]	4.71 ± 1.20
Multiple massage (96 hr)	1.75 ± 0.17
Multiple massage (24 hr)	3.77 ± 0.20

[a] Values in parentheses, times since the last massage treatment.

It has previously been reported (16, 18) that the multiple application of promoters to mouse skin can increase the magnitude of the ornithine decarboxylase change, and alter the time course for induction of enzymic activity. In these experiments, no increases in epidermal ornithine decarboxylase activity were detected at 6, 12, or 24 hr after a final skin massage preceded by biweekly massage for 3 weeks.

In a control experiment (data not shown), it was estab-

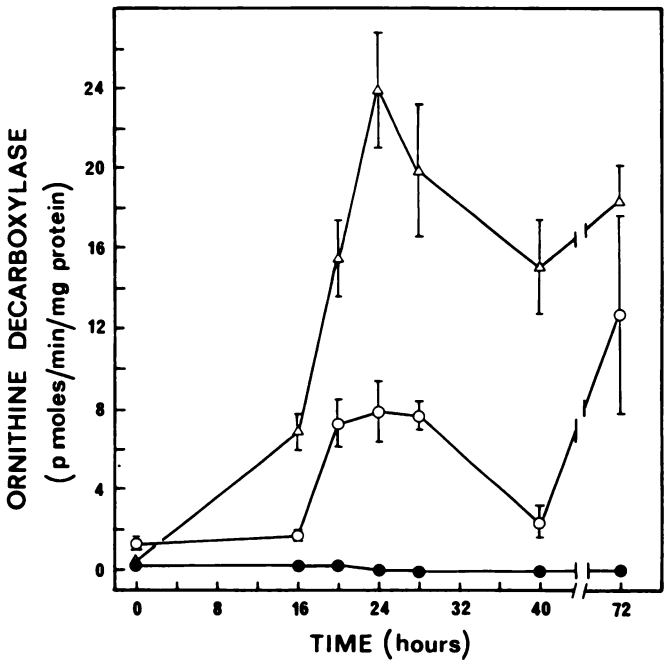

Chart 2. The effect of skin wounding or massage on epidermal ornithine decarboxylase activity. Skin was wounded under light ether anesthesia by the infliction of multiple cuts (○) or by treatment of depilated skin with silicon carbide paper (△; see "Materials and Methods"); in another group of animals the skin was massaged (●). At appropriate times after treatment, extracts were prepared and assayed for ornithine decarboxylase activity. Each *point* represents the mean ± S.E. of determinations made on 5 separate extracts.

lished that the treatment of multiply wounded skin with antibiotic powder did not alter the elevation in epidermal ornithine decarboxylase activity after 24 hr. Antibiotic powder was not applied in the experiments reported in Chart 2 but was used in the promotion experiment summarized in Chart 1. Additional control experiments were carried out to examine the possibility that the changes in ornithine decarboxylase activity were associated with nonepidermal cells accumulating in the wounded area as a part of an inflammatory response. Although extracts were made from epidermal preparations, some contamination with other cells in wounded skin was possible. Histological examination of wounded skin showed a marked inflammatory response after 6 hr, but ornithine decarboxylase activity was not increased until 16 to 20 hr (Chart 2). Thus, cellular contamination of the epidermal preparations could not explain the increases in ornithine decarboxylase activity, unless the activity of the decarboxylase increased at some time subsequent to the accumulation of cells at the wounded area. In separate experiments it was shown that the inflammation associated with silicon carbide paper wounding could be markedly depressed by the topical application of indomethacin (0.2 mg in 0.2 ml of acetone) at 0, 3, 6, 9, 12, and 24 hr (histological examination was carried out at 27 hr). Such treatment led to some increase in ornithine decarboxylase specific activity measured 27 hr after wounding. Thus, the 27-hr enzymic activities after indomethacin or acetone treatments were 20.7 ± 2.3 and 9.3 ± 2.0 pmoles/min/mg of protein, respectively. Consequently, although the possibility of some contribution of nonepidermal cells to the enzyme activities measured is difficult to eliminate completely, we believe that a major contribution is unlikely.

DISCUSSION

The present experiments are consistent with the suggestion (16) that induction of epidermal ornithine decarboxylase activity is a specific marker for tumor-promoting stimuli. Thus, epidermal ornithine decarboxylase activity was enhanced after skin wounding, but not after skin massage. Both treatments lead to increased proliferative activity in the epidermis (1, 2), but only wounding has a promoting action. It is recognized that the correlative evidence available at the moment does not establish a causal link between epidermal ornithine decarboxylase and tumor promotion. The establishment of this link, if it exists, would require more definitive information on the biological roles of ornithine decarboxylase and the polyamines. Nevertheless, we feel that the correlation is strengthened by the observation that widely differing promoting stimuli (both mechanical and chemical) induce decarboxylase activity.

The apparent specificity of ornithine decarboxylase as a marker for tumor promoters in mouse skin is unexpected, in view of the wide range of physiological stimuli which are known to modify ornithine decarboxylase activity in tissues other than skin (21). Many of these stimuli are associated with the induction of increased cellular proliferation, and it has been generally concluded that ornithine decarboxylase (12) and/or polyamines (3, 4, 7, 11) are involved in growth regulation. The data obtained thus far with mouse skin, however, suggest that, at least in this tissue, the relationship between the polyamines and proliferation is complex. Although some basal level of the polyamines may be required, there seems to be no doubt that hyperplasia and enhanced proliferative activity can be chemically induced without detectable increases in ornithine decarboxylase activity (16); the same is true of proliferation induced by mechanical massage (this study). In some cells stimulated to proliferate, there is an early peak of ornithine decarboxylase activity which precedes DNA synthesis and mitosis (3, 5, 6, 11, 23). In these instances it is possible to envisage the increase as part of a biochemical cascade leading to cell proliferation. This is not the situation in wounded skin, where the major peaks in DNA-synthetic activity and ornithine decarboxylase activity occur at about 15 (1) and 20 to 24 hr, respectively. Consequently, there is need for caution in assuming that there is a causal relationship between the induction of ornithine decarboxylase activity and enhanced cell proliferation in all tissues.

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