Correlation of the Inhibition by Retinoids of Tumor Promoter-induced Mouse Epidermal Ornithine Decarboxylase Activity and of Skin Tumor Promotion

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ABSTRACT

Application of the potent tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), to mouse skin leads to a more than 200-fold increase in epidermal ornithine decarboxylase (EC 4.1.1.17) activity, a phenotypic change proposed to be essential for skin tumor promotion. The correlation between TPA-induced ornithine decarboxylase activity and skin tumor promotion received additional support from our finding that vitamin A acid and its analogs inhibit both TPA-induced ornithine decarboxylase activity and formation of skin papillomas.

The induction of ornithine decarboxylase activity was investigated following multiple applications of TPA to mouse skin initiated with dimethylbenz[a]anthracene, the regimen followed in initiation-promotion experiments. Ornithine decarboxylase activity was increased to about 600-fold during repeated applications of 17 nmol of TPA. Application of 1.7 nmol of retinoic acid 1 hr prior to each treatment with TPA inhibited TPA-induced ornithine decarboxylase activity by 60 to 80%. In tumor induction experiments, application of 1.7 or 17 nmol of retinoic acid 1 hr before each promotion with 17 nmol of TPA reduced by 57 and 75%, respectively, the number of papillomas per mouse. In contrast, retinoic acid treatment 24 hr after each TPA treatment did not suppress the formation of skin papillomas. Furthermore, application of retinoic acid at various times relative to the time of initiation with dimethylbenz[a]anthracene did not alter the development of skin tumors.

The application of the trimethylmethoxyphenyl analog of ethyl retinoate, 13-cis-retinoic acid, or the dimethylmethoxyethylcyclopentenyl analog of retinoic acid 1 hr prior to each TPA application inhibited TPA-induced ornithine decarboxylase activity as well as formation of skin papillomas. The trimethylhydroxyphenyl analog of ethyl retinoate or the 13-trifluoromethyltrimethylmethoxyphenyl analog of ethyl retinoate altered neither TPA-induced ornithine decarboxylase activity nor development of skin papillomas. Treatment with retinoids did not result in any sign of local toxicity; also, the average weight of the control mice was identical to the average weight of those treated with retinoids. Furthermore, retinoid treatment specifically inhibited TPA-induced ornithine decarboxylase activity and the resultant increases in putrescine levels, but it did not inhibit TPA-induced S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) activity and the accumulation of spermidine and spermine.

The results indicate that the possible mechanism of prevention of skin papillomas by retinoids involves their ability to inhibit TPA-induced epidermal ornithine decarboxylase activity and the associated elevated putrescine levels. These findings suggest that the assay of the inhibition of TPA-induced ornithine decarboxylase activity by retinoids may be a simple, rapid screen for antipromoting properties of new synthetic retinoids.

INTRODUCTION

The 2-stage model of mouse skin carcinogenesis provides a system in which the biochemical events unique to either initiation or promotion can be studied and related to cancer formation. In recent years, remarkable progress in the understanding of the biochemical mechanism of the promotion phase of skin tumor formation has been accomplished (7-9).

Application of the potent tumor-promoting agent TPA3 to mouse skin leads to about a 200-fold increase in the activity of epidermal ODC, the enzyme proposed to be a marker for growth and cancer (1, 12, 13, 22, 29). An increasing body of literature indicates that this phenotypic change may be essential for skin tumor promotion. Thus, the degree of induction of ODC activity is dependent on the dose of TPA and correlates with the ability of the dose to promote skin tumor formation. The degree of induction of ODC activity by a series of phorbol esters as well as by a number of structurally unrelated tumor promoters correlates well with their tumor-promoting ability (18, 20). Furthermore, ODC activity is induced only following tumor promoter treatment to mouse skin and not after treatment with nonpromoting hyperplastic agents. In contrast, both tumor promoters and hyperplastic agents induce the activity of S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50), a second enzyme in the pathway of polyamine biosynthesis (18-20). These findings suggest that TPA-induced ODC activity may be a marker for tumor promoters. Further support to this proposal was provided by our recent work on vitamin A analogs and skin tumor promotion (30, 33). Thus, vitamin A acid (retinoic acid), which is required for the maintenance of normal differentiation and function of epithelial tissues, inhibits both TPA-induced ODC activity and the formation of skin papillomas. Furthermore, the degree of inhibition of TPA-induced ODC activity by various natural vitamin A analogs.

Received August 31, 1978; accepted October 30, 1978.
1 The work was supported by NIH Grants CA 07175 and CA 22484.
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3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate (Chemical Abstracts Registry No. 20839, 11-6); ODC, ornithine decarboxylase; DMBA, 7,12-dimethylbenz[a]anthracene; TMMP, trimethylmethoxyphenyl; TMHP, trimethylhydroxyphenyl.

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analogs correlates with the degree of inhibition of formation of skin papillomas (30). These results indicate that inhibition of TPA-induced epidermal ODC activity by retinoids may be a rapid screen for evaluating the potential prophylactic activities of retinoids.

Recently, we have reported the results of the test of a number of synthetic retinoids for their ability to inhibit TPA-induced ODC activity and, interestingly, the activity of the retinoids in the ODC test system appears to agree with the activity of retinoids in a number of previously reported in vitro test systems (33). In these studies, the proposal that inhibition of TPA-induced ODC activity by retinoids may be a screen for their antipromoting properties is further examined by studying the correlative effects of a number of synthetic retinoids on induction of epidermal ODC activity as well as their effect on formation of skin papillomas promoted with TPA. It is also shown in this presentation that retinoic acid prevents formation of skin papillomas by a mechanism which involves the promotion and not the initiation step of mouse skin carcinogenesis.

MATERIALS AND METHODS

Materials. Female Charles River CD-1 mice, 7 to 9 weeks old, were maintained and treated as described previously (30). The dorsal skin was shaved 3 to 4 days before experimentation, and only those mice not exhibiting hair regrowth over this period were used. TPA was obtained from Dr. Peter Borchert, University of Minnesota, Minneapolis, Minn., and DMBA was purchased from Eastman Organic Chemicals, Rochester, N. Y. β-Retinoic acid was from Sigma Chemical Co., St. Louis, Mo. The retinoids used in this study were prepared by Hoffmann-La Roche Inc., Nutley, N. J., and F. Hoffmann-La Roche and Co. AG, Basel, Switzerland, and were generously supplied by Dr. M. B. Sporn, Lung Cancer Branch, National Cancer Institute, Bethesda, Md., and by Dr. B. A. Pawson, Hoffmann-La Roche Inc. DL-[1-14C]Ornithine hydrochloride (specific activity, 49.9 mCi/mmol) and S-adenosyl-L-[carboxyl-14C]methionine (specific activity, 54.6 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. The solutions of TPA and retinoids were prepared in acetone and were delivered to the shaved areas of individual mice in a volume of 0.2 ml. Control mice were treated with the same volume of acetone.

Tumor Induction Experiments. Tumors were initiated in all mice by application of 0.2 μmol (51.2 μg) of DMBA in 0.2 ml of acetone: 14 days following initiation, all mice were promoted twice a week (on Days 1 and 4) with either 8 or 17 nmol of TPA for the duration of the experiment. Mice were treated with various retinoids 1 hr before each promotion with TPA; controls were pretreated with acetone only. There were at least 30 mice in each treatment group. Mice were housed 10/cage in screen-bottomed stainless steel cages. The incidence of papillomas was observed weekly.

Assay of ODC and S-Adenosyl-l-methionine Decarboxylase Activity. At appropriate times after treatment, mice were killed by cervical dislocation, and epidermis from individual mice was separated by a brief heat treatment (55° for 30 sec) (15). The epidermal preparations from 2 or 3 mice were pooled, homogenized in 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA, and centrifuged (30,000 × g for 30 min). ODC and S-adenosyl-L-methionine decarboxylase activities from soluble epidermal extracts were determined by measuring the release of 14CO2 from DL-[1-14C]ornithine hydrochloride and S-adenosyl-L-[carboxyl-14C]methionine, respectively, as described before (30). Protein content in the soluble epidermal extracts was determined by the method of Lowry et al. (14).

RESULTS

The Effects of Retinoic Acid on TPA-induced ODC Activity and Formation of Skin Tumors. Since development of skin tumors requires repeated applications of a tumor promoter to the initiated skin, the level of ODC induction was determined following multiple applications of TPA to mouse skin initiated with DMBA. It has been shown previously that a single application of 17 nmol of TPA in 0.2 ml of acetone leads to a rapid, transient induction of epidermal ODC activity. Maximum activity, about 200-fold over basal level, occurs about 4.5 hr following TPA treatment, and the enzyme activity returns to the original level about 12 hr after treatment (30). If mouse skin is treated with 1.7 nmol of retinoic acid 1 hr before a single TPA treatment, the degree of induction of ODC activity by TPA is greatly depressed (30). The effect of the application of 1.7 nmol of retinoic acid 1 hr before each of several applications of 17 nmol of TPA to the initiated skin is shown in Chart 1. In this and subsequent experiments, the interval between each TPA application was alternately 3 and 4 days, and ODC activity was measured 4.5 hr after the last application of TPA. The multiple applications of TPA led to a greater degree of induction of ODC activity than that which was observed after the first or second application of TPA. About a 400-fold induction of ODC activity was observed after the third application of TPA and ODC induction plateaued at about 4.5 hr following TPA treatment, and the enzyme activity returns to the original level about 12 hr after treatment (30). If mouse skin is treated with 1.7 nmol of retinoic acid 1 hr before a single TPA treatment, the degree of induction of ODC activity by TPA is greatly depressed (30). The effect of the application of 1.7 nmol of retinoic acid 1 hr before each of several applications of 17 nmol of TPA to the initiated skin is shown in Chart 1. In this and subsequent experiments, the interval between each TPA application was alternately 3 and 4 days, and ODC activity was measured 4.5 hr after the last application of TPA. The multiple applications of TPA led to a greater degree of induction of ODC activity than that which was observed after the first or second application of TPA. About a 400-fold induction of ODC activity was observed after the third application of TPA and ODC induction plateaued at about
The incidence of papillomas was observed weekly. Mice were treated with either 1.7 or 17 nmol of retinoic acid in 0.2 ml of acetone 1 hr prior to each treatment with 17 nmol of TPA for 5 days starting 7 days after initiation with DMBA (a) or for 7 days following initiation (○); 68 nmol of retinoic acid were applied every day for 5 days starting 7 days after initiation with DMBA (△). Treatment of mice concurrently with 68 nmol of retinoic acid and DMBA did not inhibit the incidence of formation of papillomas (not shown). Two weeks following initiation, all mice were promoted twice a week with 17 nmol of TPA. Mice were treated with either acetone (●) or 68 nmol of retinoic acid (●) 1 hr before each promotion with TPA.

To test further the relevance of ODC induction to skin tumor promotion, groups of mice were treated with 34 nmol of retinoic acid 24 hr after each treatment with 8 nmol of TPA, the time point when the time of maximum ODC induction has passed and ODC levels have completely returned to original control values. The results (Chart 4) indicate that retinoic acid treatment 24 hr after TPA treatment did not affect the incidence of papillomas, whereas retinoic acid applied 1 hr before each promotion produced an 84% reduction in the number of papillomas per mouse, and there were only 42% papilloma-bearing mice.

The Effects of Other Retinoids on TPA-induced ODC Activity and Formation of Skin Papillomas. The effect of a number of retinoids on TPA-induced ODC and S-adenosyl-L-methionine decarboxylase activities as well as TPA-caused accumulation of products of these enzymes (putrescine, spermidine, and spermine) was determined. In this experiment (Table 1), the analyses were made after the seventh application of 8 nmol of TPA to mouse skin initiated with DMBA, and retinoids were applied 1 hr prior to each treatment with TPA. Application of 34 nmol of retinoic acid, 34 nmol of 13-cis-retinoic acid, or 140 nmol of the TMMP analog of ethyl retinoate inhibited TPA-induced ODC activity, whereas treatment with 140 nmol of either the TMHP analog of ethyl retinoate or the 13-trifluoromethyl-TMMP
All mice were initiated with 0.2 μmol of DMBA in 0.2 ml of acetone; 2 weeks after initiation, mice were treated twice a week with either 0.2 ml of acetone or a retinoid in 0.2 ml of acetone 1 hr before each treatment with 8 nmol of TPA. The analyses were made after the seventh TPA treatment. For determination of ODC and S-adenosyl-L-methionine decarboxylase activities, mice were killed 4.5 and 24 hr after TPA treatment, respectively. Each value represents the mean ± S.E. of determinations of enzyme activity from 3 groups of mice, and each group contained the combined supernatant prepared from 3 or 4 mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>CO₂/30 min/mg protein</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>0.02</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Acetone</td>
<td>34</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>17</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>13-cis-Retinoic acid</td>
<td>17</td>
<td>0.14 ± 0.01</td>
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<td>TMMP analog of ethyl retinoate</td>
<td>140</td>
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<td>TMHP analog of ethyl retinoate</td>
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<tr>
<td>13-Trifluoromethyl-TMMP analog of ethyl retinoate</td>
<td>140</td>
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A correlation between the ability of a number of natural retinoids (retinoic acid, retinol, retinyl acetate, and retinyl palmitate) to inhibit TPA-induced ODC activity and their ability to inhibit formation of skin papillomas has been reported previously (30). To examine this correlation further, a number of synthetic retinoids (Table 1) were tested for their effect on the incidence of skin papillomas. The results are shown in Chart 5. Application of 34 nmol of retinoic acid, 34 nmol of 13-cis-retinoic acid, or 140 nmol of the TMMP analog of ethyl retinoate 1 hr before each TPA treatment that effectively inhibited TPA-induced ODC activity (Table 1) also reduced the number of papillomas per mouse and the number of papilloma-bearing mice. In contrast, treatment with 140 nmol of either the TMHP analog of ethyl retinoate or the 13-trifluoromethyl-TMMP analog of ethyl retinoate affected neither TPA-induced ODC activity (Table 1) nor formation of skin papillomas (Chart 5). Furthermore, the application of 32 nmol of the dimethylmethoxyethylcyclopentenyl analog of retinoic acid, the most potent inhibitor of TPA-induced ODC activity among the retinoids (33), 1 hr before each promotion with 17 nmol of analog of ethyl retinoate did not affect ODC induction. Retinoid treatment did not inhibit TPA-induced S-adenosyl-L-methionine decarboxylase activity (Table 1). Application of the retinoids which inhibited TPA-induced ODC activity also inhibited accumulation of putrescine following TPA treatment. In contrast, the levels of spermidine and spermine were not affected by prior treatment with retinoids (data not shown), results which are in accord with our previous findings (33).

Chart 4. The effect of retinoic acid treatment on formation of skin tumors. Mice were initiated and promoted as described in "Materials and Methods." Retinoic acid, 17 nmol, (□) or acetone (●) was applied 1 hr before each promotion with 8 nmol of TPA; retinoic acid, 17 nmol, was applied 24 hr after each TPA treatment (▲).

Chart 5. The effect of various retinoids on skin tumor promotion. All mice were initiated and promoted as described in "Materials and Methods." Retinoids were applied 1 hr before each promotion with 8 nmol of TPA. Doses for retinoids were 34 nmol for retinoic acid and 13-cis-retinoic acid and 140 nmol for the TMMP analog of ethyl retinoate, the TMHP analog of ethyl retinoate, and the 13-trifluoromethyl-TMMP analog of ethyl retinoate. The control mice (curve 6) were pretreated with acetone only.
TPA reduced the number of papillomas per mouse to 23% of the control incidence (3.1 versus 13.7 papillomas/mouse), and only 57% of the mice bore papillomas at the 14th week after promotion (data not shown).

DISCUSSION

Among the pleiotropic changes (7-9, 17, 31) elicited in epidermis by treatment of mouse skin with the tumor promoter TPA, the induction of ODC activity is the largest phenotypic change that has been specifically related to promotion (9, 18). Convincing evidence relating ODC induction to promotion is the observation that retinoic acid treatment prior to each promotion with TPA inhibits TPA-induced ODC activity as well as formation of skin papillomas. Structure-activity studies indicate that retinooids, the TMHP analog of ethyl retinoate, and the 13-trifluoromethyl-TMMP analog of ethyl retinoate, which do not inhibit TPA-induced ODC activity, are virtually devoid of a prophylactic effect on skin papilloma formation. A further support for the role of ODC induction by TPA in skin tumor promotion is lent by the observation that retinoic acid applied 24 hr after TPA treatment, when ODC activity has returned to its original level, does not inhibit papilloma formation.

Recently, Clark-Lewis and Murray (10) have shown that wounding, which provides a promoting stimulus, induced epidermal ODC activity. In contrast, skin massage induced cell proliferation but induced neither ODC activity nor skin tumor formation. Furthermore, the fact that retinooid treatment inhibits TPA-induced ODC activity and the formation of skin papillomas without affecting the induction of S-adenosyl-L-methionine decarboxylase activity emphasizes the importance of ODC induction by TPA in skin tumor promotion and eliminates cytotoxicity as a causal factor for the reduction in the incidence of the skin papillomas.

The evidence is overwhelming that TPA-induced ODC activity is an essential component of the mechanism of skin tumor promotion. However, there are biochemical events elicited by TPA other than ODC induction that appear to be necessary for promotion. For instance, enhanced activity of plasminogen activator in certain cultured cells following TPA treatment has been reported (34). Furthermore, dexamethasone and fluorocinolone acetonide do not inhibit TPA-induced ODC activity, but they are very potent inhibitors of promoter-stimulated DNA synthesis and formation of skin tumors. It is thus suggested that ODC induction and DNA synthesis may be independent biochemical events and are not causally related (35). Thus, it seems highly likely that steroids and retinooids exert their antipromoting effect by modifying independent biochemical events which are necessary for skin tumor promotion.

Application of retinoic acid during initiation of mice with DMBA does not affect formation of skin papillomas. Prevention of skin papillomas is observed only when retinoic acid is applied 1 hr before each promotion with TPA. These findings indicate that retinoic acid exerts its prophylactic effect by interfering with promotion and does not appear either to inhibit initiation or to inactivate initiated cells.

The results presented demonstrate the prophylactic effect of topically applied retinooids on skin tumor formation. The therapeutic effect of the retinooids was not determined in this study. Furthermore, we did not study the effect of the retinooids on the incidence of skin carcinomas. Bollag (3-6) has reported that systemic administration of retinooids leads to the regression of skin papillomas as well as a reduced incidence of papillomas and carcinomas. We have observed that p.o. administration of retinoic acid inhibits ODC induction by topically applied TPA (33), but we do not know about the relative prophylactic activities of retinooids when administered systemically. Bollag (6) has shown that systemic administration of the TMMP analog of ethyl retinoate, in which the cyclohexyl ring of retinoic acid was replaced by an aromatic ring and the polar end group was esterified, prevented formation of skin papillomas more effectively than did all-trans-retinoic acid. In contrast, when topically applied the TMMP analog of ethyl retinoate was less potent than retinoic acid; more than 4 times the dose of all-trans-retinoic acid was required to achieve equivalent inhibition of formation of skin papillomas.

The molecular mechanism of inhibition of TPA-induced ODC activity and prevention of skin papillomas (4, 6, 25, 30) by retinooids is unknown. Recently, we have reported a role for prostaglandins in induction of ODC activity by TPA (32). Retinoic acid treatment presumably modifies gene expression, thereby inhibiting the induction of ODC by TPA, altering glycoprotein synthesis in cultured epidermal cells (11), and suppressing production of interferon produced by virus (2). It is not known whether prostaglandins are the mediators for retinoic acid action. Since retinoic acid is required for the maintenance of normal differentiation of epithelial tissues, it is quite likely that retinoic acid may function by maintaining the normal differentiation of epidermis altered by TPA and that putrescine, the product of the ODC reaction, may be a key factor in determining differentiation. In addition, retinoic acid-binding protein has been detected in many tissues (21, 23, 24). The retinoic acid-binding protein may be involved in the expression of biological and anticarcinogenic activities of retinooids.

Currently, tremendous interest has been expressed in the study of retinooids in carcinogenesis (16, 26, 28). A number of new synthetic retinooids have proved useful in the prevention of cancers of various organs without noticeable appearance of hypervitaminosis syndrome (27, 28). It is clear that retinooids are potent inhibitors of skin tumor promotion and that one of the possible mechanisms of their action is inhibition of TPA-induced ODC activity and the resultant increase in putrescine levels (33). It is suggested that inhibition of TPA-induced ODC activity by retinooids may be a rapid, preliminary screen for testing the potential prophylactic activity of retinooids.

REFERENCES

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Figs. 1 and 2. Representative mice were photographed at the end of the experiment illustrated in Chart 2. Mice in Fig. 1 were treated with 0.2 ml of acetone 1 hr prior to each treatment with 17 nmol of TPA (Chart 2, •; 100% incidence of papillomas, 18 papillomas/mouse). Mice in Fig. 2 were treated with 17 nmol of retinoic acid 1 hr prior to each treatment with 17 nmol of TPA (Chart 2, □; 85% incidence of papillomas, 5 papillomas/mouse).
Correlation of the Inhibition by Retinoids of Tumor Promoter-induced Mouse Epidermal Ornithine Decarboxylase Activity and of Skin Tumor Promotion


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