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EFFECTS OF VITAMIN A AND RELATED RETINOIDS ON THE BIOCHEMICAL PROCESSES LINKED TO CARCINOGENESIS

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INTRODUCTION

The introduction will be divided into three sections. The first will deal with the two-step model for skin tumor formation in mice. The second will review the role of the induction of ornithine decarboxylase activity in skin tumor formation. Finally, the evidence for a role for Vitamin A in tumor formation will be presented.

The ultimate goal of this research program is to learn the biochemical changes that are essential for the transformation of a normal cell to a tumor cell using the initiation-promotion (two-step) model system for the production of skin tumors in mice. Toward this goal we will show herein that those retinoids which effectively inhibit skin carcinogenesis also inhibit the induction of ornithine decarboxylase by the promoting component of the carcinogenic process, a correlation which strengthens the probability that increased ornithine decarboxylase activity is essential to carcinogenesis.

The Two-step Model for Skin Tumor Formation

Skin tumors are easily produced in an appropriate animal by repeated applications to the skin of a chemical carcinogen such as benzpyrene. However, there is a model system using mice devised by Mottram (1) in which the carcinogen need be administered only once and when followed by twice weekly applications of croton oil or its active principle, 12-O-tetradecanoylphorbol-13-acetate, many tumors may result. Neither the single application of the carcinogen nor the multiple applications of croton oil cause tumors when one is applied without the other. Furthermore, the single application of the carcinogen must precede the multiple applications of croton oil; if the order is reversed, no tumors result. Thus the terminology, initiation for the event accomplished by the single application of the carcinogen and promotion for the process accomplished by the croton oil, is generally accepted. Further evidence for the qualitative difference in the nature of these stages arises from the fact that the initiation step is irreversible in contrast to the reversible nature of the action of promoting agents. The biology of the initiation-promotion phenomenon has been reviewed (2,3). The two-stage model for the development of skin tumors is a remarkably quantitative model for the study of the process of carcinogenesis and is excellent for the investigation of factors modifying the process of tumor formation.

The Induction of Ornithine Decarboxylase by Tumor Promoters

The biochemistry of the process of tumor promotion has been extensively studied (4). A prominent biochemical response to treatment of mouse skin with a potent tumor promoter such as 12-O-tetradecanoyl-phorbol-13-acetate is the induction of ornithine decarboxylase activity (EC 4.1.1.17). Decarboxylation of ornithine to putrescine is catalyzed by ornithine decarboxylase and is the first and rate-limiting step in the pathway of polyamine biosynthesis. S-Adenosylmethionine decarboxylase (EC 4.1.1.50) is the second enzyme in polyamine biosynthesis and catalyzes the production of the propylamino group which condenses with putrescine to form spermidine and a second propylamino group condenses with spermidine to form spermine. In general terms these polyamines control the synthesis of nucleic acids and proteins and are involved in cell proliferation and differentation (5).

In a typical experiment, 17 nmol of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate is applied to the skin of the back of a mouse and 4.5 hr later mice are killed, a supernatant solution prepared from the epidermal cells of the treated skin, and the activity of the polyamine biosynthetic enzymes is measured (6). Results of a typical experiment are shown in Fig. 1. Ornithine decarboxylase activity in normal mouse skin is very low, but activity increases rapidly reaching a peak of 250-fold or more of the control level by 4.5 hr and thereafter rapidly returns to normal. The activity of S-adenosylmethionine decarboxylase rises more slowly reaching a plateau about 6 to 7-fold above the control level which is maintained for perhaps 24 hr before declining to control level over a period of several days.

Tumor promoters cause typical pleiotypic effects; they act as reversible derepressors of gene activity.

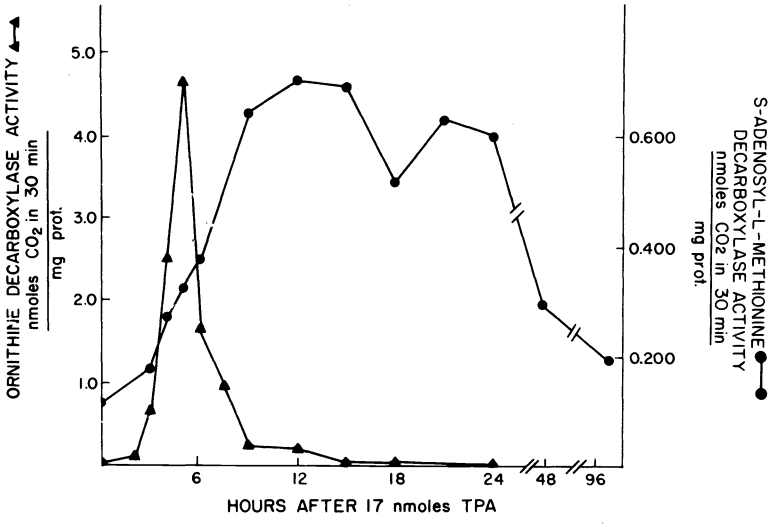


Fig. 1 The effect of a single topical application of 17 nmol of 12-O-tetradecanoylphorobol-13-acetate on the activities of epidermal ornithine decarboxylase and S-adenosylmethionine decarboxylase. Groups of 5 mice were treated once with either 0.2 ml acetone or the phorbol ester dissolved in acetone and killed at the times indicated. The points represent the means of triplicate determinations of enzyme activity; the variation was less than 10%.

Evidence from dose-response and structure-response studies showed a good correlation between the ability of an agent to induce ornithine decarboxylase activity and to promote tumor formation. In contrast, non-promoting hyperplasiogenic agents induced S-adenosylmethionine decarboxylase activity without inducing ornithine decarboxylase activity. Thus increased activity of ornithine decarboxylase may be an essential part of the mechanism of promotion, but not so in the case of the second enzyme (S-adenosylmethionine decarboxylase) in the polyamine biosynthetic pathway.

Retinoids and Tumor Promotion

Further evidence on a possible obligatory role for increased ornithine decarboxylase activity in tumor formation can be obtained by observing the effect on the activity of the enzyme of agents capable of inhibiting tumor promotion. Specifically, Vitamin A has been shown to inhibit tumor promotion (7). Does vitamin A inhibit the ability of the phorbol ester to induce activity of the polyamine biosynthetic enzymes? This question is the subject of this paper.

Vitamin A is essential for controlling the normal differentiation of many epidermal tissues (8). In the trachea and bronchi, squamous metaplasia occurs in vitamin A deficiency, a condition mimicing premalignancy. In fact those tissues which are most prone to cancer (skin, breast, lung, colon, rectum, pancreas, prostate, and bladder) require vitamin A for normal differentiation.

There are a number of examples of enhanced susceptibility to chemical carcinogenesis in vitamin A deficiency (8). Although these observations suggest that increased vitamin A levels might preclude the development of tumors, this is not always the case (8). The major problem is that when administered systemically, the natural retinoids accumulate in the liver with toxic manifestations and without increased plasma retinoid level. Thus problems of toxicity and tissue distribution limit the usefulness of natural forms of vitamin A for the prevention of cancer. However, vitamin A acid (retinoic acid) is not converted to vitamin A (retinol) in the animal body, it is not stored in the liver, and is much less toxic than retinol and its esters. It, together with an analog of retinoic acid, were used successfully by Bollag (7,9) to inhibit the promotion of skin tumors in mice. Because the mouse skin tumor model which we employ precludes problems of toxicity of retinoids since the retinoids

can be applied directly to the site, the model lends itself to the study of the effect of retinoids on tumor formation. Moreover, as described in the previous section, the effect of retinoids on the biochemical response of mouse skin to tumor initiators and promoters may be easily determined. Thus the relevance of biochemical observations to the process of tumor formation may be established.

EXPERIMENTS AND RESULTS

The experimental procedures have been fully described (6,10,11) and some of the results have been published (10,11).

The basic observation (shown in Fig. 2) is that a very small dose of retinoic acid applied to the skin inhibits the induction of ornithine decarboxylase activity in the epidermis by the tumor promoting phorbol ester and, using the same protocols, also inhibits the promotion of epithelial tumors. The data on the inhibition of enzyme induction by retinoic acid is shown in Fig. 2 and the inhibitory effect on tumor formation is shown in Fig. 3.

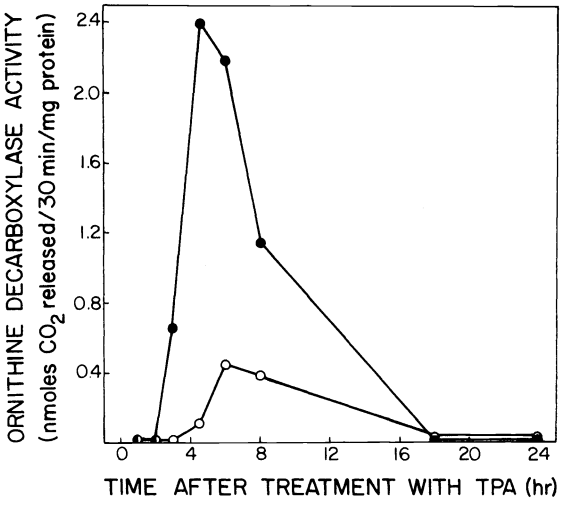


Fig. 2 The effect of pretreatment with retinoic acid on phorbol ester-induced epidermal ornithine decarboxylase activity. Groups of mice were treated with 1.7 nmoles of retinoic acid (○) or acetone (●) 1 hr before treatment with 17 nmol of tetradecanoylphorbol acetate. Mice were killed for enzyme assay at the indicated times after application of the phorbol ester. Each point in the graph represents the average of triplicate determinations of enzyme activity from soluble epidermal extracts prepared from 4 mice.

As shown in Fig. 2 (also see Fig. 1), application of 17 nmol of the phorbol ester to mouse skin caused about a 250-fold increase in ornithine decarboxylase activity of the epidermis between 4 and 6 hours after treatment. In contrast, a much smaller increase in enzyme activity resulted in those mice treated topically with 1.7 nmol of retinoic 1 hour before 17 nmol of the phorbol ester was applied to the same area of the skin of the back. Assays for enzyme activity are made on epidermis isolated from the treated skin area in all cases; the area consists of about 12 sq. cm. and is the area over which the test materials are spread by the solvent, 0.2 ml acetone. In contrast to the ability of retinoic acid to inhibit the induction of ornithine decarboxylase, the increase in S-adenosylmethionine decarboxylase activity which is induced by the phorbol ester is not inhibited by retinoic acid (data not shown, see ref. 10). The fact that the second enzyme in the polyamine biosynthetic pathway is not inhibited shows a high degree of specifcity for the retinoic acid effect. The specificity is emphasized by the observation that the increased incorporation of trititiated leucine into the total soluble proteins of epidermis which is caused by the phorbol esters is not blocked by retinoic acid. Only a very specific, small part of the total stimulation is blocked, namely ornithine decarboxylase.

Fig. 3 shows the inhibition of tumor formation by retinoic acid. The mice were initiated by a single application to the skin of the back of 0.2 nmol of 7,12-dimethylbenz[a]anthracene in 0.2 ml of acetone per mouse. After one week, all mice were treated twice a week with 17 nmol of the phorbol ester to the same area of the back. In addition the mice of one group were

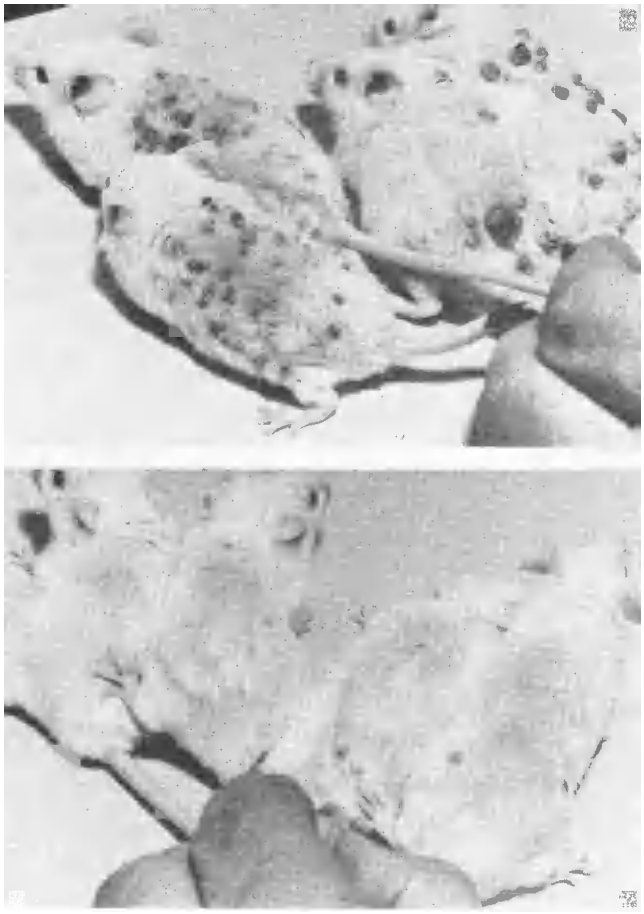


Fig. 3 Representative mice were photographed at the end of a tumor induction experiment (20 weeks). The mice shown in the top photograph were treated with 0.2 ml of acetone 1 hour prior to each treatment with 17 nmol of 12-O-tetradecanoylphorbol-13-acetate (100% incidence of tumors, 18 tumors/mouse average). The mice shown in the bottom photograph were treated with 17 nmol of retinoic acid one hour prior to each treatment with 17 nmol of the phorbol ester (85% incidence of tumors, 4.5 tumors/mouse average).

treated with 17 nmol of retinoic acid one hour before each application of phorbol esters. Typical mice from each group are shown. Many tumors were elicited in the control mice in contrast to the impressive reduction in the size and number of tumors in the mice treated with retinoic acid.

The ability of retinoic acid to inhibit the induction of ornithine deacarboxylase was limited to a definite period of time before and after application of the inducing agent (10). At the dose level of 1.7 nmol, no inhibitory effect was observed if the applications of retinoic acid were made 16 hours or longer before the phorbol ester. At shorter time intervals, the inhibitory effect increased to a maximum which was observed with applications given 1 hour before application of the phorbol ester until about 1 hour after the phorbol esters. An inhibition was still detectable by retinoic acid given 3 hours after the phorbol ester (only 1.5 hours before killing the mice for the assay of enzyme activity). Retinoic acid added to the enzyme assay flasks had no inhibitory effect on the enzyme. Because retinoic acid applied to the skin at times removed from application of the phorbol ester had no inhibitory effect on ornithine decarboxylase activity, the following tumor induction experiment was done to gain

further circumstantial evidence on the question of the relevance of high levels of the enzyme and the formation of tumors. All mice were initiated as described and then treated with the 8 nmol of the phorbol ester twice a week. Mice given no further treatment served as the controls and many tumors developed. Mice in a second group that were initiated and promoted were treated with 17 nmol of retinoic acid 1 hour before each promoter treatment and there was a dramatic reduction of tumors in these mice (equivalent to that shown in Fig. 3). In contrast, a third group of mice were treated with retinoic acid 24 hours after each promoter treatment (a regimen which does not inhibit the induction by phorbol ester of ornithine decarboxylase) and the tumor incidence was high, the same as in those mice not treated with retinoic acid. These results show that the time at which the retinoic acid is applied relative to the time of phorbol esters is critical to the inhibition of tumors; the timing correlates with the timing required to inhibit ornithine decarboxylase induction and, therefore, the results support an obligatory role for the induction of the enzyme in the biochemical mechanism of skin carcinogenesis.

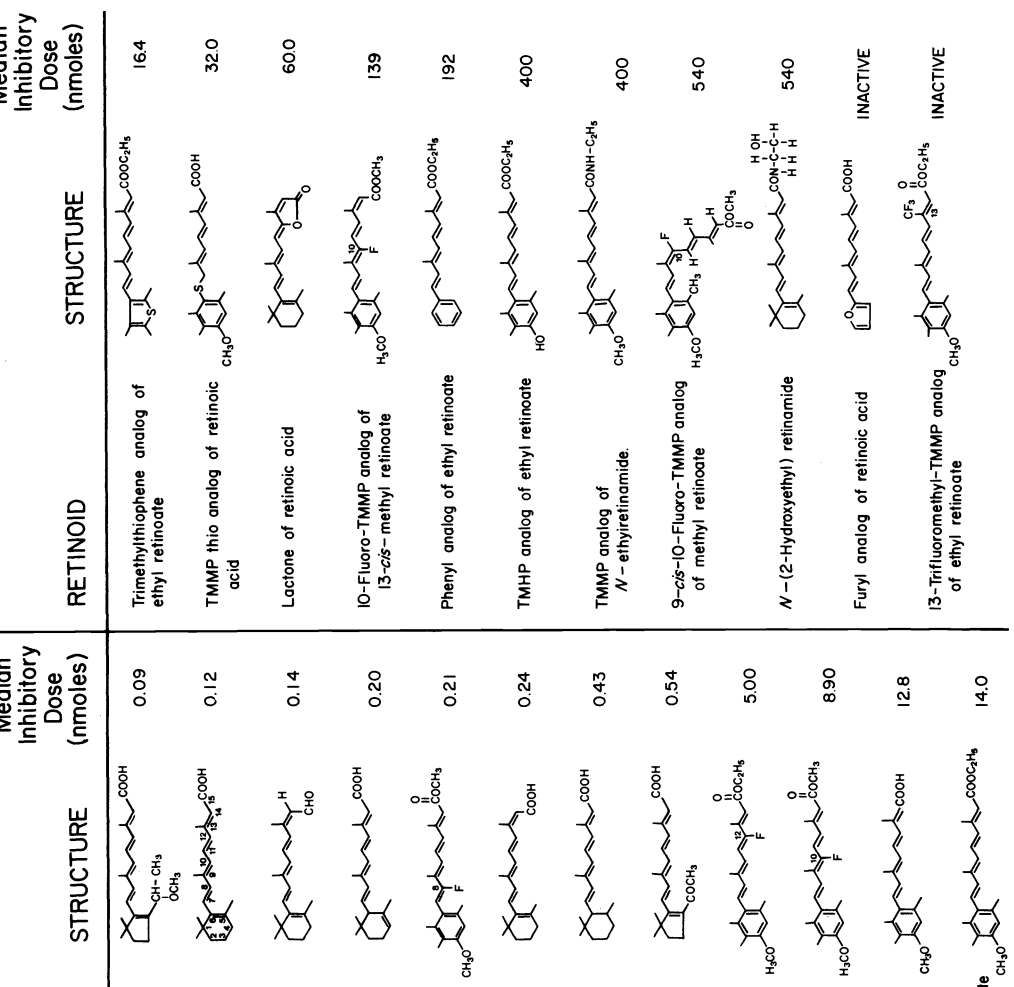
Dose-response studies showed that the inhibitory effect of retinoic acid on ornithine decarboxylase induction was barely discernable at 0.0034 nmol of retinoic acid. Increasing doses, applied one hour before the phorbol ester, caused an increasing inhibition of induction and at doses of 3-4 nmol essentially no induction was detectable (10). A 50% inhibition was achieved with a dose of about 0.17 nmol of retinoic acid applied in 0.2 ml of acetone to the skin of the back in the same area to which 17 nmol of 12-O-tetradecanoylphorbol-13-acetate in 0.2 ml of acetone was applied one hour later.

In contrast to the broad range of effective dose of retinoic acid applied topically, the dose-response curve for orally administered retinoic acid was much sharper (10). As in the topically applied tests, the retinoic acid administered by stomach tube (in 0.2 ml corn oil) was given one hour before the phorbol ester was applied to the skin. Mice were killed for ornithine decarboxylase assay 4.5 hr after the phorbol ester. Doses of retinoic acid below 0.034 µmol were ineffective but between 0.034 µmol and 0.34 µmol there was a dose-dependent inhibition of enzyme with a 50% inhibitory dose at about 0.17 µmol retinoic acid. In summary, orally administered retinoic acid effectively inhibited enzyme induction when administered only one hour before the topical application of the inducing agent; the amount of retinoic acid required for 50% inhibition was 1000-fold larger by the oral route and the dose-response curve was much steeper for orally-administered retinoic acid.

A number of natural and synthetic retinoids were tested for their ability to inhibit the induction of ornithine decarboxylase activity and for their inhibitory effect on tumor formation. Dose response curves were determined for the inhibitory effect of each of the retinoids, topically applied, on the enzyme induction (11). This allowed a rational choice of dose for the tumor studies. The results of these dose-response studies on the inhibition of enzyme induction by 23 synthetic retinoids together with the structure of the compounds is presented in Fig. 4. The mean inhibitory dose for each retinoid is given.

A number of representative synthetic analogs of retinoic acid were tested for their effect on the incidence of skin papillomas when administered one hour before each of the twice weekly applications of 8 nmol of the phorbol ester. Results of a typical experiment are shown in Fig. 5 (12). The conclusion from these experiments is that there is a close parallel in the ability of the synthetic retinoids to inhibit the induction of ornithine decarboxylase activity and to inhibit tumor formation. None of these retinoids inhibited the induction of S-adenosylmethionine decarboxylase activity.

There was a similar parallel in the ability of the natural retinoids to inhibit both the induction of the enzyme and the promotion of tumors by the phorbol ester. The inhibition of the phorbol ester-induced ornithine decarboxylase activity is shown in Table 1. The ability of these retinoids to inhibit tumor formation was proportional to their ability to inhibit the induction of the enzyme (10).



RETINOID	STRUCTURE	Median Inhibitory Dose (nmoles)
DMECP analog of retinoic acid		0.09
β-Retinoic acid		0.12
13-*cis*-Retinal		0.14
α-Retinoic acid		0.20
8-Fluoro-TMMP analog of methyl retinoate		0.21
13-*cis*-Retinoic acid		0.24
5,6-Dihydroretinoic acid		0.43
DACP analog retinoic acid		0.54
12-Fluoro-TMMP analog of ethyl retinoate		5.00
10-Fluoro-TMMP analog of methyl retinoate		8.90
TMMP analog of retinoic acid		12.8
TMMP analog of ethyl retinoate		14.0
Trimethylthiophene analog of ethyl retinoate		16.4
TMMP thio analog of retinoic acid		32.0
Lactone of retinoic acid		60.0
10-Fluoro-TMMP analog of 13-*cis*-methyl retinoate		139
Phenyl analog of ethyl retinoate		192
TMHP analog of ethyl retinoate		400
TMMP analog of *N*-ethyiretinamide.		400
9-*cis*-10-Fluoro-TMMP analog of methyl retinoate		540
N-(2-Hydroxyethyl) retinamide		540
Furyl analog of retinoic acid		INACTIVE
13-Trifluoromethyl-TMMP analog of ethyl retinoate		INACTIVE

Fig. 4 Doses of retinoids that, when administered topically one hour before 17 nmol of 12-O-tetradecanoylphorbol-13-acetate, inhibited by 50% the phorbol ester-induced ornithine decarboxylase activity in mouse epidermis.

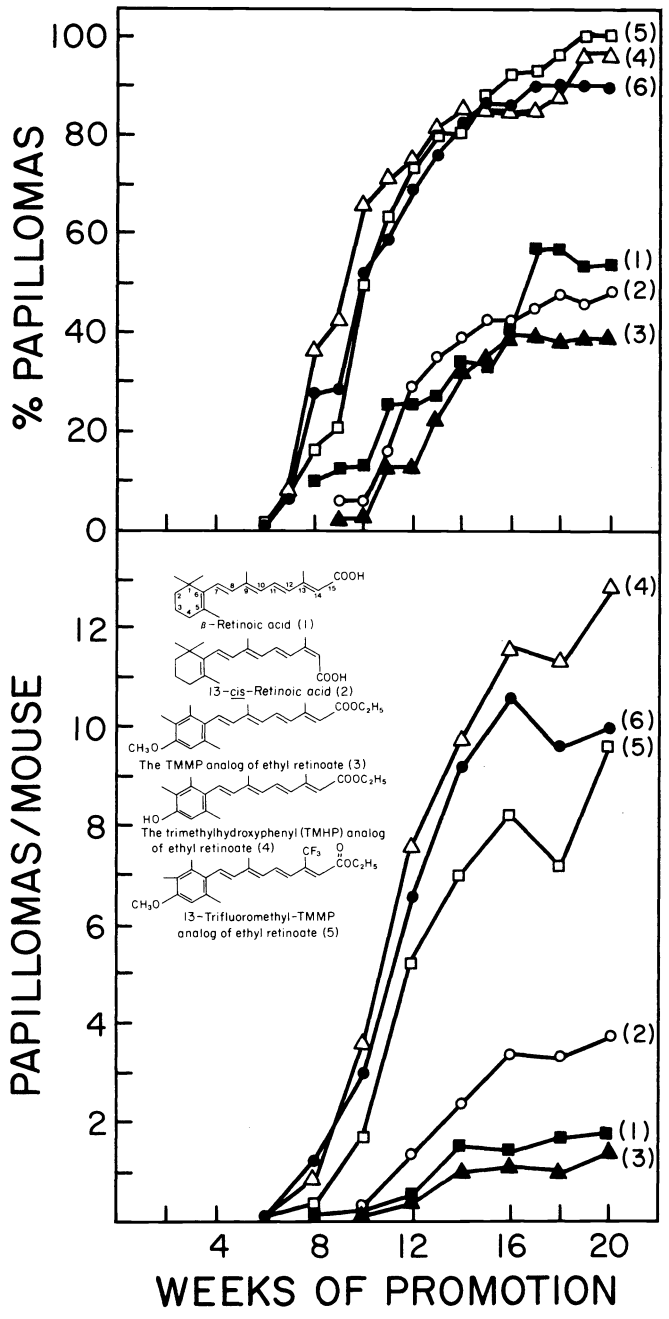


Fig. 5 The effect of various retinoids on skin tumor promotion. All mice were initiated with a single application of 0.2 μmol of 7,12-dimethylbenz[a]anthracene. After 2 weeks all mice were promoted with twice weekly applications of 8 nmol of the phorbol ester for 20 weeks. Retinoids were applied one hour before each phorbol ester application, except the control group of mice (group 6) were pretreated with acetone solvent only. Doses for retinoids were 34 nmol for β-retinoic acid and 13-cis-retinoic acid, and 140 nmol for the trimethylmethoxyphenyl (TMMP) analog of ethyl retinoate, the trimethylhydroxyphenyl (TMHP) analog of ethyl retinoate, and the 13-trifluoromethyl-TMMP analog of ethyl retinoate.

TABLE 1 The Inhibition by Natural Retinoids of 12-O-Tetradecanoylphorbol-13-acetate-Induced Activity in Mouse Epidermis

Retinoid, nmoles[1]	Enzyme Activity
None	100[2]
Retinoic Acid (all trans), 1.7	2
Retinal, 1.7	42
Retinol, 1.7	62
Retinyl acetate, 17	18
Retinyl palmitate, 17	42
Retinyl palmitate, 1.7	100
β-Carotene, 1.7	100

1. The retinoids were applied to the skin in 0.2 ml of acetone 1 hour before the application of 17 nmoles of the phorbol ester in 0.2 ml acetone.

2. The phorbol ester-induced level of ornithine decarboxylase activity was set at 100.

DISCUSSION

There is considerable evidence that the induction of epidermal ornithine decarboxylase activity and the resultant increase in tissue level of the polyamines, particularly putrescine, may be an essential metabolic component of the process of skin carcinogenesis (13). All tumor promoting stimuli that have been tested accomplish the induction, particularly 12-O-tetradecanoylphorbol-13-acetate. The degree of induction parallels the ability of the test substance to elicit skin tumors in initiated skin. Hyperplasiagenic agents that do not promote do not induce the enzyme. The correlation is strengthened by the observation that retinoids inhibit the induction, and in addition the inhibition parallels the inhibition of tumor formation by the retinoids which have been tested.

Retinoids have been long associated with the control of normal differentiation of epithelial tissue (8). In contrast, the process of tumor promotion, accomplished so effectivey by microgram quantities of 12-O-tetradecanoylphorbol-13-acetate, has been thought to involve a block of terminal differentiation. The increased levels of putrescine observed after phorbol ester treatment may be responsible. In any case, the fact that vitamin A-active compounds are capable of antagonizing the biochemical response that appears to be characteristic of an agent which blocks terminal differentiation and whose biochemical and morphological consequence is to cause cells to mimic the tumor cell phenotype after a single dose, suggests that the observed metabolic effect of retinoids may be a strong clue to their biochemical mechanism of action. It is very important to pursue the role of retinoids in controlling the putrescine level of epithelial tissues and the biological significance of that control.

In addition to contributing to the understanding of the mechanism of carcinogenesis, these studies suggest that a simple, rapid, and inexpensive method for testing the antipromoting properties of synthetic retinoids as well as retinoid metabolites may be developed based on inhibition of ornithine decarboxylase induction. One of the advantages of the proposal is that it is a test using intact mice in an organ system in which the retinoids actually function to inhibit tumor formation. A number of test systems now exist based on reversal of the metaplasia of vitamin A deficient hamster trachea in organ culture (13), reversal of carcinogen-induced hyperplasia of mouse prostate organ cultures, and other biological systems. The activity of a number of retinoids have been assayed in both the hamster trachea test (13, and personal communication) and the ornithine decarboxylase test. Agreement is good. There may be discrepancies in a few retinoid derivatives that are not metabolized to active forms when topically applied to mouse skin; for example, we found that an amide derivative of retinoic acid was a very effective inhibitor of phorbol ester-induced ornithine decarboxylase activity if administered orally but the same derivative was at least three orders of magnitude less effective than retinoic acid if applied topically (11). We conclude that some retinoids should be tested by the oral route as well as by local application to provide a valid assay of potency.

Currently there is great interest in the role of retinoids in the inhibition of carcinogenesis, both as useful agents to study for elucidating the biochemical mechanism of carcinogenesis and for practical application for prophylaxis against certain human cancers (8, 15). However, there have been two recent reports in which it has been suggested that the prophylactic application of retinoids in human neoplasia may be ill-advised (16, 17). Certainly when one is dealing with agents that are as powerful as the retinoids, capable of altering basic processes of differentiation, caution and full understanding are essential before human use. In both cases which led to the suggestion for caution, there is the possibility that the authors overinterpreted their results. The model systems used cultured fibroblasts

rather than epithelial cells, the levels or retinoids were high (1000-fold or more higher than the levels shown to inhibit metaplasia in the master trachea) and probably toxic, and the effects were not correlated with effects on tumor formation. In contrast, differentiation of teratocarcinoma stem cells by retinoic acid was reported in a paper (18) published immediately adjacent to one of those urging caution (17) and the ability of retinoids to block phenotypic cell transformation caused by sarcoma growth factor (19) was published immediately adjacent to the other (16). Obviously, more research is essential to resolve the role of retinoids in controlling differentiation and preventing cancer.

SUMMARY

A diterpene, 12-O-tetradecanoylphorbol-13-acetate is capable of eliciting many tumors of the skin of mice previously exposed to a carcinogen at a noncarcinogenic dose. The same agent also causes an increase of 250-fold or more in the activity of ornithine decarboxylase activity in the epidermis of mice within 5 hours after the application of 8.5 nmoles of the diterpene to the skin. Structure-activity and dose-response studies show that the induction of the enzyme may be an essential component of the biochemical mechanism of skin carcinogenesis. Further evidence that the induction of epidermal ornithine dearboxylase actvitiy may be one of the biochemical events essential for skin tumor formation was obtained from studies on Vitamin A and its analogs (retinoids). The ability of a large number of retinoids to inhibit the induction of ornithine decarboxylase activity in mouse epidermis was studied. Several of the same retinoids were tested for their ability to inhibit phorbol ester-promoted skin tumors. It was found that those retinoids that are the most effective as inhibitors of the enzyme induction are also the most effective inhibitors of tumor formation. The possibility that increased levels of the polyamine biosynthetic enzyme, ornithine decarboxylase, may play an obligatory role in the mechanism of carcinogenesis was strengthened by these data on the effects of retinoids. In addition, it is proposed that inhibition of the induction of this enzyme by retinoids in intact mouse skin may provide a simple and rapid test system for determining the tumor inhibitory activity of new synthetic retinoids.

ACKNOWLEDGEMENTS

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