Differential Effects of Retinoic Acid and 7,8-Benzoflavone on the Induction of Mouse Skin Tumors by the Complete Carcinogenesis Process and by the Initiation-Promotion Regimen

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ABSTRACT

The present study was designed to elucidate differences in the mechanism of the induction of mouse skin tumors either by the initiation-promotion regimen or by the complete carcinogenesis process. The protocols used to elicit skin tumors were: (a) by the initiation-promotion regimen, a single application of 0.2 μmol of 7,12-dimethylbenz[a]anthracene (DMBA) followed by twice-weekly applications of 12-O-tetradecanoylphorbol-13-acetate (TPA); and (b) by the complete carcinogenesis process, a single application of 3.2 μmol of DMBA without TPA treatment, or 0.2 μmol of DMBA applied once weekly, or 1, 10, 50, or 100 nmol of DMBA applied twice weekly without application of TPA. The biology of tumor formation by the initiation of tumors with a single application of 0.2 μmol of DMBA followed by twice-weekly applications of TPA differs from tumor induction by once-weekly 0.2-μmol doses of DMBA. In the latter case, papillomas developed more slowly and were less common (the tumor induction time was long; tumor yield was less), but carcinomas appeared much earlier. Retinoic acid, a potent inhibitor of tumor promotion by TPA, failed to inhibit and under some experimental conditions significantly (p < 0.001) enhanced tumor formation by DMBA. Also, retinoic acid did not inhibit ornithine decarboxylase induction by DMBA. In contrast, 7,8-benzoflavone, which did not inhibit the induction of ornithine decarboxylase activity by TPA, inhibited the induction of ornithine decarboxylase activity and tumor formation by DMBA.

The results indicate that the nature and the mechanism of the biochemical events elicited by the presumed promoting component of carcinogenesis by a complete carcinogen are different from those of the tumor promoter TPA.

INTRODUCTION

Mouse skin provides a useful system for studying the biochemical mechanism of carcinogenesis because it allows correlation between the biochemical change and the development of cancer. Factors that modify carcinogenesis are useful to obtain clues regarding the biochemical mechanism of the process. There are 2 commonly used protocols for the induction of mouse skin tumors: (a) by the initiation-promotion model system (2, 3, 19); and (b) by the complete carcinogenesis process (15, 16, 20). In the 2-stage model system, initiation is accomplished by topical application of a single small dose of a carcinogen such as DMBA. An initiating dose of a carcinogen per se will not lead to the development of visible tumors. Visible tumors will result only after prolonged and repeated applications of a tumor-promoting agent, such as TPA, to the initiated skin (2, 3, 19). In the complete carcinogenesis process, mouse skin tumors are induced by a single application of a large dose (15, 16) or by repeated applications of a smaller dose of a carcinogen such as the polycyclic hydrocarbon DMBA (20).

It is not clear whether tumor formation accomplished by the complete carcinogenesis process involves a promoting component with a mechanism analogous to that of TPA (11, 20). We have shown that retinoic acid, which is a potent inhibitor of the induction of ODC activity and tumor promotion by TPA, fails to inhibit the induction of ODC activity and tumor formation by DMBA (20). Thus, the nature of the presumed promoting component in DMBA carcinogenesis may differ from TPA promotion. In this paper, we have investigated further the effects of retinoic acid and benzoflavone on the induction of mouse skin tumors by the complete carcinogenesis process and by the initiation-promotion regimen to obtain clues on the mechanism of these 2 processes.

MATERIALS AND METHODS

Materials. Female Charles River CD-1 mice were purchased from Charles River Breeding Laboratory, Wilmington, Mass., and were used for experimentation at 8 weeks of age. TPA was obtained from Peter Borchert, Eden Prairie, Minn. DMBA was purchased from Eastman Organic Chemicals, Rochester, N. Y. Retinoic acid was obtained from Sigma Chemical Co., St. Louis, Mo. α-Naphthoflavone (7,8-benzoflavone) was purchased from Aldrich Chemical Co., Milwaukee, Wis. α-[1-14C]Ornithine hydrochloride (specific activity, 49.9 mCi/mmoll was purchased from New England Nuclear, Boston, Mass.

Treatment of Mice. All mice were housed in screen-bottomed stainless steel cages in light- and temperature-controlled rooms; food and water were available ad libitum. The dorsal skin of the mice was shaved 3 to 4 days before treatment, and only those mice in the resting phase of the hair cycle were used for experimentation. The solutions of all agents to be applied topically were prepared in acetone and were delivered to the shaved backs of individual mice in a volume of 0.2 ml. Control mice were treated with the same volume of acetone.

Assay of ODC Activity. At appropriate times after treatment, mice were killed by cervical dislocation, and epidermis from individual mice was separated by a brief heat treatment (57° for 30 sec). The epidermal preparations from 2 to 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 at 30,000 x g for 30 min to...
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give a soluble supernatant (20).

ODC activity from soluble epidermal extracts was determined by measuring the release of $^{14}$CO$_2$ from DL-[1-$^{14}$C]ornithine hydrochloride (20). The protein content in the soluble epidermal extracts was determined by the method of Lowry et al. (8).

**Tumor Induction Experiments.** Tumors were induced either by a single topical application of 3.2 μmol or by weekly applications of 0.2-μmol, or lower, doses of DMBA without further TPA treatment. Tumors were also induced by the initiation-promotion regimen (19). A single initiating dose of 0.2 μmol of DMBA in 0.2 ml acetone was applied topically to the shaved backs of mice; 2 weeks later, mice were treated twice weekly with 5 or 10 nmol of TPA for the duration of the experiment. There were at least 30 mice in each treatment group. Mice were weighed every second week.

The tumor incidence was determined weekly, and the total number of papillomas appearing on each mouse were counted at every other week of promotion treatment. The life history of individual papillomas was not recorded; hence, no data are available to reveal the rate of regression. The data reveal only the balance between the appearance and disappearance of papillomas in terms of the total number present at each counting. The term carcinoma is used for lesions grossly invading into the dermis or panniculus carnosus. These were confirmed by light microscopic examination. Carcinoma-bearing mice were killed by euthanasia after diagnosis was established. Carcinoma data are expressed as percentage of the effective total. The effective total is defined as the number of mice in each group at the time of appearance of the first carcinoma in any group.

**Statistical Analysis of Tumor Data.** The significance of the difference in the tumor multiplicity data obtained from the control and retinoic acid-treated mice was determined with the Wilcoxon rank sum test (6). An advantage of the Wilcoxon test is that it is valid without any assumption about the shape of the distribution of tumor multiplicities. Therefore, a significant result cannot be attributed to inappropriate distributional assumptions.

**RESULTS**

**Induction of Mouse Skin Tumors by the Complete Carcinogenesis Process**

Application of a single initiating dose of a complete carcinogen did not elicit tumors (Chart 1). However, weekly applications of a small dose of a complete carcinogen did lead to tumor formation. Once-weekly applications of 0.2 μmol of DMBA in 0.2 ml of acetone to the shaved backs of CD-1 mice resulted in 11.0 papillomas/mouse at 28 weeks after DMBA treatment; 100% of the mice had tumors. Furthermore, a single sufficiently large dose of the carcinogen led to a low incidence of tumors; application of a single 0.2-μmol dose of DMBA in 0.2 ml of acetone to the shaved backs of CD-1 mice did not result in tumors. When a single 3.2-μmol dose of DMBA was applied, a 19% tumor incidence occurred, and there was 0.28 papilloma/mouse (Chart 1).

**Kinetics of the Induction of Skin Papillomas and Carcinomas by the Complete Carcinogenesis Process and by the Initiation-Promotion Regimen**

In this experiment, mice were treated either once weekly with 0.2 μmol of DMBA without TPA treatment or with 0.2 μmol of DMBA followed by twice-weekly treatments with 10 nmol of TPA. Mice receiving repetitive treatments with DMBA did not develop papillomas until 14 weeks after treatment; the papilloma incidence plateaued after 32 weeks of DMBA treatment. In contrast, mice receiving promotion treatment with TPA developed papillomas at 8 weeks, and tumor response leveled off at 22 weeks after TPA treatment. However, carcinomas appeared earlier in mice in which tumors were promoted with DMBA than in mice in which tumors were promoted with TPA (Chart 2).

**Effects of Retinoic Acid on DMBA Carcinogenesis**

**Effect of Retinoic Acid Dose When Applied Once before DMBA.** Application of retinoic acid in conjunction with an initiating dose of DMBA has no effect on the incidence of tumors elicited by multiple applications of the tumor-promoting phorbol ester, but when retinoic acid is applied in conjunction with promotion treatment with TPA in the initiation-promotion regimen it inhibits the formation of both papillomas and carcinomas (17, 18, 21). The effect of topical application of retinoic acid on tumors elicited by once-weekly applications of DMBA in the complete carcinogenic regimen is shown in Chart 3. In this experiment, mice were treated with 0.2 μmol of DMBA; 2 weeks later, mice were treated with 1.7, 17, or 34 nmol of retinoic acid 1 hr before application of 0.2 μmol of DMBA or with 17 nmol of retinoic acid 1 hr before twice weekly applications of 5 nmol of TPA. Application of retinoic acid at any of the doses tested failed to inhibit the appearance of papillomas by the repeated DMBA treatment but inhibited the formation of skin papillomas by the DMBA-TPA protocol (Chart 3). Furthermore, application of retinoic acid 1 hr before each weekly application of 0.2 μmol of DMBA did not inhibit the development of carcinomas; application of 0.2 ml of acetone or 1.7, 17, or 34 nmol of retinoic acid in 0.2 ml acetone 1 hr before DMBA treatment resulted in 30, 29, 25, and 29% carcinomas, respectively, at 32 weeks of DMBA treatment.
Modulation of Mouse Skin Carcinogenesis

Effect of Retinoic Acid When Applied Once before and Once after DMBA Treatment. DMBA must be metabolized to its reactive form(s) for its carcinogenicity (4, 9). Thus, there is presumed to be a lag period before the effects of DMBA appear. For instance, there is a lag period of about 24 hr before DMBA induces ODC activity in mouse epidermis (10, 20). Consequently, because of the short half-life of retinoic acid in mouse skin (18, 21), the possibility that retinoic acid may inhibit DMBA carcinogenesis if applied more frequently was explored. As shown in Chart 4, application of retinoic acid 1 hr before and 24 hr after each once-weekly application of 0.2 \( \mu \)mol of DMBA did not inhibit tumor formation but rather potentiated papillomas per mouse by 64% at 28 weeks following DMBA treatment (\( p < 0.006 \)). In a separate repeat experiment, application of retinoic acid, under identical conditions in conjunction with DMBA, potentiated DMBA-induced skin papillomas by 80%. Mice treated once weekly with 0.2 \( \mu \)mol of DMBA developed 7.16 \( \pm \) 0.92 (S.E.) papillomas/mouse, whereas mice treated with 17 nmol of retinoic acid 1 hr before and 24 hr after once weekly applications of 0.2 \( \mu \)mol of DMBA resulted in the formation of 12.97 \( \pm \) 1.3 papillomas/mouse. The difference was statistically significant (\( p < 0.001 \)).

Effect of Retinoic Acid When Administered i.p. Systemic administration of retinoic acid has been shown to inhibit skin...
tumor promotion by TPA (17). As shown in Chart 5, i.p. administration of 3.3 μmol of retinoic acid in corn oil 30 min before each application of DMBA did not inhibit but rather augmented the carcinogenic action of DMBA.

Effect of Retinoic Acid When Applied before Various Doses of DMBA. The lack of an inhibitory effect of retinoic acid on DMBA carcinogenesis was not found to be due to a high dose of DMBA. As shown in Chart 6, 34 nmol of retinoic acid applied 1 hr before twice-weekly applications of 1, 10, or 50 nmol of DMBA failed to inhibit tumor formation by DMBA; rather, increases in yield and in incidence of skin papillomas were observed.

The effect of retinoic acid pretreatments on the formation of skin carcinomas elicited by increasing doses of DMBA was also observed. Mice treated with retinoic acid prior to treatment with DMBA developed carcinomas earlier than did mice treated with the vehicle (acetone) prior to each DMBA application. However, the incidence of carcinomas in the retinoic acid and control groups plateaued at the same level. The results are described for the groups treated with DMBA at 3 levels.

Application of 1 nmol of DMBA twice weekly to the shaved backs of mice did not result in the formation of carcinomas in the acetone pretreated group. Mice pretreated with 34 nmol of retinoic acid 1 hr prior to each application of 1 nmol of DMBA had 7.4% carcinomas at 37 weeks (not significantly different but perhaps indicative of an enhancing effect).

The times of appearance of the first carcinoma in mice treated with acetone or 34 nmol of retinoic acid 1 hr prior to each twice-weekly application of 10 nmol of DMBA were 24 and 17 weeks, respectively. The incidences of carcinomas at 31 weeks of DMBA treatment in the acetone and retinoic acid-pretreated groups were 18.5 and 48%, respectively. However, the carcinoma incidence of the 2 groups plateaued at the same level, 59% at 37 weeks; treatment with retinoic acid shortened the development time of carcinomas but did not increase the total number.

At the level of 50 nmol of DMBA twice weekly, the times of appearance of the first carcinoma in mice pretreated with acetone or retinoic acid were 17 and 21 weeks, respectively. There was no real difference in the incidence of carcinomas related to retinoic acid pretreatment; the incidence was 60 to 70% at 25, 27, 29, and 31 weeks in both groups. At the 50-nmol level, the carcinogenic potency of DMBA appeared to overwhelm the influence of retinoic acid.

Effect of Retinoic Acid on Tumor Formation by B(a)P or 3-MC. The inability of retinoic acid to inhibit complete carcinogenesis was not confined to DMBA. As shown in Chart 7, retinoic acid at a 34-nmol dose applied topically 1 hr before each application of 100 nmol of B(a)P or 3-MC did not affect skin papillomas elicited with 3-MC but significantly enhanced (p < 0.001) papilloma formation with B(a)P. Retinoic acid pretreatment also enhanced the formation of skin carcinomas with B(a)P; carcinoma incidences in acetone- and retinoic acid-pretreated groups were 38 and 66%, respectively.

Effect of 7,8-Benzoflavone on DMBA Carcinogenesis

In this experiment (Chart 8), 367 nmol of 7,8-benzoflavone
Chart 7. Effect of retinoic acid (RA) on skin tumor formation by B(a)P and 3-MC. Acetone or retinoic acid (34 nmol in 0.2 ml acetone) was applied 30 min before twice-weekly applications of 100 nmol of B(a)P or 3-MC in 0.2 ml of acetone. Retinoic acid neither inhibited nor enhanced tumor formation with 3-MC but enhanced significantly (p < 0.001) tumor formation with B(a)P (28 weeks).

or 17 nmol of retinoic acid were applied topically 30 min before each weekly application of 0.2 /¿mol of DMBA. 7,8-Benzoflavone inhibited the formation of skin papillomas by 74%, but retinoic acid treatment did not inhibit tumor formation by DMBA. 7,8-Benzoflavone has been shown to inhibit formation of skin tumors elicited by repeated applications of DMBA (4, 7, 12).

Effect of Retinoic Acid and 7,8-Benzoflavone on the Induction of ODC Activity by DMBA

As shown previously (10, 20), a single application of 3.6 /¿mol of DMBA to mouse skin led to epidermal ODC induction with peak activity (50-fold) at about 36 hr following treatment. A dose of retinoic acid that inhibited by about 90% the induction of ODC activity by TPA failed to inhibit the induction of ODC activity by DMBA (20). A single application of various doses (0.017, 1.7, and 17.0 nmol) of retinoic acid 1 hr before application of 3.6 /¿mol of DMBA to mouse skin was without effect, but applications of 36.7 or 367 nmol of 7,8-benzoflavone inhibited the induction by 3.6 /¿mol DMBA of ODC activity by 62 and 80%, respectively (data not shown). Benzoflavone, which inhibited the induction of ODC activity by DMBA, failed to inhibit the induction of ODC activity by TPA (20).

A more frequent application of retinoic acid in conjunction with DMBA did not inhibit the induction of ODC activity by DMBA. Application of 17 nmol of retinoic acid 1 hr before and 24 hr after topical application of 3.6 /¿mol of DMBA to mouse skin or application of 0.017, 0.17, or 1.7 nmol retinoic acid 4 times at 3-hr intervals immediately preceding and 4 times at 2-hr intervals immediately following application of 3.6 /¿mol of DMBA did not inhibit the induction of ODC activity, as determined 36 hr after DMBA application (20). Furthermore, 3.3 /¿mol retinoic acid administered by stomach tube did not inhibit ODC induction by DMBA (20).

DISCUSSION

Polycyclic hydrocarbons such as B(a)P, 3-MC, and DMBA are complete carcinogens; their application to skin, at appropriate doses, results in tumor formation. It is known that polycyclic hydrocarbons are metabolized by the mixed-function oxidases to reactive electrophiles, which react with nucleophilic centers in cellular macromolecules to initiate carcinogenesis (9). When skin carcinogenesis is considered as a multistage process, it is tentatively assumed that there is also a promoting component of carcinogenesis by, for example, DMBA. We now present evidence that the promotion of skin tumorigenesis by the complete carcinogen DMBA may be mechanistically different from that of the tumor promoter TPA.

The application of TPA to DMBA-initiated mouse skin is perhaps the most effective protocol for the development of papillomas (benign lesions); tumor induction time is short, and a large number of papillomas are elicited. However, some of the papillomas regress, and the carcinomas develop late from papillomas (Chart 2; Ref. 19). In contrast, in a complete carcinogenesis process, papillomas are fewer and appear later, but carcinomas appear much earlier (Chart 2). Furthermore, the difference in the kinetics of tumor development by these 2 protocols indicates that they may involve different mechanisms.

Further clues about the different nature of the promoting component in complete carcinogenesis emerged from the differential effects of retinoic acid on the 2 different methods of eliciting skin tumors. Retinoic acid applied in conjunction with TPA following initiation inhibits the formation of skin papillomas.
In contrast, retinoic acid applied in conjunction with each weekly application of 0.2 μmol DMBA did not inhibit DMBA-induced skin tumors (Chart 3). The difference cannot be attributed to differences in retinoic acid, since the same solution of retinoic acid was applied before either TPA or DMBA; the groups were a part of the same internally controlled experiment. Furthermore, the application of a wide dose range (0.17 to 68 nmol) of retinoic acid with various doses (1 to 200 nmol) of DMBA did not inhibit the formation of skin tumors (Charts 3 and 6). Systemic administration of retinoic acid and more frequent applications of retinoic acid in conjunction with DMBA resulted in a paradoxical potentiation of DMBA-induced skin tumors (Charts 4 and 5). Application of as much as 66 nmol of retinoic acid twice weekly to DMBA-initiated mouse skin did not elicit tumors (Chart 3). Furthermore, application of 17 nmol of retinoic acid alone, either in acetone or methanol vehicle, twice or 3 times weekly, to DMBA-initiated (0.2 μmol) skin did not elicit tumors at 39 weeks of retinoic acid treatment. Thus, retinoic acid is not a mouse skin tumor promoter at doses (17 or 68 nmol) that do not induce ODC activity in mouse epidermis but do cause enhanced thymidine incorporation into epidermal DNA at 32 and 42 hr following treatment (17).

As shown previously (20), application of either TPA or a single completely carcinogenic dose of DMBA leads to a dramatic induction of epidermal ODC activity. However, there is a considerable difference in the time course and in the degree of ODC induction (10, 20). Retinoic acid inhibits ODC induction by TPA but not the induction of ODC by DMBA; the mechanism of such a differential effect of retinoic acid is not clear. Evidence indicates that ODC induction by TPA may involve new protein and RNA synthesis and that retinoic acid may inhibit ODC induction at the transcriptional and/or posttranscriptional levels (17). It is likely that DMBA may not have its pleiotypic effect on chromatin in a sharp time focus as does TPA but rather has a moderate effect over a longer period of time in proportion to the availability of metabolically active forms.

7,8-Benzoflavone, an inhibitor of the metabolism of DMBA when applied in conjunction with DMBA (4, 12), inhibits the induction of ODC activity and the formation of skin tumors by DMBA. This indicates a requirement for the metabolic activation of DMBA for ODC induction and for tumor formation. 7,8-Benzoflavone did not affect ODC induction by TPA. Furthermore, these findings, together with the fact that retinoic acid inhibits neither the induction of ODC activity nor tumor formation by DMBA, indicate that DMBA-induced ODC activity may be an important component of the mechanism of DMBA carcinogenesis.

The mechanism of complete carcinogenesis by DMBA remains speculative. A single complete carcinogenic dose of DMBA results, after an initial depression for 30 hr, in enhanced DNA synthesis (data not shown). Ulceration and wounding are also observed after a single large dose (3.2 μmol) or repeated small doses (0.2 μmol) of the carcinogen. It is possible that such treatments with the carcinogen may induce considerable cell damage and cell death in the basal epidermal cell layer, and that leads to regenerative epidermal hyperplasia. It is likely that regenerative hyperplasia of epidermis associated with wounding is a promoting stimulus (1). Alternatively, in addition to the reaction of the electrophile with bases in DNA, reaction with protein components of chromatin may result in a pleiotypic response that provides the promoting component of the complete carcinogen. However, on the basis of the retinoic acid effect and the nature of the kinetics of tumor induction, it appears that whatever is the nature of the promoting component in DMBA carcinogenesis it is accomplished by a pathway different from TPA promotion.

Retinoic acid has been shown to inhibit tumor formation in a variety of model systems (13, 14). Characteristic of most of these models is the fact that retinoic acid treatment was begun after exposure to the carcinogen was terminated; treatment with retinoids was restricted to a later period in the process of tumor formation. These later processes progress in the absence of carcinogen, are susceptible to inhibition by retinoids, are perhaps analogous to promotion by TPA, and may be accomplished by endogenously produced promoters (e.g., hormones). We can cite at least 2 examples in which retinoic acid treatment concurrent with a carcinogenic stimulus is either without effect or causes increased tumor incidence. Two of these are UV-induced tumors (5) and the multiple applications of certain polycyclic hydrocarbons reported herein. In each case, retinoic acid does not prevent the induction of ODC by the carcinogenic agent.

We conclude that the prevention of cancer by retinoids is not universal. One cannot extrapolate the results of the effect of an inhibitor on a carcinogen or a promoter in one organ to another carcinogen or promoter in the same or another organ. As another example, 7,8-benzoflavone inhibits DMBA carcinogenesis but not BaP carcinogenesis in mouse skin (7).

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