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Effects of Retinoids and Inhibitors of Arachidonic Acid Metabolism on Tumor-promoter-induced Soft Agar Colony Formation of Mouse Epidermal Cells and Rat Urinary Bladder Cells

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Effects of retinoids and inhibitors of arachidonic acid metabolism on tumor-promoter-induced soft agar colony formation of mouse epidermal cells and rat bladder cells were evaluated. Topical application of retinoic acid, an anti-tumor-promoter, to female SENCAR mouse skin inhibited 12-O-tetradecanoylphorbol-13-acetate-induced soft agar colony formation of mouse epidermal cells, an event proposed to be essential for tumor promotion. Effects of dietary retinyl acetate, nordihydroguaiaretic acid (NDGA) and quinacrine hydrochloride on colony formation of rat bladder cells were then examined. Male Fischer 344 rats were given 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine for 3 weeks, followed immediately by the administration for 9 weeks of 5% sodium saccharin supplemented with or without 0.05% retinyl acetate, 0.1% NDGA or 0.01% quinacrine hydrochloride. Saccharin-induced colony growth was significantly inhibited by the administration of retinyl acetate or NDGA, suggesting that these two agents have anti-tumor-promoting effects on rat bladder carcinogenesis. Thus, the colony-forming assay might be useful for early detection of anti-tumor-promoters of skin and bladder.

Key words: Colony-forming assay — Anti-tumor-promoter — Bladder cancer — Retinoids — Nordihydroguaiaretic acid

A strong correlation has been observed between anchorage-independent growth *in vitro* and tumorigenic properties of cells, and colony formation in soft agar has been used as a specific indicator of phenotypic changes associated with malignant transformation.[1,2]

We previously reported that colony formation of mouse epidermal cells in soft agar occurred earlier than papilloma development in the skin of mice treated with DMBA*[2] and TPA.[3] Subsequently, colony formation in soft agar was also shown with rat bladder cells prior to papilloma development in rats treated with BBN. Furthermore, sodium saccharin and several amino acids, which act as rat bladder cancer promoters, induced colony formation of rat bladder cells after treatments with subcarcinogenic doses of BBN, suggesting a relationship between tumor-promoting activity and colony-inducing activity.[4] Thus, we considered that the application of the colony-forming assay could be useful to analyze early events in carcinogenesis and could provide a short-term screening method for detecting new carcinogens, promoters and anti-tumor-promoters.

Here we report that retinoids, which have been shown to prevent cancer of the skin, bladder, lung and breast in experimental animals exposed to carcinogens,[5] inhibit soft agar colony formations of mouse epidermal cells and rat bladder cells induced by TPA and sodium saccharin, respectively, after treatment with suboptimal doses of carcinogens. We also evaluated the effects of inhibitors of arachidonic acid metabolism, which were reported to inhibit TPA-induced ODC activity or tumor promotion in mouse skin,[6,7] on saccharin-induced colony formation of rat bladder cells.

MATERIALS AND METHODS

Animals Female SENCAR mice at 7 weeks old and male Fischer 344 rats at 6 weeks old were

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*[2] The abbreviations used are: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; DMBA, 9,10-dimethyl-1,2-benzanthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; NDGA, nordihydroguaiaretic acid; ODC, ornithine decarboxylase; MEM, minimum essential medium; FANFT, N-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide.

purchased from Shizuoka Experimental Animal Farm, Shizuoka. They were housed 3 or 4 per cage, with softwood chips as bedding, in an air-conditioned room at 24° and 50% humidity, on a 12-hr light-dark cycle, and water and feed were provided *ad libitum*.

Chemicals DMBA, TPA, phorbol, retinoic acid, sodium saccharin, retinyl acetate, NDGA, quinacrine hydrochloride were obtained from Sigma Chemical Company, St. Louis, MO. DMBA, TPA, phorbol and retinoic acid were dissolved in 0.1 ml of acetone, and topically applied to the shaved backs of individual mice using a micropipette. Sodium saccharin, retinyl acetate, NDGA and quinacrine hydrochloride were added to the powdered diet, CE-2 (CLEA, Osaka), on a weight/weight basis. BBN was obtained from Nakarai Chemical Co., Kyoto and was given at a concentration of 0.05% in the drinking water.

Experiment I The dorsal skin of the mice was shaved 2 days before treatment, and only those in the resting phase of the hair cycle were used. The mice were given a single topical application of DMBA (390 nmol). One week later, the mice were divided into three groups. In group I, the mice were treated with twice-weekly applications of phorbol (4.1 nmol). Phorbol at this dosage showed no tumor-promoting effect in the skin of SENCAR mice (unpublished data). Group II mice were treated twice weekly with TPA (4.1 nmol). In group III, retinoic acid (41 nmol) was applied topically 1 hr before each TPA treatment. Five mice in each group were sacrificed for the colony-forming assay before DMBA treatment and after 0, 2 and 4 weeks of TPA (or phorbol) treatment.

Experiment II The rats, weighing approximately 165 g, were randomly divided into 7 groups. They were given drinking water with (groups I–V) or without (groups VI and VII) 0.05% BBN for 3 weeks, and then for 9 weeks they were given powdered diet containing 5% sodium saccharin (groups I and VI), 5% sodium saccharin plus 0.05% retinyl acetate (group II), 5% sodium saccharin plus 0.1% NDGA (group III), 5% sodium saccharin plus 0.01% quinacrine hydrochloride (group IV) or powdered diet alone (group VII). The biopotency of the retinyl acetate was 500,000 IU/g. Therefore, 0.05% retinyl acetate corresponded to 250 IU per g of diet. Body weight and food consumption were measured every 3 weeks. All rats in each group were killed at the end of week 12 for the colony-forming assay.

Double Soft Agar Colony-forming Assay The colony-forming assay was done as previously described.[4] Briefly, the mouse skin or rat bladders were removed and minced with a sterile scalpel. The pieces were digested for 1 hr at 37° in Eagle's MEM (Flow Laboratories) containing 0.14% collagenase type-1 and 0.05% DNase (Sigma), then decanted through two layers of sterile gauze and washed once in 0.03% DNase solution. Cells were collected by centrifugation and suspended in MEM supplemented with 15% heat-inactivated fetal bovine serum (Flow), penicillin (100 units/ml, Sigma) and streptomycin (100 μg/ml, Sigma), then passed through 26G syringes and a 60 μm nylon mesh. The cells were then suspended in 0.3% agar (Sigma) containing MEM and plated over a 1.0-ml underlayer of MEM in 0.5% agar in 35×10 mm plastic Petri dishes (Flow). The final concentration of cells in each culture was 2×10^4 viable cells per dish, as determined by trypan blue dye exclusion. Triplicate cultures were used in each experiment. The dishes were then cultured at 37° in a humidified atmosphere of 5% CO_2 in air. One milliliter of 10% formalin was poured over the top layer of one dish and the preparation was stored at 4°. The number of colonies with diameters of 50 μm or more was counted using an inverted phase-contrast microscope (Nikon), at 2 weeks after plating. The colony numbers were corrected for background colony counts by subtracting the colony counts on the formalin-added dishes.

Results and Discussion

The results of the colony-forming assay with SENCAR mouse epidermal cells after a single application of DMBA and twice-weekly treatments with TPA (or phorbol) and the effect of retinoic acid on colony formation are shown in Table I. Treatment with phorbol (group I) did not significantly increase the colony number as compared to that before DMBA application. In contrast, significant colony growth was obsreved in group II as compared to group I after 2 and 4 weeks of treatment with TPA. On the other hand, colony numbers of mice treated with retinoic acid (group III) were significantly fewer than those in group II at both 2 and 4 weeks of TPA treatment.

Retinoic acid is known to prevent TPA-promoted tumor formation in mouse skin carcinogenesis.[8] In Experiment I, an application of retinoic acid to mouse skin inhibited soft agar colony formation of mouse epidermal cells induced by TPA. We have previously reported that TPA induced colony formation in soft agar medium of mouse epidermal cells during a two-stage mouse skin carcinogenesis experiment, and also showed that colony-inducing activity correlated well

Table I. Effect of Retinoic Acid on Soft Agar Colony Formation of Mouse Epidermal Cells (Experiment I)

Group	Treatment	Number of colonies (mean±SD)			
		Before DMBA	Time after treatment with TPA (or phorbol) (weeks)		
			0	2	4
I	DMBA − phorbol	2.9±3.3[a]	1.8±2.0[b]	0.4±0.7[c]	4.5±3.2[d]
II	DMBA − TPA			13.0±6.7[e]	25.2±3.6[f]
III	DMBA − TPA+retinoic acid			5.0±3.1[g]	7.4±4.0[h]

a-b, a-c, a-d: Not significant.
c-e: $P<0.02$, *e-g*: $P<0.05$, *d-f, f-h*: $P<0.001$.

Table II. Effect of Retinyl Acetate and Inhibitors of Arachidonic Acid Metabolism on Soft Agar Colony Formation of Rat Bladder Cells (Experiment II)

Group	Treatment		No. of rats	Colony No. (mean±SD)
	Initial	Second		
I	BBN	saccharin	12	20.8±5.9[a]
II	BBN	saccharin+retinyl acetate	12	5.3±2.4[b]
III	BBN	saccharin+NDGA	11	9.8±5.4[c]
IV	BBN	saccharin+quinacrine	12	16.2±6.3[d]
V	BBN	control diet	7	4.2±1.4[e]
VI	control diet	saccharin	5	4.5±1.9[f]
VII	control diet	control diet	5	4.2±1.9[g]

a-b, a-c, a-e: $P<0.001$.
a-d, e-f, e-g: Not significant.

with tumor-promoting activity in rat urinary bladders.[3,4] Our present data lend further support to the above correlation. These findings also led us to consider that anti-tumor-promoting activities of various agents may be assessed by evaluating their inhibitory activities on soft agar colony formation. Therefore, Experiment II was designed to examine the effects of three compounds on saccharin-induced colony formation of rat bladder cells.

The results of colony formation of rat bladder cells are shown in Table II. Rats treated with 0.05% BBN for 3 weeks followed by 5% sodium saccharin (group I), as previously reported,[4] showed significant colony growth compared to those treated with BBN alone (group V). Colony growth was significantly inhibited by the addition of 0.05% retinyl acetate (group II) or 0.1% NDGA (group III) to the diet supplemented with sodium saccharin, while it was not inhibited by 0.01% quinacrine hydrochloride (group IV). No significant differences were observed in food consumption or in weight gain of the rats, among any of the groups.

Retinoids have been shown to inhibit chemical carcinogenesis in urinary bladders of experimental animals.[9,10] However, the mechanism by which retinoids prevent bladder carcinogenesis is unclear. The inhibitory effect of retinyl acetate on saccharin-induced colony formation in Experiment II could show that their chemo-preventive effect might be in part due to their anti-tumor-promoting activities.

It has been reported that phospholipase A_2 inhibitors, such as quinacrine or *p*-bromophenacyl bromide, and also lipoxygenase inhibitors, such as NDGA and phenidone, inhibit the epidermal ODC induction and the formation of skin papillomas by TPA.[6,7] Thus, the products of arachidonic acid metabolism appear to be essential for skin tumor

promotion. In the present study, NDGA inhibited saccharin-induced colony formation, suggesting that it has anti-tumor-promoting activity in rat bladder carcinogenesis. It has also been reported in the two-stage rat urinary bladder carcinogenesis model that aspirin, a cyclooxygenase inhibitor, inhibits both FANFT initiation and saccharin promotion of bladder carcinogenesis.[11] Taken together, the products of arachidonic acid metabolism seem to play an important role in bladder carcinogenesis, as they do in skin carcinogenesis.

The reason why quinacrine did not show any inhibitory effect on colony formation of bladder cells is not clear. One of the explanations for this may be its relatively low concentration in the diet. Quinacrine is known to be toxic to the heart of F344 rats.[12] Therefore, optimal concentrations might not be reached *in vivo* at a dose which would be well tolerated by the rats. Furthermore, little is known of its metabolism in rats.

It is very important to develop cancer chemo-preventive agents, and several short-term assays, such as the determination of mouse epidermal ODC activity[7] or the measurement of agglutinability of rat bladder cells by concanavalin A,[13] have been utilized to examine the anti-tumor-promoting effects of various compounds. We think that the method presented here might be useful for early screening of anti-tumor-promoting agents, as well as tumor promoters, although further studies are needed to confirm its validity.

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