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Anti-promoting Effect of Nordihydroguaiaretic Acid on N-Butyl-N-(4-hydroxybutyl)nitrosamine and Sodium Saccharin-induced Rat Urinary Bladder Carcinogenesis

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The effects of oral administration of nordihydroguaiaretic acid (NDGA), an antioxidant and inhibitor of arachidonic acid metabolism, on rat bladder carcinogenesis were examined. Six-week-old male Fischer 344 rats were given drinking watar containing 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine for 4 weeks. Following this 4-week period, diet containing 5% sodium saccharin (SS) with or without 0.1% NDGA supplement was given to the rats for 36 weeks. The incidences of papillary or nodular (PN) hyperplasia and of papilloma in the group treated with SS plus NDGA were significantly lower than those in the group treated with SS alone. The number of PN hyperplasic foci per 10 cm of basement membrane in rats treated with SS plus NDGA was also lower than that in the group treated with SS alone. These results suggest that NDGA has an anti-tumor-promoting effect on rat bladder carcinogenesis.

Key words: NDGA — Antioxidant — Inhibitor of arachidonic acid — Antipromoter — Bladder carcinogenesis

Nordihydroguaiaretic acid (NDGA[2]) is a constituent of an evergreen shrub, *Larrea divaricata* (creosote bush),[1,2] and a potent antioxidant[3] which was formerly used as a food and pharmaceutical additive to prevent development of rancidity.[4] It has also been reported that NDGA is an inhibitor of arachidonic acid metabolism, and that NDGA inhibits the epidermal ornithine decarboxylase (ODC) induction and formation of skin papilloma induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mice.[5,6]

We previously reported that the colony formation of mouse epidermal cells in soft agar was observed earlier than papilloma development in the skin of mice treated with 7,12-dimethylbenz[*a*]anthracene and TPA.[7] Subsequently, colony formation in soft agar was also shown with rat bladder cells taken prior to papilloma development in rats treated with N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN).[8] Thus, we developed a short-term screening method for detecting carcinogenic activities as well as promoter and antipromoter effects of chemicals. We then evaluated the inhibitory effect of NDGA on BBN and sodium saccharin (SS)-induced colony formation, and found that it may have anti-tumor-promoting activity in rat bladder carcinogenesis.[9] Therefore, in the present study, we examined the anti-tumor-promoting effect of NDGA in a long-term *in vivo* carcinogenesis experiment.

MATERIALS AND METHODS

Animals Six-week-old male Fischer 344 rats purchased from Shizuoka Experimental Animal Farm were used. Two or three rats were housed in each cage and maintained at 24°C and 50% humidity on a 12-h light-dark cycle. They were given food (CE-2; CLEA, Osaka) and water *ad libitum*.

Chemicals and media BBN (Nakarai Chemical Co., Kyoto) was added to drinking water at the concentration of 0.05%. Further, 0.1% NDGA (Sigma Chemical Company, St. Luis, MO) and 5% SS (Nakarai Chemical Co.) on a weight/weight basis were added to the diet.

Experiment A total of 130 rats were divided into 8 groups which were treated with chemicals according to the schedule shown in Fig. 1. All surviving animals were killed at week 40. Urinary bladders of the rats were distended with 10% formaldehyde solution, and a ligature was placed around the bladder neck to maintain proper distention. Each of the bladders was cut into 6 slices, which were then embedded in paraffin and stained with hematoxylin and eosin for histological examination. Histological changes in the urinary bladder were classified as simple hyperplasia, papillary or nodular (PN)

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[2] The abbreviations used are: NDGA, nordihydroguaiaretic acid; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; SS, sodium saccharin; PN hyperplasia, papillary or nodular hyperplasia; ODC, ornithine decarboxylase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HETES, hydroxyeicosatetraenoic acid derivatives.

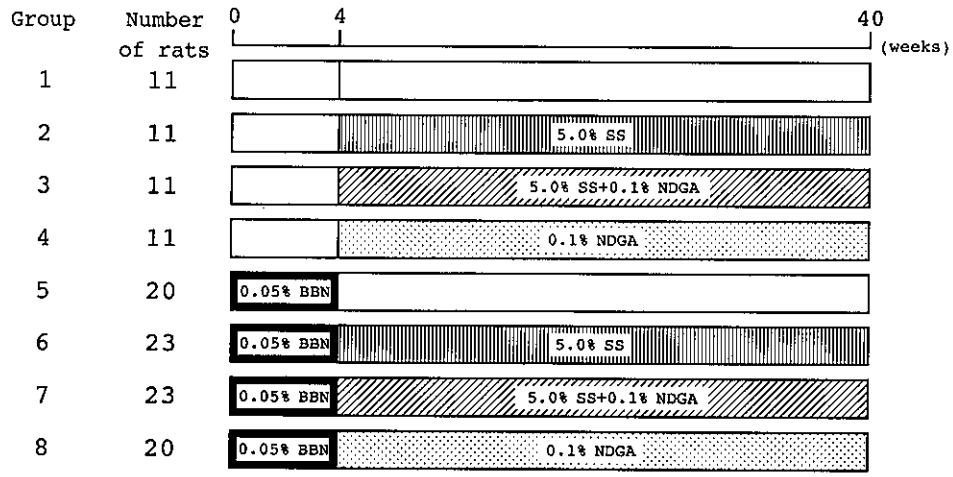

Fig. 1. Experimental design. A total of 130 rats were divided into the following 8 groups; those given drinking water without (groups 1–4) and with (groups 5–8) 0.05% BBN for 4 weeks, of which groups 3 and 7 were then given diet containing 5% SS with 0.1% NDGA and groups 2 and 6 5% SS without 0.1% NDGA for 36 weeks. Groups 4 and 8 were given diet containing 0.1% NDGA only for 36 weeks, and groups 1 and 5 control diet.

hyperplasia, papilloma, or carcinoma.[10] In addition, 6 sections of each urinary bladder were prepared for light microscopy and measurement of the total length of the basement membrane using a Digitizer kw-3300 (Graphtec Corporation, Yokohama). The incidence and average number of foci of histological change per 10 cm of basement membrane were recorded. Statistical analyses were done using the Bonferroni method for comparison of incidences and mean numbers of foci of histological change.[11]

RESULTS

The length of the urinary bladder basement membrane (BM) was about 8–12 cm in each group. The four types of histological changes of the urinary bladder, simple hyperplasia, PN hyperplasia, papilloma and cancer, were observed in groups 5–8. These changes were observed together or singly. All carcinomas were of the transitional cell type, and some also showed squamous cell metaplasia and invasive growth into the submucosa. No metastasis was found in any rat. Groups 1 through 4 did not show any abnormalities of the bladder epithelium. Incidences and numbers of carcinoma and precarcinomatous lesions in the rat urinary bladder are shown in Tables I and II, respectively. Incidence (23/23, 100%) of simple hyperplasia and incidence (19/23, 82.6%) and average number of foci (3.60 ± 3.31/10 cm BM) of PN hyperplasia in group 6 were significantly ($P<0.05$, $P<0.01$) higher than those in group 5 (14/20, 70%; 6/20 30%; and 0.73 ± 1.51/10 cm BM, respectively). In addition, incidence (12/22, 54.5%) and average number of foci (1.39 ± 2.35/10 cm BM) of PN hyperplasia and incidence (0/22, 0%) of papilloma in group 7 were

Table I. Incidences of Carcinomas and Precarcinomatous Lesions in the Rat Urinary Bladder

Group	Treatment	No. of rats		Simple hyperplasia (%)	PN hyperplasia (%)	Papilloma (%)	Carcinoma (%)
		Initial	Effective				
5	BBN→CE-2	20	20	14 (70)	6 (30)	2 (10)	1 (5)
6	BBN→SS	23	23	23 (100) *	19 (82.6) **	5 (21.7) *	2 (8.7)
7	BBN→SS+NDGA	23	22	21 (95.5)	12 (54.5) *	0 (0)	1 (4.5)
8	BBN→NDGA	20	20	16 (80)	7 (35)	3 (15)	4 (20)

* $P<0.05$. ** $P<0.01$.

Table II. Numbers of Carcinomas and Precarcinomatous Lesions in the Rat Urinary Bladder

Group	Treatment	PN hyperplasia No. per 10 cm of BM	Papilloma No. per 10 cm of BM	Carcinoma No. per 10 cm of BM
5	BBN→CE-2	0.73±1.51 ┐**	0.18±0.57	0.05±0.23
6	BBN→SS	3.60±3.31 ┘	0.29±0.49	0.24±0.79
7	BBN→SS+NDGA	1.39±2.35 ┐*	0	0.05±0.21
8	BBN→NDGA	0.61±0.92	0.17±0.55	0.40±0.90

The total length of basement membrane (BM) was measured using a Digitizer kw-3300 (Graphtec Corporation, Yokohama) and the number of lesions per 10 cm of BM was determined.
* $P<0.05$. ** $P<0.01$.

significantly ($P<0.05$) lower than those in group 6 (19/23, 82.6%; 5/23, 21.7%; and 3.60±3.31/10 cm BM, respectively). However, incidence of carcinoma in each of groups 6 through 8 was not significantly different from that in group 5.

The average consumption of BBN in groups 5–8 was similar, being about 1.53 g/kg/day, and the total weight of food consumed in groups 1–8 was approximately the same. The final body weights were 449±14 g in group 1, 442±31 g in group 2, 412±19 g in group 3, 428±15 g in group 4, 448±20 g in group 5, 447±17 g in group 6, 389±18 g in group 7, and 428±19 g in group 8. Body weight gain was significantly smaller in group 7 than those in group 5 and in group 3 compared to group 1. One rat in group 7 died of an unknown cause at week 37.

DISCUSSION

The potential role of tumor suppressor genes in human cancer has recently received considerable attention.[12] DNA marker studies have revealed that in many tumors genes located on specific chromosomes have undergone reductions to homo- or hemizygosity. In bladder cancer also, nonrandom chromosome losses and genetic deletion have been found.[13, 14] Studies of oncogenes and tumor suppressor genes have revealed that cancer cells exhibit alteration of genes, and multiple genetic alterations may account for multiple-step carcinogenesis.

Active oxygen species are known to be involved in the development of various human diseases, including cancer. The process of cancer development consists of multiple steps, and oxygen free radicals are thought to be involved in at least two of these steps, initiation and promotion.[15, 16] Results of various studies give some clues as to how promoters might act; they disrupt the mitotic apparatus, causing hemizygosity and expression of recessive genes.[17, 18] Phorbol esters generate oxygen radicals, which cause chromosome breaks[19] and increase gene copy number.[20] Promoters also cause formation of the peroxide hormones of the prostaglandin and leukotriene family by oxidation of arachidonic acid, and inhibitors of this process appear to act as antipromoters.[21, 22] These hormones are intimately involved in cell division, differentiation, and tumor growth, and might have arisen in evolution as signal molecules warning the cell of oxidative damage. The effect on the cell membrane has also been suggested as an important factor in promotion, causing inhibition of intercellular communication or protein kinase activation.[23] Suzuki *et al.* investigated the inhibitory effect on rat urinary bladder carcinogenesis of 2-*O*-octadecylascorbic acid, a scavenger of active oxygen species, using an agglutination assay with concanavalin A, and suggested that active oxygen species are involved in various steps of bladder carcinogenesis including the promotion phase.[24]

Eicosanoids, derived from arachidonic acids, are metabolized via two pathways; the enzyme cyclooxygenase produces prostaglandins, prostacyclins, and thromboxanes, while lipoxygenase produces hydroxyeicosatetraenoic acid derivatives (HETES) and the leukotrienes. It has been reported that aspirin, a cyclooxygenase inhibitor, inhibits both N-[4-(5-nitro-2-furyl)-2-thiazoyl]-formamide (FANFT) initiation and saccharin promotion in the two-stage rat urinary bladder carcinogenesis model.[25] It has been also reported that lipoxygenase inhibitors, such as NDGA and phenidone, inhibit the epidermal ODC induction and the formation of skin papillomas induced by TPA.[5, 6] Recent work on *in vivo* inhibition of the lipoxygenase pathway demonstrated suppression of chemically induced carcinogenesis in the rat mammary gland[26] and large bowel.[27] Arachidonic acid metabolism seems to play an important role in bladder carcinogenesis, as it does in carcinogenesis in other organs. HETES and the leukotrienes are known to be products of metabolism of arachidonic acid by lipoxygenase. Further study is required to demonstrate which metabolites have promoting activity.

Saccharin has been shown to act as a powerful promoting agent in 2-stage urinary bladder carcinogenesis in rats following initiation with BBN. Cohen *et al.*[28] reported

that 5% SS was considerably more potent as a promoting agent than 2% DL-tryptophan, inducing higher incidence of rat bladder tumor. In the present study, NDGA was found to inhibit saccharin promotion of bladder carcinogenesis. This finding suggests that NDGA may also exert an anti-tumor-promoting effect against some other promoters of chemically induced bladder carcinogenesis.

The development of cancer chemo-preventive agents is essential, and several short-term assays, such as the determination of mouse epidermal ODC activity[5] and measurement of agglutinability of rat bladder cells by concanavalin A,[24, 29] have been utilized to examine the anti-tumor-promoting effects of various compounds. The present results confirm that the soft agar colony-forming assay is also useful for early screening of anti-tumor-promoting agents, as well as of tumor promoters.

In the present study, weight gain in the NDGA-treated rats was observed to be smaller than that in the other groups. However, the amounts of food consumption by NDGA-treated groups did not differ significantly from those in the other groups, and no significant toxicities of NDGA were observed. NDGA had been used as a food and pharmaceutical additive to prevent rancidity until 1967, when it was replaced with better food antioxidants. Its toxicity is low; 800 mg/kg is the 50% lethal dose in mice when given intraperitoneally.[30] No significant toxicity was associated with 0.1, 0.5 or 1.0% NDGA supplement to the diet of rats for 2 years and NDGA had little or no effect on growth or food intake except at the highest concentration in rats, where there was a temporary decrease in growth associated with a decreased food intake.[31] Because NDGA is relatively non-toxic, its use in chemoprevention of human bladder cancer could be feasible, after additional studies.

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