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Inhibitory Effect of 2-*O*-Octadecylascorbic Acid in Agglutination Assay with Concanavalin A; Short-term Examination of Rat Urinary Bladder Carcinogenesis

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A derivative of ascorbic acid, 2-*O*-octadecylascorbic acid (CV-3611), is a strong scavenger of active oxygen species. We examined the effect of CV-3611 on a short-term test of bladder carcinogenesis, using concanavalin A (Con A)-dependent agglutination of isolated bladder epithelial cells. Rats were given 0.01% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BHBN) for 1 week, and then 5% sodium saccharin or 2% DL-tryptophan or 0.01% BHBN alone or with 0.002, 0.006 or 0.02% CV-3611 for 3 weeks. Treatment with CV-3611 reduced the effects of the bladder tumor promoters sodium saccharin and DL-tryptophan by 48–86 and 65–87%, respectively. CV-3611 also reduced the number of aggregates of bladder epithelial cells from rats treated with BHBN for 4 weeks. These results suggest that CV-3611 has a suppressive effect on rat bladder carcinogenesis.

Key words: CV-3611 — Active oxygen scavenger — Antipromoter — Con A-dependent agglutination — Bladder carcinogenesis

Active oxygen species are known to be involved in the development of various kinds of human diseases including cancer.[1-3] 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, for the following reasons: 1) they cause DNA-strand breaks,[4] and also modify bases to yield oxidation products including 8-hydroxydeoxyguanosine[5,6] 2) an oxygen free radical-generating compound, benzoyl peroxide, has tumor-promoting activity in skin carcinogenesis of mice,[7] and 3) many antioxidants, including 2(3)-*tert*-butyl-4-hydroxyanisole, α-tocopherol and quercetin, inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in mouse skin.[8-10] Thus, antioxidants may be important for suppressing the development of cancer in humans.

We found that agglutination of isolated bladder epithelial cells of rats by concanavalin A (Con A) increased after treatment of the animals with bladder carcinogens for 1 week.[11] Moreover, bladder tumor promoters, such as sodium saccharin and DL-tryptophan, maintained the increased agglutinability of the cells when administered continuously after treatment with bladder carcinogen.[12,13] Thus, Con A-agglutination assay is very useful for screening bladder carcinogens and bladder tumor promoters in short-term tests. In fact, L-isoleucine and L-leucine were found to have tumor-promoting activity by this method,[14] and were later proved to be bladder tumor promoters in a long-term carcinogenicity test.[15]

Recently, many 2-*O*-alkylascorbic acids have been synthesized and 2-*O*-octadecylascorbic acid (CV-3611), shown in Fig. 1, was demonstrated to be very effective for suppressing formation of lipid peroxide *in vitro* and damage to cardiac tissue caused by active oxygen species.[16,17] CV-3611 shows very low toxicity in rodents, and we, therefore, investigated the effect of CV-3611 on bladder tumor promotion by sodium saccharin and DL-tryptophan, and carcinogenesis by continuous treatment with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BHBN), using the Con A-agglutination test in rat urinary bladder.

MATERIALS AND METHODS

Chemicals CV-3611 was provided by Takeda Chemical Industries, Ltd. (Osaka). The purity of this compound was more than 98%. BHBN was obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo). Sodium saccharin and DL-tryptophan were from Wako Pure Chemicals Ind. (Osaka) and Nippon Rikagaku Yakuhin Co. (Tokyo), respectively. Con A and α-methyl mannoside were obtained from Sigma Chemical Co. (St. Louis, MO).

Treatment of animals BHBN was given to rats at a concentration of 0.01% in deionized water. The BHBN solution was freshly prepared every three or four days. Sodium saccharin and DL-tryptophan were given to rats at concentrations of 5% and 2%, respectively, in CE-2 powder diet (CLEA Japan, Tokyo). CV-3611 was added at concentrations of 0.002, 0.006 and 0.02% to sodium saccharin diet, DL-tryptophan diet or basal diet. CV-3611 was stable in the powder diet in the absence or presence of sodium saccharin or DL-tryptophan: the presence of

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more than 93% of the added CV-3611 in the diet was confirmed by methanol extraction followed by HPLC analysis, after storage of the prepared diet for 1 month at 22°C.

Male F344 rats of 5 weeks old obtained from Charles River Japan (Atsugi, Kanagawa) were randomly divided into 20 groups consisting of 9 rats each as shown in Fig. 2. The rats in Groups A-1 to -4 were given 0.01% BHBN in their drinking water and basal powder diet for 1 week. For the next three weeks, rats in Group A-1 were given water and powder diet containing sodium saccharin alone and those in Groups A-2 to -4 were given sodium saccharin plus 0.002%, 0.006% and 0.02% CV-3611, respectively, as shown in Fig. 2. Groups B-1 to -4 were given DL-tryptophan instead of sodium saccharin, other conditions being as for Groups A-1 to -4. Groups C-1 to -4 were given water containing BHBN for 4 weeks, and Groups C-2 to -4 were given diet containing

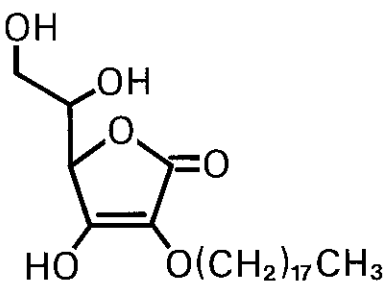

Fig. 1. Structure of CV-3611

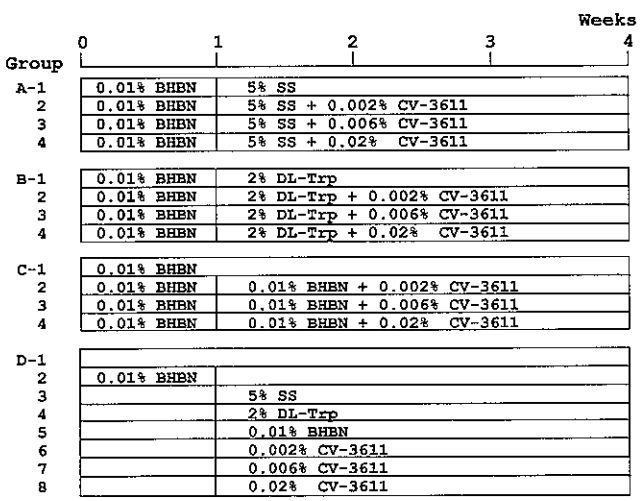

Fig. 2. Experimental design for examining the effect of CV-3611 on agglutinability of bladder cells with Con A. BHBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; SS, sodium saccharin; DL-Trp, DL-tryptophan.

CV-3611 from the second to fourth week. Group D-1 was not given any chemical throughout the experiment. Group D-2 was given BHBN for 1 week and then water and CE-2 diet for three weeks. Groups D-3 to -8 were given sodium saccharin, DL-tryptophan, BHBN and three different concentrations of CV-3611, respectively, for three weeks from the second to fourth week (Fig. 2). Rats had free access to diet and water.

Agglutination assay All rats were killed in week four and the urinary bladder was excised. The Con A-agglutination assay was performed as reported previously.[11, 12, 18] Briefly, the urinary bladder was washed with saline solution, everted, and incubated in 0.15 *M* NaCl containing 5 m*M* EDTA. Epithelial cells were separated by sonicating and squashing the bladder mucosa. In each group, the epithelial cells from three animals were combined and collected by centrifugation. This procedure resulted in three composite cell suspensions from each group of 9 rats. The cell suspension (2 to 5×10^6 cells/ml) was incubated with Con A (200 μg/ml) in a total volume of 40 μl of phosphate-buffered saline (pH 7.4) with or without α-methyl mannoside (100 μg/ml), a specific inhibitor of Con A binding, for 30 min at 37°C. Then the number of aggregates of more than three cells per 200 total cells was measured using a hemocytometer. The number of Con A-dependent aggregates was calculated as the difference in the numbers with and without α-methyl mannoside. Three assays, of each composite from three rats, were performed in each group of 9 animals. The statistical significance of differences of agglutination values between CV-3611-treated and untreated groups was examined by using Student's *t* test.

RESULTS AND DISCUSSION

The average body weights of rats in Groups A-1 and B-1 fed sodium saccharin and DL-tryptophan, respectively, were 26 and 18% less than that of the untreated rats, Group D-1. That of rats in Group C-1, given BHBN for four weeks was almost the same as the average body weight of rats in Group D-1. Treatment with CV-3611 in Groups A, B and C caused a slight increase in body weight within each group, although there were no differences in food consumption.

The effects of CV-3611 on agglutination of bladder epithelial cells in the presence of 200 μg/ml Con A are summarized in Table I. No significant increase in the number of aggregates was observed after treatment with BHBN for 1 week (Group D-2). However, on treatment with sodium saccharin for three weeks after treatment with BHBN for 1 week (Group A-1), the number of Con A-dependent aggregates increased markedly (14.0 aggregates per 200 cells). Additional treatment with 0.002% CV-3611 (Group A-2) suppressed this agglutina-

Table I. Inhibitory Effect of CV-3611 on Con A-agglutination of Bladder Epithelial Cells

Group	No. of aggregates[a]			Inhibition (%)
	without α-MM	with α-MM	Con A dependent	
A-1	19.3±7.6[b]	5.3±0.6[b]	14.0	
2	11.3±1.5	4.0±1.0	7.3	48
3	7.0±1.0	5.0±1.0	2.0	86
4	6.3±1.5[c]	3.0±0.6	3.3	76
B-1	10.7±2.5	3.0±1.0	7.7	
2	6.0±1.7	3.3±1.5	2.7	65
3	4.0±2.0[d]	2.3±0.6	1.7	78
4	3.3±1.5[d]	2.3±0.6	1.0	87
C-1	15.3±3.0	2.7±1.2	12.6	
2	5.3±2.3[e]	2.3±1.5	3.0	76
3	3.3±0.6[f]	2.7±1.2	0.6	95
4	3.0±1.0[f]	1.7±0.6	1.3	90
D-1	1.0±1.0	0.7±0.6		
2	2.7±2.1	2.0±1.0		
3	3.3±2.0	2.7±1.2		
4	3.7±3.0	2.2±1.0		
5	15.0±1.0	5.0±1.0		
6	1.3±0.6	1.7±0.6		
7	1.7±0.6	1.3±0.6		
8	0.3±0.6	0.7±0.3		

Experimental conditions for each group are shown in Fig. 2. Agglutination of urinary bladder cells in each group was induced by 200 μg/ml Con A with or without 100 μg/ml α-methyl mannoside (α-MM). Three assays, each a composite cell suspension from three rats, were carried out in each group of 9 rats.

a) The number of Con A-dependent aggregates was calculated as the difference between the numbers with and without α-MM.
b) Mean±SD.
c) $P<0.05$ compared with Group A-1.
d) $P<0.05$ compared with Group B-1.
e) $P<0.05$ compared with Group C-1.
f) $P<0.01$ compared with Group C-1.

tion by 48%, while treatments with 0.006 and 0.02% CV-3611 (Groups A-3 and A-4) suppressed the agglutination by 86% and 76%, respectively.

Treatment with DL-tryptophan after BHBN increased the number of Con A-dependent aggregates to 7.7 per 200 cells (Group B-1 in Table I). This effect of DL-tryptophan was suppressed by 65–87% in the presence of 0.002, 0.006 and 0.02% CV-3611 (Groups B-2, B-3 and B-4).

Similarly, agglutination of bladder epithelial cells of rats treated with BHBN alone for four weeks was suppressed by CV-3611 administration for the last three weeks: 0.002% CV-3611 caused 76% decrease in the agglutination (Group C-2), and 0.006 and 0.02% CV-3611 caused decreases of 95 and 90%, respectively (Groups C-3 and C-4).

In the present study, CV-3611 at concentrations of 0.002–0.02% clearly suppressed Con A-agglutinability of bladder cells from rats treated with BHBN followed by the bladder tumor promoters sodium saccharin and DL-tryptophan. Inhibition of Con A-agglutinability by CV-3611 was also observed when the complete carcinogen BHBN was administered continuously. Previously, using the same method, we found that ascorbic acid at concentrations of 0.25 and 0.5% inhibited the effect of sodium saccharin on Con A-agglutination of bladder cells.[18] The effective concentration of ascorbic acid was about 100 times that of CV-3611. Another lipophilic ascorbic acid derivative, ascorbic acid 6-palmitate was also reported to be a much more potent inhibitor than ascorbic acid of ornithine decarboxylase induction and tumor promotion by TPA in mouse skin.[19] Thus, introduction of lipophilic groups into ascorbic acid seems to be effective for increasing the antipromoting activity. In addition, the above observations suggest that active oxygen species are probably involved in various steps of bladder carcinogenesis including the promotion phase.

In Con A-agglutination assay, α-difluoromethylornithine, which is an inhibitor of ornithine decarboxylase, was shown to decrease the effect of sodium saccharin on agglutinability of bladder cells at concentrations of more than 0.1%,[18] and these concentrations were also effective for inhibiting BHBN-induced urinary bladder carcinogenesis in long-term experiments.[20,21] These observations suggest that CV-3611 may suppress bladder carcinogenesis by BHBN. However, it can not be expected that CV-3611 can necessarily inhibit the carcinogenicity at the concentrations of 0.002–0.02% at which it inhibited Con A-agglutination, because there is no strict quantitative correlation between Con A-agglutinability and carcinogenic potencies of various bladder carcinogens, including initiators and promoters.[11,13,20,22] The toxicity of CV-3611 is very low and its LD_{50} is more than 3 g/kg in mice and rats. Therefore, CV-3611 could be an effective chemopreventive compound for urinary bladder cancer. A test of the effect of CV-3611 in a long-term experiment on bladder carcinogenesis in rats is now in progress in our laboratory.

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