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The Modifying Effects of Indomethacin or Ascorbic Acid on Cell Proliferation Induced by Different Types of Bladder Tumor Promoters in Rat Urinary Bladder and Forestomach Mucosal Epithelium

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The effects of indomethacin (IM) or L-ascorbic acid (AsA) on cell proliferation induced by bladder tumor promoters such as butylated hydroxyanisole (BHA), sodium L-ascorbate (Na-AsA), sodium citrate (Na-Cit), and diphenyl (DP) in rat bladder and forestomach epithelium were investigated. Treatment with IM in combination with BHA or Na-AsA diminished DNA synthesis levels of bladder epithelium as compared to the BHA or Na-AsA alone values. On the other hand, AsA further amplified the increase of bladder epithelial DNA synthesis caused by Na-Cit treatment. Histopathologically, administration of Na-AsA in combination with IM reduced the incidence of simple hyperplasia. In contrast, simultaneous treatment with Na-Cit and AsA caused an increase of the hyperplasia development. No apparent combination effects were observed in the DP-treated groups. In forestomach epithelium, AsA enhanced the BHA-induced increase in DNA synthesis and epithelial hyperplasia, characterized by marked basal cell proliferation. The present results thus suggested that IM may exert inhibitory effects on promotion of bladder carcinogenesis by certain tumor promoter types, and AsA may enhance BHA forestomach carcinogenesis.

Key words: Indomethacin — Ascorbic acid — Modifying effect — Cell proliferation — Bladder tumor promoter

Following the original report of Jaffe,[1] a number of investigators have detected high levels of prostaglandin (PG*), especially PGE_2, in human cancer tissues,[2-4] cultured neoplastic cells,[5-7] and experimentally induced tumors,[8] as well as in bladder epithelium following application of tumor promoters or carcinogens *in vitro* and *in vivo.*[9-12] Furthermore, antitumor activity of the non-steroid anti-inflammatory drugs indomethacin (IM) and aspirin has been demonstrated in transplantable tumors and chemically-induced tumors in animals.[13-18] These drugs are known to block the biosynthesis of PG and therefore it has been postulated that diminished PG levels might inhibit tumor development. In the bladder, treatment with tumor promoter sodium L-ascorbate (Na-AsA) or butylated hydroxyanisole (BHA) increases levels of PGE_2 and, in addition, ascorbic acid in the bladder tissue.[12] L-Ascorbic acid (AsA; vitamin C) has been reported to act as an amplifier of tumor promotion (co-promoter) in rat bladder carcinogenesis, i.e., AsA in combination with tumor promoters such as sodium salts amplified their promoting potentials[19-21] in association with elevated DNA synthesis.[22] Thus, the possible role of the above biochemical parameters in promotion of rat bladder carcinogenesis requires elucidation. The present study was therefore designed to assess combination effects of IM or AsA with bladder tumor promoters of three different types, i.e., antioxidant type (BHA), sodium salt type (Na-AsA or sodium citrate (Na-Cit)), and microcalculi-inducer type (diphenyl (DP)), on epithelial cell proliferation in the urinary bladder. Since BHA was found to be a forestomach carcinogen in rodents,[23, 24] the influence of these agents on forestomach epithelium was also investigated.

MATERIALS AND METHODS

Test chemicals AsA, Na-AsA, Na-Cit, BHA and IM were obtained from Wako Pure Chemical Industries Co., Osaka; DP was purchased from Tokyo Kasei Industries Co., Ltd., Tokyo.

Animals A total of sixty-five male Fischer 344 rats, 6 weeks old at the commencement (from Charles River Japan, Inc., Atsugi), were used. They were housed, five rats to a plastic cage, on hardwood chip bedding in an environment-controlled room maintained at 22±2°C and 60±10% relative humidity and artificially illuminated for 12 h each day.

Experimental procedure The animals were randomly allocated to 13 groups consisting of 5 rats each. They were given powdered basal diet (Oriental MF, Oriental

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Yeast Co., Tokyo) with no supplement (control) or containing one of the following: 2% BHA; 2% BHA+IM (20 ppm in drinking water); 2% BHA+5% AsA; 5% Na-AsA; 5% Na-AsA+IM (20 ppm); 5% Na-Cit; 5% Na-Cit+5% AsA; 0.5% DP; 0.5% DP+IM (20 ppm); 0.5% DP+5% AsA; IM (20 ppm); 5% AsA. IM, dissolved initially in absolute ethanol and then in tap water, was renewed at intervals of 3 days. The rats were observed daily, and body weights and food and water consumption were measured weekly.

Urinalysis At week 8, fresh urine specimens were obtained from rats of each group by forced micturition at 8.00 a.m. to avoid diurnal differences. For pH determination (using a pH meter, model F-8DP, Hitachi-Horiba, Tokyo), the portion voided first was excluded, because of possible contamination with bacteria and foreign matter from the external genitalia. Urine samples were also collected from rats placed in individual metabolic cages, over a 4-h period (09.00–13.00 h), without food or water. The urine volume was measured (by weighing) and its osmolality determined (by freezing-point depression using an Osmett A, Precision Systems Inc., MA, USA). Aliquots were then used for measurements of mineral ion concentration. The remainder of the samples were centrifuged and examined microscopically for sediment.

DNA synthesis and histopathology of bladder and forestomach epithelium At week 8, the five rats in each group were injected intraperitoneally with 100 mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU, Sigma Chemical Co., MO, USA). Circadian rhythms have been demonstrated for DNA synthesis and mitosis[25, 26] and therefore, in order to avoid any variance between groups, the animals were sequentially injected with BrdU and killed one hour later in such a way that one rat from each of the groups was simultaneously killed by exsanguination. This was then repeated with the second, third, fourth and fifth animals from each of the groups at 15 min intervals. The bladders were ligated at the neck, inflated by intraluminal injection of 10% phosphate-buffered formalin (0.5 ml), removed quickly, and immersed in the fixative. They were subsequently divided in half sagittally, one half being cut longitudinally into 6 strips. The stomachs were also taken, inflated with 5 ml of fixative and cut into 6 strips of forestomach tissue. Bladder and stomach sections were prepared for assessment of histopathology (stained with hematoxylin and eosin) and for measurement of DNA synthesis (immunohistochemical BrdU staining). Forestomach epithelial thickness, from the basal cell layer to the superficial cell layer without keratotic layer, was measured with the aid of a color image processor (SPICCA-II, Nippon Avionics Co., Ltd., Tokyo). Immunohistochemical BrdU staining was performed as previously described[27] using a Vectastain Elite ABC Kit in combination with an anti-BrdU monoclonal antibody (Dako Japan Co., Ltd., Kyoto). The numbers of bladder epithelial cells incorporating BrdU into the DNA per 1,000 cells were counted. Similarly, 1,000 basal cells each from the prefundic region of the lesser curvature and the mid-region of the greater curvature in the forestomach epithelium were counted. Labeling indices were expressed as percentage values.

Statistical analyses Significances of differences between the means of treated and control group body weight, and DNA synthesis values were assessed by the use of Student' s *t* test. Insufficient homogeneity of variance was corrected with respect to the degree of freedom according to the method of Welch. The incidences of lesions were analyzed by the chi-square test.

RESULTS

Clinical observation did not reveal any abnormalities or differences between the groups, and no deaths occurred during the treatment period. As shown in Table I, significantly reduced weight gains were observed for the BHA, BHA+IM, BHA+AsA, DP+IM, DP+AsA and AsA groups at week 8 as compared to the control values. Food consumption showed a tendency for increase in the BHA, BHA+IM, BHA+AsA, Na-AsA, Na-Cit, Na-Cit+AsA, DP+AsA and AsA groups, although in these cases a degree of overestimation was produced by excess diet spillage. Water consumption showed an increased trend in rats receiving BHA, Na-AsA, Na-AsA+IM, Na-Cit, Na-Cit+AsA and DP+AsA.

Urinalysis The results of urinalysis data are shown in Table II. A significant increase or trend towards increase in urine volume was observed in all treated groups, with the exception of the IM and AsA groups, as compared to control values. Increased urine volume in these groups was associated with hypotonic urine (decreased osmolality). Urinary pH was significantly elevated in rats given Na-AsA or Na-Cit, either alone or in combination with IM or AsA. Treatment with DP+AsA or AsA itself brought about a drop in pH. All groups treated with sodium salts demonstrated increase in Na^+ levels and $MgNH_4PO_4$ crystalluria. Urinary microcalculi were microscopically observed after DP ingestion with or without IM or AsA. The urine characteristics in all treated groups were independent of combination with IM or AsA.

Cell proliferation in the bladder epithelium Macroscopically, no luminal surface abnormalities were observed in the urinary bladder of any treated groups. Histopathological changes and labeling indices (%) as assessed by incorporation of BrdU into nuclear DNA of bladder epithelial cells in the S phase of treated and control rats are shown in Table III. Simple hyperplasia consisted of

Table I. Mean Body Weights, and Food and Water Consumption of Rats Given Test Chemicals for 8 Weeks[a)]

Test chemical	No. of rats	Body weights (g) at wk:				Food consumption (g/rat/day)	Water consumption (g/rat/day)
		0	2	4	8		
BHA	5	115	162*	213*	273*	17.6	24.1
BHA+IM	5	112	159**	214**	270**	18.9	23.4
BHA+AsA	5	114	147**	193**	238**	17.4	22.2
Na-AsA	5	113	184	247	309	15.8	24.2
Na-AsA+IM	5	114	181	240	301	14.5	25.9
Na-Cit	5	114	188	245	307	16.2	31.1
Na-Cit+AsA	5	112	182	229	286	17.5	27.0
DP	5	115	177	225	281	13.3	21.1
DP+IM	5	114	173	228*	282**	13.6	21.9
DP+AsA	5	112	170*	208**	263**	17.3	25.6
IM	5	113	185	245	309	14.2	21.6
AsA	5	111	178	233	298**	17.8	22.2
None	5	114	188	245	311	13.3	19.2

a) Values are means (SDs not shown for sake of simplicity).
* Significantly different from control value, $P<0.05$.
** Significantly different from control value, $P<0.01$.

Table II. Urine Characteristics of Rats Treated with Test Chemicals for 8 Weeks[a)]

Test chemical	No. of rats	Urine volume (g)	Urine osmolality (mOsmol/kg H_2O)	Urine pH	Urine level (mEq/liter) of:			Urine level (mg/dl) of:		Crystals[b)]
					Na	K	Ca	P	Mg	
BHA	5	2.7±0.8*	1259±135**	6.9±0.2	103±43	164±60	8±4	10±13	41±24	±
BHA+IM	5	2.9±0.5**	1093±232**	6.8±0.2	90±15	149±25	9±4	18±13	37±7	±
BHA+AsA	5	1.8±0.2	1323±342*	6.7±0.4	60±44	119±51	14±2	16±9	64±19	±
Na-AsA	5	2.2±0.5*	1355±383*	7.8±0.2**	239±82*	114±35*	13±7	6±3	65±24	++
Na-AsA+IM	5	2.0±0.4*	1419±363*	7.8±0.1**	218±61*	96±18**	11±3	21±15	29±8	++
Na-Cit	5	2.0±0.4*	1419±326*	8.1±0.1**	355±68**	70±24**	10±8	11±9	46±30	++
Na-Cit+AsA	5	1.8±0.6	1443±315*	7.9±0.4**	375±65**	94±16**	8±2	29±18	34±11	++
DP	5	2.0±0.7	1117±103**	6.8±0.4	121±26	150±16	10±5	14±15	35±13	+++[c)]
DP+IM	5	2.1±0.3*	1234±141**	6.7±0.4	104±33	164±27	10±4	18±7	35±9	+++[c)]
DP+AsA	5	2.3±0.4**	987±213**	6.1±0.1**	91±29	113±32*	6±2	81±23**	30±12	+++[c)]
IM	5	1.5±0.4	1827±176	6.8±0.3	83±49	273±55*	15±3	29±22	54±5*	+
AsA	5	1.5±0.4	1871±116	6.1±0.3*	107±52	256±79	8±2	83±37	69±8	±
None	5	1.4±0.4	1920±264	6.7±0.3	91±76	182±42	8±6	23±21	40±97	±

a) Values represent mean±SD.
b) Grading (mean of group): ±, trace; +, slight; ++, moderate; +++, severe.
c) Numerous microcalculi were seen among the crystals.
* Significantly different from control value, $P<0.05$.
** Significantly different from control value, $P<0.01$.

diffuse or focal thickening of the epithelium with 4 to 8 layers of transitional epithelial cells.[28)] Simple hyperplasia development was not evident light-microscopically in the BHA group, as previously described.[12)] However, development of this lesion was significantly increased in the Na-AsA and Na-Cit+AsA groups. Although administration of IM in combination with Na-AsA reduced the incidence of simple hyperplasia as compared to Na-AsA

Table III. Influences of Indomethacin (IM) or L-Ascorbic Acid (AsA) on Rat Bladder Epithelial Proliferation Induced by Various Bladder Tumor Promoters

Test chemical	No. of rats	No. of rats with simple hyperplasia (%)	Labeling index[a] (%)
BHA	5	0	2.76±1.16[c]
BHA+IM	5	0	0.66±0.55[e]
BHA+AsA	5	1 (20)	2.84±1.39[c]
Na-AsA	5	4 (80)[b]	2.44±1.85[b]
Na-AsA+IM	5	1 (20)	1.02±0.61[b]
Na-Cit	5	1 (20)	0.82±0.45[b]
Na-Cit+AsA	5	4 (80)[b]	2.04±0.94[c, d]
DP	5	2 (40)	2.00±1.57[b]
DP+IM	5	2 (40)	1.62±1.65
DP+AsA	5	1 (20)	0.24±0.27
IM	5	0	0.14±0.16
AsA	5	0	0.06±0.08
None	5	0	0.08±0.13

a) Values are means±SD.
b) Significantly different from control value, $P<0.05$.
c) Significantly different from control value, $P<0.01$.
d) Significantly different from Na-Cit group, $P<0.05$.
e) Significantly different from BHA group, $P<0.01$.

alone (Fig. 1a and b), demonstration of statistical significance was precluded by the low number of rats used. In contrast, administration of Na-Cit in combination with AsA showed an increase in simple hyperplasia development as compared to the Na-Cit alone. Significant increases in DNA synthesis were observed in the groups given BHA, BHA + AsA, Na-AsA, Na-AsA + IM, Na-Cit, Na-Cit+AsA and DP as compared to control values. IM administration significantly diminished the DNA synthesis in bladder epithelium caused by BHA (Fig. 2a and b). In the Na-AsA+IM treatment case, although the values was less than half that of Na-AsA alone, the difference was not significant. Taken together with the difference between incidence of simple hyperplasia for Na-AsA alone and in combination with IM, albeit again lacking statistical significance, this suggests a potential for reduction or inhibition which requires further investigation. AsA significantly amplified the increase in DNA synthesis caused by Na-Cit treatment. Combination treatment with AsA and DP tended to decrease DNA synthesis in bladder epithelium as compared to DP treatment alone, but this was not significant and was not associated with changes in hyperplasia development.

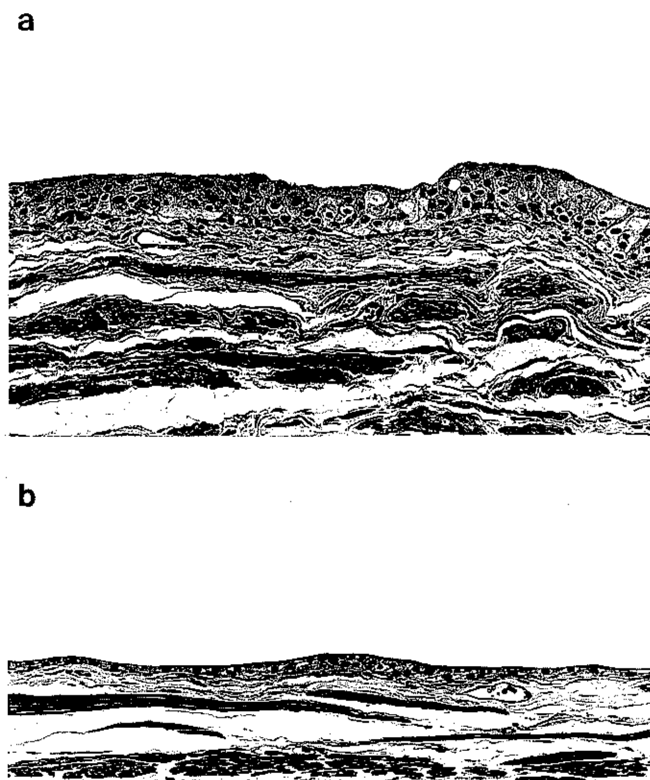

Fig. 1. (a) Simple hyperplasia in the urinary bladder epithelium of a rat given Na-AsA for 8 weeks. H&E. ×200. (b) Almost normal bladder epithelium of a rat given Na-AsA+IM for 8 weeks. H&E. ×200.

Cell proliferation in the forestomach epithelium Grossly, nodular lesions were observed in the forestomach mucosa of rats given BHA with and without IM or AsA. The extent and degree of lesion development in the BHA+AsA group was more severe than in the BHA or BHA+IM groups. The results of assessment of proliferative indices in forestomach epithelial cells are shown in Table IV. Histopathologically, forestomach epithelial hyperplasias were observed in all rats given BHA, BHA+IM or BHA+AsA. Combination treatment with BHA and AsA induced more severe changes than did treatment with BHA alone (Fig. 3a and b), characterized by marked basal cell proliferation and increment of epithelial thickness (Table IV). Since it has been reported that DNA synthesis shows a different response in the prefundic region and mid-region of the rat forestomach epithelium to certain chemical treatments,[29] both areas

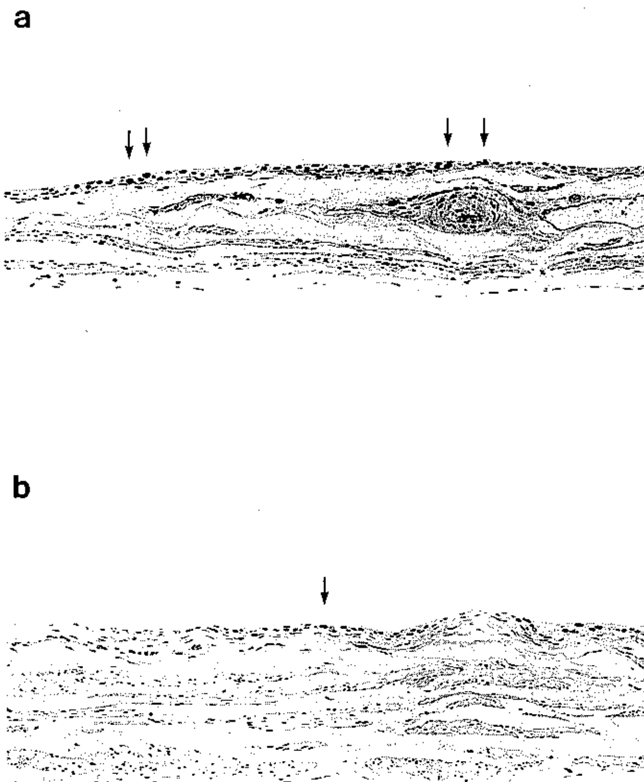

Fig. 2. (a) An increase in BrdU-incorporating cells (arrows) but no development of simple hyperplasia in the bladder epithelium of a rat given BHA. (b) Decrease in BrdU-incorporating cells (arrow) is evident in the BHA+IM group. Immunohistochemical staining for BrdU. ×150.

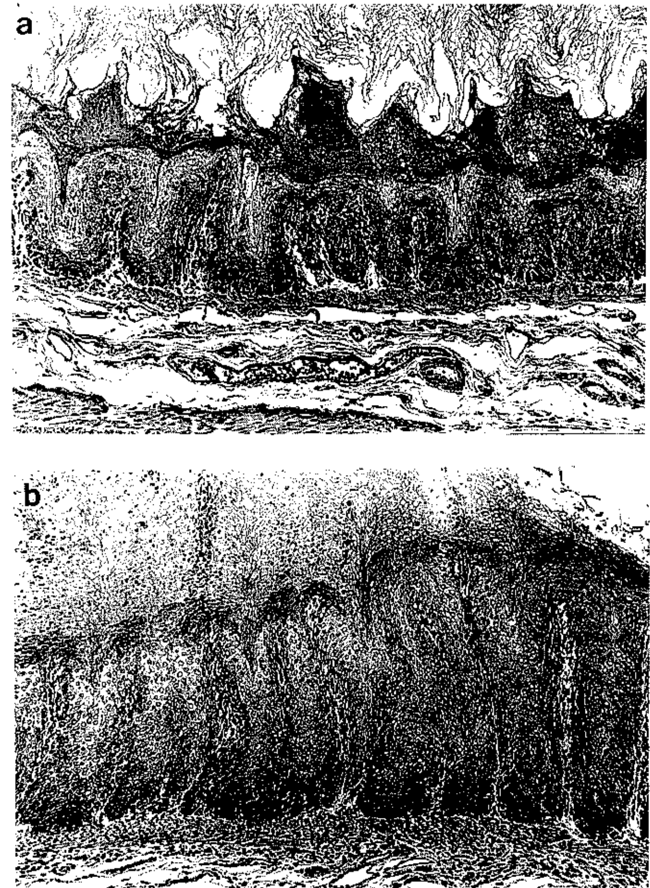

Fig. 3. (a) Mild hyperplasia of the forestomach epithelium in a rat given BHA for 8 weeks. H&E. ×100. (b) Severe hyperplasia of the forestomach epithelium in a rat given BHA+AsA for 8 weeks. Note marked basal cell proliferation. H&E. ×100.

of tissue were separately assessed. In the BHA-treated groups, significant elevation of DNA synthesis was observed in both the prefundic region and the mid-region of the forestomach epithelium. In the combination BHA and AsA case, DNA synthesis in both areas of forestomach epithelium was further significantly increased. DNA synthesis in forestomach epithelium was in all cases more prevalent in the prefundic region of the forestomach than in the mid-region, as previously described.[29, 30] No other treatments induced any significant changes in forestomach epithelium.

DISCUSSION

The present study demonstrated that induction of bladder epithelial proliferation by BHA or Na-AsA treatment can be alleviated by IM. While in the latter case strict statistical significance was lacking, such a conclusion nevertheless appears warranted from the present data. In addition, AsA was found to enhance the bladder epithelial proliferation associated with Na-Cit treatment and BHA-induced forestomach hyperplasia. There have been many experimental reports on participation of Na^+ and urine alkalinity in cell proliferation and promotion of bladder carcinogenesis.[19, 22] However, in the present study, since the urine characteristics in all treated groups were independent of combination with AsA or IM, other mechanisms require discussion.

Elevated levels of PGs, especially PGE_2, have been found in various malignant tissues[2-8] as well as in bladder epithelium treated with tumor promoters such as BHA and Na-AsA.[10-12] While the normal bladder epithelium

Table IV. Influences of Indomethacin (IM) or L-Ascorbic Acid (AsA) on Rat Forestomach Epithelial Cell Proliferation Induced by Various Bladder Tumor Promoters

Test chemical	No. of rats	Hyperplasia		Labeling index (%)[a]	
		No. (%)	Thickness (μm)[a]	Prefundic region	Mid-region
BHA	5	5 (100)[b]	557±282[b]	23.3±2.0[c]	6.8±1.1[c]
BHA+IM	5	5 (100)[b]	416±203[b]	23.2±7.3[c]	5.7±2.5[b]
BHA+AsA	5	5 (100)[b]	958±173[c,e]	32.0±5.7[c,d]	16.4±4.7[c,d]
Na-AsA	5	0	39±5	5.8±2.5	2.1±0.7
Na-AsA+IM	5	0	40±4	7.5±2.4	1.7±0.5
Na-Cit	5	0	40±7	7.0±2.8	1.6±0.8
Na-Cit+AsA	5	0	38±6	6.0±0.7	1.6±0.5
DP	5	0	33±9	7.3±4.3	1.7±0.6
DP+IM	5	0	35±5	7.6±2.4	1.5±0.7
DP+AsA	5	0	41±7	6.9±2.7	1.7±0.7
IM	5	0	39±6	6.6±1.9	2.2±0.3
AsA	5	0	35±5	4.2±2.1	1.6±0.7
None	5	0	43±6	5.3±1.4	1.8±0.7

a) Values are means±SD.
b) Significantly different from control value, $P<0.05$.
c) Significantly different from control value, $P<0.01$.
d) Significantly different from BHA group, $P<0.05$.
e) Significantly different from BHA group, $P<0.01$.

of rats, rabbits and dogs itself has substantial PG synthetic activity,[17, 31, 32] PGs may participate in the promotion process of carcinogenesis in the bladder.

The antitumor activity of IM has been investigated with transplantable tumors and chemically induced primary tumors in animals.[13-16] The mechanism(s) by which IM inhibits tumor growth or cell proliferation remains unknown. A previous study demonstrated a parallelism in the ability of IM to inhibit both cell growth and PG production, but IM does not have any direct inhibitory action on protein or on nucleic acid synthesis.[33] A further study showed that IM reduces the rate of propagation of tumor cells, possibly by interfering with cell growth at the G_1 phase of the cell cycle.[34] Other suggested possibilities relating to anti-tumor or anti-cell proliferation activity of IM are: (i) the immunologic status of the host is elevated by blocking PG production[35]; (ii) the effect of IM may not be dependent on blockage of PG synthesis alone, because this drug affects other biosynthetic processes. With regard to the latter, the fact that IM depresses the induction of ornithine decarboxylase (ODC) activity, which is involved in tumor promotion and cell proliferation,[36, 37] may be important. Indeed, several experiments have indicated that α-difluoromethylornithine, a specific irreversible inhibitor of ODC, suppresses tumor development in the rat bladder.[38, 39] IM did not alleviate the urothelial hyperplasia associated with mechanical irritation of DP-induced microcalculi.

It has been suggested that high doses of AsA can help prevent human cancer. On the other hand, *in vivo* studies demonstrated that AsA, in combination with tumor promoters of sodium salt type such as $NaHCO_3$, is associated with amplification of promoting potential in two-stage rat bladder carcinogenesis[19-21] and with elevated DNA synthesis.[22] This appears to be promoter type-specific, since in the current study, AsA caused an increment of DNA synthesis in the bladder epithelium over that with the sodium salt-type promoter Na-Cit alone, whereas it did not affect the bladder treated with the antioxidant-type promoter BHA.

Previously, it was reported that bladder epithelial cells of rats exposed to the tumor promoter sodium saccharin show an increase in membrane potential and related cellular Na/K ion pump activity,[40] sodium influx perhaps acting as a trigger for DNA synthesis.[41, 42] It is possible that Na^+ transport into bladder epithelium of rats treated with Na-Cit+AsA is similarly increased accompanied by a transmembrane movement of AsA. In fact, our recent experiment indicated that Na-AsA treatment for 16 weeks causes increases in total ascorbic acid as well as PGE_2 levels in bladder epithelial tissue.[12] Other studies have also shown that AsA may be concentrated in malignant tissues of experimental animals as well as in man.[43] In addition, AsA was reported to stimulate epidermal growth factor (EGF) production and result in induction of cell proliferation *in vivo*.[44] The responsibil-

ity for the observed promoting activity of normal urine for rat bladder carcinogenesis may also lie with the EGF component.[45] However, AsA did not amplify DP-induced hyperplasia. Therefore, it is probable that the action of AsA is dependent on the underlying urinary condition of high Na^+ levels.

The present investigation also demonstrated that AsA possesses marked enhancing potential, characterized by increased basal cell proliferation for BHA-induced forestomach epithelial hyperplasia. Previously, it was reported that BHA-induced upward proliferation is reversible, while basal cell proliferation is persistent and therefore of greater importance for BHA forestomach carcinogenesis, although the exact significance of this in terms of malignant potential remains unclear. The mechanism(s) by which AsA enhances BHA-induced forestomach hyperplasia is currently unknown. However, it was earlier shown that diethylmaleate, a tissue glutathione-depleting agent, inhibits BHA-induced rat forestomach hyperplasia when administered simultaneously,[46] suggesting a direct involvement of tissue glutathione. AsA, a potent reducing agent, can modulate the reduction of GSSG to GSH, possibly resulting in elevation of tissue GSH levels and this could therefore explain the amplification of BHA-induced hyperplasia. It should be mentioned here that Na-AsA can also enhance the generation of rat forestomach hyperplasia by BHA.[47]

In conclusion, the present results suggested that IM may exert inhibitory effects on promotion of bladder carcinogenesis, and AsA may promote BHA forestomach carcinogenesis.

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REFERENCES

1) Jaffe, B. M. Prostaglandins and cancer: an update. *Prostaglandins,* **6,** 453–461 (1974).
2) Bennett, A., Carroll, M. A., Stamford, I. F., Whimster, W. F. and Williams, F. Prostaglandins and human lung carcinomas. *Br. J. Cancer,* **46,** 888–893 (1982).
3) McLemore, T. L., Hubbard, W. C., Litterst, C. L., Liu, M. C., Miller, S., McMahon, N. A., Eggleston, J. C. and Boyd, M. R. Profiles of prostaglandin biosynthesis in normal lung and tumor tissue from lung cancer patients. *Cancer Res.,* **48,** 3140–3147 (1988).
4) Rolland, P. H., Martin, P. M., Jacquemier, J., Rolland, A. M. and Toga, M. Prostaglandin in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. *J. Natl. Cancer Inst.,* **64,** 1061–1070 (1980).
5) Trevisani, A., Ferretti, E., Capuzzo, A. and Tomasi, V. Elevated levels of prostaglandin E_2 in Yoshida hepatoma and the inhibition of tumor growth by non-steroidal anti-inflammatory drugs. *Br. J. Cancer,* **41,** 341–347 (1980).
6) Owen, K., Gomolka, D. and Droller, M. J. Production of prostaglandin E_2 by tumor cells *in vitro. Cancer Res.,* **40,** 3167–3171 (1980).
7) Hubbard, W. C., Alley, M. C., McLemore, T. L. and Boyd, M. R. Profiles of prostaglandin biosynthesis in sixteen established cell lines derived from human lung, colon, prostate, and ovarian tumors. *Cancer Res.,* **48,** 4770–4775 (1988).
8) Tan, W. C., Privett, O. S. and Goldyne, M. E. Studies of prostaglandins in rat mammary tumors induced by 7,12-dimethylbenz(a)anthracene. *Cancer Res.,* **34,** 3229–3231 (1974).
9) Wise, R. W., Zenser, T. V., Kadlubar, F. F. and Davis, B. B. Metabolic activation of carcinogenic aromatic amines by dog bladder and kidney prostaglandin H synthase. *Cancer Res.,* **44,** 1893–1897 (1984).
10) Danon, A., Zenser, T. V., Thomasson, D. L. and Davis, B. B. Eicosanoid synthesis by cultured human urothelial cells: potential role in bladder cancer. *Cancer Res.,* **46,** 5676–5681 (1986).
11) Zenser, T. V., Thomasson, D. L. and Davis, B. B. Characteristics of bradykinin and TPA increases in the PGE_2 levels of human urothelial cells. *Carcinogenesis,* **9,** 1173–1177 (1988).
12) Shibata, M.-A., Yamada, M., Asakawa, E., Hagiwara, A. and Fukushima, S. Responses of rat urine and urothelium to bladder tumor promoters: possible roles of prostaglandin E_2 and ascorbic acid synthesis in bladder carcinogenesis. *Carcinogenesis,* **10,** 1651–1656 (1989).
13) Lynch, N. R. and Salomon, J.-C. Tumor growth inhibition and potentiation of immunotherapy by indomethacin in mice. *J. Natl. Cancer Inst.,* **62,** 117–121 (1979).
14) Pollard, M. and Luckert, P. H. Effect of indomethacin on intestinal tumors induced in rats by the acetate derivative of dimethylnitrosamine. *Science,* **214,** 558–559 (1981).
15) Narisawa, T., Sato, M., Tani, M., Kudo, T., Takahashi, T. and Goto, A. Inhibition of development of methylnitrosourea-induced rat colon tumors by indomethacin treatment. *Cancer Res.,* **41,** 1954–1957 (1981).
16) Takahashi, M., Furukawa, F., Toyoda, K., Sato, H., Hasegawa, R., Imaida, K. and Hayashi, Y. Effects of

various prostaglandin synthesis inhibitors on pancreatic carcinogenesis in hamsters after initiation with *N*-nitrosobis(2-oxopropyl)amine. *Carcinogenesis,* **11,** 393–395 (1990).

17) Cohen, S. M., Zenser, T. V., Murasaki, G., Fukushima, S., Mattammal, M. B., Rapp, N. S. and Davis, B. B. Aspirin inhibition of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide-induced lesions of the urinary bladder correlated with inhibition of metabolism by bladder prostaglandin endoperoxide synthetase. *Cancer Res.,* **41,** 3355–3359 (1981).
18) Sakata, T., Hasegawa, R., Johansson, S. L., Zenser, T. V. and Cohen, S. M. Inhibition by aspirin of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide initiation and sodium saccharin promotion of urinary bladder carcinogenesis in male F344 rats. *Cancer Res.,* **46,** 3903–3906 (1986).
19) Fukushima, S., Shibata, M.-A., Shirai, T., Tamano, S. and Ito, N. Roles of urinary sodium ion concentration and pH in promotion by ascorbic acid of urinary bladder carcinogenesis in rats. *Cancer Res.,* **46,** 1623–1626 (1986).
20) Fukushima, S., Shibata, M.-A., Shirai, T., Kurata, Y., Tamano, S. and Imaida, K. Promotion by L-ascorbic acid of urinary bladder carcinogenesis in rats under conditions of increased urinary K ion concentration and pH. *Cancer Res.,* **47,** 4821–4824 (1987).
21) Fukushima, S., Imaida, K., Shibata, M.-A., Tamano, S., Kurata, Y. and Shirai, T. L-Ascorbic acid amplification of second-stage bladder carcinogenesis promotion by $NaHCO_3$. *Cancer Res.,* **48,** 6317–6320 (1988).
22) Shibata, M.-A., Tamano, S., Kurata, Y., Hagiwara, A. and Fukushima, S. Participation of urinary Na^+, K^+, pH, and L-ascorbic acid in the proliferative response of the bladder epithelium after the oral administration of various salts and/or ascorbic acid to rats. *Food Chem. Toxicol.,* **27,** 403–413 (1989).
23) Ito, N., Fukushima, S., Hagiwara, A., Shibata, M. and Ogiso, T. Carcinogenicity of butylated hydroxyanisole in F344 rats. *J. Natl. Cancer Inst.,* **70,** 343–352 (1983).
24) Masui, T., Hirose, M., Imaida, K., Fukushima, S., Tamano, S. and Ito, N. Sequential changes of the forestomach of F344 rats, Syrian golden hamsters, and $B6C3F_1$ mice treated with butylated hydroxyanisole. *Jpn. J. Cancer Res.,* **77,** 1083–1090 (1986).
25) Scheving, L. E., Mayersbach, H. V. and Pauly, J. E. An overview of chronopharmacology. *J. Eur. Toxicol.,* **7,** 203–227 (1974).
26) Beland, F. A., Dooley, K. L., Sheldon, W. G. and Delongchamp, R. R. Circadian variation in the induction of intestinal tumors by *N*-methyl-*N*-nitrosourea in male C57BL/6N mice. *J. Natl. Cancer Inst.,* **80,** 325–330 (1988).
27) Morstyn, G., Hsu, S.-M., Kinsella, T., Gratzner, H., Russo, A. and Mitchell, J. B. Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. *J. Clin. Invest.,* **72,** 1844–1850 (1983).
28) Fukushima, S., Murasaki, G., Hirose, M., Nakanishi, K., Hasegawa, R. and Ito, N. Histopathological analysis of preneoplastic changes during *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine-induced urinary bladder carcinogenesis in rats. *Acta Pathol. Jpn.,* **32,** 243–250 (1982).
29) Clayson, D. B., Iverson, F., Nera, E., Lok, E., Rogers, C. and Rodrigues, C. Histopathological and radioautographical studies on the forestomach of F344 rats treated with butylated hydroxyanisole and related chemicals. *Food Chem. Toxicol.,* **24,** 1171–1182 (1986).
30) Hirose, M., Masuda, A., Imaida, K., Kagawa, M., Tsuda, H. and Ito, N. Induction of forestomach lesions in rats by oral administrations of naturally occurring antioxidants for 4 weeks. *Jpn. J. Cancer Res.,* **78,** 317–321 (1987).
31) Brown, W. W., Zenser, T. V. and Davis, B. B. Prostaglandin E_2 production by rabbit urinary bladder. *Am. J. Physiol.,* **239,** 1435–1440 (1980).
32) Wong, Y. H., Zenser, T. V. and Davis, B. B. Regulation of dog urothelial cell arachidonic acid release and prostaglandin E_2 synthesis. *Carcinogenesis,* **10,** 1621–1627 (1989).
33) Hial, V., de Mello, M. C. F., Horakova, Z. and Beaven, M. A. Antiproliferative activity of anti-inflammatory drugs in two mammalian cell culture lines. *J. Pharmacol. Exp. Ther.,* **202,** 446–454 (1977).
34) Bayer, B. M. and Beaven, M. A. Evidence that indomethacin reversibly inhibits cell growth in the G_1 phase of the cell cycle. *Biochem. Pharmacol.,* **28,** 441–443 (1978).
35) Pelus, L. M. and Strausser, H. R. Prostaglandins and the immune response. *Life Sci.,* **20,** 903–914 (1977).
36) Verma, A. K., Ashendel, C. L. and Boutwell, R. K. Inhibition by prostaglandin synthesis inhibitors of the induction of epidermal ornithine decarboxylase activity, the accumulation of prostaglandins, and tumor promotion caused by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.,* **40,** 308–315 (1980).
37) Narisawa, T., Hosaka, S. and Niwa, M. Prostaglandin E_2 counteracts the inhibition by indomethacin of rat colon ornithine decarboxylase induction by deoxycholic acid. *Jpn. J. Cancer Res.,* **76,** 338–344 (1985).
38) Homma, Y., Kakizoe, T., Samma, S. and Oyasu, R. Inhibition of N-butyl-N-(4-hydroxybutyl)nitrosamine-induced rat urinary bladder carcinogenesis by α-difluoromethylornithine. *Cancer Res.,* **47,** 6176–6179 (1987).
39) Uchida, K., Seidenfeld, J., Rademaker, A. and Oyasu, R. Inhibitory action of α-difluoromethylornithine on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced rat urinary bladder carcinogenesis. *Cancer Res.,* **49,** 5249–5253 (1989).
40) Imaida, K., Oshima, M., Fukushima, S., Ito, N. and Hotta, K. Membrane potentials of urinary bladder epithelium in F344 rats treated with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine or sodium saccharin. *Carcinogenesis,* **4,** 659–661 (1983).
41) Moolenaar, W. H., Tsien, R. Y., van Der Saag, P. T. and de Laat, S. W. Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature,*

304, 645–648 (1983).

42) Allemain, G. L., Paris, S. and Pouyssegur, J. Growth factor action and intracellular pH regulation in fibroblasts. *J. Biol. Chem.,* **259,** 5809–5815 (1984).

43) Moriarty, M. J., Mulgrew, S., Malone, J. R. and O'Connor, M. K. Results and analysis of tumour levels of ascorbic acid. *Ir. J. Med. Sci.,* **146,** 74–78 (1977).

44) Carpenter, G. and Cohen, S. Human epidermal growth factor and the proliferation of human fibroblasts. *J. Cell Physiol.,* **88,** 227–238 (1976).

45) Yura, Y., Hayashi, O., Kelly, M. and Oyasu, R. Identification of epidermal growth factor as a component of the rat urinary bladder tumor enhancing urinary fractions. *Cancer Res.,* **49,** 1548–1533 (1989).

46) Hirose, M., Inoue, T., Masuda, A., Tsuda, H. and Ito, N. Effects of simultaneous treatment with various chemicals on BHA-induced development of rat forestomach hyperplasia — complete inhibition by diethylmaleate in a 5-week feeding study. *Carcinogenesis,* **8,** 1555–1558 (1987).

47) Hirose, M., Masuda, A., Tsuda, H., Uwagawa, S. and Ito, N. Enhancement of BHA-induced proliferative rat forestomach lesion development by simultaneous treatment with other antioxidants. *Carcinogenesis,* **8,** 1731–1735 (1987).