Korean J. Toxicol.
Vol. 12, No. 1, pp. 21-27, 1996

Inhibitory Effect of Vitamin C on Mutagenicity of 6-Sulfooxymethylbenzo[a]pyrene

Young-Sik Cho, Sun-Taek Hong and An-Sik Chung*

Department of Biological Sciences, KAIST, Taejeon 305-701, Korea

(Received May 9, 1996)
(Accepted May 20, 1996)

ABSTRACT : *Vitamin C has been well known to be a potential chemopreventive agent for several toxic compounds. It reduced the mutation frequencies of 6-sulfooxymethylbenzo[a]pyrene (SMBP) and 6-hydroxymethylbenzo[a]pyrene (HMBP) in Salmonella typhimurium TA98 and TA100, indicating that ascorbic acid affects both frameshift and base-pair substitution mutations. A similar type of dose-response relationship was shown in the V79 cells, although the inhibitory effect was less dramatic compared with that in S. typhimurium. However, SMBP or HMBP binding to calf thymus DNA was not affected by the presence of vitamin C, suggesting that SMBP seems to be much more reactive to calf thymus DNA than vitamin C. This was supported by migration pattern and fluorescence intensity of SMBP-modified plasmid on the gel. These results were not correlated with mutation tests in bacterial and mammalian cell systems. It has been already reported that vitamin C inactivates SMBP through the formation of covalently bound adduct. It was found from HPLC analysis that the reaction between vitamin C and SMBP was accomplished within just 5 min and then produced the several products. These findings indicate that the beneficiary of vitamin C is not merely derived from the covalent adducts. On the other hand, the addition of DNA to incubation mixture reduced the amounts of vitamin C adducts while the magnitude of HMBP peak increased, suggesting that DNA accelerates the SMBP hydrolysis to intercept the interaction between SMBP and vitamin C or forms rapidly complex with SMBP.*

Key Words : *6-Sulfooxymethylbenzo[a]pyrene, Vitamin C, Mutagenicity, Adducts*

I. INTRODUCTION

There are many constituents acting as anticarcinogens and carcinogens in food. Among them, vitamin C has been considered to be effective in preventing cell damage caused by several carcinogens. It has to be ingested through diet by human and guinea pigs, which are not capable of synthesizing ascorbic acid from glucose by lack of L-gluconolactone oxidase activity. Ascorbic acid deficiency results in scurvy, which can be prevented in humans by administration of a small amount much as 10 mg of ascorbic acid per day (Hodges *et al.*, 1969). It has been also reported that vitamin C is effective to prevent and treat cancer, the common cold and AIDS (Block, 1991; Cook *et al.*, 1977; Harakeh *et al.*, 1990). Furthermore, vitamin C is positive in all assays using 6 chemoprevention-associated biochemical end points as a screening method (Sharma *et al.*, 1994). Polycyclic aromatic hydrocarbons (PAHs) are a class of chemicals that contain many known carcinogens, including benzo[a]pyrene (BP), aflatoxin and benz[a]anthracene. Certain occupations, smoking and charcoal-broiled foods contribute to the exposure of these chemicals for human. A variety of mammalian cells can metabolize polycyclic aromatic hydrocarbons to polycyclic phenols, dihydrodiols, epoxides, quinones, and water-soluble conjugates by a series of carcinogen-metabolizing enzymes. A carcinogen, SMBP has been known to the ultimate metabolite of *meso*-methyl group of benzo[a]pyrene, which induces the carcinoma in mice as a dose-dependent manner (Surh *et al.*, 1990). In recent, it is also reported that ascorbic acid forms mutagenically inactive covalent adduct with SMBP, which appears to be a protective mechanism against this reactive sulfuric acid ester (Surh *et al.*, 1994). The antimutagenicity of ascorbic acid was investigated with SMBP using Chinese hamster V79 cell and

S. typhimurium strains. Effect of vitamin C on the binding potential of SMBP or HMBP was also examined with calf thymus DNA or pUC19 plasmid. The protective mechanism of vitamin C was reexamined by comparing the hydrolysis products of SMBP in the presence or absence of vitamin C.

II. MATERIALS AND METHODS

1. Chemicals

Fetal bovine serum was purchased from Gibco, Grand Island, NY. Dulbecco's modified Eagle's medium (DME), 6-thioguanine (6-TG), dimethyl sulfoxide (DMSO), calf thymus DNA, and agarose were obtained from Sigma Chemical Co., St. Louis, MO. Proteinase K was obtained from Boehringer-Mannheim, Indianapolis, IN. HMBP and SMBP were synthesized as described previously (Natarajan and Flesher, 1973; Surh *et al.*, 1989). Other reagents were of analytical purity.

2. Preparation of Hepatic Cytosolic Fractions

Rat liver cytosol was prepared from the livers of 4-week-old female Sprague-Dawley rat (Korea Research Institute of Bioscience and Biotechnology, Taejeon, Korea). Livers were minced and homogenized with Potter-Elvehjem-teflon homogenizer in 3 vol of ice-cold 0.154 M KCl, 50 mM Tris-HCl buffer (pH 7.4). These preparative steps were carried out at 4°C. Homogenates were centrifuged at 12,000×g for 20 min. The pellets were discarded and the supernatants were recentrifuged at 105,000×g for 60 min in a Beckman L8-M ultracentrifuge with a type 70 Ti fixed angle rotor. The supernatants obtained were stored at -80°C until use. The protein content of the cytosol was determined by the Lowry assay with bovine serum albumin as a standard (Lowry *et al.*, 1951).

3. Cell Growth Assay

Chinese hamster V79 cells were grown in monolayer culture in DME medium containing 5% heat-inactivated (56°C for 30 min) fetal bovine serum at 37°C in an incubator humidified to 95-100% in an atmosphere of 5% CO_2 in air. Subculture was performed by use of 0.05% trypsin solution (1 : 250) for cell detachments, and the cell number was determined by use of a haemacytometer. Cytotoxicity was evaluated by measuring the cell growth. Chinese hamster V79 cells (5×10^5 or 1×10^6) were inoculated into 60-mm tissue culture dishes and allowed to attach. After 6 hr the original medium was removed and replaced with serum-free medium containing various concentrations of vitamin C. Treatments with 1.5 μM SMBP were performed for 30 min at 37°C. The cells were then allowed to grow for 48 hr to count the cell number.

4. Mutagenicity Assay

The tester strains, *S. typhimurium* TA98 and TA 100 were supplied from Korea Research Institute of Chemical Technology, Taejeon, Korea. Mutagenicity studies were performed based on the modification of Ames standard assay (Maron and Ames, 1983). A relatively high number ($3\text{-}4\times10^9$) of bacteria were used to increase the sensitivity of assays. HMBP (7.2 nmol) was incubated for 60 min at 37°C together with a series of doses of vitamin C in a final volume of 1.1 ml of 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.4) containing the rat liver cytosol and 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-generating system (5 mM ATP, 5 mM sodium sulphate, 3 mM magnesium chloride and 0.1 mM EDTA). The cytosolic fraction and PAPS-generating system were omitted from the mixture when 1 nmol of SMBP was used instead of HMBP. The treatment of SMBP was for 30 min at 37°C. After incubation, the mixtures were diluted with soft agar, poured onto hard agar plate and further incubated for 48 hr to allow the growth of His^+ revertant colonies. Otherwise indicated, the data were corrected for the number of spontaneous revertants or for the number obtained in solvent-treated controls.

The technique for utilizing the V79 cells in assaying mutagenicity at hypoxanthine: guanine phosphoribosyltransferase (*hgprt*) locus has been described earlier (Chu, 1971; Jenssen, 1984). SMBP at a concentration of 1.5 μM was tested together with various concentrations of vitamin C for 1 hr. For selection of mutants, a final concentra-

tion of 0.2 mM 6-TG was added after the cells were subcultured 3 times. The medium was replaced every 2 days with fresh medium containing 6-TG. Two hundred cells were seeded onto each plate for the determination of cloning efficiency under the condition used for the selection of mutants. Mutation frequency was expressed as 6-TG resistant mutants/10^6 survivors, which was corrected for plating efficiency.

5. Covalent Binding of HMBP and SMBP to Calf Thymus DNA

The covalent binding of HMBP to DNA was determined after incubation at 37°C for 90 min in a final volume of 500 μl of 0.1 M Tris-HCl buffer (pH 7.4) containing 500 μg DNA, 10 nmol HMBP, 50 μl of liver cytosol (~50 mg/ml protein) and PAPS-generating system. The cytosolic fraction and PAPS-generating system were omitted from the mixture when SMBP (1 nmol) dissolved in 10 μl of DMSO was used instead of HMBP. Protein was removed from the mixture by protease digestion and subsequent phenol extraction. The DNA was precipitated by the addition of ethanol in the presence of 0.2 M sodium acetate, and then washed with organic solvents as previously reported (Watabe *et al.*, 1985). The washed nucleic acid was dried *in vacuo*, dissolved in Tris-EDTA buffer (pH 8.0) and determined by absorbance at 260 nm. The adducts were measured by their fluorescence at 418 nm (excitation at 360 nm) and expressed in terms of fluorescence intensity/mg DNA.

6. Electrophoresis

The plasmid, pUC19 was purified from *E. coli* according to the procedure described previously (Maniatis *et al.*, 1982). SMBP (1 nmol) was incubated in the reaction mixtures containing pUC19 plasmid and various concentrations of vitamin C for 30 min at 37°C and then the aliquots were subjected to electrophoresis on 0.8% agarose gel at 100 V for 50 min. Other antioxidants were also tested against SMBP binding to plasmid. Several antioxidants at fixed dose (0.1 μmol) were incubated with 1 nmol SMBP and the reaction was repeated under the same condition as described before. SMBP-modified DNA was visualized under UV light as a violet fluorescence spot, characteristic of BP chromophore. DNA band was also identified under UV light after staining with ethidium bromide (EtBr).

7. HPLC Analysis of Products Formed from Vitamin C and SMBP

The HPLC system was equipped with Waters a Model 510 pump and a Model 486 tunable absorbance detector. The analysis of SMBP hydrolysates was performed using 4.6×250 mm Ultremex 5 C_{18} column at a 15 min linear gradient of 80% methanol in water to 100% methanol. SMBP (0.1 mM) dissolved in DMSO was reacted with ascorbic acid (1 or 5 mM) in 200 μl of a final volume of 0.4 M Tris-HCl buffer (pH 7.4). The equal volume of methanol was mixed with reaction mixture and then small aliquots were analyzed.

To determine the effect of DNA on products formed from vitamin C and SMBP, the reaction was started as soon as 0.1 mM SMBP was added to incubation mixture consisting of 100 μg calf thymus DNA and 1 mM vitamin C. DNA was precipitated by the addition of equal volume of isopropanol and its supernatant was subjected to HPLC analysis under the same condition as described previously (Surh *et al.*, 1994).

III. RESULTS

As an initial approach to assess the plausible protective effect of vitamin C against SMBP, the Ames-His^+ reversion assay was performed using *S. typhimurium* TA98 and TA100 strains. The dose-dependent inhibition of SMBP- or HMBP-induced mutagenicity by vitamin C was shown in Fig. 1. SMBP and HMBP were highly mutagenic toward both TA98 and TA100 strains, which are sensitive to the frameshift and base-pair substitution mutations, respectively. No significant mutagenicity of HMBP was detected without cytosol or the PAPS-generating system. Vitamin C effectively inhibited the mutagenicity of both SMBP and HMBP in a dose-dependent manner up to 10 μmol in the

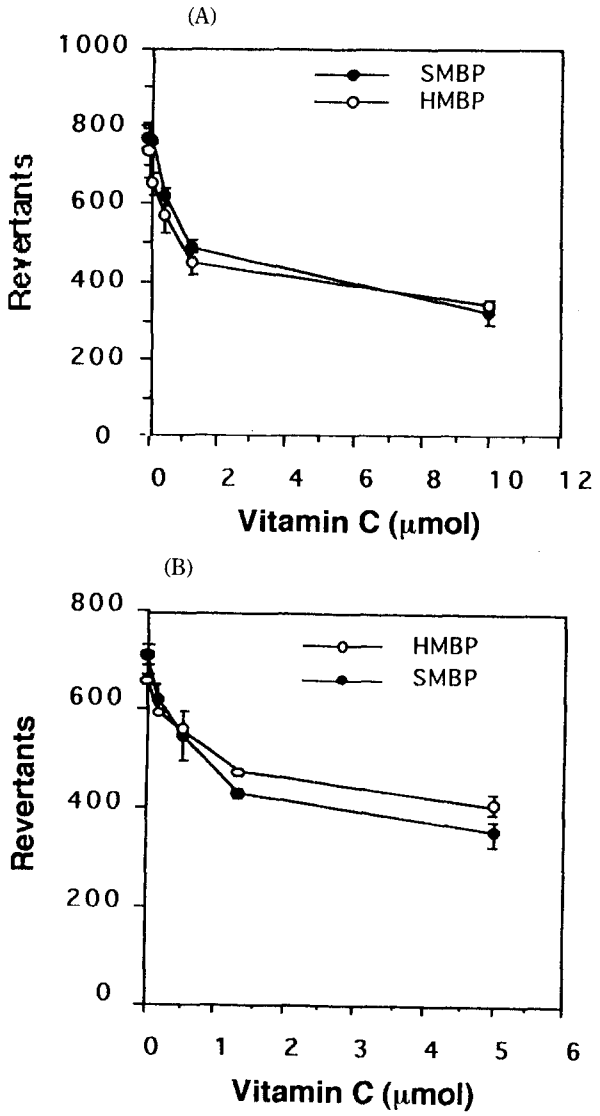

Fig. 1. The inhibitory effect of vitamin C on mutagenesis induced by HMBP and SMBP in *S. typhimurium* strains TA98 (A) and TA100 (B). Assays were performed with the indicated amounts of HMBP or SMBP as described in Materials and Methods. Each point represents the means of three replicate determinations. Spontaneous revertants were subtracted.

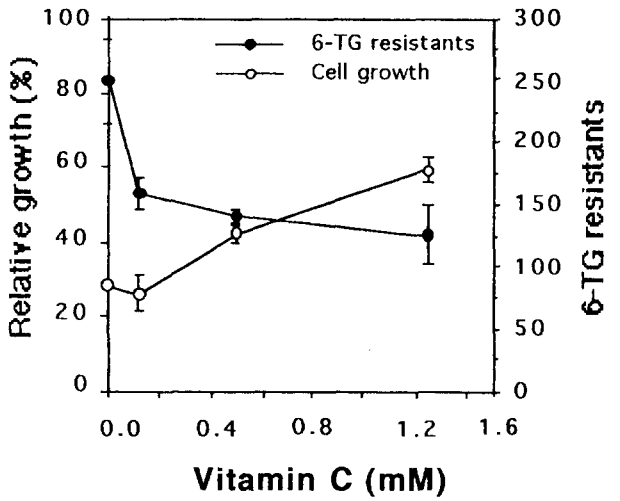

Fig. 2. The dose-response curves of vitamin C for cell growth inhibition and mutation frequency caused by SMBP. SMBP (1.5 μM) was treated to cultured Chinese hamster V79 cells in DME media containing various concentrations of vitamin C. The resistant mutants for 6-thioguanine were determined by counting the colonies and mutagenicity frequency was expressed as mutants/10^6 survivors. For the determination of cell growth, exponentially growing cells were exposed to 1.5 μM of SMBP for 30 min in the presence of various concentrations of vitamin C. The relative growth was calculated as compared to the solvent control.

strain TA98. The similar type of dose-response of Vitamin C against both chemicals was also shown in the strain TA100 assay. The chemopreventive activity of vitamin C on SMBP was also confirmed in the mammalian cell culture system. The inhibition of SMBP-induced cytotoxicity and mutagenicity by vitamin C was exhibited in Fig. 2. In the presence of vitamin C, there were an enhancement of cell growth and a reduction of mutation frequency. The recovery of the survival rate was closely related to the concentration of vitamin C within the range used. The growth of SMBP-treated cells was 30% of that of untreated control, and treatment of 1.25 mM vitamin C recovered the cell growth to about 60%. SMBP at a 1.5 μM induced 250 mutants/10^6 survival cells in *hgprt* locus. The mutation frequencies of SMBP were reduced to 50% by the treatment of 1.25 mM vitamin C.

However, the treatment of vitamin C did not affect the covalent binding of SMBP to calf thymus DNA as shown in Fig. 3. The binding potential of SMBP to the plasmid was evaluated by the fluorescence intensity and migration pattern of the SMBP-modified DNA on agarose gel (Fig. 4). The plasmid modified with SMBP emitted the fluorescent light of BP-chromophore and migrated slowly on the gel. Although altering the migration pattern slightly, vitamin C did not attenuate the fluorescence intensity of SMBP-modified DNA. Other antioxidants including retinol, (±)-α-tocopherol and *trans*-β-carotene did not affect DNA binding of SMBP.

The products formed from reaction of vitamin C

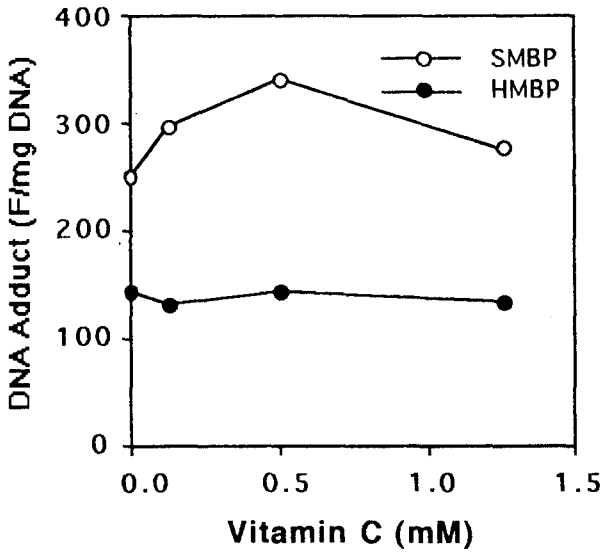

Fig. 3. Effect of vitamin C on the binding of SMBP or HMBP to calf thymus DNA. SMBP (1 nmol) and HMBP (10 nmol) was treated to incubation mixture consisting of various concentrations of vitamin C and DNA for 20 min at 37°C. DNA binding experiments were performed as described in Materials and Methods. DNA adducts were measured by their fluorescence at 418 nm (exciting at 360 nm) and expressed in terms of fluorescence intensity/mg DNA.

and SMBP were analyzed by HPLC (Fig. 5). The HPLC profiles showed that at least four intensive peaks were detected with linear gradient from methanol-water (8 : 2, v/v) to 100% methanol at retention times, 6.5 (I), 7.2 (II), 8.1 (III) and 10.4 min (IV). All of the peaks showed a typical fluorescent spectra just same as HMBP itself (data not shown). The isolated peaks I and IV were assumed to be vitamin C adduct and HMBP, respectively, from the elution patterns of the previous result (Surh *et al.*, 1994). Peaks II and III has not been identified but may be derived from the oxido-reduction reaction of vitamin C. In the presence of DNA, elution profiles of products formed from the reaction of vitamin C and SMBP were shown (Fig. 6). The adduct formation of vitamin C and SMBP was decreased by the addition of DNA.

IV. DISCUSSION

Vitamin C was found to be quite effective in suppressing the mutagenicities of SMBP and HMBP in *S. typhimurium* TA98 and TA100 strains, indicating that vitamin C affects both frameshift

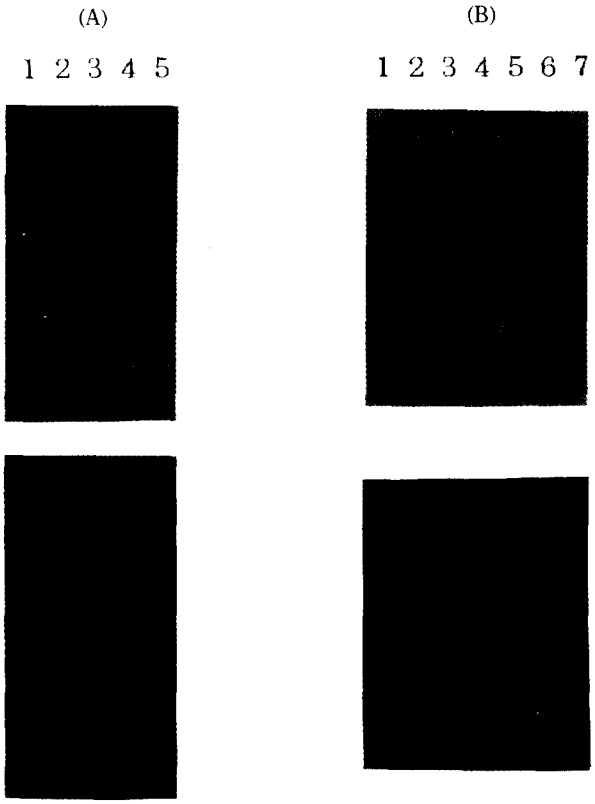

Fig. 4. Comparison of SMBP binding to pUC19 plasmid as a function of ascorbic acid concentrations (A) and various antioxidants (B). All treatments were performed for 20 min at 37°C under the conditions described in the text. (A) lane 1 was control DNA and lanes 2-5 were the result of SMBP treatment in the absence or presence of ascorbic acid. Ascorbic acid concentrations used were: lane 2, no; lane 3, 0.2 μmol; lane 4, 0.5 μmol; lane 5, 1 μmol. (B) 1 and 2 lanes were untreated and SMBP-treated control, respectively. 3-7 lanes were combination of following antioxidants with SMBP (lane 3, retinol; lane 4, vitamin C; lane 5, cholecalciferol; lane 6, (±)-α-tocopherol; lane 7, trans-β-carotene). Upper and lower panels are photographs of SMBP-modified DNA and EtBr-stained DNA visualized under UV illumination, respectively.

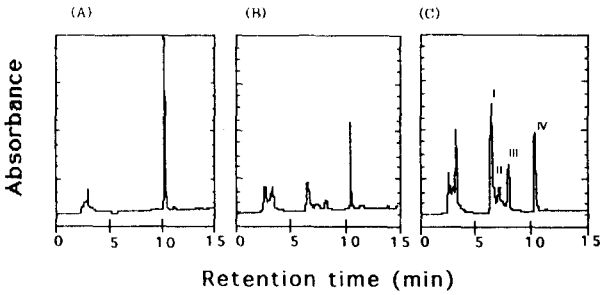

Fig. 5. HPLC analysis of products formed from SMBP in aqueous solution in the absence (A) or presence of 1 mM (B) or 5 mM (C) ascorbic acid. Aliquots (100 μl) were analyzed by reverse-phase HPLC with a 15 min linear gradient of 80% methanol in water to 100% methanol. The products were monitored by UV absorbance of its chromophore at 394 nm.

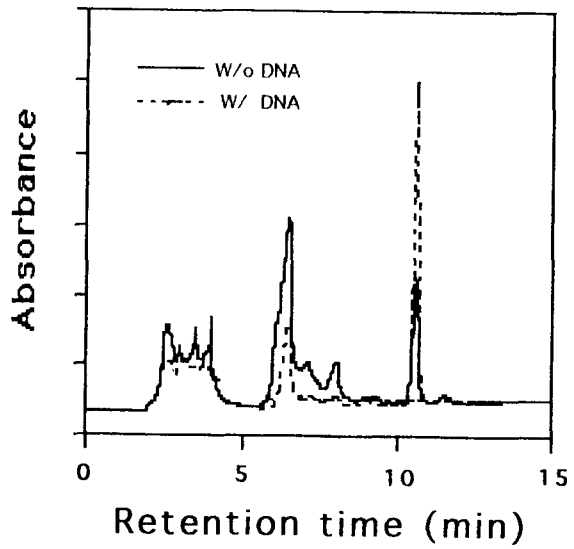

Fig. 6. Effect of calf thymus DNA on HPLC profiles of products formed from SMBP and vitamin C. SMBP at a 0.1 mM concentration was incubated at 37°C with 1 mM vitamin C in the absence or presence of 100 μg of calf thymus DNA. The chromatographic conditions are the same as in Fig. 5.

and base-pair substitution mutations, respectively. SMBP exhibited a strong mutagenic activity without further activation, while HMBP should be activated for mutagenesis by PAPS-dependent sulfotransferase activity. The dose-responses of vitamin C against SMBP were also ascertained in V 79 cells. These results indicate that vitamin C has a common protective character in both biological systems. However, the binding studies showed that covalent binding of SMBP to calf thymus DNA was not affected by high concentrations of vitamin C, suggesting that SMBP has very high affinity for DNA. A similar reactivity was shown in the electrophoresis of SMBP-modified pUC19 plasmid in the presence of vitamin C. These data were not correlated to the mutagenicities of SMBP in the bacterial and mammalian cell culture systems. This discrepancy may be due to its permeability, stability, metabolism and redox potential in biological system.

It has been generally suggested that ascorbic acid act as intracellular/intranuclear antioxidants, which is the ultimate defense against oxidative DNA damage or free radicals (Fischer-Nielsen, 1993; Mehlhorn, 1991). Another report suggests that the ability of ascorbate to reduce directly phenoxy radicals may be important mechanism of their protective function against the cytotoxicity of phenol/quinoid redox couples (Kagan *et al.*, 1994). Recently, it has been demonstrated that vitamin C reacts with SMBP to form adducts, thereby reducing available SMBP (Surh *et al.*, 1994). However, the role of ascorbic acid in biological system is much more sophisticated than that in *in vitro* experiments. Vitamin C, associated with glutathione, provides redox potential for chemical reaction and protein folding (Meister, 1994). The level of ascorbate is affected by glutathione concentration in the cells, reflecting the metabolic importance of such antioxidant activity. The permeability and stability of SMBP are considered to be important for toxic effect of SMBP. Chloride ion can enhance the permeability of SMBP across membrane by formation of chloromethyl derivatives, which is more hydrophobic than SMBP itself (Surh *et al.*, 1991). Moreover, our unpublished studies support strongly that serum albumin and lipoproteins are responsible for stabilizing of SMBP (data not shown here).

The several products were detected from the reaction of vitamin C and SMBP by analysis of HPLC, which may be affected by the redox potential of vitamin C. It was interesting that DNA addition to the incubation mixture of vitamin C and SMBP resulted in a reduction of vitamin C adducts and simultaneously an increase of HMBP. These results suggest that DNA accelerates the SMBP hydrolysis to HMBP, thereby inhibiting the vitamin C adduct formation with SMBP. Although protective mechanism of vitamin C against SMBP is not clear, the beneficiary effect of vitamin C against SMBP in the biological system may be derived from not only adduct formation of vitamin C but also intracellular redox potential of vitamin C such as ascorbic acid-glutathione antioxidant system.

ACKNOWLEDGEMENT

We thank Dr. K.K. Park for his helpful scientific discussions and editorial assistance in the preparation of the manuscript.

REFERENCES

Block, G. (1991): Vitamin C and cancer prevention:

The epidemiological evidence. *Am. J. Clin. Nutr.*, **53**, 270S-282S.

Chu, E.H.Y. (1971): Induction and analysis of gene mutations in mammalian cell cultures. InHollaender, A. (ed.), Chemical mutagens: Principles and Methods for their Detection. Plenum Publishing Corp., New York, pp. 411-444.

Cook, J.D. and Monsen, E.R. (1977): Vitamin C, the common cold, and iron absorption. *Am. J. Clin. Nutr.*, **30**, 235-241.

Fischer-Nelsen, A., Loft, S. and Jensen, K.G. (1993): Effect of ascorbate and 5-aminosalicylic acid on light-induced 8-hydroxydeoxyguanosine formation in V79 Chinese hamster cells. *Carcinogenesis*, **14**, 2431-2433.

Harakeh, S., Jariwalla, R.J. and Pauling, L. (1990): Suppression of human immunodeficiency virus replication by ascorbate in chronically and acutely infected cells. *Proc. Natl. Acad. Sci. USA.*, **87**, 7245-7249.

Hodges, P.E., Baker, E.M., Hood, J., Sauberlich, H.E. and March, S.C. (1969): Experimental scurvy in man. *Am. J. Clin. Nutr.*, **22**, 535-548.

Jenssen, D. (1984): A quantitative test for mutagenicity in V79 Chinese hamster cells. In Kilbey, B.J., Legator, Ramel, C. (eds.), Handbook of Mutagenicity Test Procedure, 2nd end. Elsevier, Amsterdam, pp. 269-290.

Kagan, V.E., Yalowich, J.C., Day, B.W., Goldman, R., Gantchev, T.G. and Stoyanovsky, D.A. (1994): Ascorbate is the primary reductant of the phenoxy radical of etoposide in the presence of thiols both in cell homogenates and in model systems. *Biochemistry*, **33**, 9651-9660.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982): Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, pp. 92.

Maron, D.M. and Ames, B.N. (1983): Revised method for the Salmonella mutagenicity test. *Mutat. Res.*, **113**, 173-215.

Mehlhorn, R.J. (1991): Ascorbate-and dehydroascorbic acid-mediated reduction of free radicals in the human erythrocyte. *J. Biol. Chem.*, **266**, 2724-2731.

Meister, A. (1994): Glutathione-ascorbic acid antioxidant system in animals. *J. Biol. Chem.*, **269**, 9397-9400.

Natarajan, R.K. and Flesher, J.W. (1973): Synthesis and carcinogenicity of compounds related to 6-hydroxymethylbenzo[a]pyrene. *J. Med. Chem.*, **16**, 714-715.

Sharma, S., Stutzman, J.D., Kelloff, G.J. and Steele, V. E. (1994): Screening of potential chemopreventive agents using biochemical markers of carcinogenesis. *Cancer Res.*, **54**, 5848-5855.

Surh, Y-J., Liem, A., Miller, E.C. and Miller, J.A. (1989): Metabolic activation of the carcinogen 6-hydroxymethylbenzo[a]pyrene: formation of an electrophilic sulfuric acid ester and benzylic DNA adducts in rat liver *in vivo* and in reaction *in vitro*. *Carcinogenesis*, **10**, 1519-1528.

Surh, Y-J., Liem,A., Miller, E.C. and Miller, J.A. (1990): The strong hepatocarcinogenicity of the electrophilic and mutagenic metabolite 6-sulfooxymethylbenzo[a]-pyrene and its formation of benzylic DNA adducts in the liver of infant male B6C3F_1 mice. *Biochem. Biophys. Res. Commun.*, **172**, 85-91.

Surh, Y-J., Liem, A., Miller, E.C. and Miller, J.A. (1991): 7-sulfooxymethyl-12-methylbenz[a]anthracene is an electrophilic mutagen, but does not appear to play a role in carcinogenesis by 7,12-dimethylbenz-[a]anthracene or 7-hydroxymethyl-12-methylbenz-[a]anthracene. *Carcinogenesis*, **12**, 339-347.

Surh, Y-J., Park, K-K. and Miller, J.A. (1994): Inhibitory effect of vitamin C on the mutagenicity and covalent DNA binding of the electrophilic and carcinogenic metabolite, 6-sulfooxymethylbenzo[a] pyrene. *Carcinogenesis*, **15**, 917- 920.

Watabe, T., Hiratsuka, A., Ogura, K. and Endoh, K. (1985): A reactive hydroxymethylsulfate ester formed regioselectively from the carcinogen, 7,12-dihydroxymethylbenz[a]anthracene, by rat liver cytosol. *Biochem. Biophys. Res. Commun.*, **131**, 694-699.