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Antimutagenic and Anticarcinogenic Effects of Alginic Acid Extracted from Sporophyll of Sea Mustard

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Abstract

Antimutagenic and anticarcinogenic effects of alginic acids extracted from sea mustard (SM) and sporophyll of sea mustard (SSM) were studied by *Salmonella typhimurium* assay system and cytotoxicity and transformation tests using C3H/10T1/2 cells, respectively. Alginic acid-SM and alginic acid-SSM showed antimutagenic effects on aflatoxin B_1 (AFB_1) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in *Salmonella typhimurium* TA100 strain. The antimutagenic effect showed concentration dependent manner. At the 2.5 mg/plate concentration, alginic acid-SSM exhibited 92% antimutagenicity against AFB_1, while alginic acid-SM revealed 54% antimutagenicity, showing effectiveness of the alginic acid-SSM for the antimutagenicity. Alginic acid-SSM also significantly decreased the cytotoxicity induced by 3-methylcholanthrene (MCA) and MNNG in C3H/10T1/2 cells ($p<0.05$). The type II and type III transformation foci formation by MCA and MNNG were also decreased when the alginic acid-SSM was treated, indicating that the alginic acid-SSM reduces the carcinogenesis induced by these carcinogens. The MCA-treated culture produced 10.5 foci of type II+III in C3H/10T1/2 cells, however, MCA+0.2 mg/ml alginic acid-SSM treated culture formed only 1.8 foci of the types II+III ($p<0.05$). While MNNG-treated culture formed 13.0 foci, MNNG+0.2 mg/ml alginic acid-SSM treated one produced 3.0 foci of type II+III ($p<0.05$). These results suggest that alginic acid-SSM can effectively prevent the mutagenicities and also decrease cytotoxicity and transformation induced by some carcinogens.

Key words : antimutagenic, anticarcinogenic, alginic acid, sporophyll of sea mustard, C3H/10T1/2 cell

INTRODUCTION

It is well known that diet is associated with several types of human cancer (1,2). This hypothesis stems largely from the observed incidence of cancers in various parts of the world. Cancers of the colon, breast, ovary and pancreas are more common in the United States and Western Europe than Asia (3). The specific dietary risk factors have not been identified but there appeares to be both high risks and protective compounds in diet. For example, the diets rich in seaweeds, and thus high in sodium alginates, protect against the development of breast cancer (4-7).

Several studies on *in vitro* and *in vivo* have shown that hot water extracts of seaweed inhibit the growth of certain transplantable cancers (8-11). Evidence is that tumor transplantation studies by Yamamoto and coworkers have used aqueous seaweed fractions as chemotherapeutic agents to treat Sarcoma 180, Meth-A, B-16 melanoma and L-1210 leukemia in mice (8-10). Seaweed is a source of nondigestible fiber, minerals, vitamins and highly branched glucan which have the activity to stimulate immune response (6,7).

Alginic acid is a polysaccharide found originally in brown seaweeds. The polysaccharide is a liner glycuronan consisting of (1→4)-linked residues of D-mannuronic acid and L-guluronic acid, arranged in a block fashion in the polymer chain. The sodium alginate from seaweeds shows a considerable antitumor activity against various tumors and it has the abiiity to enhance cytostatic and cytolytic activities of macrophage, and thus the antitumor effect may partly be caused by the activation of macrophage (12).

Although sporophyll of sea mustard has been traditionally used as food and also as an additive or seasoning with various foodstuffs in Korea, the antimutagenic and anticarcinogenic effects of sporophyll of sea mustard have never been elucidated until now. In this study we inves-

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tigated antimutagenicity using Ames assay system and inhibitory effects on carcinogen-induced cytotoxicity and transformation in C3H/10T1/2 cells using the active compound, alginic acid from sporophyll of sea mustard.

MATERIALS AND METHODS

Preparation of samples and extraction of alginic acids

Sea mustard and sporophyll of sea mustard were purchased from the Jagalchi market in Pusan, Korea. Alginic acids from sea mustard and sporophyll of sea mustard were extracted by the method of Kim and Cheong (13). The powdered sea mustard and sporophyll of sea mustard were soaked in 0.04% NaOH and then 0.5% HCl for 1 hour. The alginic acid was extracted with 60 volumes of 1.0% Na_2CO_3 at 80°C for 3 hours. Alginic acid gel was precipitated with 1.0% H_2SO_4, and 80% methanol was added and then dried at 105°C. The extracted alginic acid samples were then dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA) for the experiment.

Antimutagenicity test

Aflatoxin B_1 (AFB_1) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were used as mutagens. AFB_1 was purchased from Sigma Chemical Co. (St. Louis, Mo, USA) and dissolved in DMSO. MNNG was obtained from Aldrich Co. (Milwaukee, WI, USA) and dissolved in distilled water. *Salmonella typhimurium* TA100 bacterial strain, histidine requiring mutant, was provided by Dr. B.N. Ames (Univ. of California, Berkerly, CA, USA) and was maintained as described by Maron and Ames (14). The genotype of the tester strain was checked routinely for the histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvr* B mutation) and the presence of R factor. S9 mixture to activate the indirect mutagen, AFB_1, was also prepared by the method of Maron and Ames (14). Antimutagenicity test (15-17) was carried out by a modified plate incorporation test (liquid preincubation of the organism with the test compound). In preincubation test, 0.5 ml of S9 mixture (or 0.5 ml phosphate buffer for direct mutagen, MNNG) was distributed into sterilized capped tubes on ice bath and then 0.1 ml of tester strain cultured overnight ($1\sim2\times10^9$ cells/ml) and 0.1 ml of test compound (50 μl of mutagen and 50 μl of alginic acid) were added. The tubes were vortex-mixed gently and preincubated at 37°C for 20 min and then 2 ml of the top agar kept at 45°C was added to each tube and vortex-mixed. The resulting entire mixture was poured on the minimal agar plate. The plates were incubated at 37°C for 48 hours and then the revertant bacterial colonies on each plate were counted.

Dose-response test (14) of the mutagens on the tester strain was carried out to determine the regions of revealing mutagenicity induced by the mutagens. Toxicity test was also carried out. The alginic acids from sea mustard and sporophyll of sea mustard used for antimutagenicity test did not show any toxicity on the tester strain.

Cytotoxicity and transformation tests

Carcinogens/Chemicals

3-Methylcholanthrene (MCA) and Giemsa stain were obtained from Sigma Chemical Co. (St. Louis, MO, USA). N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Eagle's basal medium, fetal bovine serum (FBS), 0.05% trypsin-0.02% EDTA and penicillin-streptomycin were obtained from Gibco Chemical Co. (Grand Island, NY, USA).

Cell culture

C3H/10T1/2 cells, mouse fibroblast embryo cells, were obtained from Japanese Cell Line Collection (Tokyo, Japan). The medium used for the cells was Eagle's basal medium supplemented with 10% FBS and 100 unit/ml penicillin- streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. A medium change was made on the 5th day after seeding. The cells were transferred every 10 days, using phosphate buffered saline (PBS) and 0.05% trypsin-0.2% EDTA and new flasks were seeded with 5×10^4 cells in 5 ml of medium.

Cytotoxicity assay

Cytotoxicity was determined by measuring the inhibition of colony formation (18,19). C3H/10T1/2 cells were seeded at 2×10^3 cells/60 mm dish. After 24 hours the alginic acids from sea mustard and sporophyll of sea mustard were added to serum free Eagle's basal medium. The cells were also treated with MCA (10 μg/ml) and MNNG (1 μg/ml) in the absence or presence of alginic acid. Following the treatment for 48 hrs, the medium was

changed, and the cells were fixed with methanol, stained with Giemsa stain and counted. Cytotoxicity was expressed as the number of surviving colonies on the treated dishes divided by the number of surviving colonies on the control dishes.

Transformation test

Transformation experiment was performed by a modified method of Reznikoff et al. (20). 2×10^3 C3H/10T1/2 cells were seeded in 60 mm dish (10 dishes/group). After 24 hours, the cells were loaded with serum free Eagle's basal medium containing MCA (10 μg/ml), MNNG (1 μg/ml) and alginic acid from sporophyll of sea mustard or PBS for 48 hours. The medium medium was changed twice a week until the cells reached confluence, then once a week. At the 6 week, cells in the dishes were fixed with methanol and stained by Giemsa, and the morphologically transformed foci were counted. Transfomed foci are classified as three types (type I, II and III) by the morphological criteria initially established by Reznikoff et al. (20).

Statistical analysis

Data from individual experiment were expressed as the means±standard deviation of means. The data was submitted to analysis of varience (ANOVA) and student's *t*-test with significance at $p<0.05$.

RESULTS AND DISCUSSION

The antimutagenicity using Ames assay system and anticarcinogenicity using C3H/10T1/2 cells of alginic acids from sea mustard (alginic acid-SM) and sporophyll of sea mustard (alginic acid-SSM) were evaluated. As sea mustard grows, sporophyll of sea mustard is formed at the base of stem. While sporophyll of sea mustard is used for foods in Korea, the study on physiological effect of sporophyll of sea mustard has not been yet made which allows to evaluate the antimutagenic and anticarcinogenic effects.

Alginate is a viscous dietary fiber in algae-containing foods and the main constituents of alginate are uronic acids (mannuronic and guluronic acid), which give the pectin-like characteristics (21). The alginic acids extracted from sea mustard (alginic acid-SM) and sporophyll of sea mustard (alginic acid-SSM) showed antimutagenic activity against AFB_1 in *Salmonella typhimurium* TA100

Table 1. Effect of alginic acids from sea mustard (SM) and sporophyll of sea mustard (SSM) on the mutagenicity induced by aflatoxin B_1 (AFB_1, 0.2 μg/plate) in *Salmonella typhimurium* strain of TA100

Treatment	Concentration (mg/plate)	Revertants/ plate	Inhibition rate(%)
Spontaneous		104 ± 11	
Control(AFB_1)		745 ± 16	
AFB_1 +	0.625	622 ± 29	19
Alginic acid	1.25	505 ± 21	37
−SM	2.5	402 ± 21	54
AFB_1 +	0.625	515 ± 6*	36
Alginic acid	1.25	377 ± 6*	57
−SSM	2.5	155 ± 24*	92

*Significantly different from treatment of alginic acid-SM at each concentration at the $p<0.05$ level

Table 2. Effect of alginic acids from sea mustard (SM) and sporophyll of sea mustard (SSM) on the mutagenicity induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.5 μg /plate) in *Salmonella typhimurium* strain of TA100

Treatment	Concentration (mg/plate)	Revertants/ plate	Inhibition rate(%)
Spontaneous		109 ± 6	
Control(MNNG)		904 ± 10	
MNNG +	0.625	470 ± 24	55
Alginic acid	1.25	432 ± 20	59
−SM	2.5	409 ± 8	62
MNNG +	0.625	446 ± 31	55
Alginic acid	1.25	432 ± 26	59
−SSM	2.5	359 ± 13	69

(Table 1). The antimutagenic effect of the alginic acid showed concentration dependent manner. Alginic acid-SSM exerted stronger antimutagenicity than alginic acid-SM ($p<0.05$). At the 2.5 mg/plate concentration, alginic acid-SSM exhibited 92% of antimutagenicity, while alginic acid-SM showed 54% of antimutagenicity. Table 2 showed the antimutagenic effect against MNNG, direct mutagen. Both of the alginic acids exhibited antimutagenic effect against MNNG and there was no difference in the activity between the two. At the 2.5 mg/plate, both of them inhibited mutagenicity more than 60% induced by MNNG. These results suggest that alginic acids-SM and SSM have antimutagenic effects on AFB_1 and MNNG. Especially alginic acid-SSM shows higher antimutagenicity than alginic acid-SM against AFB_1. In previous reports, the methanol extracts of common edible sea

Table 3. Effect on the cytotoxicity of alginic acid from sporophyll of sea mustard (SSM) in C3H/10T1/2 cells when treated with 3-methylcholanthrene (MCA, 10 μg/ml) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 1 μg/ml)[1]

Treatment	Concentration (mg/ml)	Cell colony	Cytotoxicity[2]
MCA(control)		33.3±6.4	1.00
MCA+ Alginic acid-SSM	0.1	47.3±4.0*	1.42
	0.2	60.0±1.0*	1.80
MNNG(control)		36.0±4.4	1.00
MNNG+ Alginic acid-SSM	0.1	57.5±7.8*	1.60
	0.2	64.5±3.5*	1.79

[1]In 24 hours after seeding, 2×10^3 cells/60 mm dish were cultured in serum-free Eagle's basal medium in the presence of various concentration of the above alginic acid-SSM and MCA, MNNG for 48 hours. Following the treatment, the cells were allowed to grow an additional 7 days in the medium supplemented with 10% FBS. Surviving colonies were fixed with methanol, stained and counted.

[2]$\frac{\text{Number of surviving colonies on treated dishes}}{\text{Number of surviving colonies on control dishes}}$

*Significantly different from the control at the $p<0.05$ level

weeds had antimutagenic effect on AFB_1 and MNNG (4,22). In particular, sporophyll of sea mustard showed the highest antimutagenic effect against AFB_1 and MNNG among the seaweeds. It is thought that the difference in the activity between the two depends on the composition of alginate. Alginates with higher content of β-D-manuronic acid (MM-block) showed higher antitumor activity than those with lower content (23). Chiharu et al. (24) found that alginates had binding capacity for the the mutagen, Trp-P-1, Glu-P-1, dimethylnitrosoamine. Therefore, alginate is expected to decrease the occurance of cancer or tumors induced by such mutagens. Alginate is assumed to bind the amino group of carcinogens at its carboxyl group and thus may decrease the occurrence of cancer in the intestine, even if the mutagens might be released in the stomach.

Since alginic acid-SSM exerted higher antimutagenicity than alginic acid-SM, we also investigated anticarcinogenic effect of the alginic acid-SSM using C3H/10T1/2 cells. C3H/10T1/2 cells have been widely used to study mechanism of neoplastic transformation in mammalian cells and as a target indicator cell system to screen industrial chemicals for carcinogenicity (20).

Table 3 shows an inhibitory effect of alginic acid-SSM on MCA and MNNG-induced cytotoxicities in C3H/10T1/2 cells. Alginic acid-SSM supressed the MCA and MNNG-induced cytotoxicity in C3H/10T1/2 cells as the dose-dependent manner. The 0.1 mg/ml and 0.2 mg/ml of alginic acid-SSM inhibited cytotoxicity induced by MCA and MNNG by 42%, 80% and 60%, 79%, respectively. Yamamoto and cowokers found that the powder or extract from some species of brown algae significantly decreased the incidence rate of chemically induced carcinogenesis (25,26). Dietary seaweeds decrease the absorption of chemical carcinogen, thereby minimize the toxicity and carcinogenicity of carcinogens (26).

The transformation inhibition test of alginic acid-SSM induced by MCA and MNNG was also investigated (Table 4). No spontaneous morphological transformation has been observed in C3H/10T1/2 cells. In the MCA and

Table 4. Inhibitory effects of the alginic acid from sporophyll of sea mustard (SSM) on the transformation of C3H/10T1/2 cells treated with 3-methylcholanthrene (MCA, 10 μg/ml) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 1 μg/ml)[1]

Treatment	Concentration (mg/ml)	Total number/dish			
		Type I foci	Type II foci	Type III foci	Type II+III foci
MCA(control)		6.5±0.7	7.5±0.7	3.0±1.4	10.5
MCA+ Alginic acid-SSM	0.1	3.5±0.7*	2.7±0.6*	0.5±0.7*	3.2*
	0.2	3.7±0.6*	1.5±0.7*	0.3±0.6*	1.8*
MNNG(control)		16.3±1.5	10.7±1.2	2.3±1.5	13.0
MNNG+ Alginic acid-SSM	0.1	6.7±0.6*	4.3±1.2*	1.3±1.2	5.6*
	0.2	5.0±2.6*	3.0±1.0*	0.3±0.6*	3.3*

[1]In 24 hours after seeding, C3H/10T1/2 cells were treated with MCA, MNNG and alginic acid-SSM for 48 hours, at which time a medium change was made. Medium with 10% FBS was changed twice a week until the cells reached confluence, then once a week. At 6 weeks, the transformation frequency was calculated.

*Significantly different from the control at the $p<0.05$ level

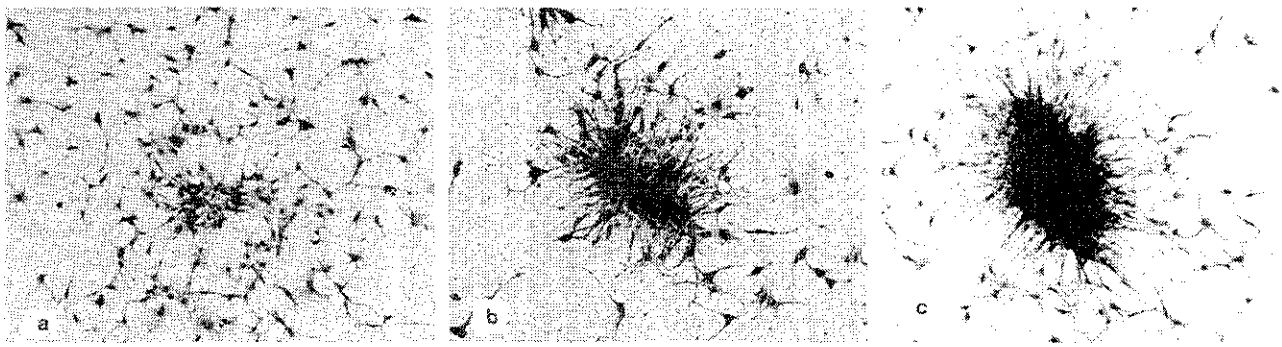

Fig. 1. Photomicrographs of various types of foci formed in the transformation test on C3H/10T1/2 cells treated with 10 μg/ml of 3-methylcholanthrene(×40)[1)]

[1)]The experimental procedure is the same as shown in Table 4.
a: Type I foci, b: Type II foci, c: Type III foci

MNNG-treated cultures, discrete dense foci were seen at 6 weeks, after confluence has been attained. Foci are defined as an area of increased cell density and/or altered cell morphology in a confluent monolayer. The foci are classified into 3 types. Type I is a focus composed of tightly packed cells (Fig. 1a), but it is not scored as malignantly transformed because these cells have not yet produced tumors after inoculation into irradiated C3H mice (20). Type II is a focus showing massive piling up into virtually opaque multilayers (Fig. 1b). Type III is a focus composed of highly polar, fibroblatic, multilayered criss-crossed arrays of densely stained. Type II and III foci are scored as malignantly transformed foci because a high percentage (50% of type II and 85% of type III) of these foci produce sarcomas after inoculation into irradiated C3H mice (20). The numbers of transformed foci (type II and III) decreased after treatment of alginic acid-SSM. As shown in Table 4, MCA-treated culture produced 10.5 foci of type II+III in C3H/10T1/2 cells. However, when alginic acid-SSM was treated at the concentration of 0.1 mg/ml and 0.2 mg/ml, only 3.2 and 1.8 foci of type II+III were observed, respectively ($p<0.05$). While MNNG-treated culture formed 13.0 foci, 0.1 mg/ml and 0.2 mg/ml of alginic acid-SSM treated culture produce 5.6 and 3.0, respectively ($p<0.05$) in the number of type II+III foci. Thus, alginic acid- SSM significantly reduced the transformation frequency (type II or III) produced by MCA and MNNG. It was published that sodium alginate from *Sagassum fulvellum* showed a considerable antitumor activity against various murine tumors by Fujihara and coworkers (12). Alginates and their components had the ability to stimulate immune response and the composition of MM- or GG- blocks in alginates may also be closely corelated with the activity.

In conclusion, this study showed that the alginic acid extracted from sporophyll of sea mustard had the antimutagenic activity on Ames test and anticarcinogenic effect in C3H/10T1/2 cells system. It is not known why the alginic acid-SSM inhibit chemically induced- carcinogenesis more effectively than alginic acid-SM. Therefore, futher study is needed to identify components and the mechanisms of the activity of alginic acid-SSM.

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