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Antimutagenic Effects of Linoleic Acid

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Abstract

In order to determine the effectiveness of linoleic acid(LA) to inhibit carcinogens/mutagens-induced mutagenesis, Ames test using *Salmonella typhimurium* TA100 and the SOS chromotest using *E. coli* PQ37, were carried out. The inhibitory effect of LA(1%) on the Ames mutagenicity test were 98%, 78% and 69% mediated by aflatoxin B_1(AFB_1), N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) and 4-nitroquinoline-1-oxide(4-NQO), respectively. LA exhibited a strong antimutagenic activity against indirect mutagen, AFB_1, whereas exhibited the same concentration of LA showed weaker inhibitory effects on direct mutagens of MNNG and 4-NQO than that of AFB_1. LA also reduced the SOS responses induced by MNNG and 4-NQO significantly. This result showed a possibility that LA can be a protective agent in the early step of cancinogenesis.

Key words: linoleic acid(LA), Ames test, SOS chromotest, antimutagenicity

INTRODUCTION

The essential fatty acids are the parents of two families: the omega 6 family and omega 3 family. The omega 6 essential fatty acid is called linoleic acid(LA). It is found in most seed oils. From LA, a healthy body makes a derivative called gamma-linolenic acid(γ-LA). γ-LA is present in human mother's milk. From γ-LA, the body makes dihomogamma-linolenic acid(DGLA). This is also found in mother's milk and it is the hormone-like prostaglandin 1(PG1) series. From DGLA, the body makes arachidonic acid(AA). AA is the parent of another group of hormone-like substances. the prostaglandin 2(PG2) series. The omega 3 essential fatty acid itself is called alpha-linolenic acid(α-LNA). From α-LNA, through cycloxygenase or lipoxygenase, the body makes a fatty acid which is called eicosapentanoic acid(EPA). EPA is the parent from which the body makes a group of hormone-like regulation substances called the prostaglandin 3(PG3) series.

The oil that contains the highest in omega 6s is safflower which contains 65%, and then corn oil 54%, sesame 45%, peanut 29%, almond 17% and olive 8% etc. The richest source of omega 3s is flax seed, whose oil contains 55~65%, perilla oil 60%, pumpkin seed oil 0~15%, and soybean oil 7~9%(1).

Deficiency of the omega 3s which keeps a handle on the production of deleterious PG2s, combined with an excess of omega 6s which pushes the arachidonic acid cascade to produce the PG2s unchecked, is one of the major imbalances in our fat nutrition. Biochemists have suggested that the ideal ratio of omega 3s to omega 6s should be 1 : 4 or 1 : 5(2).

It has been established that diets high in fat can significantly enhance mammary tumorigenesis in rats, unsaturated fat being more effective than saturated fat(3,4). Epidemiological studies have shown that the Japanese with their low-fat diet have a very low mortality from breast cancer(5,6). However, there is a growing body of evidence that the essential fatty acids, linoleic acid, alpha-linolenic acid and several of their metabolites, including gamma-linolenic acid, arachidonic acid and some of the prostaglandins, supress the proliferation rate of a variety of malignant cell lines in culture(7,8). These observations have been extended by showing that linoleic acid and its metabolites not only inhibit their growth but selectively kill human breast, lung and prostate cancer cells without damaging normal fibroblasts and animal kidney cells(9). The antineolplastic effects of essential fatty acids and their metabolites have been extensively reviewed by Begin(10). Zhu et al.(11) reported linoleic acid significantly prolonged the life span of Ehrlich ascites carcinoma-bearing mice and inhibited the growth of Ehrlich solid carcinoma in mice compared with the findings in untreated control mice. One such study was done by Siegel et al.(12), who

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reported that linoleic(18 : 2)-linolenic(18 : 3) acid combinations prolonged the life span of tumor-bearing rats significantly.

In the present study, the effect of linoleic acid was investigated for its possible antimutagenic activity on the mutagenesis induced by aflatoxin B_1(AFB_1), N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) and 4-nitroquinoline-1-oxide(4-NQO) in the *Salmonella typhimurium* TA100 in Ames test. The antimutagenic activity was also evaluated on the mutagens such as MNNG and 4-NQO mediated mutagenicities in SOS chromotest system.

MATERIALS AND METHODS

Materials

Linoleic acid(LA) of 99% purity was obtained from Sigma Chemical Co.(St. Louis, Mo., USA) and dissolved in dimethylsulfoxide(DMSO, Aldrich Co., Milwaukee, WI, USA) before it was used.

Mutagens/Carcinogens

Aflatoxin B_1(AFB_1), N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) and 4-nitroquinoline-1-oxide(4-NQO) were employed as mutagens/carcinogens for this study.

AFB_1 was purchased from Sigma Chemical Co.(St. Louis, Mo., USA) and an appropriate amount was dissolved in DMSO. MNNG and 4-NQO were obtained from Aldrich Chemical Co.(Milwaukee, WI, USA). The mutagens were dissoved in distilled water and 95% ethanol, respectively.

Ames mutagenicity test

Bacterial strains:

Salmonella typhimurium TA100, histidine requiring mutants was provided by Dr. B. N. Ames, Univ. of California, Berkeley, CA, USA and was maintained as described by Marons and Ames(13). The genotypes of tester strain were checked routinely for their histidine requirement, deep rough(*rfa*) character, UV sensitivity (*uvr* B mutation) and for the presence of R factor.

S9 fraction and S9 mix:

Sprague-Dawley male rats were injected intraperitoneally with Aroclor 1254 dissovled in corn oil(500mg/kg of body wt.). After five days of the injections, the rats were sacrificed, livers were removed and minced in 0.15M KCl, and then homogenized with a Potter-Elvehjem apparatus. The homogenates were centrifuged at 9000g for 10min in a refrigerated centrifuge and the supernatant S9 fraction was distributed in 1.8~2.0ml portions in Nunc tubes, and stored at -80°C until used for mutagenic studies. In order to prepare the S9 mix, S9 fraction was thawed immediately before being used for the preparation of S9 mix. Ten percent of S9 fraction in S9 mix was used as S9 mix for the experiment.

Antimutagenicity test:

Plate incorporation test was performed to determine the mutagenic activities of MNNG and 4-NQO(13). A modified plate incorporation test(14) in which 30min liquid preincubation of the organisms with the test compounds was employed to determine the antimutagenic effects of LA on mutagenesis of AFB_1. In the preincubation test, 0.5ml of S9 mixture was distributed in sterile capped tubes in ice bath and then 0.1ml of testers from overnight culture($1\sim2\times10^9$cells/ml), 50μl of test compounds(0.001~5.0% LA solution) and 50μl of mutagens were added. The tubes were votexed gently and preincubated at 37°C for 30min. Two ml of the top agar in each tube kept at 45°C were added and votexed for 3 seconds. The resulting entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48hrs and then the revertant bacterial colonies on each plate were counted. Dose response tests of the mutagens on the tester strains were carried out (14) to determine the regions of revealing mutagenicity

Table 1. Effect of linoleic acid(LA) on the mutagenicity in the presence of S9 mix(+S9) and phosphate buffer(-S9) in *Salmonella typhimurium* TA100

Sample	Concentration(%)	Revertants/plate
Spontaneous		100±4
LA	0.001	118±3
(+S9)	0.01	111±6
	0.1	101±8
	0.5	110±4
	1.0	97±2
	5.0	149±6
Spontaneous		97±7
LA	0.001	86±3
(-S9)	0.01	86±1
	0.1	99±4
	0.5	93±1
	1.0	88±2
	5.0	100±3

induced by the mutagens. Toxicity test for the different levels of the LA was carried out and LA samples for the toxicity test in this study did not show any toxicity to the tester strain. The LA samples(0.001~5.0%) also did not show any mutagenicities in the presence or in the absence of S9 mix(Table 1).

SOS chromotest

The modified assay method described by Quillardet and Hofnung(15), and Baik and Ham(16) was employed. 50μl of frozen stock of *E. coli* PQ37 was added to 50 ml/L medium and incubated in shaking water bath at 37°C overnight, then it was inoculated to the 5ml/L medium at 37°C and incubated in shaking water bath for 2 hrs until the absorbance at 660nm reached 0.3~0.4, the active culture. The obtained active culture was diluted to 10 folds with L medium. 100μl of the diluted culture distributed to the 2 series in the wells of 96 well plate. 20μl of LA that was treated with mutagen(10μl LA+10μl mutagen) were added, and then the SOS response was induced at 37°C for 90min. 100μl of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and 100μl of PNPP(*p*-nitrophenyl phosphate disodium) were added to each set of the wells to determine the activities of β-galactosidase(β-G) and akaline phosphatase(A-P), respectively.

After the color development for 20min, 100μl of 1.5M Na_2CO_3 and 50μl of 1M HCl were added to stop the color developments of β-G and A-P, respectively. After 5min, 50μl of 2M Tris buffer was added to the A-P to neutralize the HCl and then determine the SOS responses at 420nm. The SOS responses of the samples were calculated by the method of Miller(17).

RESULTS AND DISCUSSION

Effect of LA on antimutagenesis in Ames test

Toxicity test for the different levels of LA was also carried out and the LA samples did not show any toxicity to the tester strain.

From the dose response test of AFB_1, 1μg of AFB_1 per plate was employed to evaluate the antimutagenic effect of linoleic acid(LA) on the AFB_1 induced mutagenesis. As shown in Table 2, at the addition of 0.5% LA sample to the system, the inhibition rate for AFB_1 was 91% while the inhibition rate showed 98% at 1% LA addition. A similar inhibitory effect was observed

Table 2. Effect of linoleic acid(LA) on the mutagenicity of aflatoxin B_1(AFB_1, 1μg/plate) in *Salmonella typhimurium* TA100

Treatment(%)	Revertant/plate	Inhibition rate(%)
Spontaneous	106±13[1)]	
AFB_1(Control)	1017±69	
AFB_1+LA 0.001	825±37	21
0.01	708±14	34
0.1	533±25	53
0.5	185± 6	91
1.0	125± 2	98
5.0	112± 3	99

[1)]The values are means of 3 replicates±SD

with the increased concentrations of LA. We also compared ts to determine whether this antimutagenic effect is also effective to other carcinogens/mutagens, such as direct mutagens of MNNG and 4-NQO. Direct mutagens of MNNG and 4-NQO exhibited strong mutagenic activity toward TA100 strain without metabolic activating system. The dose response tests were performed with the plate incorporation test recommended by Ames et al.(18) and Marons and Ames(13). 0.45μg of MNNG/plate resulted in revertant numbers of 810±32 and 0.15μg of 4-NQO/plate revealed 1405±21 of revertants. These concentrations were employed to study antimutagenic effects of LA toward these mutagens. Table 3 showed that 85% of MNNG induced mutagenicity was blocked at the concentration of 5% LA. The was added to the test system. In addition, 69% and 75% of the mutagenicity induced by 4-NQO were inhibited at concentrations of 1% and 5% LA, respectively(Table 4).

Thus, it can be concluded that LA showed strong antimutagenic activity not only to AFB_1 but also to other known mutagens/carcinogens, MNNG and 4-NQO.

Nakahara(19) observed increases in the resistance of mice to several transplantable tumors following the injec-

Table 3. Effect of linoleic acid(LA) on the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.45μg/plate) in *Salmonella typhimurium* TA100

Treatment(%)	Revertant/plate	Inhibition rate(%)
Spontaneous	93±12[1)]	
MNNG(Control)	810±32	
MNNG+LA 0.01	414±59	55
0.1	361±32	63
0.5	296±18	72
1.0	251±23	78
5.0	199±37	85

[1)]The values are means of 3 replicates±SD

Table 4. Effect of linoleic acid(LA) on the mutagenicity of 4-nitro-quinoline-1-oxide(4-NQO, 0.15μg /assay) in *Salmonella typhimurium* TA100

Treatment(%)	Revertant/plate	Inhibition rate(%)
Spontaneous	102±20[1)]	
4-NQO(Control)	1425±21	
4-NQO+LA 0.01	641±35	60
0.1	604± 5	62
0.5	562±36	65
1.0	506±12	69
5.0	438±11	75

[1)]The values are means of 3 replicates±SD

tion of olive oil and unsaturated fatty acids, such as oleic, linoleic, and linolenic acid. Hayatsu et al.(20) suggested that oleic and linoleic acid decreased significantly the mutagenicities mediated by indirect mutagens of aflatoxin B_1(AFB_1), 3-amino-1-methyl-5H-pyrido[4,3-b]indole Trp-p-1) and benzo[a]pyrene. Tolnai and Morgan(21) showed that unsaturated fatty acid had antitumor activity *in vitro* against three different mouse ascites tumors, and that the compounds with the most marked antitumor activity were linoleic and linolenic acid.

Effect of LA on antimutagenesis in SOS chromotest

When enzyme activity of β-galactosidase and alkaline phosphatase were assayed, the activity of β-galactosidase had to be compared with alkaline phosphatase as a parameter by a given dose, respectively.

As shown in Table 5, we observed LA had the strong inhibitory effect on SOS response. Especially, 86% and 96% of SOS response induced by MNNG were blocked

Table 5. SOS response of N-methyl-N'-nitro-N-nitrosoguanidine(MNNG, 0.07μg/assay) treated with different levels of linoleic acid(LA)

Sample(%)	β-unit[1)]	a-unit	R[2)]	Inhibition(%)
Spontaneous	11.7	9.3	1.25	
MNNG(Control)	13.9	9.5	1.46	
MNNG+LA 0.001	13.8	9.5	1.44	5
0.01	13.8	8.8	1.56	5
0.1	12.7	8.6	1.47	55
0.5	12.6	8.7	1.47	57
1.0	11.9	9.6	1.23	86
5.0	11.6	10.5	1.10	96

[1)]Enzyme unit $= \frac{1000 \times A_{420}}{t}$

[2)]$R = \frac{\beta\text{-Galactosidase units}}{\text{Alkaline phosphatase units}} = \frac{A_{420}B \times tP}{A_{420}P \times tB}$

Table 6. SOS response of N-nitro-quinoline-1-oxide(4-NQO, 0.02μg/assay) treated with different levels of linoleic acid(LA)

Sample(%)	β-unit[1)]	a-unit	R[2)]	Inhibition(%)
Spontaneous	10.9	6.1	1.79	
4-NQO(Control)	14.6	6.4	2.30	
4-NQO+LA 0.001	14.4	6.3	2.28	9
0.01	14.3	6.2	2.29	9
0.1	13.3	6.0	2.20	37
0.5	12.9	6.0	2.20	46
1.0	11.7	6.8	1.70	80
5.0	11.0	10.9	1.00	97

[1)]Enzyme unit $= \frac{1000 \times A_{420}}{t}$

[2)]$R = \frac{\beta\text{-Galactosidase units}}{\text{Alkaline phosphatase units}} = \frac{A_{420}B \times tP}{A_{420}P \times tB}$

by adding 1% and 5% concentrations of LA to the well respectively, and a similar inhibitory effect was found by the increased concentrations of LA. In order to reconfirm the effect of LA on SOS response, another mutagen of 4-NQO was tested. 80% of the mutagenicity induced by 4-NQO was blocked by the addition of 1% of LA and 97% of the mutagenicity mediated by 4-NQO was inhibited by 5% of LA(Table 6).

From the above studies, our results showed LA had strong antimutagenic activity in the mutagenesis induced by AFB_1, MNNG and 4-NQO in both Ames test and SOS chromotest systems. Park et al.(22) reported that one of the major antimutagenic compounds found in doenjang(Korean soy paste) was linoleic acid. They also indicated that the LA in doenjang extracts exhibited strong antimutagenic activity on wide range of the carcinogens.

The antineoplastic properties of many essential polyunsaturated fatty acids(PUFA), such as linoleic acid and its metabolites, are known. Linoleic acid inhibited *in vitro* growth of all three malignant human colon adenocarcinoma cell lines(23). Ha et al.(24,25) also reported that isomeric derivates of LA which were isolated from grilled ground beef were effective in partially inhibiting the inhibition of mouse epidermal carcinogenesis by 7,12-dimethylbenzo(a)anthrancene and forestomach tumorogenesis induced by benzo(a)pyrene (26,27). LA decreased growth of various human cancer cells(28) and transplanted tumors in mice(29). LA also enhanced the phagocytic activity and NBT reduction of pertional phagocyte of mice(30).

This results showed that the inhibitory action of LA can be the physical trapping of the lipophilic mutagens by the phospholipid bilayer structure formed by the fatty acids. It is known that bilayer vesicles can be produced from unsaturated fatty acids but not from saturated ones(31,32). Other mechanisms, such as chemical reactions between the inhibitors and the mutagens in early step of carcinogenesis, are also a possibility and must be explored by further work.

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