RESEARCH COMMUNICATION

Inhibition of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine-induced Duodenal Tumorigenesis in Mice by Whole-leaf *Aloe arborescens* Miller var. *natalensis* Berger

Takeshi Chihara[1], Kan Shimpo[1], Masanori Shinzato[2], Hidehiko Beppu[1], Takaaki Kaneko[1], Chikako Ida[1], Kaoru Kawai[1], Iwao Hirono[3], Mikihiro Shamoto[2], Toshiharu Nagatsu[2], Hiroshi Kuzuya[1]

Abstract

We examined the modifying effects of freeze-dried whole-leaf *Aloe arborescens* Miller var. *natalensis* Berger (designated as 'ALOE') on *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)-induced duodenal tumorigenesis in C57BL/6 mice. Experiment 1: Male mice were given ENNG in drinking water for the first 4 weeks, and then 10% ALOE in basal diet for 16 weeks. Experiment 2: Female mice were given ENNG for 5 weeks, and then 5%, 1% or 0.2% ALOE in the diet were given for 15 weeks. In Experiment 1, the tumor incidence and tumor multiplicity (tumors per mouse) of the duodenum in the ENNG + 10% ALOE group were significantly decreased compared with that in the ENNG alone group. Erythrocyte polyamine levels in the ENNG + 10% ALOE group were also significantly decreased. In Experiment 2, the incidence of duodenal tumors in the ENNG + 5% ALOE group were significantly decreased compared with that in the ENNG alone group. These results indicated that ALOE, especially at 10% in the diet, inhibits ENNG-induced duodenal tumorigenesis in mice.

Key words: Aloe arborescens Miller var. natalensis Berger - anti-tumor promotion - mouse duodenal tumorigenesis - erythrocyte polyamines - N-ethyl-N'-nitro-N-nitrosoguanidine

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Introduction

In Japan, *Aloe arborescens* Miller var. *natalensis* Berger (Japanese name Kidachi aloe) has been used, not only as a peptic and a laxative in family medicine, but also as an ingredient in health foods (Fujita, 1994; Yagi, 1993; Yamamoto et al., 1995). Our laboratory has previously reported various pharmacological and therapeutic activities of *A. arborescens*, especially in extracts with high molecular weight components (Fujita, 1993; Koike et al., 1995a, 1995b). We also previously demonstrated that dietary freeze-dried whole-leaf *A. arborescens* inhibited induction of preneoplastic focal lesions in the rat liver (Tsuda et al., 1993). In the present report, we describe the inhibitory effects of dietary freeze-dried whole-leaf *A. arborescens* on *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)-induced duodenal tumorigenesis in mice.

Materials and Methods

Materials

Male (8 weeks old; for Experiment 1) and female C57BL/6 mice (8 weeks old; for Experiment 2) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept in groups of four or five in plastic cages on woodchip bedding and fed on basal diet, Oriental MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan), in an animal facility controlled at a temperature of 23±5°C , 60±5 % humidity, and with a 12-h

[1]Fujita Memorial Institute of Pharmacognosy, Fujita Health University, Hisai, Mie 514-1296; [2]Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192; [3]School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Correspondence to: Dr. Kan Shimpo, Fujita Memorial Institute of Pharmacognosy, Fujita Health University, Hisai, Mie 514-1296, Japan. Tel: +81 59 252 1010; Fax: +81 59 252 0710 E-mail: shimpo@fujita-hu.ac.jp

light/dark cycle. ENNG (Nacalai Tesque Co. Ltd., Kyoto, Japan) was dissolved in distilled water at 100 mg/l. The solution was freshly prepared three times a week and protected from light by storage in black bottles. Freeze-dried powder of whole-leaf *Aloe arborescens* (abbreviated as ALOE) was kindly provided by Yurika Co. Ltd. (Hisai, Japan). The powder was finely pulverized with an Oster Power Blender (Osaka Chemical Co., Ltd., Osaka, Japan) and added to the basal diet at 10% for Experiment 1 and at 5%, 1%, or 0.2% for Experiment 2, and throughly mixed using a ball mill. All other chemicals were of the highest grade available and were obtained commercially.

Experimental protocol

Animal studies were conducted in accordance with our institutional guidlines. The experiments on ENNG-induced duodenal tumorigenesis in C57BL/6 mice were performed according to the procedures of Fujita et al. (1989) and Huang et al. (1994) with slight modifications (Fig. 1). In Experiment 1, mice in Groups 1 and 2 were given basal diet and ENNG (100 mg/l) as drinking water *ad libitum* for the first 4 weeks. Then, these groups were shifted to tap water, and Groups 1 and 2 were given basal diet and 10% ALOE in basal diet *ad libitum* for 16 weeks, respectively. Mice in Groups 3 and 4 were given basal diet for the first 4 weeks, and then basal diet and 10% ALOE in basal diet *ad libitum* for 16 weeks, respectively. Mice in these 2 groups were given tap water throughout the entire experiment. In Experiment 2, mice in Groups 1, 2, 3 and 4 were given basal diet and ENNG (100 mg/l) as drinking water *ad libitum* for the first 5 weeks. Then, these groups were shifted to tap water, and Groups 1, 2, 3 and 4 were given basal diet, or 5%, 1% or 0.2% ALOE in basal diet *ad libitum* for 15 weeks, respectively. Mice in Groups 5, 6, 7 and 8 were given basal diet for the first 5 weeks, and then given basal diet, or 5%, 1% or 0.2% ALOE in basal diet *ad libitum* for 15 weeks, respectively. Mice in these 4 groups were given tap water throughout the entire experiment. Both Experiments 1 and 2 were terminated 20 weeks after the start of ENNG treatment. All mice were anesthetized with diethyl ether and exsanguinated through the heart into heparin-coated syringes. The tongue, esophagus, stomach, small intestine and large intestine were removed together. The duodenum was cut up to 4 cm distal from the pyloric ring, fixed with 1% formalin in phosphate buffered saline (PBS) for about 5 min, and opened longitudinally. The contents were flushed with PBS, and the duodenal mucosa was examined stereomicroscopically. The tumors in the duodenal mucosa were observed as polyp-like enlarged villi or crater-form tumors among the normal villi. The tissues were fixed in 10% formalin and embedded in paraffin wax, and stained with hematoxylin-eosin for histological examination. Histologically, the tumors in the duodenum consisted of atypical glands with mitotic activity, and were adenomas or adenocarcinomas.

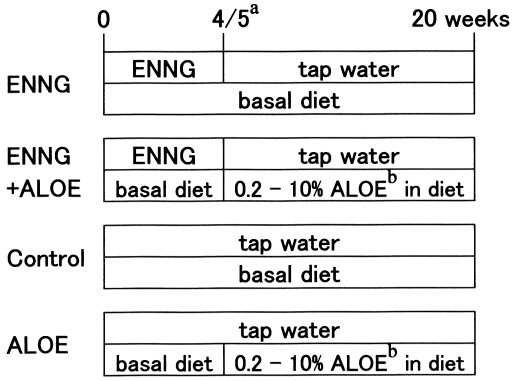

Figure 1. Experimental schedule.

[a]4 weeks (for Experiment 1) or 5 weeks (for Experiment 2). [b]10% ALOE in basal diet (for Experimental 1), or 0.2, 1, or 5% ALOE in basal diet (for Experiment 2).

Determination of erythrocyte polyamine levels

Erythrocyte polyamines were assayed by a slight modification of the method of Gerbaut (1991). Briefly, heparinized blood samples were centrifuged at 600 x g for 10 min at 4 °C. After removal of plasma and the buffy coat layer, packed erythrocytes were hemolysed with water, and extracted into 10% perchloric acid. The polyamines in the cell extracts were derivatized with dansyl chloride at 70°C for 10 min and extracted with cyclohexane. The cyclohexane phase was dried. The residues were redissolved in 70% acetonitrile and separated on a Puresil C_{18} column (5 μm, 4.6 x 250 mm; Nihon Waters K.K., Tokyo, Japan), with an acetonitrile-water gradient as the mobile phase. Eluted dansyl polyamines were detected with a fluorescence detector.

Statistical analysis

Statistical analyses of the final body weights in Experiments 1 and 2, and tumor multiplicity (average number of tumors per mouse) in Experiment 2 were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Tumor incidence (percentage of tumor-bearing mice) in Experiments 1 and 2, and tumor multiplicity in Experiment 1 were compared by Fisher's exact test, and by Welch's corrected *t* test, respectively. Erythrocyte polyamine levels were expressed as median values and ranges, and compared with the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn's multiple comparisons test. These statistical procedures were performed with InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

Body weight

Final body weights in Experiments 1 and 2 are shown in Table I. In Experiment 1, final body weights in Group 2 were significantly lower than those in Group 1 ($p<0.01$), and those in Group 4 were also lower, albeit not significantly,

Table 1. Effects of ALOE on ENNG-induced Mouse Duodenal Tumorigenesis

Group/Treatment	No. of mice	Body wt[a] (g)	Tumor incidence (%)[b] AD	ADC	Total	Tumor multiplicity[c] AD	ADC	Total
Experiment 1								
1/ ENNG + basal diet	18	37.6±1.2	39	22	44	0.44±0.15	0.39±0.23	0.83±0.31
2/ ENNG + 10%ALOE	20	30.7±0.6[d]	0[f]	15	15[g]	0[i]	0.15±0.08	0.15±0.08[j]
3/ Basal diet	5	34.9±1.6	0	0	0	0	0	0
4/ 10% ALOE	5	30.2±0.9	0	0	0	0	0	0
Experiment 2								
1/ ENNG + basal diet	34	29.5±0.6	41	15	47	0.56±0.13	0.15±0.06	0.71±0.16
2/ ENNG + 5% ALOE	30	25.8±0.3[d]	7[f]	17	17[h]	0.13±0.09[e]	0.33±0.17	0.47±0.24
3/ ENNG + 1% ALOE	30	28.7±0.5	30	23	37	0.43±0.14	0.30±0.11	0.73±0.21
4/ ENNG + 0.2% ALOE	30	29.4±0.5	33	23	47	0.40±0.11	0.40±0.15	0.80±0.22
5/ Basal diet	5	28.6±0.8	0	0	0	0	0	0
6/ 5% ALOE	5	26.3±0.4	0	0	0	0	0	0
7/ 1% ALOE	5	27.4±1.6	0	0	0	0	0	0
8/ 0.2% ALOE	5	28.9±1.4	0	0	0	0	0	0

Abbreviations: AD, adenoma; ADC, adenocarcinoma; [a]Body weight at killing (mean±SE); [b]Percentage of tumor-bearing mice; [c]Average number of tumors per mouse (mean±SE); [d,e]Significantly different from the corresponding Group 1 by one-way ANOVA with Dunnett's test ([d]$p<0.01$, [e]$p<0.05$); [f-h]Significantly different from the corresponding Group 1 by Fisher's exact test ([f]$p<0.005$; [g]$p<0.05$; [h]$p<0.01$); [i,j]Significantly different from Group 1 by Welch's corrected t test ([i]$p<0.01$, [j]$p<0.05$).

than those of the control group (Group 3). In Experiment 2, final body weights in Group 2, but not those in Groups 3 or 4, were significantly lower than those in Group 1 ($p<0.01$). The daily food consumption was similar between control and all treatment group in Experiments 1 and 2 (data not shown). Therefore, the administration of ALOE mainly resulted in a reduction of body weight in treated mice.

Duodenal tumors induced by ENNG

The effects of ALOE on ENNG-induced mouse duodenal tumorigenesis are summarized in Table 1. In Experiment 1, the incidences of adenomas, adenocarcinomas and total tumors (adenomas + adenocarcinomas) in Group 1 were 39%, 22% and 44%, respectively. The incidences of adenomas and total tumors in Group 2 were significantly lower than those in Group 1 ($p<0.005$ or $p<0.05$). The multiplicities of adenomas, adenocarcinomas and total tumors in Group 1 were 0.44±0.15, 0.39±0.23 and 0.83±0.31, respectively. The multiplicities of adenomas and total tumors in Group 2 were significantly lower than those in Group 1 ($p<0.01$ or $p<0.05$). In Experiment 2, the incidences of adenomas, adenocarcinomas and total tumors in Group 1 were 41%, 15% and 47%, respectively. The incidences of adenomas and total tumors in Group 2 were significantly lowered than those in Group 1 ($p<0.005$ or $p<0.01$). The multiplicities of adenomas, adenocarcinomas and total tumors in Group 1 were 0.56±0.13, 0.15±0.06 and 0.71±0.16, respectively. The multiplicity of adenomas in Group 2 was significantly lower than that in Group 1 ($p<0.05$). Diets containing 1% and 0.2% ALOE (Groups 3 and 4, respectively) showed no effect in this experimental system. No tumors were found in animals that did not receive ENNG in Experiments 1 and 2.

Erythrocyte polyamine levels

Table 2 shows erythrocyte polyamine levels of mice in Experiment 1. Erythrocyte spermidine levels in Group 1 were significantly greater than those in the control group (Group 3) ($p<0.01$). Erythrocyte spermine levels in Group 1 were also greater, although not significantly, than those in Group 3. Erythrocyte spermidine levels in Group 2 were significantly lower than those in Group 1 ($p<0.001$), and erythrocyte spermine levels in Group 2 were also significantly lower than those in Group 1 ($p<0.001$). In Experiment 2, erythrocyte spermidine and spermine levels in Group 1 were siginificantly greater than those in the control group (both $p<0.05$). However, these polyamine levels in Groups 2, 3 and 4 were not significantly lower than those in Group 1 (data not shown). These results indicated that administration of diet with 10% ALOE prevented the increases in erythrocyte polyamine level by ENNG-induced duodenal tumorigenesis in mice.

Table 2. Erythrocyte Polyamine Levels of Mice in Experiment 1

Group/Treatment	No.	Spermidine	Spermine
1/ ENNG + basal diet	18	80.7 (47.6-187.9)[a]	8.3 (4.3-19.2)[a]
2/ ENNG + 10%ALOE	20	50.3 (33.2-100.7)[b]	5.4 (2.9-82.9)[b]
3/ Basal diet	5	43.7 (33.1-71.0)[c]	5.7 (3.4-8.9)
4/ 10% ALOE	5	47.5 (39.5-68.2)	5.3 (2.9-7.5)

[a]Data are given as medians, with the range given in parentheses (nmol/ml packed erythrocytes); [b,c]Significantly different from Group 1 by Kruskal-Wallis test with Dunn's test ([b]$p<0.001$, [c]$p<0.01$).

Discussion

The ENNG-induced mouse duodenal tumorigenesis model was developed by Matsuyama et al. (1975). Fujita et al. (1989) used this model to examine the inhibitory effect on tumor promotion of (-)-epigallocatechin gallate in the upper gastrointestinal tract. In this model system, the tumors were induced within a shorter time in the duodenum of C57BL/6 mice than in other parts of the digestive tract, and the tumors were histologically demonstrated to be adenocarcinomas (Fujita et al., 1989). In the present study, we therefore examined the effects of ALOE on duodenal tumorigenesis induced by ENNG in C57BL/6 mice as the first step of tumorigenesis in the upper gastrointestinal tract. In Experiment 1, we demonstrated that 10% ALOE in the diet significantly inhibited both tumor incidence and tumor multiplicity in ENNG-induced duodenal tumorigenesis in male C57BL/6 mice, although the final body weights were significantly decreased.

In the present experiment, the tumor incidence of the ENNG-alone group was 44%. Our data were lower than the tumor incidence (63%) reported by Fujita et al. (1989). However, our results were similar to those (45%) of Huang et al. (1994). Ho et al. (1995) reported that female C57BL/6 mice were very susceptible to duodenal carcinogenesis following oral administration of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). Therefore, we gave ENNG to female C57BL/6 mice for 5 weeks for the initiation phase in Experiment 2. However, the incidence of duodenal tumors in the ENNG-alone group in this experiment (47%) was not different from that in Experiment 1. In this experiment, we examined the effects of lower levels of ALOE (5%, 1% or 0.1% in the diet) on duodenal tumorigenesis in mice. Feeding with 5%ALOE significantly decreased the tumor incidence, although the final body weights were also significantly decreased. However, feeding with 1% or 0.2% ALOE had no effect. To summarize the results in Experiments 1 and 2, inclusion of ALOE at 10% or 5% in the diet during the post-initiation phase exerted inhibitory effects on tumor promotion in the ENNG-induced duodenal tumorigenesis model in mice.

Most previous studies indicating the active components of various Aloe species including *A. arborescens* to have anticancer effects used transplanted tumor cells and not chemically induced tumors in experimental animals (for reviews, see Capasso et al., 1998; Reynolds and Dweck, 1999). However, we recently examined the effects of ALOE on chemically induced carcinogenesis models in rodents. Dietary administration of ALOE exerted significantly inhibitory effects on the induction of preneoplastic focal lesions in the rat liver (Tsuda et al.,1993), and azoxymethane-induced aberrant crypt foci, putative preneoplastic lesions, in the rat colorectum (Shimpo et al., 1998b). It was suggested that ALOE might have a chemopreventive effect against these types of carcinogenesis at least in the initiation phase, since feeding with ALOE during this phase inhibited the development of these two preneoplastic lesions. However, in the present study, feeding of ALOE was started when ENNG administration was completed. Thus ALOE is considered to act during the tumor promotion (post-initiation) stage. We also reported that topical application of acetone and ethylacetate extracts prepared from ALOE markedly inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in mouse skin initiated with 7,12-dimethylbenz[a]anthracene (DMBA) (Shimpo et al., 1996, 1998a). HPLC analysis of these extracts indicated that these effects were due to phenolic compounds such as barbaloin, isobarbaloin and aloenin, which are abundant in ALOE (Shimpo et al., 1996, 1998a). In fact, topical application of barbaloin (purity 97% , Sigma B6906; prepared from *Aloe barbadensis* Miller leaves) tended to suppress TPA-promoting activity (Shimpo et al., 1996). Fahim et al. (1993a, 1993b, 1997) also reported the antitumor activity of aloin (barbaloin) in experimental tumor models. In addition, *A. arborescens* was already found to have free radical scavenging action (Sato et al., 1990; Kato and Arai, 1997; Beppu et al., unpublished data) and anti-inflammatory activity (Yagi et al., 1987; Fujita, 1993; Yamamoto et al., 1991). Thus, these active compounds of ALOE might have been responsible for the inhibition of tumor promotion in the present tumorigenesis model.

In the present study, we measured erythrocyte polyamine level as a marker of cell proliferation, and we found that feeding with ALOE (10% in diet) significantly suppressed the increase in erythrocyte polyamine level in ENNG-treated mice in Experiment 1. We previously demonstrated the significance of erythrocyte polyamine determination in various carcinogenic models (Shimpo et al., 1984, 1996; Kawai et al., 1992a, 1992b). Tanaka et al. (1998) also measured blood polyamines as intermediate biomarkers in chemopreventive studies, and they showed that total levels in blood were well correlated with those in the target organ (colonic mucosa). Thus, the results of the present study on erythrocyte polyamines also provide support for a potential of ALOE to reduce the development of tumors.

In conclusion, we here found that post-initiation-phase feeding of ALOE inhibited both the development of ENNG-induced duodenal tumors and the increase of erythrocyte polyamine levels in C57 BL/6 mice.

Acknowledgements

This paper is dedicated to the late Dr. Keisuke Fujita (the Founding President and Professor of the Fujita Health University), who died on June 11, 1995. We also would like to express our appreciation to Yurika Co. Ltd. for providing the freeze-dried powder of whole-leaf *Aloe arborescens*. This study was supported in part by a Grant-in-Aid from Fujita Health University.

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Personal Profiles: Overleaf

Personal Profile: Takeshi Chihara

1986, School of Health Sciences, Fujita Health University, BSMedTech
1998, PhDMedSci
1986, Research Associate; 1989, Instructor, Institute for Comprehensive Medical Science, Fujita Health University
1992-94, Visiting Scientist, University of Florida College of Medicine
1998, Instructor, Fujita Memorial Institute of Pharmacognosy, Fujita Health University

Personal Profile: Kan Shimpo

1975, School of Health Sciences, Fujita Health University, BSMedTech
1985, PhDMedSci
1975, Research Associate; 1978, Instructor; 1985; Assistant Professor, Institute for Comprehensive Medical Science, Fujita Health University
1987-89, Visiting Scientist, University of Florida College of Medicine
1998, Assistant Professor; 2000, Associate Professor, Fujita Memorial Institute of Pharmacognosy, Fujita Health University

The **Fujita Memorial Institute of Pharmacognosy** (Director, Professor Hiroshi Kuzuya) is striving further to promote research on the pharmacological effects as well as on the therapeutic influence of Kidachi aloe (*Aloe arborescens* Miller var. *natalensis* Berger) as a healthful functional food, which project was started by the late Professor Keisuke Fujita, the Founding President of Fujita Health University. Drs. Shimpo and Chihara and their coworkers, especially, have been studying the anticancer effects of Kidachi aloe in various animal models of cancer development.

Drs. Takeshi Chihara (left) and Kan Shimpo (right) in the Aloe field of their Institute.