RESEARCH COMMUNICATION

Inhibition of Azoxymethane-induced DNA Adduct Formation by *Aloe arborescens* var. *natalensis*

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Abstract

To clarify the possible mechanisms of inhibition of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in the rat colorectum by freeze-dried whole leaves of *Aloe arborescens* var. *natalensis* (Kidachi aloe) (hereinafter referred to as ALOE) and commercial crude aloin (Sigma A-0451; from Curacao aloe) (hereinafter ALOIN), we studied the effects of ALOE and ALOIN on the formation of AOM-induced DNA adducts (O^6-methylguanine; O^6-MeG) in rats. Male F344 rats (4 weeks old) were fed a basal diet, or experimental diets containing 5%ALOE or 0.25%ALOIN for 5 weeks. All rats were injected s.c. twice with 15 mg/kg AOM, once at the end of week 1, and once at the end of week 2. The animals were sacrificed 6 hours after the second injection to analyze DNA adducts (O^6-MeG) in the colorectum. Dietary administration of ALOE significantly inhibited the O^6-MeG levels (50% reduction) compared with controls, whereas the O^6-MeG levels in the ALOIN-fed rats showed a tendency to decrease (by 30%), although not significantly. In this study, we also measured the enzyme activity and mRNA level of cytochrome (CYP) 2E1, known to be responsible for the activation of AOM, in rat liver. ALOE-fed rats showed significantly reduced CYP2E1 enzymatic activity (27% reduction) compared with controls. On the other hand, the activity in ALOIN-fed rats tended to decrease by 11%, although not significantly. The CYP2E1 mRNA levels in ALOE- and ALOIN-fed rats were slightly reduced (9.7% and 5.2%, respectively). These results may explain, at least in part, the previously observed inhibitory effects of ALOE and ALOIN, especially ALOE on AOM-induced ACF formation in the rat colorectum.

Key Words: *Aloe arborescens* var. *natalensis* (Kidachi aloe) - DNA adducts (O^6-methylguanine) - cytochrome P450 2E1 - azoxymethane

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Introduction

Aloe arborescens Miller var. *natalensis* Berger (Japanese name Kidachi aloe) is widely used in Japan, not only as a traditional remedy for gastrointestinal complaints, skin injury and burns, but also as an ingredient in health foods (Fujita, 1993; Yamamoto et al., 1995; Beppu et al., 2003).

Previously, we demonstrated that freeze-dried whole leaves of *Aloe arborescens* var. *natalensis* (hereinafter called ALOE) inhibited the development of azoxymethane (AOM)-induced aberrant crypt foci (ACF), a putative neoplastic lesion, in the rat colorectum, with increased quinone reductase activity in the liver (Shimpo et al., 2001a). We also showed that feeding Japanese Pharmacopoeia aloe and commercial crude aloin (Sigma A-0451; hereinafter ALOIN) suppressed the AOM-induced ACF formation in the rat colorectum (Shimpo et al., 1999). However, the exact mechanisms involved in the inhibitory action are still unclear.

In the present study, we examined the effects of ALOE and ALOIN on AOM-DNA adduct formation and cytochrome P450 (CYP) 2E1 activity in male F344 rats, to clarify the possible mechanisms involved in its inhibition of ACF formation

Materials and Methods

Materials

Azoxymethane (A-9517; AOM), ALOIN (A-0451; from Curacao aloe; barbaloin content: approx. 20%), guanine (G) and O^6-methylguanine (O^6-MeG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest grade available and were

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obtained commercially. ALOE (Yurikaron™) was provided by Yurika Co. Ltd., Hisai, Japan. The phenolic compounds of ALOE and ALOIN used in the present experiment were analyzed by high-performance liquid chromatography as described previously (Shimpo et al., 2003a). The contents of phenolic compounds are shown in Table 1.

Table 1. Contents (mg/g dry weight) of Major Phenolic Compounds in ALOE and ALOIN

Phenolic compounds	ALOE	ALOIN
1 Barbaloin (Aloin A)	6.3	197.1
2 Isobarbaloin (Aloin B)	6.0	182.6
1+2	12.3	379.7
3 Feruloylaloesin	2.4	20.8
4 Coumaroylaloesin	1.0	13.9
3+4	3.4	34.7
5 Aloenin	13.6	0

ALOE, freeze-dried powder of whole-leaf *Aloe arborescens*; ALOIN, Sigma A-0451 aloin (from Curacao aloe).

Animals

Four-week-old male F344 rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were housed two or three to a cage in plastic cages on woodchip bedding, in an animal facility at a controlled temperature of 23±5°C, 60±5% humidity, and with a 12-h light/dark cycle. All rats were fed ad libitum with a basal diet and tap drinking water. Oriental MF powdered diet (Oriental Yeast Co., Ltd., Tokyo, Japan) was used as the basal diet. The care and use of the animals were performed according to the 'Guidelines for the Care and Use of Laboratory Animals' of Fujita Health University.

Treatment of Animals

The experiment was performed as described by Chen et al. (1998) (Fig. 1). After acclimatization for 1 week, the rats were fed the basal diet, or experimental diets containing 5% ALOE or 0.25% ALOIN for 5 weeks. All the rats were given two s.c. injections 15 mg/kg AOM, one at the end of week 1, and one at the end of week 2. Six hours after the second injection, the animals were anesthetized with diethyl ether, sacrificed by exsanguination, and carefully autopsied. The liver and colorectum were immediately removed and rinsed in ice-cold saline. The colorectum was cut open longitudinally and washed with saline to remove the colorectal contents. Then the colorectum was laid flat on a glass plate and the mucosa was scraped off with a glass slide. These samples were kept at -80°C until analysis of DNA adducts and CYP2E1 activity.

Analysis of DNA Adducts

Tissue DNA from the liver and colorectal mucosa was isolated by DNAZOL reagent (Invitrogen Corp., Carlsbad, CA, USA) and precipitated by ethanol. The pellet was suspended in 0.1 M HCl (5 mg/ml), and hydrolyzed at 70°C for 30 min to release the purines as free bases. The DNA hydrolysates were analyzed by the method of Herron and Shank (1981). The procedure was performed by high-performance liquid chromatography (HPLC) with a Waters Model 590 Programmable Solvent Delivery Module (Nihon Waters K.K., Tokyo) equipped with a Shimadzu RF-535 fluorescence monitor (Shimadzu Corp., Kyoto) at 366 nm emission with excitation at 286 nm. An analytic column, Whatman Partisil-10 SCX, 250 x 4.5 mm (GL Sciences Inc., Tokyo), with the same type of guard column was employed. The O^6-MeG was eluted with a 0.05 M ammonium phosphate solution at pH 2.0 and a flow rate of 2.0 ml/min. Results are expressed as the ratio of O^6-MeG to guanine (G) in nmol/μmol.

Assay of CYP2E1 Activity

The liver microsomes were prepared as described previously (Verschoyle et al., 1993). CYP2E1 activity in the liver microsomes was determined by monitoring *p*-nitrophenol (PNP) hydroxylation as described previously (Reinke and Moyer, 1985). PNP hydroxylase activity was assayed as described previously (Mishin et al., 1996). Briefly, the reaction mixtures (50 μg protein of hepatic

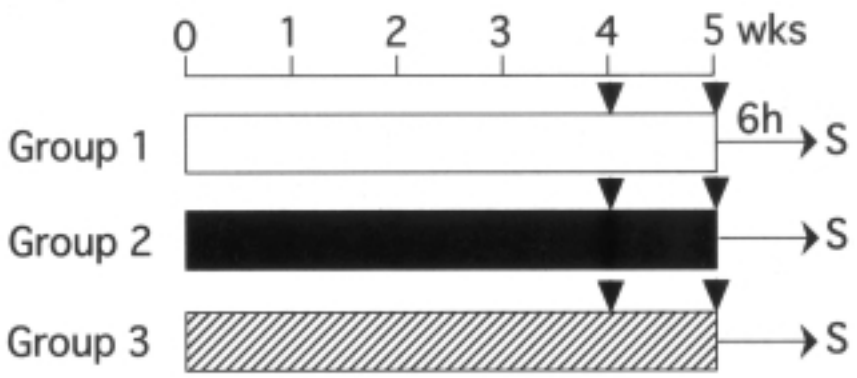

Figure 1. Experimental Protocol.

▼ AOM, 15mg/kg Body Weight, s.c. Injection.

Basal diet, Oriental MF, ALOE, 5% in diet, ALOIN (Sigma A-0451), 0.25% in diet, S, Sacrifice.

microsomes, 100 mM potassium phosphate buffer (pH 6.8), 2 mM NADPH, 0.2 mM PNP and 5 mM magnesium chloride) were incubated at 37°C for 30 min, and stopped by addition of 25 μl trifluoacetic acid. The *p*-nitrocatechol formed was then determined by HPLC with a Shimadzu LC-10AT pump, BAS LC-4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN) with a cell equipped with a glassy carbon working electrode set at +0.7 V against an Ag/AgCl reference electrode, and a Chromatocorder (Model 21; System Instruments Co., Ltd., Tokyo). A Wakopak Handy ODS 6 μm column (4.6 mm x 25 cm; Wako Pure Chemical Industries, Ltd., Osaka) was used and the mobile phase of 25% acetonitrile and 0.1% trifluoroacetic acid was pumped at 1.5 ml/min (oven temperature, 26°C).

CYP2E1 mRNA expression

The CYP2E1 mRNA level was evaluated by RT-PCR analysis (Jiang et al., 1998). Total cellular RNA was isolated from the liver using TRI ZOL reagent (Invitrogen Corp., Carlsbad, CA, USA), and then mRNA from the total RNA was isolated with an Oligotex-dT30 <Super> mRNA purification kit (Takara Bio Inc., Otsu, Japan). An aliquot of 0.5 μg of the mRNA was reverse-transcribed in 20 μl using the M-MLV reverse-transcriptase (Invitrogen Corp.) in its own buffer and random primers at 37°C for 1 hr. The cDNA product (1 μl) was amplified in 20 μl using 200 μM of each of the four deoxyribonucleoside triphosphates, 0.5μM of each primer and 1.0 unit of Takara Ex Taq in its own buffer (Takara Bio Inc.). PCR was performed using the Takara PCR thermal cycler MP (Takara Bio Inc.). The CYP2E1 primers and reaction conditions described by Jiang et al. (1998) and the β-actin primers and reaction conditions described by Yoshimi et al. (1997) were used. Those primers of CYP2E1 and β-actin were predicted to produce amplicons of 381 and 263 base pairs, respectively. After amplification, the PCR reaction product was electrophoresed on a 2.5% agarose gel. The gel was then stained with ethidium bromide and the image was digitized using FAS-III (Toyobo Co., Ltd., Osaka, Japan). The yield of CYP2E1 was normalized to β-actin after quantitative estimation using Diversity Database (PDI, New York, USA). PCR analyses were performed in triplicate.

Statistical Analysis

Statistical analysis of the final body weights was performed by one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons test. Hepatic microsomal CYP2E1 activity and the formation of AOM-induced DNA adducts (O^6-MeG) were compared with the Kruskal-Wallis test (nonparametric ANOVA) followed by the Dunn's multiple comparisons test. These statistical procedures were performed with InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA). A p value of <0.05 was considered significant.

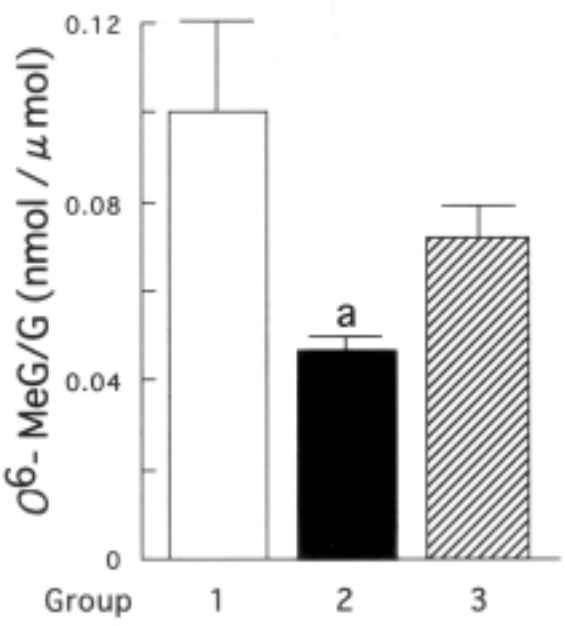

Figure 2. Effects of ALOE and ALOIN on the Formation of DNA Adducts (O^6-MeG) by AOM in the Rat Colorectum.

O^6-MeG, O^6-methylguanine; G, guanine.
Each bar represents the mean ± SE.
[a]Significantly different from group 1; $p < 0.01$.

Results

Final body weights, food consumption and intake of aloins A (barbaloin) and B (isobarbaloin) are shown in Table 2. The final body weight of rats fed the 5% ALOE diet, but not those fed the 0.25% ALOIN diet, was significantly decreased compared with control rats ($p < 0.05$). The intake

Table 2. Final Body Weight, Food Consumption and Aloin Intake

Group no.	Diet	n	Final body weight (g)	Food consumption (g/kg/day)	Aloin intake[a] (mg/kg/day)
1	Basal diet	7	240±5[b]	99.0±2.2	0
2	5% ALOE diet	6	220±3[c]	108.0±1.6	66.4±1.0
3	0.25% ALOIN diet	6	232±6	106.2±1.5	102.5±1.5

[a]Aloins A (barbaloin) +B (isobarbaloin).
[b]Mean±SE
[c]Significantly different from group 1 (basal diet) by ANOVA with the Dunnett's multiple comparisons test ($p<0.05$).

Table 3. Hepatic Microsomal CYP2E1 Activity of Rats in Each Group

Group no.	Diet	n	CYP2E1 activity[a] (*p*-nitrocatechol production, nmol/min/mg protein)	
			Mean±SE	Range
1	Basal diet	7	0.55±0.01	0.49-0.61
2	5% ALOE diet	6	0.40±0.04[b]	0.27-0.59
3	0.25% ALOIN diet	6	0.49±0.04	0.39-0.64

[a]CYP2E1 activity was measured by *p*-nitrophenol hydroxylase activity.

[b]Significantly different from group 1 (basal diet) by the Kruskal-Wallis test with the Dunn's multiple comparisons test ($p<0.05$).

of aloins A and B, the major phenolic compounds, in rats fed the 5% ALOE diet was lower than that in rats fed the 0.25% ALOIN diet. Therefore, it is thought that components other than aloins A and B of ALOE mainly resulted in the reduction of body weight in the treated rats.

Figure 2 shows the results of AOM-induced O^6-MeG formation in the colorectal mucosa in rats. The O^6-MeG levels in the rats fed the 5% ALOE diet was significantly decreased by 50% compared with those in the rats fed the basal diet ($p < 0.01$), whereas the O^6-MeG levels in the rats fed the 0.25% ALOIN diet showed a tendency to decrease (by 30%), although not significantly. In the liver, there was no significant difference in the O^6-MeG levels between the control rats and rats fed the 5% ALOE diet or 0.25% ALOIN diet (data not shown). Table 3 shows the results of CYP2E1-dependent PNP hydroxylase activity in the liver microsomes of the rats. The PNP hydroxylase activity was significantly reduced by 27% in rats fed the 5% ALOE diet compared with control rats. On the other hand, the activity in the rats fed the 0.25% ALOIN diet tended to decrease by 11%, although not significantly. The CYP2E1 mRNA levels in the rats fed the 5% ALOE diet and 0.25% ALOIN diet were reduced by 9.7% and 5.2%, respectively, compared with control rats. However, there were no significant differences between the controls and ALOE- or ALOIN-treated animals.

Discussion

We previously found that dietary administration of ALOE and ALOIN inhibited the formation of AOM-induced ACF in the rat colorectum (Shimpo et al., 1999, 2001a). The mechanisms by which ALOE and ALOIN inhibit AOM-induced ACF formation in the rat colorectum have not been clearly elucidated. However, it is possible that ALOE and ALOIN inhibit activation enzymes (phase 1 enzymes), activate detoxifying enzymes (phase 2 enzymes), or reduce DNA adduct formation. Sohn et al. (1991) showed that methylazoxymethanol (MAM), and its chemical and metabolic precursor, AOM, both strong colon carcinogens in rodents, were metabolically activated by CYP2E1 *in vitro* and *in vivo*.

In the present study, we indicated that dietary ALOE significantly decreased CYP2E1 (a phase 1 enzyme)-mediated PNP hydroxylase activity in the rat liver. Additionally, it was found that the CYP2E1 mRNA levels were slightly decreased in ALOE-treated animals. Thus, it is necessary to determine the CYP2E1 protein expression.

Previously, we also found that oral feeding with ALOE or ALOIN for 5 weeks significantly increased the activity of quinone reductase (QR), a phase 2 enzyme, in the rat liver (Shimpo et al., 2001a, 2001b).

Furthermore, we demonstrated in this study that ALOE significantly reduced the formation of DNA adducts by AOM in the rat colorectum. However, ALOIN feeding (0.25% in diet) failed to inhibit the enzyme activity and mRNA expression of CYP2E1 and AOM-induced DNA adducts, although intake of aloins A and B in the ALOIN-fed group was 1.5-fold larger than that in the ALOE-fed rats. It is suggested that ALOE contains more effective chemopreventive components, except for aloin, than ALOIN.

Recently, we reported that ALOE contains aloenin, feruloylaloesin and coumaroylaloesin as well as aloins A and B, as major phenolic compounds, and feruloylaloesin and coumaroylaloesin have higher radical-scavenging activity than aloin and aloenin (Shimpo et al., 2003a). On the other hand, Kim and Lee (1997) reported that *Aloe barbadensis* Miller (polysaccharide fraction) inhibits benzo[a]pyrene-DNA adduct formation *in vitro* and *in vivo*. ALOE also contains abundant polysaccharides (Beppu et al., 2003). Therefore, various components of ALOE, such as the phenolic compounds and polysaccharides may act additively to inhibit CYP2E1 activity in the liver and DNA adduct formation in the colorectum of AOM-treated rats.

ALOE was also found to inhibit the initiation stage of hepatocarcinogenesis induced by 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQ) (Tsuda et al., 1993). In a related study, ALOE reduces the CYP1A2 protein levels, which may be directly linked to a reduction of IQ-DNA adduct formation (Uehara et al., 1996). Recently, Furukawa et al. (2002) also demonstrated that ALOE proved markedly efficacious in inhibiting pancreatic tumorigenesis at the initiation stage in hamsters treated with *N*-nitrosobis-(2-oxopropyl)amine, in good agreement with the reduction of DNA adduct formation.

Most recently, we tried to investigate the inhibitory effect of ALOE on 1,2-dimethylhydrazine (DMH)-induced colorectal tumorigenesis in ICR mice in a long-term study. As a result, ALOE feeding (1% in diet) significantly reduced colorectal proliferative lesions including adenomatous hyperplasias, adenomas and adenocarcinomas in mice (Shimpo et al., 2003b). Contrastively, it has already been reported that aloin (a gift from Madaus AG, Germany; 0.03% in the diet) did not inhibit DMH-induced colorectal tumors in mice (Siegers et al., 1993).

Taking our results and those of previous studies together, ALOE might have a chemopreventive effect against some kinds of carcinogenesis models at least in the initiation stage.

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