Extract of Vinegar "Kurosu" from Unpolished Rice Inhibits the Development of Colonic Aberrant Crypt Foci Induced by Azoxymethane

Y. Shimoji[1], S. Sugie[2], H. Kohno[3], T. Tanaka[3], K. Nanda[1], Y. Tamura[1], Y. Nishikawa[1], R. Hayashi[4], K. Uenakai[1], and H. Ohigashi[4]

Research Center[1], Tamanoi Vinegar Co. Ltd., Yamatokoriyama, Nara; Dept. of Pathology[2], Gifu University School of Medicine, Gifu; Dept. of Pathology[3], Kanazawa Medical University, Uchinada, Ishikawa; Division of Food Science and Biotechnology[4], Graduate School of Agriculture, Kyoto University, Kitashirakawa Sakyo-ku, Kyoto; Japan

The modifying effects of administrating an ethyl acetate extract of "Kurosu" (EK), a vinegar made from unpolished rice, on development of azoxymethane (AOM)- induced colonic aberrant crypt foci (ACF) were investigated in male F344 rats. We also assessed the effects of EK on proliferating cell nuclear antigen (PCNA) index in ACF, prostaglandin (PG) E_2 expression in the colonic mucosa and activities of detoxifying enzymes of glutathione *S*-transferase (GST) and quinone reductase (QR) in the liver. To induce ACF, rats were given two weekly subcutaneous injections of AOM (20 mg/kg body wt). They also received drinking water containing 0, 0.05, 0.1 or 0.2% EK for 4 weeks, starting 1 week before the first dosing of AOM. AOM exposure produced 140 ± 23 ACF/rat at the end of the study (week 4). EK administration dose-dependently inhibited ACF formation and inhibition by 0.2% EK was statistically significant ($P<0.002$). Treatment with EK significantly lowered PCNA index in ACF and reduced PGE_2 content in the colonic mucosa, while EK elevated liver GST and QR activities. These findings suggest that EK may be effective for inhibiting colonic ACF, through induction of liver GST and QR and possibly alteration of PGE_2 production.

Key Words: "Kurosu", Vinegar, Extract, Chemoprevention, ACF, Rat

Vinegar, which can be made from rice, apple, wine and various other materials, is a widely used acidic seasoning. "Kurosu" produced from unpolished rice containing rice bran, which contains potentially chemopreventive phenols (1), through static surface acetic acid fermentation is one of the most common traditional vinegars in Japan (2). It is characterized by the higher contents of amino acids and organic acids than other vinegars. An extract of "Kurosu" has recently been shown to suppress lipid peroxidation in both *in vitro* and *in vivo* studies (3). It has also been found to possess a stronger antioxidative activity in a 1,1-diphenyl-2-picryllhydrazyl (DPPH) radical scavenging system than those of other vinegars, and an anti-promoting activity in mouse skin carcinogenesis (3). Moreover, a water extract of rice bran contained in unpolished rice, contains two types of hydroxy acids, which possess antitumor activity against a variety of cultured tumor cells (4, 5). We recently have isolated antioxidative compounds, dihydroferulic acid (DFA) and dihydrosinapic acid (DSA), from an ethyl acetate extract of "Kurosu" (EK)(6). DFA and DSA are derivatives of ferulic acid (FA), a well-known antioxidant in rice (7) and fermented rice product (8). FA, a phenolic compound with antioxidative activity (9), is widely present in bran from rice, wheat and barley, vegetables, and several other species of plants (10-12). FA has been reported to inhibit chemically induced carcinogenesis in various organs including colon (13, 14). FA is able to inhibit both the development of chemically induced aberrant crypt foci (ACF) and colonic adenocarcinoma (13, 14), the former being a putative precancerous lesion for colonic adenocarcinoma (15). A synthetic geranylated derivative of FA, ethyl 3-(4'-geranyloxy-3-methoxy-phenyl)-2-propenoate (EGMP), suppresses ACF formation (13) and colon carcinogenesis (14). EK could be considered to contain various antioxidative compounds such as FA and its derivatives, which are reported to inhibit carcinogenesis. However, the content of FA in EK is too low to explain the strong antioxidant activity of EK (6).

These findings lead us to examine possible inhibitory effects of EK on chemically induced ACF formation.

In the present study, we investigated the inhibitory effects of EK, which contains a part of the extract previously reported (3), on the development of ACF to predict the possible inhibitory effect on chemically induced colon carcinogenesis. In addition, the effects of EK on the activities of several enzymes, that involve in early stage of colon tumorigenesis (16) were investigated.

Materials and Methods

Animals, chemicals and diets. Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) aged 5 weeks were used for an ACF assay. All animals were housed in plastic cages (four or five rats/cage) with free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), lighting (12 h light/dark cycle) and temperature (23 ± 2°C). They were quarantined for 7 days and randomized by body weight into experimental and control groups. AOM for ACF induction was purchased from Sigma Chemical Co. (St Lois, MO, USA). "Kurosu" was made by Tamanoi Vinegar Co. (Nara, Japan). Amberlite XAD-4 was purchased from Organo Co., Ltd. (Tokyo, Japan).

Extraction of "Kurosu". "Kurosu" (150 L, unpolished rice vinegar) was loaded onto Amberlite XAD-4 column equilibrated with ion-exchanged water. Adsorbed fraction was eluted with methanol and concentrated under reduced pressure and the concentrate was partitioned between ethyl acetate and water. The ethyl acetate soluble part was concentrated under reduced pressure. Then, about 60 g of EK was obtained from 150 L of "Kurosu".

Experimental procedure for ACF assay. Forty-two male F344 rats were divided into five experimental and control groups. Groups 1-4 were initiated with AOM by two weekly s.c. injections (20 mg/kg body wt). Rats in groups 2, 3 and 4 were given distilled water containing EK at 0.05, 0.1, and 0.2%, respectively, for 4 weeks, starting 1 week before the first dosing of AOM. Group 5 was given drinking water containing 0.2% EK alone. Group 6 served as an untreated control. Rats were killed at week 4 by CO_2 asphyxiation to analyze colonic ACF. They underwent careful necropsy, with emphasis on the colon, liver, and kidney. All grossly abnormal lesions in any tissue, and the organs such as liver and kidney were fixed in 10% buffered formalin solution.

Determination of ACF. The frequency of ACF was determined according to the method described in our previous report (17). At necropsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. They were cut and fixed in 10% buffered formalin for at least 24 hrs. Fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 30 sec, briefly washed with distilled water and placed on a microscope slide with the mucosal surface up. Using a light microscope at a magnification of x 40, ACF were distinguished from the surrounding 'normal- appearing' crypts by their increased size (15).

Proliferating cell nuclear antigen (PCNA) immunohistochemistry. Immunohistochemical staining for PCNA was performed by the avidin-biotin complex method (Vecstain Elite ABC kit; Vector, Burlingame, CA). Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series, immersed in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to block endogenous peroxidase activity and then washed in phosphate-buffered saline (pH 7.2). Following incubation with normal rabbit serum at room temperature for 10 min to block background staining, the sections were incubated with an anti-PCNA antibody (mouse monoclonal PC10; Dako, Kyoto, Japan; a 1:100 dilution) for 12 hrs in a humidified chamber at room temperature. They were then reacted with 3,3'-diaminobenzidine and counterstained with Harris' hematoxylin. For determination of PCNA-positive index, 10 full-length crypts (aberrant crypts, 'normal-appearing' crypts or normal crypts) of each colon were examined. The number of PCNA positively stained nuclei in each crypt column was recorded. The PCNA-positive index (number of positive stained nuclei x 100/total number of nuclei counted) was then calculated. The scorer was unaware of the group to which the specimens belonged.

Assay of liver GST and QR activities. To determine whether EK can modify liver GST and QR activities, livers were excised immediately from all rats at necropsy. The livers were perfused with saline to remove blood and minced into small pieces. Aliquots from minced livers were processed to obtain the cytosolic fraction as described previously (18). The activities of GST with 1-chloro-2, 4-dinitrobenzene

(CDNB) and/or 1,2-dichloro-4-nitrobenzene (DCNB) as substrates, and QR with NADH and menadione as substrates were determined (19, 20). All assays were performed by spectrophotometer at 340 nm and all samples were measured in triplicate. One unit of enzyme activity is the amount of enzyme catalyzing the conversion of 1 μmol of substrate to product per min at 25°C. Cytosolic protein concentrations were determined by the Bradford method (21) using bovine serum albumin (BSA) as the standard.

Measurement of colonic PGE_2. For PGE_2 determination, the scraped colonic mucosa of 3 rats from each group was divided in half and homogenized in a 100 μL of ice-cold phosphate-buffered saline (PBS) on ice. After centrifugation at 21500 x g for 10 min, the supernatants thus obtained were diluted at the ratio of 1:1000, and measured for the PGE_2 concentration using a commercial experimental kit (Cayman Chemical Company, Ann Arbor, MI) according to the protocol of the manufacturer.

Statistical evaluation. Where applicable, data were analyzed using one-way ANOVA, followed by a Bonferroni/Dunn post-hoc test or Student's t-test or Welch's t-test with $P<0.05$ as the criterion of significance.

Results

General observations. All animals remained healthy throughout the experimental period. Body, liver and relative liver weights (g/100 g body weight), and intake of drinking water with or without EK (ml/day/rat) in all groups are shown in Table I. Water intake and body weight gain did not significantly differ among the groups, suggesting that water containing 0.05%, 0.1% or 0.2% EK were well tolerated and supported normal growth in rats without any adverse effects. At the end of the study, mean body, liver and relative liver weights of all groups were not significantly different.

Frequency of ACF. Table II summarizes the data on colonic ACF formation. ACF developed in all rats belonging to groups 1-4, which were initiated with AOM. In group 1, AOM induced 140 ± 23 ACF per rat. In groups 2-4, given water containing EK caused significant inhibition in the ACF incidence: 110 ± 14 at a dose of 0.05%, 88 ± 20 at a dose of 0.1% and 46 ± 23 at a dose of 0.2% ($P<0.002$). The inhibition by EK was dose-dependent ($r = -0.997$, $P=0.0015$). Furthermore, the percentage of ACF consisting of more than four crypts in group 4 was significantly smaller than that of group 1 ($P<0.001$). In groups 5 (0.2% EK alone) and 6

Table I - Body, liver, and relative liver weights and intakes of drinking water and EK

Group no.	Treatment (no. of rats examined)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	Daily intake of:	
					Drinking water (mL/day/rat)	EK (mg/day/rat)
1	AOM alone (8)	194 ± 15[a]	8.75 ± 0.83	4.51 ± 0.30	26.8 ± 1.8	-
2	AOM + 0.05% EK (8)	210 ± 10	9.75 ± 0.43	4.66 ± 0.24	22.3 ± 3.0	11.13
3	AOM + 0.1% EK (8)	197 ± 9	8.75 ± 1.20	4.44 ± 0.48	22.9 ± 3.3	22.88
4	AOM + 0.2% EK (8)	196 ± 17	9.25 ± 0.97	4.83 ± 0.76	22.1 ± 4.7	44.20
5	0.2% EK (5)	223 ± 17	10.80 ± 0.75	4.87 ± 0.40	24.6 ± 3.4	49.20
6	None (5)	210 ± 8	9.60 ± 0.49	4.58 ± 0.17	27.7 ± 1.6	-

[a] Mean ± SD.

Table II - Effects of EK on the development of AOM-induced ACF

Group no.	Treatment (no. of rats examined)	Total no. of ACF/colon	Total no. of aberrant crypts/colon	No. of aberrant crypts/focus	No. of ACF with more than 4 aberrant crypts
1	AOM alone (8)	140 ± 23[a]	326 ± 40	2.35 ± 0.14	22 ± 6
2	AOM + 0.05% EK (8)	110 ± 14	254 ± 47	2.29 ± 0.15	15 ± 7
3	AOM + 0.1% EK (8)	88 ± 20	205 ± 44[b]	2.35 ± 0.26	14 ± 6
4	AOM + 0.2% EK (8)	46 ± 23[c]	105 ± 51[d]	2.28 ± 0.33	5 ± 3[e]
5	0.2% EK (5)	0	0	0	0
6	None (5)	0	0	0	0

[a] Mean ± SD. [b-e] Significantly different from group 1 by one-way ANOVA, followed by a Bonferroni/Dunn post-hoc test ([b]P<0.05, [c]P<0.002, [d]P<0.001, and [e]P<0.01).

(untreated), there were no microscopically observable changes, including ACF, in colonic morphology.

PCNA-labeling indices in ACF and 'normal-appearing' colonic crypts. The PCNA-labeling indices in ACF and 'normal-appearing' crypts are shown in Fig. 1. The mean PCNA-labeling indices in ACF of groups 2-4 were lower than group 1 and the value of group 4 was significantly lower than that of group 1 (P<0.05). The PCNA-labeling indices in 'normal-appearing' crypts of groups 2-4 were also significantly decreased by the administration of EK (P<0.05, P<0.01 and P<0.05, respectively) compared with that of group 1. Administration of EK did not affect the PCNA-labeling index in normal crypts of rats in group 5, when compared with that of untreated rats (group 6).

Liver GST and QR activities. Liver GST and QR activities at the end of the study are shown in Fig. 2. GST activities using CDNB as a substrate in groups 3 and 4 were significantly greater than that of group 1 (P<0.001). Similarly, GST activities using DCNB as a substrate in groups 3 and 4 were significantly greater than that of group 1 (P<0.05). QR activities in groups 2, 3 and 4 were also significantly greater than that of group 1 (P<0.01, P<0.05 and P<0.05, respectively). GST and QR activities in groups 5 (0.2% EK alone) and 6 (untreated) were comparable.

PGE_2 level. Colonic mucosal PGE_2 levels in all groups at the end of the study are illustrated in Fig. 3. Mean colonic PGE_2 content (± SE) of group 1 (13.41 ± 1.63) was significantly greater than that of group 6 (4.81 ± 0.13). The PGE_2 contents of groups 3 (2.89 ± 0.36) and 4 (6.50 ± 0.52) were significantly smaller than that of group 1 (P<0.001 and P<0.05, respectively). The PGE_2 content of group 2 (10.56 ± 2.58) was also smaller than group 1, but the difference was insignificant. The PGE_2 level of group 5 (6.47 ± 1.21) was slightly higher than that of group 6.

Discussion

In the present study, administration of EK in drinking water dose-dependently suppressed AOM-induced ACF formation in the colon. The number of large ACF consisting of four or more aberrant crypts, which reflects the incidence of colonic adenocarcinoma (15), was significantly reduced by EK exposure at a dose level of 0.2%. Such effect of EK is thought to be the "blocking" effect (22) of carcinogenesis. Importantly, EK administration together with or without AOM did

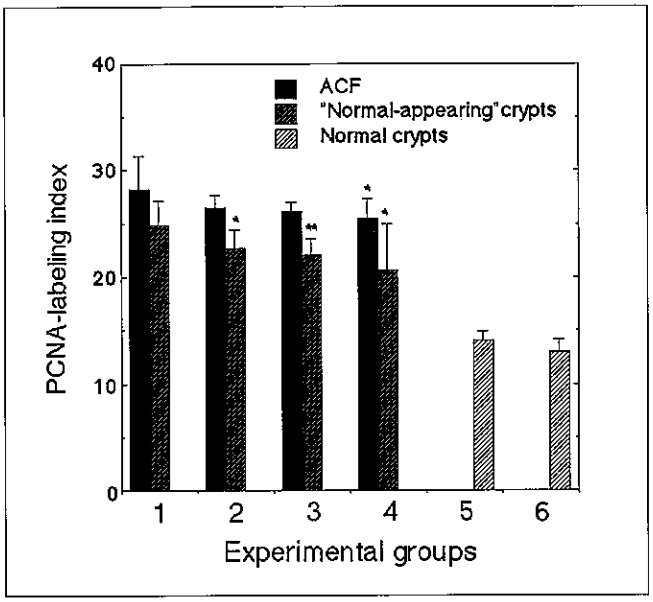

Fig. 1 - PCNA-labeling index in normal crypts, "normal-appearing" crypts, and ACF. Each column represents mean ± SD. Significant values are indicated by asterisks: *P<0.05 and **P<0.01 as compared with group 1.

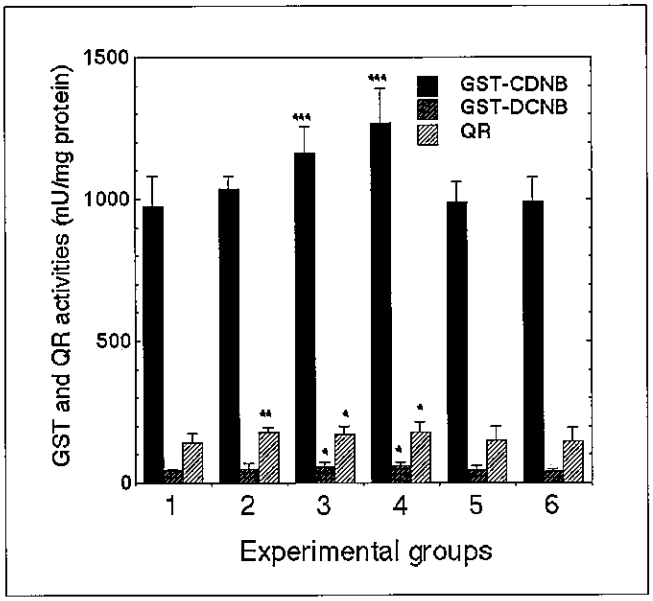

Fig. 2 - GST and QR activities in the liver of rats given AOM and/or EK. Each column represents mean ± SD. Significant values are indicated by asterisks: *P<0.05, **P<0.01 and ***P<0.001 as compared with group 1.

not cause any growth retardation and clinical toxicity.

"Kurosu" is known to be one of the traditional health foods in Japan (2). Our previous study revealed an anti-tumor promotion activity of EK in mouse two-stage tumorigenesis, in which the production of malondialdehyde in the mouse epidermis was reduced by topical application of EK (3). Auraptene possessing a powerful chemopreventive activity in multi-organ is reported to lower the production of aldehydic lipid peroxidation [malondialdehyde and 4-hydroxy-2(*E*)-nonenal] and inhibit rat colon carcinogenesis (23). The chemical also inhibited AOM-induced ACF, elevated GST and QR activities in the liver, and suppressed cell proliferation activity in rat colon (17). EK similarly inhibited the development of ACF and modified the activities of GST and QR, and cell proliferation. Thus, it may be possible that EK inhibits development of colonic adenocarcinoma. The results in our recent study suggest that FA derivatives, DFA and DSA, are responsible for the antioxidative activity of EK, since the amount of FA is extremely low when compared with that of DFA and DSA (6). DFA and DSA in EK, therefore, may contribute to the inhibitory effect of EK on ACF in the current study.

The mechanisms of chemopreventive ("blocking") effect (22) of EK against ACF are not known, but possible mechanisms can be considered. EK may modify phase I and/or phase II enzymes in liver. AOM exerts its carcinogenic activity through activation of CYP2E1 (24). In the current study, we did not investigate the effect of EK on this enzyme. However, in this study, EK induced phase II detoxification enzymes GST and QR in the liver, as did auraptene (17, 23). This biological function may contribute to its "blocking" effect (22) on AOM-induced ACF formation.

Cell proliferation plays an important role in multi-stage carcinogenesis with multiple genetic changes (25). Modulation of cell proliferation activity in target organs is one of the important actions of cancer chemoprevention agents (26). In this study, EK treatment lowered the PCNA-labeling indices in ACF, suggesting that EK may inhibit the growth of ACF through suppression of cell proliferation. AOM was reported to cause oxidative stress, which influences cell proliferation (27), in the colonic mucosa (23). EK possesses antioxidative activity (3). Therefore, the ACF inhibitory effect of EK may be due to modification of cell hyper-proliferation in the colonic mucosa exposed to AOM through suppression of oxidative stress (23).

In general, PGE_2 content in colon cancer tissues is much greater than that in their surrounding tissues and normal tissues (28). PGE_2 level is also high in colon with a number of preneoplastic lesions in early stage of colon carcinogenesis (29), leading to hyper-cell proliferation (30). Certain chemopreventive agents can decrease the PGs contents (31, 32). In the current

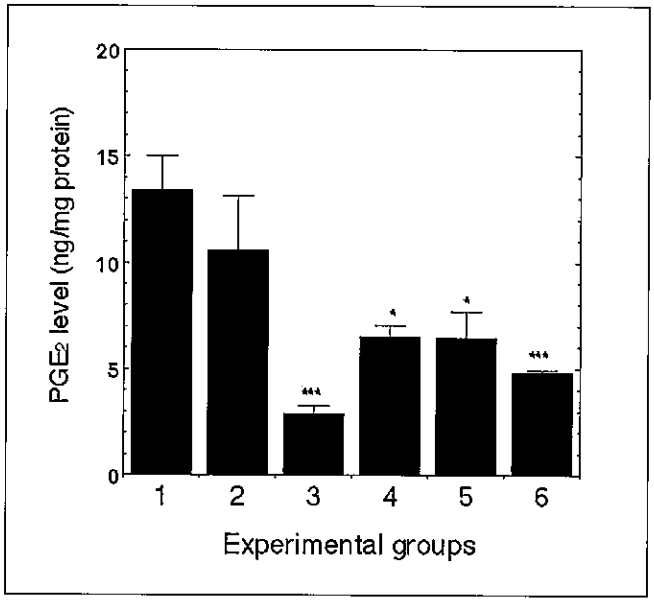

Fig. 3 - PGE_2 level in the colonic mucosa. Each column represents mean ± SE. Significant values are indicated by asterisks: *P<0.05 and ***P<0.001 as compared with group 1.

study, EK inhibited PGE_2 production in a dose-dependent manner. EK may inhibit hyper-cell proliferation by control of PGE_2 biosynthesis. This also explains the chemopreventive effect of EK against ACF formation.

In conclusion, the results described here suggest that EK has an anti-initiation effect on rat colon carcinogenesis in addition to an anti-tumor promoting activity in mouse skin tumorigenesis (3). EK has a beneficial effect on chemically-induced colonic preneoplastic progression through multifactorial mechanisms in rats, and provides an effective dietary chemopreventive approach to disease management. A long-term bioassay to confirm the results is on-going in our laboratory.

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Dr. Yumi Shimoji,
Research Center,
Tamanoi Vinegar Co. Ltd.,
100 Nishimachi, Yamatokoriyama,
Nara 639-1038, Japan
Tel.:+81-743-56-1338; Fax:+81-743-56-4777;
E-mail: research@tamanoi.co.jp