Quercetin Enhances Tumorigenicity Induced by N-Ethyl-N'-Nitro-N-Nitrosoguanidine in the Duodenum of Mice*

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Abstract

Quercetin, a flavonoid, widely distributed in many fruits and vegetables, is well known to have an anti-tumor effect despite its mutagenicity. In this study, we examined the effect of dietary quercetin on duodenum-tumorigenicity of mice induced by a chemical carcinogen, N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG). Eight-week-old male C57BL/6 mice were divided into 4 groups; ENNG without quercetin (group A), ENNG with 0.2% quercetin (group B), ENNG with 2% quercetin (group C), and 2% quercetin without ENNG (group D). ENNG was given in drinking water for the first 4 weeks, and thereafter quercetin was given in a mixed diet. At week 20, the average number of duodenal tumors per mouse was significantly higher in group C (mean±SE, 7.26±1.75, $p<0.05$) than in group A (2.32±0.31). The size of the duodenal tumors increased significantly in group B (1.79±0.09 mm, $p<0.001$) compared with group A (1.43±0.09 mm). In contrast, no duodenal tumor was induced in group D. The present findings suggest that excessive intake of quercetin occasionally is a risk factor for carcinogenesis of some specific organs such as the upper intestine.

Key words: flavonoids, quercetin, mice, duodenal tumor, ENNG

Introduction

Epidemiological studies have elucidated that the consumption of vegetables reduces the incidence and mortality of human stomach, colon, and breast cancer¹⁻³). Therefore, compounds in edible plants have been extensively studied for inhibition of tumor development. Among them, quercetin, a flavonoid, is a pigment of edible plants, and it has been estimated that an average person consumes 50 mg 'quercetin-equivalent' per day⁴). Quercetin shows various biological activities and it reduces the proliferation of malignant tumor cells, such as leukemia cells, Ehrlich ascites tumor cells, and breast cancer cells^{5,6}). In addition, our previous studies showed that quercetin inhibits the proliferation of human gastric and colon cancer cells and arrests their cell cycle at the late G1 phase^{7,8}), which is the major point for control of cancer cell

proliferation. The following study confirms the inhibitory effects of quercetin on intestinal cancer cells⁹).

In vivo studies have shown that quercetin inhibits carcinogenesis induced by chemical carcinogens in the organs of rodents as follows. We found that dietary quercetin at a dose of 2% reduces azoxymethane-induced colon aberrant crypt foci of rats¹⁰), and other investigators reported that 2% or 5% quercetin shows dose-dependent inhibition of rat mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene and N-nitrosomethylurea¹¹). In two-stage carcinogenesis experiments, quercetin markedly inhibited the promoter effect of teleocidin or 12-O-tetradecanoylphorbol-13-acetate on skin tumor formation in mice initiated by 7,12-dimethylbenz(*a*)anthracene^{12,13}). These previous findings suggest that quercetin has a chemopreventive activity *in vivo*. Furthermore, quercetin did not cause retardation in body weight gain of experimental animals treated with the chemical carcinogens, and exhibited no toxicity in the short- and long-term studies reported by Ambrose et al. on the low toxicity of quercetin for rodents¹⁴).

On the other hand, quercetin is one of the most mutagenic flavonoids. Quercetin exhibits frameshift mutagenic activity in bacterial strains¹⁵) and chromosomal aberration and sister chromatid exchange in mammalian cells¹⁶). Since mutagens have a good correlation with carcinogens, the carcinogenicity of quercetin has

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been examined intensively. Limited information exists about positive aspects of the carcinogenesis of this flavonoid. Pamucku et al. reported that rats fed 0.1% quercetin develop multiple tumors of the ileal intestine and bladder in a long-term study¹⁷⁾, although these findings could not be confirmed in other studies using quercetin at up to 10% of the diet^{18,19)}. Therefore, the carcinogenicity of quercetin is considered unlikely despite its mutagenicity and quercetin is expected as an anti-proliferative and anti-carcinogenic chemopreventive agent.

To clarify its risk potential for human beings, it is important to examine in more detail non-carcinogenesis or anti-carcinogenesis of quercetin in experimental animals. Alkylating carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is well known to cause malignant tumors in the stomach of rats by oral administration²⁰⁾. Similarly, its ethyl homolog, ENNG, induces gastric tumors in mice, rats, dogs, or monkeys^{21–23)}. ENNG is more effective than MNNG for the induction of duodenal tumors when continuous doses were administered orally to mice^{21,24)}. Duodenal tumors by ENNG were induced within a short time and under a stereomicroscope they were observed as an enlarged villus, fusion of several villi, and a crater formed or a bulged lesion²¹⁾. It is easy to compare the incidence of carcinogenesis during the early stage by counting the number and the size of tumors. C57BL/6 mice, which bear duodenal tumors more specifically with ENNG treatment than other strains of mice, are suitable experimental models developed by Matsuyama et al. in 1975 to detect the early stage of ENNG carcinogenesis²¹⁾. In the present study, we examined the effect of dietary quercetin on ENNG carcinogenesis in the duodenum of C57BL/6 mice.

Materials and Methods

Chemicals and animals

Quercetin and ENNG were purchased from Nacalai Tesque Ltd., Kyoto, Japan. ENNG was dissolved in tap water at 100 mg/liter (0.01%) and its solution was freshly prepared two times per week and bottles were covered with foil to prevent deterioration of carcinogen by light. The basic diet, a commercial laboratory mouse chow (MF chow) and mouse chow containing 0.2% or 2% quercetin were prepared and pelleted by Oriental Yeast Co., Ltd., Tokyo, Japan. The quercetin content of the basic diet was less than 0.0005% by HPLC analysis and negligible.

Experimental animals were 8-week-old male C57BL/6 mice with an average weight of 23.8 g at the start of the experiment. Animals were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka, Japan. They were housed in plastic cages in a temperature- and humidity- controlled

environment with a 12-h day/12-h night cycle.

Effect of quercetin on tumorigenicity

Eighty mice were divided into four groups of 20 mice each: ENNG without quercetin (group A), ENNG with 0.2% quercetin (group B), ENNG with 2% quercetin (group C), and 2% quercetin without ENNG (group D). For the first 4 weeks, mice were administered 0.01% ENNG freely in drinking water (A–C), or tap water alone (D), and they were fed the basic diet. Thereafter, the mice were given tap water and were fed the basic diet continuously (group A), 0.2% quercetin in the basic diet (group B), or 2% quercetin in diet (groups C, D) for 16 successive weeks. Tap water and food consumption were measured one or two times per week and the body weight of mice was recorded every 5 weeks. All mice were killed by cervical dislocation at week 20. The esophagus, stomach, duodenum and jejunum were removed together. The upper digestive tract was ligated at the esophagus and 10% formalin was poured into the jejunum followed by fixation for about 10 minutes. The duodenum was opened longitudinally and stretched out on a cork mat. The duodenal mucosa was examined stereomicroscopically up to 4 cm distal from the pyloric ring.

Statistics

All values are presented as means±SE. Statistical analysis was performed using χ^2 -test and one way analysis of variance with Bonferroni's multiple comparison. Differences at $p<0.05$ were considered to be significant.

Results

Effects of ENNG and/or quercetin on the body weight of mice

Until the 5th week of the study, the consumption of water including ENNG did not show any significant differences among groups A–C (data not shown). The total cumulative dose of ENNG ingested by each mouse was 13.5 mg–14.0 mg (Table 1). Until the 5th week of the study, the average consumption of food by mice in each group was not significantly different (data not shown). In the period between the 5th week and the 20th week, the daily consumption of the basic diet without quercetin per mouse was 3.58 ± 0.09 g in group A, and that of each dose in the quercetin-supplemented diet was 4.01 ± 0.11 g in group B, 3.91 ± 0.06 g in group C, and 3.63 ± 0.15 g in group D. During the experimental period, the body weights of animals increased as follows: group A, from $24.5\pm(\text{SE}) 0.3$ to 37.8 ± 0.8 g; group B, from 22.8 ± 0.3 to 36.7 ± 0.9 g; group C, from 24.5 ± 0.3 to 32.5 ± 0.6 g; group D, from 23.2 ± 0.3 to 39.9 ± 0.8 g. While the food consumption in groups B and C increased compared with that in group A, the average body

Table 1 Estimated cumulative dosage of ENNG, and the incidence, multiplicity, or size of the duodenal tumors produced in male C57BL/6 mice

Experimental group	Treatment	Estimated cumulative dosage of ENNG (mg/mouse)	Tumor incidence		Tumor multiplicity per mouse (mean±SE)	Diameter of tumor (mean±SE, mm)
			No. of mice	% of mice		
A	ENNG	13.5	19/19 ^a	100	2.32 ± 0.31	1.28 ± 0.09
B	ENNG+0.2% quercetin	13.9	20/20	100	3.95 ± 0.67	1.79 ± 0.09^c
C	ENNG+2% quercetin	14.0	18/19 ^a	95	7.26 ± 1.75^b	1.43 ± 0.06^d
D	2% quercetin	0	0/20	0		

^a One accidental death occurred during the experiment.

^b Values significantly different from ENNG-treated group A ($p<0.05$).

^c Values significantly different from ENNG-treated group A ($p<0.001$).

^d Values significantly different from ENNG-treated group B ($p<0.005$).

weight for mice in group C was significantly lower ($p<0.05$) than that in group A after 10 weeks (data not shown).

Effects of quercetin on duodenal tumorigenicity induced by ENNG

All mice were killed at the 20th week for stereomicroscopic observation of the duodenum. Stereomicroscopy is able to visualize extremely small lesions in the regularly situated and high villi of duodenal mucosa. As shown in Fig. 1, small tumors were observed as an enlarged villus, or fusion of several villi (A), and most of the large tumors were observed as an elevation with a central crater (B). Only in group B, the other large tumors ($n=2$) were seen as a bulged lesion without a crater. These morphological changes have been noted previously^{21,24}. The enlarged villus or the

fusion of several villi has been suggested to be the initial morphological change caused by ENNG and these abnormal villi develop further into the large crater-shaped lesion²¹. Histologically, atypical glands have been reported to be situated in the enlarged villus or in the fusion of several villi, and the crater-shaped or bulged lesion consists of adenocarcinoma cells^{21,24}.

Table 1 shows the incidence, multiplicity, and size of duodenal tumors per mouse in each group. There was no significant difference ($p<0.05$) in the incidence of tumors among groups A–C. In contrast, the mice in group D treated with 2% quercetin without initial ENNG exposure bore no duodenal tumors. The average number of tumors per mouse was significantly higher in group C than in group A, and high dose quercetin was more effective in

Fig. 1 Stereomicroscopic findings of mice duodenal tumors induced by ENNG at week 20.
(A) Fusion of several villi in the regularly situated and high villi of duodenal mucosa.
(B) Large tumor with a depression at the center.

Fig. 2 Effect of quercetin on different types of mice duodenal tumors induced by ENNG. The duodenal mucosae of C57BL/6 mice were observed stereomicroscopically at week 20. White bars, ENNG-treated group A; gray bars, ENNG- and 0.2% quercetin-treated group B; black bars, ENNG- and 2% quercetin-treated group C. Columns, average number of tumors per mouse; bars, SE. *, values significantly different from ENNG-treated group A ($p < 0.05$).

developing ENNG-induced duodenal tumors than low dose administration. On the other hand, the average tumor size (mean of long and short diameter) increased significantly in group B compared with group A ($p < 0.001$), whereas the tumor size in group C was significantly lower than in group B ($p < 0.005$). This discrepancy was perhaps due to the smaller number of enlarged villi and fusion of several villi in group B compared with groups A and C, although both the average numbers of these small lesions and of the crater-formed or bulged lesions increased in group C compared with group A (Fig. 2). As Fig. 2 shows, these findings suggest that quercetin may shorten the mean periods for the development of duodenal tumors induced by ENNG.

Discussion

The daily average intake of flavonoids in the United States of America amounts to about 1 g from a normal mixed diet²⁵). A representative flavonoid, quercetin is found in the majority of edible plants and their products, such as tea, fruit juice, and wine²⁶). Quercetin administered orally is poorly absorbed via the alimentary tract; a proportion of the quercetin is degraded by intestinal microflora, and the remainder is excreted in feces in unchanged form²⁷). Therefore, dietary quercetin may have a more significant influence on the gastro-intestinal tract than on other organs. The average intake of quercetin per mouse by a dietary supplement of 0.2% quercetin in this research (approximately 0.28 g/kg/day) was about 280-fold higher than the estimated human oral daily consumption of quercetin (about 0.001 g/kg/day)⁴). The present results suggest that a high dose of quercetin occasionally might be one of the risk factors for carcinogenesis of some specified organs such as the upper intestine, although duodenal tumors are rarely observed in humans.

The present findings support the pleiotropic effects of dietary

quercetin on the toxicity and carcinogenesis in experimental animals. First, there have been several studies on the low- or non-toxicity of quercetin as stated above¹⁰⁻¹⁴), whereas in the present study 2% quercetin caused a retardation in the body weight gain of experimental animals treated with ENNG despite the increase in food consumption. Quercetin might reveal the toxicity or enhance the metabolism in the mice under the pretreatment of ENNG. The retardation of body weight gain by quercetin was also observed in other studies^{17,19}), and the life span of mice was reduced by a dietary supplement of 0.1% quercetin²⁸). Second, the present findings suggest that dietary quercetin enhances the tumorigenicity of ENNG in mice duodenum in a dose-dependent manner, although the anti-carcinogenic or anti-promoting effects of quercetin have been reported earlier in the colon, mammary, or skin tumors in experimental animals¹⁰⁻¹³). On the other hand, in the present study dietary quercetin alone showed no carcinogenesis in group D, as expected from earlier studies^{18,19}).

Quercetin is known to be an inhibitor of both lipoxygenase and cyclo-oxygenase of arachidonic acid metabolism^{29,30}). Another inhibitor of cyclo-oxygenase, aspirin, also shows pleiotropic effects on tumorigenesis. Aspirin increases the incidence of rat gastric tumors induced by MNNG³¹), although aspirin reduces the growth of colon tumors and fibrosarcomas in rodents^{32,33}). Moreover, aspirin is not a carcinogen alone³¹), similar to quercetin. As one explanation for the enhancement of rat gastrointestinal tumors by aspirin, aspirin causes gastric mucosal lesions such as edema, congestion, or erosion³¹), and therefore it may reduce the capacity of the gastric mucosa to resist the action of MNNG. In contrast, dietary quercetin shows antiulcer and cytoprotective effects on mucosal injury produced by absolute ethanol or indomethacin³⁴). On the other hand, ENNG causes neither erosion nor ulcers in mice duodenum after the initial treatment with ENNG²¹). Consistently, histologic damages in the duodenal mucosa of mice treated with quercetin and/or ENNG were not observed in the present study (data not shown). Thus, the mechanism of the cocarcinogenic action of quercetin may be different from that of aspirin.

One possibility is that both quercetin and ENNG exhibit potent mutagenicity in bacteria cells and mammalian cells^{15,16,35}). An enhancing or suppressing effect of quercetin on mutagens was observed in *Salmonella typhimurium*³⁶). The discrepant effects of quercetin on carcinogenesis might be deduced from its pleiotropic interactions with mutagens, although we have not obtained any direct proof. Another possibility is that ENNG acts as an initiator, and that bile acids act as a promoter. In the present study, quercetin was administered after ENNG treatment, so that the direct interaction between ENNG and quercetin can be regarded as negligible. Thus, quercetin might act as a co-promoter with bile acids in duodenal carcinogenesis with ENNG, because quercetin alone showed no carcinogenesis. Further examinations are required with regard to the risk potential of quercetin in humans.

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