Chemoprevention studies of the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse colon

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In this study we investigated the chemopreventive effects of quercetin and rutin when added to standard AIN-76A diet and fed to normal and azoxymethane (AOM)-treated mice. Early changes in colonic mucosa were analyzed, including colonic cell proliferation, apoptotic cell death, cyclin D₁ expression and focal areas of dysplasia (FAD). The findings show that the number of colonic epithelial cells per crypt column increased (P < 0.01) in each normal mouse group fed the flavonoids; AOM administration increased colonic crypt cell proliferation and resulted in a marked rise of bromodeoxyuridine-labeled cells in the lower proliferative zone of the crypt. Both supplementary dietary quercetin and rutin increased the apoptotic index and caused a redistribution of apoptotic cells along the crypt axis in normal mice fed a standard AIN-76A diet. The number of apoptotic cells/column and apoptotic indices markedly increased (P < 0.01) in the AOM-treated group compared with untreated animals; apoptotic cells expanded throughout the colonic crypts after flavonoid supplementation and AOM administration. Positive cyclin D₁ expression was detected in mice on diets supplemented either with quercetin (P < 0.01) or rutin (P < 0.05). AOM administration resulted in the formation of FAD. Both the number of mice exhibiting FAD and the total numer of FAD observed were significantly reduced (P < 0.01) in AOM-treated animals fed flavonoids compared with mice maintained on the standard AIN-76A diet. Surprisingly, however, quercetin alone was able to induce FAD in 22% of normal mice fed the standard AIN-76A diet.

Introduction

Flavonoids are polyphenolic compounds found in various foods of plant origin (1-3). It has been estimated that humans consuming high fruit and vegetable diets may ingest up to 1 g of these compounds daily (1-3). Quercetin, the major representative of the flavonol subclass of flavonoids, is an integral part of the human diet and the average human intake has been estimated to be ~25 mg/day.

Quercetin is present mostly in the form of glycosides such as rutin, in the outer layer of ingested fruits, the outer leaves of vegetables, the leaves of unprocessed tea and in wine (2,3). Rutin is hydrolyzed to its aglycone quercetin by the β -glucosidase activity of obligate anaerobes in the gastrointestinal tract, particularly by the glycosidase enzymes of the colonic microflora (1–4). Both quercetin and rutin have demonstrated chemopreventive activity in a variety of laboratory animal models, including azoxymethane (AOM)-induced colonic tumorigenesis in mice and rats (5–8), dimethylbenz[*a*]anthracene (DMBA) and *N*-nitrosomethylurea (NMU)-treated mammary glands of rats (9) and DMBA-treated skin of mice (10). Quercetin exerts antiproliferative effects *in vitro* on cancer cell lines of diverse lineages (11–14), including colonic cancer cells (15–17). The chemopreventive action of quercetin and rutin in colonic neoplasia has been reviewed recently (18).

In this study we investigated the effects of supplementary dietary quercetin and rutin in normal and AOM-treated CF1 mice fed a standard AIN-76A diet. AOM-treated animals were killed at 15 weeks of age, at an early stage of colonic preneoplasia. Cell proliferation, apoptotic cell death, expression of cyclin D_1 and focal areas of dysplasia (FAD) were evaluated as intermediate endpoint biomarkers in colonic cells examined at various positions along the crypt continuum.

Materials and methods

Animals and diets

Sixty CF1 mice 3 weeks of age were purchased from Charles River Laboratories (Kingston, NY). They were maintained at the Rockefeller University Animal Facility, an American Association for Accreditation of Laboratory Animal Care facility complying with USDA regulations and NIH Guidelines for the Care and Use of Laboratory Animals. On arrival, all mice were fed the control AIN-76A diet (Teklad Diets, Madison, WI) and water *ad libitum*. The animals were maintained on a 12 h light–dark cycle and weighed weekly. At 6 weeks of age, all mice were randomly allotted into three diet groups (20 animals per group with an equal number of male and female mice) and fed one of three different diets: unmodified AIN-76A or AIN-76A supplemented with either 2% quercetin or 4% rutin. Quercetin and rutin were purchased from Freeman Industries Inc. (Tuckahoe, NY). Both compounds had a purity of $\geq 98\%$.

At 8 weeks of age, 10 mice from each diet group were injected subcutaneously once per week for 6 weeks with AOM at a dose/kg body wt of 5 mg (first week), 7 mg (second week) and 10 mg for the remaining 4 weeks. The untreated controls of 10 animals in each dietary group received 0.9% saline in the same volume and at the same time of carcinogen administration. A total of three mice died during the study for unknown reasons, one from the two quercetin groups and the remainder from the AOM-treated rutin group.

Tissue preparation

One week after the last AOM or saline administration (at 14 weeks of age), all mice were killed by cervical dislocation 1 h after an intraperitoneal injection of bromodeoxyuridine (BrdU; 20 μ g/g body wt). All mice were killed within the same time period (i.e. in the morning and not later than 1 pm on the same day) and kept under controlled lighting and feeding conditions to minimize possible circadian variations in colonic cell proliferation (19–21).

The colon was removed and 1 cm of rectum was cut into two equal parts. One segment of the colonic tissue was fixed in 10% neutral-buffered formalin and the remaining segment was fixed overnight in 80% ethanol, then transferred to 95% ethanol for an additional 12 h. Slides with 5 μ m thick serial sections of formalin-fixed, paraffin-embedded tissues were stained with hematoxylin and eosin (H&E). A total of 200 μ m thickness was evaluated for FAD and apoptotic cells. Tissue sections for cyclin D₁ immunohistochemistry were also sectioned from formalin-fixed tissue specimens. The specimens fixed in

Abbreviations: AOM, azoxymethane; BrdU, bromodeoxyuridine; FAD, focal areas of dysplasia; LI, labeling index; NMU, *N*-nitrosomethylurea; NSAIDS, non-steroidal anti-inflammatory drugs.

Table I. Cell proliferation in colonic mucosa of normal and AOM-treated CF1 mice fed rutin and quercetin							
Group	п	No. of colonic cells/column	No. of labeled cells/column	LI			
Saline							
AIN	10	16.84 ± 1.46	1.27 ± 0.26	0.0756 ± 0.0150			
AIN + quercetin	9	20.35 ± 3.20^{a}	1.80 ± 0.78	0.0884 ± 0.0280			
AIN + rutin	10	17.58 ± 1.72^{a}	1.45 ± 0.53	0.0827 ± 0.0270			
AOM							
AIN	10	21.92 ± 2.00^{b}	$2.15 \pm 0.77^{\circ}$	0.0980 ± 0.0330			
AIN + quercetin	9	23.76 ± 1.96^{b}	$2.46 \pm 0.80^{\circ}$	0.1031 ± 0.0330			
AIN + rutin	9	23.35 ± 3.74^{b}	$1.79 \pm 0.57^{\circ}$	0.0766 ± 0.0220			

Data represent means ± SD. AIN, AIN-76A diet.

 $^{a}P < 0.01$ compared with the AIN-76A diet group.

 $^{b}P < 0.01$ compared with the AIN-76A diet group.

 $^{c}P < 0.02$ compared with the corresponding group without AOM treatment.

ethanol were used for cell proliferation studies. The remaining colonic tissue was opened, washed in phosphate-buffered saline, fixed in 10% buffered formalin and examined under a dissecting microscope for neoplasms.

Determination of cell proliferation

Following BrdU administration, immunostaining of colonic specimens was performed using a monoclonal antibody against BrdU (Becton-Dickinson, San Jose, CA) and the standard avidin-biotin peroxidase method. The incorporation of the thymidine analogue into cell DNA, evidenced by a brownish nuclear staining, was evaluated in vertically oriented colonic crypts (50 crypt columns). The number and position of BrdU positive cells were recorded in addition to the total number of epithelial cells from each crypt column. Epithelial cell proliferation was also expressed as labeling index (LI) calculated as the number of BrdU-labeled nuclei divided by the total number of colonic nuclei scored.

Evaluation of apoptosis

Apoptotic cells were identified in the flat colonic mucosa by morphological features. These included: (i) nuclear and cytoplasmic condensation and (ii) apoptotic body forming with a halo in the cytoplasm of a host cell appearing either as a large single body or as cluster of small bodies. The apoptotic index was calculated as described for BrdU.

Determination of cyclin D_1

Cyclin D₁ expression was identified by immunohistochemistry. A specific monoclonal antibody to cyclin D1 (Novocastra/Vector, Burlingame, CA) was applied to formalin-fixed tissue sections following heat pre-treatment. Fifty well-oriented crypts were scored from each mouse. Cyclin D1 expression in nuclei was considered positive. The number of cyclin D1 positive cells was recorded for each crypt studied.

Assessment of FAD

The identification of FAD in colonic tissue (5,6) was based on the following histopathological features: (i) nuclei enlarged and hyperchromatic, with variation in size; (ii) nuclear stratification; and (iii) depletion or reduction of mucin. FAD number and size were recorded.

Statistical methods

This study consisted of 57 mice, randomly housed in groups of five animals/ cage and maintained on AIN-76A, AIN + rutin or AIN + quercetin diet. Half the mice in each diet group were injected with AOM and half with the AOM vehicle. The following parameters were analyzed: number of colonic cells/column, number of labeled cells/column and LI (number of labeled cells/ number of colonic cells). FAD incidence was analyzed using a binomial test, conditional on the total number of FAD observed. FAD size was analyzed using the Wilcoxon test. Analyses of colonic cells, labeled cells and LI were carried out using a fixed nested model. The model considers a main effect due to diet (AIN-76A versus AIN-76A + rutin versus AIN + quercetin), a main effect due to AOM (with or without carcinogen) and a nested cage effect. All parameters (colonic cells, labeled cells and LI) were log-transformed before analysis. Significant difference was considered when P < 0.05.

Results

Cell proliferation

The results shown in Table I indicate that (i) the number of colonic epithelial cells per crypt column was increased in each mouse group supplemented with quercetin and rutin compared with the control animals which were maintained on the AIN-76A diet (P < 0.01); and (ii) following AOM administration, the number colonic cells/column and the number of colonic BrdU-labeled epithelial cells per crypt column increased (P <0.01 and 0.02, respectively) in the different diet groups compared with the corresponding controls which received the AOM vehicle only. In control mice not receiving the carcinogen, the expected differential distribution of S-phase cells mainly in the lower crypt was observed. AOM administration led to the increase of labeled cells in the lower proliferative zone of the colonic crypts (P < 0.0.2) compared with the corresponding diet group without carcinogen treatment (data not shown).

Apoptosis

Table II shows the frequency and distribution of apoptotic cells in colonic mucosa of mice kept on various dietary regimens. In the control mice without AOM, significant increase was observed in animals fed rutin and quercetin compared with the animals maintained on the AIN-76A diet only (P < 0.05). The number of apoptotic cells/column and apoptotic indices were markedly increased (P < 0.01) in the AOM-treated group compared with untreated animals.

The distribution of apoptotic cells along the colonic crypt and mucosal surface was markedly different in all treated groups compared with that observed in mice fed the AIN-76A diet only. In the normal mice most apoptotic cells were found on the mucosal surface (74%) and the remainder the upper crypt zone (22%). Administration of either rutin or quercetin resulted in a significant increase (P < 0.001) in apoptotic cells along the crypt axis, including the middle and basal crypt zones. Following AOM treatment, there was an additional rise in the number of apoptotic cells/column in all mice groups. Cells exhibiting apoptotic death were distributed in all colonic crypt compartments. The modified distribution of apoptotic cells was also demonstrated in the ratio of apoptotic cells in mucosal surface and crypt.

Cyclin D_1

Cyclin D_1 expression was evaluated by immunohistochemistry of flat colonic mucosa (Table III). All mice fed the AIN-76A diet only were negative for cyclin D_1 expression. A positive response, however, was noted in animals kept on a diet supplemented either with quercetin (67%, P < 0.01) or with rutin (50%, P < 0.05). The percentages of colonic crypts with

Group	п	Apoptotic index (mean ± SD)	Apoptotic cells per column	Distribution of apoptotic cells (%)					
				Colonic crypt			BC	MS/CC	
				MS	Total	UC	MC		
Saline									
AIN	10	0.017 ± 0.008	0.48	0.36 (73.7)	0.12 (26.3)	0.10 (22.1)	0.01 (2.1)	0.01 (2.1)	3:1
AIN + quercetin	9	0.026 ± 0.009^{a}	0.85 ^b	0.22 ^a (26.2)	0.63 ^c (73.8)	0.24 ^a (28.9)	0.22^{c} (25.6)	0.17 ^c (19.3)	0.35:1
AIN + rutin	10	0.026 ± 0.009^{a}	0.81 ^a	0.05° (6.5)	0.76 ^c (93.5)	0.22 ^b (27.1)	0.25° (30.5)	0.29 ^c (35.9)	0.07:1
AOM									
AIN	10	0.037 ± 0.015^{d}	1.27 ^e	0.23 (17.8)	1.04 ^e (82.2)	0.27 ^d (21.2)	0.36^{e} (28.1)	0.41^{e} (32.9)	0.22:1
AIN + quercetin	9	0.038 ± 0.018	1.49	0.15 (10.3)	1.34 ^f (89.7)	0.35 (23.3)	$0.47^{\rm f}$ (31.5)	$0.52^{\rm f}$ (34.9)	0.11:1
AIN + rutin	9	0.036 ± 0.012	1.45 ^d	0.24 ^e (16.3)	1.21 ^f (83.7)	0.41 ^f (28.3)	0.42 ^f (29.1)	0.38 (26.3)	0.20:1

Table II. Apoptosis in colonic crypts of normal and AOM-treated CF1 mice fed quercetin and rutin

MS, mucosal surface; UC, upper crypt; MC, middle crypt; BC, basal crypt; MS/CC, ratio of apoptotic cells in the mucosal surface and colonic crypt; colonic column includes colonic crypt and the mucosal surface.

 $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ between groups of mice kept on control and supplemental AIN-76A diet with and without AOM administration by Mann–Whitney test.

 $^{d}P < 0.01$, $^{e}P < 0.001$ and $^{f}P < 0.05$ between groups of mice fed the same diet with and without AOM administration by Mann–Whitney test.

Table III. Cyclin D₁ expression in epithelial cells and incidence of FAD in flat colonic mucosa of normal and AOM-treated CF1 mice fed quercetin and rutin

Group	n	Cyclin D ₁	Focal areas of dysplasia		
			No. of mice (%)	No. of mice (%) Total number	
Saline					
AIN	10	0 (0)	0 (0)	0	
AIN + quercetin	9	6 (67) ^a	2 (22)	2	
AIN + rutin	10	5 (50) ^b	0 (0)	0	
AOM					
AIN	10	9 (90%)	10 (100%)	34	
AIN + quercetin	9	7 (78%)	5 (56%) ^c	8	
AIN + rutin	9	8 (89%)	6 (67%) ^c	7	

AIN, AIN-76A diet.

 $^{a}P = 0.003$ compared with AIN-76A diet by Fisher exact probability test. After Bonferroni adjustment for multiple comparison significant difference was seen between AIN-76A and the quercetin groups only.

 $^{b}P = 0.03$ compared with AIN-76A diet by Fisher exact probability test. After Bonferroni adjustment for multiple comparison significant difference was seen between AIN-76A and the quercetin groups only.

 $^{c}P < 0.01$ compared with the AIN group with AOM administration. FAD incidence was analyzed using a binomial test, conditional on the total number of FAD observed.

cyclin D₁-positive cells in these groups were 4.4 and 3.8, respectively. In the AOM-treated group, positive immunohistochemical staining was detected in 90% of animals. There was a slight reduction in the number of mice exhibiting a positive mucosal staining in the AOM groups supplemented with quercetin. The number of cyclin D₁-positive cells/crypt in the AOM group was 0.222; the numbers of cyclin D₁-positive cells/crypt in the rutin and quercetin groups were 0.053 and 0.067, respectively, but with large variability.

Focal areas of dysplasia

Table III shows the incidence of FAD in the colons of CF1 mice maintained on different diets with and without AOM administration. No FAD were identified in saline-treated mice except for the animal group supplemented with dietary querce-tin: two out of nine mice (22%) developed one FAD (Figure 1). All AOM-treated mice kept on the standard AIN-76A diet exhibited multiple FAD in the colon. The incidence of FAD was decreased in AOM mice supplemented with quercetin and rutin (P < 0.01 compared with the AIN-76A group). Both the

number and size of FAD were reduced in the AOM-treated animal groups fed flavonoids.

Discussion

Previous studies in mice and rats have reported that the dietary flavonoids quercetin and rutin protect colonic epithelial cells from carcinogenesis induced by AOM (5–8). In this study, examination of the colonic mucosa was carried out before the development of grossly visible neoplasia. All AOM-treated mice developed FAD, which have been considered precancerous lesions in the colon (5,6). Dietary supplementation of flavonoids to AOM-treated animals resulted in a decrease in the frequency of FAD and in the number of FAD per mouse. These results are consistent with those reported in previous studies by Deschner *et al.* (5,6). Matsukawa *et al.* (7) and Tanaka *et al.* (8) have recently shown that dietary quercetin significantly reduced the number of aberrant crypt foci in AOM-treated rats.

Notwithstanding these findings indicating an inhibitory effect



Fig. 1. FAD in one colonic crypt (arrow) in a CF1 mouse fed quercetin for 9 weeks. High magnification in the inset demonstrates characteristic FAD features: enlarged, hyperchromatic nuclei with initial stratification, variation in size and mucin depletion. H&E, $200 \times (400 \times \text{inset})$.

of the flavonoids in early stages of colorectal carcinogenesis, quercetin alone was able to induce FAD in the normal colonic mucosa of 22% of mice. However, these mice, which did not receive AOM, exhibited only one FAD lesion compared with a larger increase of FAD number in AOM-treated animals maintained on the AIN-76A diet.

The stimulatory effect of AOM on cell proliferation was evident 1 week after the last carcinogen dose: the number of colonic epithelial cells and labeled cells/column was increased compared with untreated animals. Moreover, proliferative cells were increased in number in the middle and upper crypt compartments. These results are consistent with an expansion in the size of the colonic crypt proliferative compartment following carcinogen treatment (22).

An increase in colonic cell number was noted in all groups supplemented with the flavonoids. In contrast, Yoshida *et al.* (23,24) and Hosokawa *et al.* (17) reported that quercetin arrests gastric, colonic and leukemic cell growth in cancer cell lines at the late G_1 phase. Ranelletti *et al.* (25) have shown recently that quercetin inhibits the expression of p21 *ras* in human colonic cancer cells.

Cyclin D_1 protein has been classified as a G_1 cyclin and occupies a nodal position in the regulation of the cell cycle (26–28). Recently, evidence has been provided that overexpression of cyclin D_1 may play a contributory role in the multistep

process of colorectal carcinogenesis (29–31). While normal colonic crypt cells in mice kept on AIN-76A diet were devoid of appreciable cyclin D_1 expression, AOM administration resulted in a marked rise in immunochemical positive cells. Surprisingly, positive staining was also detected in a significant number of control mice fed the AIN-76A diet supplemented with quercetin and rutin, 50 and 67% respectively.

An interesting finding of this study was that dietary quercetin and rutin perturb the growth program of normal colonic epithelial cells by enhancing the apoptotic process. Apoptosis in mice fed the AIN-76A diet was observed predominantly in colonic cells resident in the mucosal surface and in the crypt upper zone. This characteristic zonal distribution of colonic cells in spontaneous apoptosis has been observed by us and others (32,33). Dietary addition of quercetin or rutin in the absence of AOM resulted in an increase in the number of apoptotic cells throughout the colonic crypts, and in a decrease at the mucosal surface.

Of particular interest was the redistribution of apoptotic cells along the colonic crypt axis; a significant number of apoptotic cells was scored in the lower colonic crypt zones. This observation is consonant with reports indicating that drugs and bioactive molecules capable of inducing apoptosis exert their action on colonic cells resident in the crypt proliferative compartment (34).

The precise molecular modes of action of quercetin and rutin in altering the spatial and temporal death program of colonic cells remain to be elucidated. The apoptosis-inducing action of quercetin has been also observed in vivo in regenerating liver after partial hepatectomy (35) in cells of disparate lineages (13,35,36), including colonic adenocarcinoma cell lines (16,37). Wei et al. (38) and Nagasaka and Nakamura (39) have observed that the apoptotic action of the flavonoid on tumor cells is related to the inhibition of the synthesis of heat-shock proteins, a class of bioactive molecules known to play an important role in cell survival. Since quercetin was shown to be an effective protein kinase inhibitor (37-42) an alternative possibility is that the death-inducing action of this compound resides, at least partly, in promoting dephosphorylation of key proteins involved in the control of apoptotic events. Consistent with this notion is the recurring observation that extensive tyrosine dephosphorylation is a contributory determinant in the decision of cells to die an apoptotic death, including normal murine cells of the large intestine (33).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to increase apoptosis in intestinal cancer cells of mice (43). Since quercetin inhibits cyclooxygenase activity (44), a tenable hypothesis is that NSAIDs and the flavonoid share a common path of action in promoting the apoptotic process.

Notwithstanding the apoptotic death induced by quercetin and rutin in colonic cells, the flavonoids elicited hyperplasia expression in normal flat colonic mucosa and dietary administration of quercetin resulted in focal areas of dysplasia. It is noteworthy, however, that hyperplasia in the flavonoid-treated animal groups was not associated with an increase in the number of labeled cells/crypt or with changes in regional crypt cell proliferation. In contrast, the significant rise in the total cell number in AOM-treated mice was consistently accompanied by cell hyperproliferation and expansion of the proliferative compartments. The number of mice fed quercetin that exibited FAD was small (22%), while FAD lesions were more uniformly found in AOM-treated animals. Of note, both the number of mice exhibiting FAD and the total number of FAD observed were significantly reduced in AOM-treated animals fed flavonoids. However, the cellular changes present in the FAD lesions in all groups, including enlarged and hyperchromatic nuclei, variation in size and nuclear stratification are serious alterations involved in carcinogenesis and therefore should be viewed carefully.

We found that supplementation of quercetin or rutin in a standard diet resulted in the expression of cyclin D_1 in normal colonic cells. This is an intriguing observation since altered cyclin D_1 expression and activity have been related, although not consistently, to early stages of colonic tumorigenesis (23–31,45). It may be that the sustained *in vivo* exposure of colonic epithelium to the flavonoids was a promoting factor.

Previous work has shown that quercetin causes frameshift mutations in bacteria (46). *In vivo* administration of the flavonoid resulted in micronucleus formation (47); this observation was not confirmed in another study (48). Pamukcu *et al.* (49) reported that rats fed 0.1% quercetin exibited multiple tumors of the ileum and bladder: these results could not be reproduced by other investigators (50).

In contrast to observations in mice (5,6), dietary treatment with quercetin resulted in a dose-dependent increase in the yield of colonic tumors in AOM-treated rats (51). Recently, Barotto *et al.* (52) have shown that quercetin enhances pretumorous lesions in the rat NMU model of pancreatic carcinogenesis. Sugimara (53) recently reviewed the conflicting evidence for a genotoxic activity of quercetin and concluded that the flavonoid may be mutagenic but is not a carcinogenic agent.

In view of the antioxidant and anticancer effects exhibited by quercetin (18,54–61) studies focused on the absorption kinetics, half-life and bioavailability of the ingested flavonoid have been performed in humans (62–64). Quercetin has also been used in clinical trials in cancer patients (65). Since cancer is a major public health issue and flavonoids are consumed daily, further clarification of the full biological and biochemical effects of quercetin and rutin in humans is warranted.

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