

Preventive effects of chrysin on the development of azoxymethane-induced colonic aberrant crypt foci in rats

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Abstract. The modifying effects of dietary feeding with chrysin (5,7-dihydroxyflavone) on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) were investigated in male F344 rats. We also assessed the effect of chrysin on mitosis and apoptosis in 'normal appearing' crypts. To induce ACF, rats were given two weekly subcutaneous injections of AOM (20 mg/kg body weight). They also received an experimental diet containing chrysin (0.001 or 0.01%) for 4 weeks, starting 1 week before the first dose of AOM. AOM exposure produced a substantial number of ACF (73±13/rat) at the end of the study (week 4). Dietary administration of chrysin caused significant reduction in the frequency of ACF: 0.001% chrysin, 37±17/rat (49% reduction, $P<0.001$); and 0.01% chrysin, 40±10/rat (45% reduction, $P<0.001$). In addition, chrysin administration significantly reduced the mitotic index and significantly increased the apoptotic index in 'normal appearing' crypts. These findings might suggest a possible chemopreventive activity of chrysin in the early step of colon tumorigenesis through modulation of cryptal cell proliferation activity and apoptosis.

Introduction

Colorectal cancer is one of the leading causes of cancer death in Western countries. Globally, colorectal cancer accounted for approximately 1 million new cases in 2002 (9.4% of the world) and mortality is approximately one half that of incidence (~529,000 deaths in 2002) (1). In Japan, its incidence has been increasing and colonic malignancy is now the third leading cause of cancer death. In this context, primary prevention,

including chemoprevention, is important for fighting this malignancy.

Flavonoids are plant secondary metabolites ubiquitously distributed throughout the plant kingdom, and numerous reports have shown their biological effects, such as anti-oxidative and anti-inflammatory activity. They also act as inhibitors of several enzymes that are activated in certain inflammatory conditions (2), while a variety of cell types associated with the immune system are down-regulated by certain flavonoids *in vitro* (3). Further, most flavonoids show potent anti-oxidative/radical scavenging effects (4). A natural flavonoid, chrysin (5,7-dihydroxyflavone, Fig. 1), which is a potent inhibitor of the enzyme, CYP1A (5), and aromatase (6), is present in many plants, honey, and propolis (7,8). Studies have shown that chrysin suppresses lipopolysaccharide (LPS)-induced cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) expression through the activation of peroxisome proliferator-activated receptor (PPAR)- γ (9). In our previous studies, a polymethoxy flavonoid, nobiletin (5,6,7,8,3',4'-hexamethoxyflavone), suppressed the expression of proinflammatory genes, such as iNOS and COX-2, *in vitro* (10) and inhibited azoxymethane (AOM)-induced rat colon carcinogenesis (11). In addition, pomegranate (*Punica granatum L.*) seed oil, which contains more than 70% conjugated linolenic acids, in the diet suppressed AOM-induced colon carcinogenesis in rats through an up-regulation of PPAR γ protein in the non-tumorous colonic mucosa (12). Thus, proinflammatory genes and PPAR γ are good targets for chemoprevention of colon carcinogenesis.

Recently, several *in vitro* studies have shown that chrysin is able to inhibit the growth of Hela cells by downregulating the expression of proliferating cell nuclear antigen (PCNA) (13), induce apoptosis via caspase activation and Akt inactivation in U937 leukemia cells (14), and cause cell-cycle arrest in human colon cancer cells (15), and C6 glioma cells (16). However, there are few reports investigating whether chrysin has cancer chemopreventive effects on the colon in experimental animal studies.

In the current study, we investigated the possible suppressing effect of chrysin on the occurrence of AOM-induced aberrant crypt foci (ACF), which are putative preneoplastic lesions for colonic adenocarcinoma (17-19), with a short-term rat ACF bioassay. In addition, we assess

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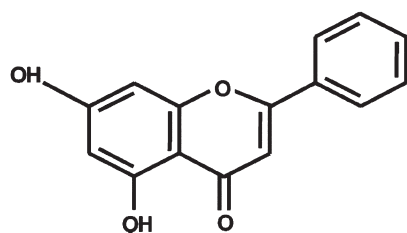


Figure 1. Chemical structure of chrysin.

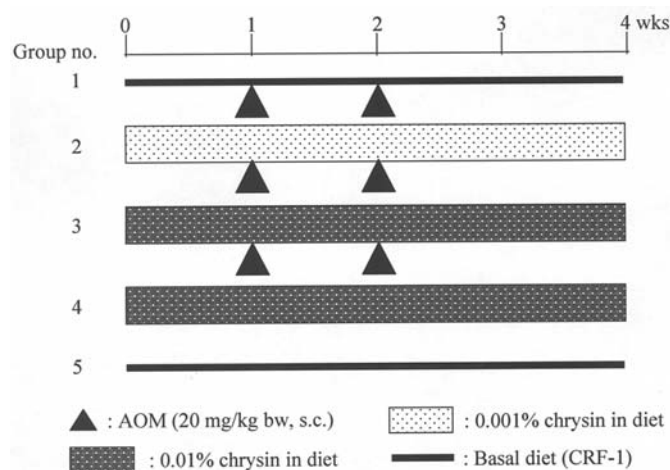


Figure 2. Experimental protocol.

whether dietary chrysin affects cell proliferation activity and induces apoptosis in the colonic epithelium, since certain chemopreventive agents exert cancer inhibitory action through reduction of cell proliferating activity (20) and induction of apoptosis (21) in the target tissue.

Materials and methods

Animals, chemicals and diet. Male F344 rats (Charles River Japan, Inc, Kanazawa, Japan), aged 4 weeks, were used for an ACF assay. The animals were maintained in Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (4 rats/cage) with free access to tap water and a basal MF diet (Oriental Yeast, Co., Ltd., Nagoya, Japan) under controlled conditions of humidity ($50 \pm 10\%$), lighting (12-h light/dark cycle), and temperature ($23 \pm 2^\circ\text{C}$). They were quarantined for 7 days after arrival, and randomized by body weight into experimental and control groups. AOM for ACF induction was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Chrysin was obtained from Funakoshi Co. (Tokyo, Japan).

Experimental procedure for ACF. A total of 32 male F344 rats were divided into five experimental and control groups (Fig. 2). Animals in groups 1 through 3 were initiated with AOM by two weekly subcutaneous injections (20 mg/kg body weight) to induce colonic ACF. Rats in groups 2 and 3 were fed diets containing 0.001% and 0.01% chrysin for 4 weeks, respectively, starting one week before the first dose of

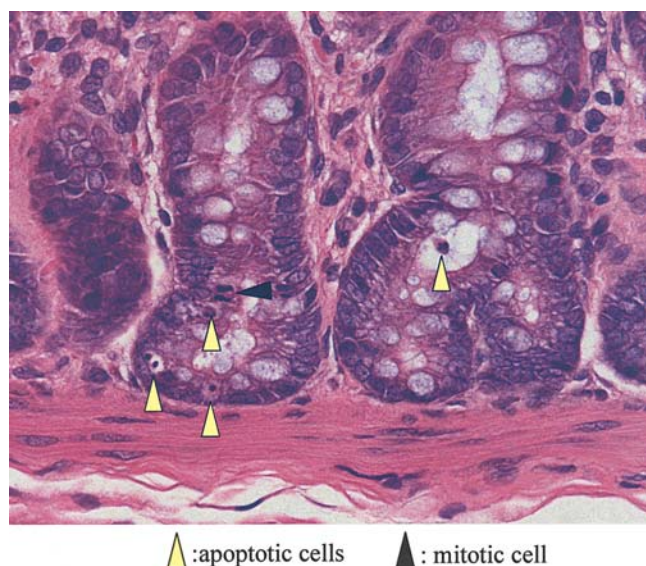



Figure 3. Apoptotic and mitotic cells in the crypt from the distal colon, which was stained with hematoxylin and eosin, from a rat in group 3 (AOM+0.01% chrysin). Apoptotic cells are identified by cell shrinkage, homogeneous basophilic and condensed nuclei, nuclear fragments (apoptotic bodies), marked eosinophilic condensation of cytoplasm and sharply delineated cell borders surrounded by a clear halo. Yellow arrowheads indicate apoptotic cells and the black arrowhead indicates a mitotic cell.

AOM. Group 4 did not receive AOM and were given the diet containing 0.01% chrysin. Group 5 served as an untreated control. At week 4, rats were sacrificed under ether anesthesia to assess the occurrence of colonic ACF and we performed a careful necropsy, with emphasis on the colon, liver, kidney, lung, and heart. All grossly abnormal lesions in any tissue and the organs, e.g. liver (caudate lobe), kidney, lung, and heart, were fixed in 10% buffered formalin solution for histopathology.

Determination of ACF. The frequency of ACF was determined according to the method described in our previous report (22). 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 h. The fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 30 sec, and placed on a microscope slide to count the ACF.

Counting mitotic and apoptotic cells. To identify intramucosal apoptotic and mitotic cells in the crypts, the distal colon (2 cm from the anus) was cut out, embedded in paraffin, and 4 μm -thick serial sections were made. The paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and evaluated under a light microscope for apoptotic and mitotic cells at a magnification of 400 (Fig. 3). Apoptotic cells were identified by cell shrinkage, homogeneous basophilic and condensed nuclei, nuclear fragments (apoptotic bodies), marked eosinophilic condensation of the cytoplasm, and sharply delineated cell borders surrounded by a clear halo (23). The apoptotic and mitotic indices in the colonic crypts were determined on longitudinal sections that allowed evaluation of the whole crypt from the top to the base.

 SPANDIDOS PUBLICATIONS body, liver, and relative liver weights.

Group no.	Treatment (no. of rats examined)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM alone (8)	194±8 ^a	9.7±0.7	5.00±0.68
2	AOM+0.001% chrysin (8)	192±7	10.5±1.1	5.47±0.45
3	AOM+0.01% chrysin (8)	195±5	9.9±0.5	5.10±0.18
4	0.01% chrysin (4)	203±7	10.5±0.9	5.14±0.28
5	No treatment (4)	196±9	9.4±0.5	4.80±0.17

^aMean ± SD.

Table II. Effect of chrysin on AOM-induced ACF formation in male F344 rats.

Group no.	Treatment (no. of rats examined)	Incidence (%)	Total no. of ACF/colon	Total no. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM alone (8)	8/8 (100%)	73±13 ^a	145±28	1.98±0.10
2	AOM+0.001% chrysin (8)	8/8 (100%)	37±17 ^b	67±29 ^b	1.81±0.14 ^c
3	AOM+0.01% chrysin (8)	8/8 (100%)	40±10 ^b	69±21 ^b	1.73±0.09 ^b
4	0.01% chrysin (4)	0/4 (0%)	0	0	0
5	No treatment (4)	0/4 (0%)	0	0	0

^aMean ± SD, ^bsignificantly different from group 1 by one-way ANOVA with Bonferroni correction ($P<0.001$), ^csignificantly different from group 1 by one-way ANOVA with Bonferroni correction ($P<0.05$).

Table III. Effect of chrysin on size of ACF induced by AOM.

Group no.	Treatment (no. of rats examined)	% of ACF containing:			
		1 crypt	2 crypts	3 crypts	≥ 4 crypts
1	AOM alone (8)	43.1±4.7 ^a	27.9±5.9	19.1±5.2	9.9±2.7
2	AOM+0.001% chrysin (8)	44.2±3.8	38.4±4.7 ^b	0.3±4.1 ^b	7.1±6.1
3	AOM+0.01% chrysin (8)	47.3±5.5	34.4±6.4	16.3±3.9 ^c	2.0±3.1 ^b

^aMean ± SD, ^bsignificantly different from group 1 by one-way ANOVA with Bonferroni correction ($P<0.01$), ^csignificantly different from group 2 by one-way ANOVA with Bonferroni correction ($P<0.05$).

Randomly chosen crypts (28-57 crypts/colon) with well-oriented crypt structure from the mouth to the base were evaluated for counting apoptosis and mitosis. The apoptotic and mitotic indices were determined by dividing the total number of apoptotic or mitotic cells by the number of epithelial cells evaluated.

Statistical evaluation. Where applicable, data were analyzed using one-way ANOVA with Bonferroni correction (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA, USA) with $P<0.05$ as the criterion of significance.

Results

General observation. All animals remained healthy throughout the experimental period. Food consumption (g/day/rat) did

not differ significantly among the groups (data not shown). As shown in Table I, the mean body, liver and relative liver weights (g/100 g body weight) in all groups did not differ significantly at the end of the study. Further, no significant pathological alternations were found in organs other than the colon.

Frequency of ACF. Table II summarizes the data on colonic ACF formation. All rats belonging to groups 1 through 3, which were treated with AOM, developed ACF. In groups 4 and 5, there was no microscopically observable change, including ACF, in colonic morphology. The mean number of ACF/colon in group 1 was 73±13. Dietary administration of chrysin (groups 2 and 3) significantly reduced the ACF incidence when compared to group 1: 49% reduction by 0.001% chrysin (group 2), $P<0.001$; and 45% reduction by

Table IV. Epithelial proliferative kinetics in the distal colon.

Group no.	Treatment (no. of crypts examined)	Mitotic index (%)	Apoptotic index (%)	Crypt column height
1	AOM alone (44)	4.3±2.5 ^{a,b}	1.2±1.6	44.2±10.1 ^b
2	AOM+0.001% chrysin (38)	3.2±2.5	3.2±2.3 ^c	43.3±6.9
3	AOM+0.01% chrysin (57)	1.4±1.4 ^c	3.7±2.1 ^c	55.4±10.2 ^c
4	0.01% chrysin (56)	1.8±1.4	1.2±1.2	54.0±11.1 ^d
5	No treatment (28)	1.3±1.4	0.8±1.0	62.0±11.7

^aMean ± SD, ^bsignificantly different from group 5 by one-way ANOVA with Bonferroni correction (P<0.001), ^csignificantly different from group 1 by one-way ANOVA with Bonferroni correction (P<0.001), ^dsignificantly different from group 5 by one-way ANOVA with Bonferroni correction (P<0.01)

0.01% chrysin (group 3), P<0.001. In addition, there were significant decreases in the total number of aberrant crypts (ACs) per colon (P<0.001), and in the number of ACs per focus in group 2 (9% reduction, P<0.05) and group 3 (13% reduction, P<0.001) when compared to group 1. The size distribution of ACF induced by AOM in groups 1-3 showed in Table III. The percentages of ACF consisting of one crypt did not significantly differ among these three groups. Although the percentage of ACF with 2 crypts in group 2 was significantly greater than that in group 1 (P<0.01), the values of ACF with 3 crypts in groups 2 and 3 were significantly smaller than in group 1 (P<0.01 and P<0.05, respectively). As for the percentage of ACF with ≥ 4 crypts, the value in group 3 was significantly lower than that in group 1 (P<0.01).

Indices of mitosis and apoptosis in colonic crypts. The data on the epithelial proliferative kinetics in 'normal appearing' distal colon are summarized in Table IV. The mitotic index was significantly higher in group 1 (4.3±2.5, 331% increase, P<0.001) than in group 5. The dietary administration of chrysin (groups 2 and 3) reduced the mitotic index in a dose-dependent manner when compared to group 1: 26% reduction by 0.001% chrysin (group 2); and 67% reduction by 0.01% chrysin, P<0.001 (group 3). Feeding with 0.01% chrysin alone (group 4) did not affect the mitotic index in the crypts. The apoptotic indices of groups 1, 4 and 5 were comparable, but the values in groups 2 and 3 were significantly increased when compared to group 1 (P<0.001). As for the crypt column height (no. of cells/crypt), the value in group 1 was significantly smaller than in group 5 (P<0.001). The crypt column height of group 3 was significantly larger than that of group 1 (P<0.001). The value in group 4 was significantly lower than in group 5 (P<0.01).

Discussion


The results described here clearly indicate that dietary administration of chrysin at dose levels of 0.001% and 0.01% significantly inhibited AOM-induced ACF formation in male F344 rats. Moreover, the percentage of ACF that consisted of 4 or more aberrant crypts was significantly reduced by feeding with the diet supplemented with 0.01% chrysin. These findings indicate that dietary chrysin effectively suppresses the early phase of chemically-induced rat colon tumorigenesis. Also,

the inhibitory effect of chrysin (0.001%) in the diet on the formation of large ACF may suggest suppression of the late stage of AOM-induced colon carcinogenesis, since the number of large ACF is well correlated with the incidence of colonic adenocarcinoma induced by a colonic carcinogen, AOM (18,19,24). Our results are the first to show the chemopreventive ability of chrysin in ACF formation in an *in vivo* study with a colon carcinogenesis model.

The oral disposition of the dietary flavonoid, chrysin, in humans has been reported (25). Seven healthy subjects were administered 400 mg chrysin orally and the areas under the plasma concentration-time curves (AUCs) and urinary recoveries of chrysin and metabolites were measured. As a result, peak plasma chrysin concentrations were only 3-16 ng ml⁻¹ with AUCs of 5-193 ng ml⁻¹ h, whereas chrysin sulphate concentrations were 30-fold higher (AUC 450-4220 ng ml⁻¹ h). In urine, chrysin and chrysin glucuronide accounted for 0.2-3.1 mg and 2-26 mg, respectively. Most of the dose appeared in faeces as unchanged chrysin. These findings, together with our data, might suggest that unchanged chrysin exists, not in plasma but in intestine, and directly affects the proliferation activity of cryptal cells.

Chrysin is a natural flavonoid that is contained in many plants, honey and propolis. Flavonoids are dietary polyphenols derived from fruits and vegetables (26). Epidemiological observations strongly suggest flavonoids to be preventive in coronary heart disease (27,28), stroke (29) and certain cancers (30). In this study, dietary administration of chrysin reduced the number of mitotic cells and increased the number of apoptotic cells. Recent studies have shown that chrysin induces apoptosis through caspase activation and Akt inactivation in U937 leukemia cells (14), and G2/M cell-cycle arrest in human colon carcinoma SW480 cells (15). Our results are in accordance with those in these *in vitro* studies. Certain components, such as caffeic acid esters and artemillin C, of propolis, which is used as a traditional medicine with a long history in Eastern Europe and Brazil, have been reported to exert antimutagenic and anticarcinogenic effects (31-33). The findings in this study suggest that other components, like chrysin in propolis (0.8 mmol chrysin/100 g of Brazilian propolis) (34), may serve as cancer chemopreventive agents.

In conclusion, this study demonstrates for the first time that dietary administration of chrysin significantly inhibits the development of AOM-induced colonic ACF in rats.

 **SPANDIDOS PUBLICATIONS** the exact mechanisms by which chrysin inhibits development remain to be elucidated, it would appear that the modulation of colon tumorigenesis by chrysin in diet is associated with the alteration of cell proliferation activity and apoptosis.

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