The effect of dietary apigenin on colonic ornithine decarboxylase activity, aberrant crypt foci formation and tumorigenesis in different experimental models.

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Running Title: Dietary Apigenin in Colon Cancer Prevention

Abstract

The efficacy of dietary apigenin, a dietary flavonoid, in colon cancer prevention was investigated by evaluating the inhibition of the ornithine decarboxylase (ODC) activity and the formation of aberrant crypt foci (ACF) and by studying the ability of apigenin to block colon carcinogenesis in two mouse models. First, the activity of ODC was measured in colon cancer cells (Caco-2) and in the colon epithelium of CF-1 mice. Apigenin at 10 and 30 μM significantly inhibited the ODC activity of Caco-2 cells by 26 and 57% respectively. Colonic ODC activity in CF-1 mice was reduced with 0.1% dietary apigenin by 42% compared with the control but this difference was not statistically significant. Second, ACF formation was evaluated in azoxymethane (AOM)-induced CF-1 mice. Female CF-1 mice at 6 weeks of age were i.p. injected with 5 mg/kg body weight (BW) AOM once to induce ACF. ACF formation in CF-1 mice was reduced by 50% ($p<0.05$) with 0.1% dietary apigenin fed for 6 weeks when compared with the control. Dietary apigenin inhibited ACF only in the distal region of the CF-1 mouse colon. Finally, tumorigenesis studies were conducted using two different mouse models: AOM-induced CF-1 mice and Min mice with mutant adenomatous polyposis coli (APC) gene. Female CF-1 mice at 6 weeks of age were i.p. injected with 10 mg/kg BW AOM weekly for 6 (AOM Study I) or 4 (AOM Study II) weeks to induce tumors. CF-1 mice were fed diets containing 0.025 or 0.1% apigenin for 23-25 weeks. Female Min mice were fed diets for 10 weeks beginning at 5 weeks of age. In two AOM-treated mouse colon tumor studies 0.025% and 0.1% dietary apigenin modestly reduced tumors in the group fed 0.025% apigenin (25% incidence in comparison with 65% in the controls) in a non-dose response manner. Apigenin failed to inhibit adenoma formation in the Min mouse study. These results suggest that dietary apigenin showed promise in cancer prevention by reducing the ODC activity and ACF formation, however, clear evidence of cancer

prevention was not obtained in mouse tumor studies. Further investigation of the potential chemopreventive effect of apigenin in carcinogenesis is warranted.

Introduction

Colon cancer is the second leading cause of cancer-related deaths in the United States, and prevention and early detection are two important strategies to protect against this disease (1). Diet has been shown to be a key factor in cancer prevention. Epidemiological data, together with research findings from animal and cell culture studies, provide strong evidence that dietary constituents in fruits and vegetables may contribute to reducing the risk of cancer including colon, breast and lung cancers (2). A high intake of plant foods is associated with reduced risk of colon cancer in humans (3). The classes of phytochemicals with cancer prevention properties include flavonoids, indoles, thiocyanates, isothiocyanates, and polyphenolic acids (4-6). Among these chemicals, flavonoids are relatively nontoxic and several of them have been identified and studied extensively to reveal their important role in chemoprevention of carcinogenesis (4).

Studies in our laboratory have focused on apigenin [5,7,4'-trihydroxyflavone], a plant flavonoid, which is found ubiquitously in plants including parsley, onions, apples and tea (7, 8). It is relatively nontoxic and nonmutagenic when compared with other flavonoids such as quercetin (7). Initial work has shown that apigenin is a promising skin cancer preventive agent. Wei et al. (9) demonstrated that apigenin significantly inhibited TPA-induced epidermal omithine decarboxylase (ODC) activity and the development of papillomas and carcinomas in a two-stage DMBA/TPA mouse model when applied topically to the SENCAR mouse skin tissue. In addition, apigenin also induced G_2/M arrest in keratinocytes (10) as well as in human colon carcinoma cell lines, in particular, SW 480 cells (11). These results together with the strong correlation between high intakes of fruits and vegetables and reduced cancer risk have prompted our laboratory to further study the efficacy of apigenin in the chemoprevention of colon

carcinogenesis using both preneoplastic markers and tumor protocols. The objective of the current series of experiments was to test the hypothesis that apigenin would inhibit colon carcinogenesis in mouse models.

In the studies described in this report the activity of omithine decarboxylase (ODC) and the formation of aberrant crypt foci (ACF) were assessed to evaluate the inhibitory effect of apigenin on colon cancer. An elevated level of ODC has been associated with the increased rate of cell proliferation in colon tumors (12). In the present study, colonic ODC activity was evaluated in Caco-2 cells, a human colon carcinoma cell line, and in colonocytes from CF-1 mice. ODC was included in the present study as an early marker of colon cancer because of its prior use in studies of apigenin and skin cancer prevention (7,9). The efficacy of apigenin in chemoprevention of colon cancer was evaluated in vivo by the assessment of ACF in a short-term feeding study (6 weeks) followed by a long-term feeding tumorigenesis study (23 or 25 weeks) using the azyoxymethane (AOM)-induced CF-1 mouse model.

Aberrant crypt foci (ACF) are early preneoplastic lesions induced in a dose-dependent manner in colonic mucosa of carcinogen-treated mice (13) and are found in human colonic mucosa of patients with colon cancer (14). Methodology has been developed to detect ACF as a preneoplastic marker of colon cancer risk and ACF have been widely used to assess the impact of dietary factors on colon cancer risk in animal models.

Mutations of the adenomatous polyposis coli (APC) gene have been observed at an early stage of human sporadic colorectal carcinogenesis, which then leads to over-expression of its downstream effector, beta-catenin (15). The AOM model involves activating mutations in beta-catenin comparable biologically to the end result of APC mutations (16). On the other hand, the germline

mutation in codon 850 of the APC gene in Min (multiple intestinal neoplasia) mice causes them to spontaneously develop a large number of intestinal polyps that are reminiscent of familial adenomatous polyposis (FAP) (17). In addition to the mutations of APC observed in large proportions of sporadic colorectal tumors (18), mutations in the K-ras proto-oncogene involved in the subsequent transformation of benign adenomas to malignant adenocarcinomas are also quite commonly found in ACF and colon tumors as seen in the AOM-induced colon tumors (19).

Therefore, the comparison of the impact of apigenin on colon carcinogenesis in these two mouse strains may provide important information on the genetic lesions in colon that increase sensitivity to apigenin growth regulation.

Materials and Methods

Chemicals

Apigenin was purchased from Fluka (Milwaukee, WI) for the study of ODC. The purity of apigenin was estimated to be at least 95% by HPLC. For the ACF and tumorigenesis studies apigenin was purchased from Toronto Research Chemicals (Ontario, Canada) because of availability and improved purity. The purity was estimated to be at least 98% as measured by high field Nuclear Magnetic Resonance and Thin Layer Chromatography. Since purity was above 95% with both sources we did not adjust for any small difference in purity in the studies reported here. DMSO and AOM were obtained from Sigma Chemical Company (St. Louis, MO).

Diets

The control diet was modified from AIN-93 by removing the antioxidant and replacing the carbohydrate from sucrose with a glucose-dextrin mixture (20). The carbohydrates were changed from the AIN formulation to reduce the intake of simple sugars by the mice. The control diet did not contain apigenin. Apigenin was added to the supplemented diet by grinding it with increasing amounts of control diet until the desired dilution was achieved. Both control and apigenin supplemented diets were made from ingredients purchased from Teklad Test Diets (Madison, WI), mixed in a Hobart mixer and mixed into a slurry by the addition of deionized water, dried and cut into blocks for administration to the mice. Diets were prepared monthly and stored at refrigerated temperature for no longer than one month.

Animals

Female CF-1 mice at 6 weeks of age were obtained from Charles River (Portage, MI). Female Min mice at 5 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). Females were used for these studies because of the possible estrogenitity of apigenin that could be assessed by measuring uterine weights. Upon arrival, mice were randomly grouped and kept under standard conditions (12-h light/12-h dark cycle, humidity- and temperature-controlled). They were fed the control diet during the week of acclimatization. At the end of the experiment, mice were sacrificed by cervical dislocation. Colon tissue was excised for the analysis of ODC activity, ACF or tumor formation in CF1 mice and small intestine and colon was excised for adenoma evaluation in the Min mice study.

ODC Studies

Two ODC studies were performed; one with CaCo2 cells that were incubated with and without apigenin for up to 39 hours and the other with mice fed diets with or without apigenin for one

week. ODC activity was measured by assessing the release of $^{14}CO_2$ from 1-C^{14}-ornithine after 30 min of reaction at 37°C. Results were expressed as nmol CO_2/mg protein/30 min (9).

Caco-2 cells, a human colon carcinoma cell line, were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Medium (DMEM, Sigma Chemical Company, St. Louis, MO) with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) at 37°C in 5% CO_2. At 50-80% confluence, the cells were treated with apigenin at 0-30 μM for 15-39 hours. Apigenin was dissolved in DMSO and the final concentration of DMSO in all cultures was kept at 0.2%. After treatment with apigenin for 15-39 hr, Caco-2 cells were washed twice with cold PBS containing 0.01% trypsin inhibitor. Then cells were suspended in 1 ml homogenization buffer and homogenized with a Polytron homogenizer for 15 sec at level 10. The cell extract was then centrifuged at 15,000 rpm for 15 mm. Supernatant was saved for protein determination by the BCA method (Pierce, Rockford, IL) and ODC was assayed as described above.

CF-1 mice were injected with a single i.p. injection of AOM at 5 mg/kg BW or saline and were fed control diet or control diet containing apigenin at 0.05% or 0.1% for one week (3 mice per group). Mouse colons were excised and stools were removed and the luminal colon was flushed with saline containing 0.015% DTT (Dithiothreitol). The colon lumen was then filled with Joklin's MEM and was incubated in a beaker of PBS at 37°C for 6 mm, it was then filled with Weiser B solution (PBS containing 0.04% EDTA and 0.008% DTT) and was incubated at 37°C for 12 mm. The colon cells were collected in the wash and diluted with 1 ml Weiser B solution before centrifugation at 1000-1500 rpm. The supernatant was discarded and an equal volume of homogenization buffer was added for sonication. Protein determination and ODC assay were conducted as described above.

ACF Study

An ACF study was conducted to assess the impact of dietary apigenin on this preneoplastic lesion induced by AOM. Seven to eight weeks old female CF-1 mice received a single 5mg/kg B.W. i.p. injection of saline or AOM. Three days after the administration of AOM or saline, mice were fed basal diet (8 AOM treated, 5 saline treated) or diet containing apigenin at 0.025% (8 AOM treated) or 0.1% (8 AOM treated, 5 saline treated) for 6 weeks. The time of sacrifice was determined based on the earlier observations of Bird (13) and on our observations with 4 sentinel AOM treated mice that were fed basal diet for 5 weeks to determine if adequate ACF were developing in the positive control group. Upon termination at 6 weeks, a section of 5-cm distal colon was excised, flushed with phosphate buffered saline (PBS), fixed flat longitudinally in 10% buffered formalin at least overnight and stained with methylene blue (0.2% in PBS) for 20 minutes for the assessment of aberrant crypts (AC) and aberrant crypt foci (ACF). AC were identified by their increased size, thicker epithelial lining, elongated luminal opening and increased pericryptal zone and were counted under a microscope using l0X objective (13). A cluster of AC was counted as an ACF. The number of ACF observed per colon, the number of AC per focus, the size of focus and the location of the focus were examined. The size was determined by counting the number of grids in the eyepiece of the light microscope at 10X magnification where each grid represented 10^{-2} mm^2. The location of ACF was assessed in three regions — distal colon (D), midcolon (M) and proximal colon (P) (13). The D region is the first 2 cm proximal to the rectum, M region is the next 2 cm from D, and P region is the next 1 cm from M. This distal 5 cm segment of the colon accounted for on average 50% of the tissue but all ACF were expected in this region based on earlier studies with mice (21). One observer who was blinded to the treatment group assessed all the ACF.

Tumorigenesis Studies

Three tumorigenesis studies were conducted using two mouse models: chemically-induced CF-1 mice (AOM Study I and AOM Study II) and Min mice with an APC mutation (Experimental designs are summarized in Table 1).

AOM Study I used CF-1 mice injected at 7-8 weeks of age with a single i.p. injection of AOM in saline at 10 mg/kg BW or saline alone for the controls and this was repeated weekly for 6 weeks. Beginning 3 days after AOM or saline treatment of 14-week old mice the experimental diets were fed as described in the ACF study. The initial number of mice for AOM Study I fed basal diet was 30 AOM treated, 8 saline treated; fed diet containing 0.025% apigenin was 30 AOM treated and fed diet containing 0.1% apigenin was 31 AOM treated, 8 saline treated. These diets were fed for 25 weeks in. AOM Study 1 used a treatment protocol anticipated to induce colon tumors in 80-100% of the mice fed basal diet. Decisions on the termination of mice were made as described below. Colon tissues from CF-1 mice were then collected and saved for histology as described below. For the AOM-induced CF-1 mouse studies, the uterus was also excised and weighed to evaluate estrogenicity of apigenin. The ratio between uterus weight and body weight was used to assess potential estrogenicity.

AOM Study II used CF-1 mice injected at 7-8 weeks of age with a single i.p. injection of AOM in saline at 10 mg/kg BW or saline alone for the controls and this was repeated weekly for 4 weeks. Beginning 3 days after AOM or saline treatment of 11-week-old mice the experimental diets were fed as described in the ACF study. The initial number of mice for AOM Study II fed basal diet was 41 AOM treated, 10 saline treated; fed diet containing 0.025% apigenin was 31 AOM treated and fed diet containing 0.1% apigenin was 31 AOM treated, 8 saline treated These

diets were fed for 23 weeks. AOM Study II used a shorter treatment protocol to determine if apigenin could prevent a more modest induction of colon tumors. Decisions on termination of mice and preparation of tissues were as described for AOM Study I.

Min mice in groups of 10 mice each were fed basal and basal diet containing apigenin at 0.1% for 10 weeks following acclimatization. Only the highest dose of apigenin was used because of the limited number of min mice in the study. Decisions on termination of mice were made as described below. Intestinal tissue from the Min mice were then collected and saved for histology and tissue was evaluated based on three sections (colon, distal small intestine, and proximal small intestine).

In all three tumorigenesis studies mice were checked daily. Body weight and food consumption was recorded weekly. Rectal prolapse and bloody feces were initially observed at 14 - 16 weeks after AOM initiation in the CF-1 mice. Criteria for euthanasia were established in conjunction with the veterinarians from Laboratory Animal Resources at Iowa State University for both the AOM and Min mouse studies. The criteria included 1) the appearance of rectal prolapse for more than two days, 2) lack of activity or responsiveness accompanied by over 15% loss in body weight within a week. As soon as either of these criteria was verified, the mouse was euthanized. In addition, fecal blood was tested using Hemoccult Kit (Hawkeye Medical Supply, Iowa City, IA) as a marker for the termination of the study. At the end of the experiments, all mice were killed by cervical dislocation and intestinal tissue from the proximal end of the rectum to the proximal small intestine was excised. All adenomas were examined visually, and the distance from the rectum, size and color were recorded. The examiner was blinded to the treatment group of each specimen. The intestinal tissue was removed, fixed in 10% buffered formalin for 12 h and subsequently transferred to 80% ethanol for storage until histology was performed. An

average of four longitudinal slides were made from each sample and they were stained with hematoxylin\eosin. Tumors were scored by microscopic evaluation of the colon by an observer blinded to treatment group. Gross observation was performed to identify any abnormality of major organs such as lungs, livers, stomachs, spleens and kidneys.

Statistical Analyses

Results the from ODC, ACF, and Min mice studies were analyzed using analysis of variance. If an F-test demonstrated significant differences in the mean responses, t-tests were used to compare results for each apigenin supplemented diet against the basal diet, otherwise no differences were declared significant. Similarly, in each tumorigenesis study, an exact randomization test was used to compare survival rates or tumor incidence rates, across diets. Separate tests were done for the AOM induced and saline treated groups. If an overall test demonstrated a significant difference in rates, Fisher exact tests were used to compare the rate for each apigenin supplemented diet against the rate for the basal diet. All tests were performed at the 0.05 level of significance. These procedures guarantee that the overall probabilty of falsely declaring a significance difference does not exceed 0.05 in any study. Microsoft Excel was used for the statistical analysis of the ODC and ACF studies and the SAS statistical package was used to analyze data from the tumorigenesis study.

Results

ODC studies

Caco-2 cells: The ODC activity was significantly inhibited by 10 μM or 30 μM apigenin in Caco-2 cells when compared with the control (Figure 1).

CF-1 mice: In vivo, dietary apigenin at 0.1% reduced the colonic ODC activity in CF-1 mice by 42% when compared with the control diet, but the difference was not statistically significant. Whereas mice fed apigenin at 0.05% in the diet clearly exhibited the same colonic ODC activity as the controls (Figure 2).

ACF study

Neither dietary apigenin nor the carcinogen treatment affected the mean body weight. The mean body weight of AOM- or saline-treated groups at the end of the study was 32 ± 1 and 33 ± 1 g, respectively. As shown in Table 2, dietary apigenin at 0.1% significantly reduced the total number of ACF per colon by 50% when compared with the control. With respect to the distribution of ACF, significant inhibition was observed only in the distal region (D) of the colon in comparison with the control. On the other hand, dietary apigenin at 0.025% and 0.1%, respectively, did not significantly affect the number of AC per focus and the size of foci when compared with the vehicle control, respectively (Table 2). In addition, dietary apigenin did not promote the formation of ACF in the saline groups (Table 2).

Tumorigenesis studies

AOM-induced mouse model – AOM Study I

In the first AOM tumorigenesis study, CF-1 mice received a single weekly i.p. injection of AOM in saline for 6 weeks. The average body weight of the AOM-treated mice decreased significantly at 0.1% dietary apigenin with respect to the basal diet group. However, no statistical difference was observed between 0.1% dietary apigenin and basal diet groups for the saline-treated mice (Table 3). This suggests that dietary apigenin at 0.1% alone did not affect the body weights of

CF-1 mice, but dietary apigenin at 0.1% with the AOM treatment decreased body weight. In addition, food consumption was not significantly different among any of the groups (Table 3).

Bloody feces and prolapse were first seen in the AOM-treated mice at week 12. In the remainder of the study, these symptoms continued to appear until they were observed in 50% of the AOM-treated mice. Some AOM-treated mice died before week 12 probably due to AOM toxicity and they did not have signs of bloody feces or prolapse. Nevertheless, there were no significant differences in average time of death among dietary treatments for AOM-treated groups in AOM Study I (Table 4). Tumor formation was first noted between week 14 and 18 in the colons of those AOM-treated mice that died or were euthanized due to the severity of body weight loss or prolonged prolapse. Overall, tumors were found only in the colons of AOM-treated mice but not in the saline mice. A modest, but not statistically significant, decrease in tumor incidence was observed in the AOM treated 0.025% and 0.1% apigenin groups (Table 4, AOM Study I). According to our gross observation, nodularity of lungs, enlarged spleens and/or enlarged kidneys were observed in the majority of AOM-treated mice (80%) regardless of dietary treatments (data not shown). These observations were believed to be evidence of AOM toxicity but were not related to dietary apigenin. Among the samples examined histologically, no significant differences were observed (Table 4) in the mean number of adenomas per colon. Gross observation of tumors was consistent with histology in terms of the presence of tumors in colons. Of the colons in which tumors were observed grossly, at least 90% were also identified histologically and verified tumors or adenomas. Among the adenomas, more than 90% of them were found in the distal colon. As expected, no tumors were found in the colons of saline groups. Estrogenicily of apigenin was assessed by the ratio of uterus weight/body weight. The mean ratio was 4.3 ± 0.6 ($X\ 10^{-3}$) and 5.4 ± 0.4 ($X\ l0^{-3}$) for saline- and AOM-treated groups, respectively.

No statistical differences were observed within the groups suggesting the increase in uterus weight/body weight was attributed to the treatment of AOM, not dietary apigenin.

AOM-induced mouse model – AOM Study II

In the second AOM tumorigenesis study, CF-1 mice received four weekly injections of AOM. Unlike the first AOM tumorigenesis study, AOM treatment did not significantly lower the mean body weights of mice when compared with the saline groups throughout the experiment. No significant difference was observed in terms of food consumption among any of the groups (Table 3). About 20% of the AOM-treated mice developed bloody feces and/or prolapse. These lesions started to appear at week 14 after the final AOM treatment and they continued to appear until the end of the study. Euthanasia and deaths after the AOM treatments with the incidence of bloody feces and prolapse were observed as early as week 12-14 for the AOM-treated mice fed basal and 0.025% apigenin diets. The AOM-treated 0.1% apigenin-fed group all survived through the end of the experiment and the survival rate was significantly improved in comparison with the other two AOM-treated groups (Table 4). Fecal blood was tested at week 22. There were 38%, 21% and 44% of mice in the basal, 0.025% and 0.1% apigenin-fed groups with evidence of fecal blood. Mice fed dietary apigenin at 0.025% had a significant lower rate of tumor incidence when compared either with 0.1% apigenin or with basal diet groups. Mice with bloody feces exhibited a significant, two-fold, increase in tumor incidences (23 mice with tumors/28 mice with bloody feces (82%) compared with 16 mice with tumors/52 mice without evidence of bloody feces (31%) ($\chi^2 = 19.23$, $P<0.0001$). However, no significant diet differences were observed among AOM-treated mice with respect to the incidence of fecal blood. As expected, tumors were found in the colons of AOM-treated mice only. In this study, histology was not performed.

Min mice model

The body weight of Min mice increased for the first six weeks and then started to decline slightly probably due to the increased adenoma burden (data not shown). The initial and final body weights for the controls were 15.7 ± 0.3 and 19.1 ± 1.0 g, respectively, whereas those for apigenin fed group were 15.5 ± 0.2 and 19.7 ± 0.8 g, respectively. Food consumption for both groups during the 10-week period decreased from an average of 2.8 ± 0.1 to 1.7 ± 0.1 g per day, and no significant difference was observed between the basal diet and apigenin-fed groups throughout the study. Gross observation showed that greater than 99% of tumors were found in the small intestine. All the tumors were adenomas by histology. The total number of adenomas in the small intestine was shown in Figure 3. A predominance of adenomas was found in the distal small intestine for both diet groups, less than 26% were located in the proximal small intestine and less than 1% in the colon. Overall, dietary apigenin at 0.1% resulted in a 15% reduction in total number of adenomas, however, the variability among animals was high and no statistical differences were observed.

Discussion

The focus of this study was to investigate the efficacy of apigenin in the chemoprevention of colon carcinogenesis using cultured cells incubated with apigenin and dietary administration of apigenin in mouse models. Models were used representing early markers (ODC), preneoplastic lesions (ACF) and fully developed adenomas (AOM induced and Min mouse adenomas). In prior studies, apigenin was shown to inhibit the epidermal ODC activity induced by TPA in SENCAR mice. In addition, topical treatment of apigenin on DMBA-initiated and TPA-

promoted SENCAR mouse skin also inhibited the development of papillomas and decreased the conversion of papillomas to carcinomas (9). In this study, we found that apigenin concentrations as low as 10 μM significantly inhibited the ODC activity in Caco-2 cells whereas dietary apigenin at 0.1% didnot significantly reduce the activity of ODC in AOM treated CF-1 mouse colonic mucosa, although the value was reduced to 42% of the control activity. Furthermore, we observed that dietary apigenin fed at 0.1% for five weeks significantly inhibited the formation of ACF by 50% and most of the inhibition took place in the distal region of the colon. Because colonic ODC activity has been suggested to be a good cellular proliferation marker to identify patients at risk of colorectal cancer (22) and ACF are a preneoplastic surrogate marker for colon tumors (23, 24), our results suggested that the apigenin dosage that we used exhibited some promise in the prevention of colon cancer. It is noteworthy that these studies of early lesions used both carcinogen induced (ODC and ACF in mice) and carcinogen free (ODC in cancer cells) systems.

In order to further investigate the potential inhibitory effect of apigenin and its long-term feeding effect in the prevention of colon carcinogenesis, we conducted three tumorigenesis studies. Two different mouse models were used: AOM-induced CF-1 mouse model and the genetically induced Min mouse model. Two trials (AOM Study I and II) were conducted using the AOM-induced tumorigenesis model with six and four treatments with AOM, respectively. In the first trial (AOM Study I), a high mortality rate was associated with AOM treatment and evidence of AOM toxicity was observed in internal organs in CF-1 mice. Moreover, all of the AOM-treated control-fed mice had colon tumors and a modest reduction in incidence was observed in AOM-treated, apigenin-fed mice. In considering body weight, it is possible that an interaction took place between dietary apigenin at 0.1% and the amount of AOM administered. This was

suggested by the observation that the mean body weight of mice fed dietary apigenin at 0.1% was significantly lower than that of the vehicle control in the AOM Study I. One possible explanation of the modest reduction in colon tumors in this study was that the dosage of AOM was excessive and this high AOM dose made it difficult for apigenin to exert a strong chemopreventive impact on tumor formation.

Thus in the second trial the duration of AOM treatment was reduced. The survival rate of mice treated with AOM improved, especially with 0.1% apigenin, and the rate of tumor incidence in AOM-treated groups decreased, especially in the 0.025% apigenin-fed group. In comparison with the vehicle control, dietary apigenin at 0.025% yielded a significantly lower rate of tumor incidence, on the other hand, dietary apigenin at 0.1% did not reduce tumor incidence. Furthermore, the significant reduction in the mean body weight was not observed in the second study with the reduced dose of AOM administered. In addition, dietary apigenin at 0.1% alone did not affect the body weight as seen in the saline group. In summary, in the second study with low dose AOM some benefits of apigenin on survival (0.1% dietary apigenin) and on colon tumor (0.025% dietary apigenin) were observed.

Unfortunately, our results from these tumorigenesis studies did not show strong and consistent colon cancer prevention as was suggested by our previous ACF and ODC studies. Imperfect concordance between ACF and adenoma studies was previously observed in reviewing data in rats (24). The agents that were most effective in reducing ACF were not necessarily the most effective in inhibiting colon carcinogenesis. Overall, we found that the number of AOM administrations had a considerable effect on mortality rate, tumor incidence and body weights between the two studies. An inhibitory effect of dietary apigenin was not observed in a dose-dependent manner in either study, although a modest reduction in tumor rate was observed with

both 0.025% and 0.1% apigenin in AOM Study I and the tumor incidence in the AOM Study II was significantly lower at 0.025% dietary apigenin but not at 0.1 % apigenin. However, an earlier study with apigenin injected s.c.(0.75 or 1.5 mg/kg body weight every other day) into rats following AOM treatment reported no evidence of colon cancer prevention by this mode of administration. In addition, although apigenin injections did not inhibit bombesin enhanced AOM induced colon cancers, apigenin administration in this manner did inhibit cancer metastasis (including lymphatic vessel invasion) in AOM and bombesin injected rats (25).

For the genetically-induced Min mouse model, dietary apigenin at 0.1% did not show an inhibitory effect. The germline mutation in codon 850 of the adenomatous polyposis coli (APC) tumor suppressor gene in Min mice initiates genetic events as observed at an early stage of human colorectal carcinogenesis. Nevertheless, the validity of this model for studying colon carcinogenesis is questionable. Our data, in agreement with other literature findings (26-28), showed that the majority of adenomas were present in the small intestine whereas less than 1% were found in the colon. Thus the adenomas in the Min mouse study developed in the absence of a colonic microenvironment. Besides the earliest mutation of APC gene, mutations in the K-ras proto-oncogene are also involved in the subsequent transformation of benign adenomas to malignant adenocarcinomas as seen in the AOM-induced colon tumors (19). Therefore, the Min mouse model may be most relevant in the study of familial adenomatous polyposis (FAP) patients (17), but may be not the case for sporadic human colon cancer.

Perhaps, the efficacy of chemopreventive properties of flavonoids is attributed to the different combinations of flavonoids. Numerous epidemiological studies have indicated that increased consumption of fruits and vegetables is correlated with the reduced risk of cancers including colon cancer. There are thousands of flavonoids found ubiquitously in fruits and vegetables. The

physiological interactions of flavonoids undoubtedly have to be taken into account. Although we failed to illustrate a strong protective effect of dietary apigenin in isolation against tumor formation, the effect of a combination of flavonoids is warranted to study the long-term effect of these flavonoids in prevention of colon cancer.

In conclusion, further investigation of the potential chemopreventive effect of apigenin in colon carcinogenesis is warranted as apigenin has been shown to be an inhibitor of cultured colon cancer cell ornithine decarboxylase and aberrant crypt foci formation in mice. However, administering an optimal dose of AOM to induce tumor development and, at the same time, to allow apigenin in isolation or in combination with other potential flavonoids to exert its chemopreventive properties in colon carcinogenesis may prove to be challenging.

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Table 1. Experimental Design of the tumorigenesis studies in mice			
Study	AOM dose	Apigenin Dose	Weeks of apigenin feeding
AOM Study I	10 mg/kg body weight 6 weekly treatments	0.025 and 0.1 % in the diet	25 weeks, beginning at 14 weeks of age
AOM Study II	10 mg/kg body weight 4 weekly treatments	0.025 and 0.1 % in the diet	23 weeks, beginning at 11 weeks of age
Min mouse Study	none	0 0.1 % in the diet	10 weeks, beginning at 6 weeks of age

Table 2. The effect of dietary apigenin on the formation of aberrant crypt foci induced by azoxymethane in CF-1 mice.

Diet	Incidence	Average # AC/focus (n1)	Total ACF/mouse[1]	ACF Distribution/mouse			Average size of foci at 10X (x 10-2 mm^2)
				D[2]	M[3]	P[4]	
AOM							
Basal	8/8	1.2 ± 0.1	6.6 ± 0.7	2.9 ± 0.7	3.8 ± 0.6	0	1.7 ± 0.5
0.025% Apigenin[5]	5/6	1.3 ± 0.3	6.0 ± 2.1	2.5 ± 1.0	3.3 ± 1.2	0.2 ± 0.2	2.4 ± 0.6
0.1% Apigenin[5]	6/8	1.1 ± 0.3	3.3 ± 1.0*	0.8 ± 0.3*	2.5 ± 0.9	0	1.8 ± 0.4
Saline							
Basal	1/5	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0	0	0.5 ± 0.5
0.1% Apigenin	0/5	0	0	0	0	0	0

[1]Mean±SE.

[2]D = the first 2 cm from the distal end.

[3]M = the next 2 cm from D.

[4]P =the next cm from M.

*$p<0.01$ compared with the AOM, Basal group using Student's t-test.

Table 3. Body weight and food consumption data for AOM-induced colon tumor studies in CF-1 mice.

Treatment	Diet	Final Body Weight[3,4] (n)	Food consumption[3] Initial	Food consumption[3] Final
AOM STUDY I[1]		*g*		*g/d*
AOM	Basal	38±1[1](24)	3.8±0.1	3.5±0.1
	0.025% Apigenin	36±1(21)	3.6±0.3	3.7±0.1
	0.1% Apigenin	35±1* (22)	3.5±0.2	3.6±0.1
Saline	Basal	39±2 (6)	3.6±0.3	3.7±0.1
	0.1% Apigenin	40±1(7)	3.5±0.1	3.9±0.4
AOM STUDY II[2]				
AOM	Basal	40±1(29)	3.5±0.2	3.9±0.3
	0.025% Apigenin	42±2 (24)	3.6±0.1	3.8±0.4
	0.1% Apigenin	38±1 (27)	3.6±0.1	3.8±0.2
Saline	Basal	42±2 (10)	3.7±0	4.4±0.2
	0.1% Apigenin	38±3 (8)	3.6±0.1	3.8±0.4

[1]For AOM Study I, mice received a single i.p. injection of AOM weekly for 6 weeks and were fed experimental diets for 25 weeks beginning after the final AOM treatment. Initial and final measurements were taken before any AOM treatments and 25 weeks after the final AOM treatment, respectively. The average daily consumption of diet was presented.

[2]For AOM Study II, mice received 4 weekly AOM injections and were fed experimental diets for 23 weeks after the final AOM treatment. Initial and final measurements were taken before any AOM treatments and 23 weeks after the final AOM treatment, respectively. The average of daily consumption of diet was presented.

[3]All values are given as mean±SE.

[4]Initial body weight ranged between 21.6 - 22.4 g for AOM Study I and between 21.2 - 22.0 g for AOM Study II. The final numbers of mice are presented in parentheses.

*$p<0.05$ when compared with the Basal group using Student's t-test.

Table 4. Data from gross observation and histology for AOM-induced tumorigenesis studies in CF-1 mice. Au 30

				(Tumor incidence)	
Diet	n	Total # dead	Survival rate through the end (%)	Total # mice with colon tumors diagnosed at death / total # assessed (%)	Avg week[1] on expt at death with colon tumors diagnosed
AOM STUDY I					
Basal	30	6	24/30 (80)	28/28 (100)	24.1
0.025% Apigenin	29	8	21/29 (72)	22/27 (82)	24.0
0.1% Apigenin	29	7	22/29 (76)	23/29 (79)	23.8
Basal	8	2	6/8 (75)	0/8 (0)	NA
0.1% Apigenin	8	1	7/8 (88)	0/7 (0)	NA
AOM STUDY II					
Basal	34	5	29/34 (85)	22/34 (65)	21.8
0.025% Apigenin	29	5	24/29 (83)	8/28 (29)[#]	20.5
0.1% Apigenin	27	0	27/27 (100)*	16/27 (59)	23.0
Basal	10	0	10/10 (100)	0/10 (0)	NA
0.1% Apigenin	8	0	8/8 (100)	0/8 (0)	NA

[1]Number of weeks after the final AOM treatment.

[2]Mean±SE

[3]Mice received a single i.p. injection of AOM at 10 mg/kg BW weekly for 6 weeks. Mice were killed 25 weeks after the last AOM injection.

[4]Mice received a single i.p. injection of AOM at 10 mg/kg BW weekly for 4 weeks. Mice were killed 23 weeks after the last AOM injection.

*$p<0.05$ when compared with the AOM, Basal group using Chi-square test.

[#]$p<0.05$ when compared with the AOM, Basal group or with the 0.1% AOM apigenin group using Chi-square test.

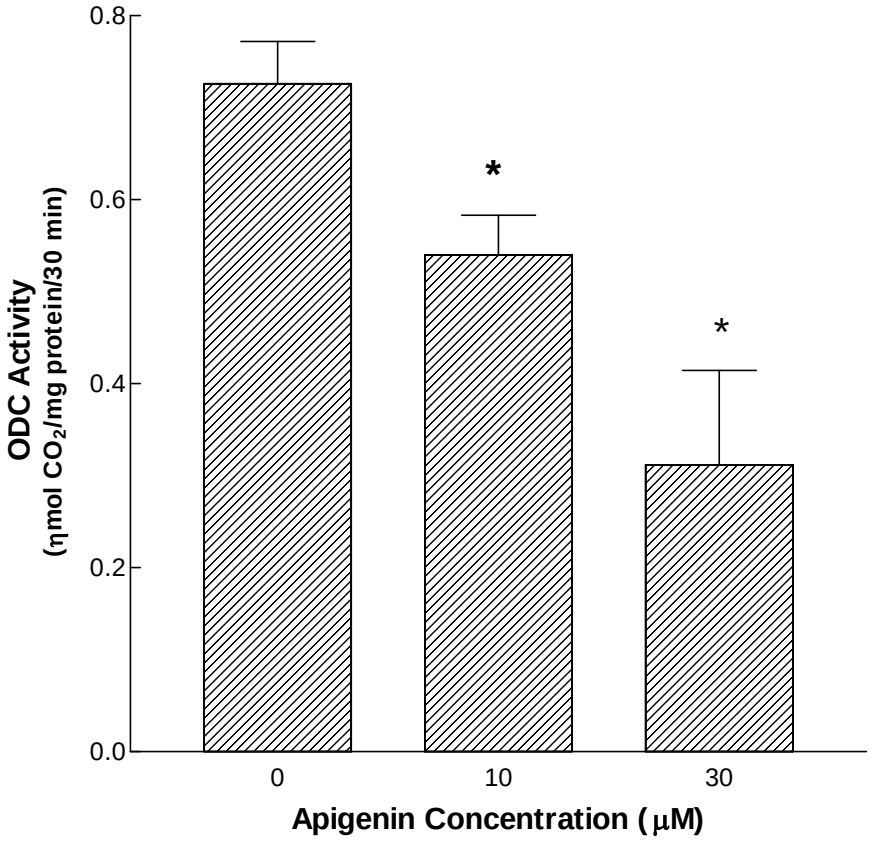
Table 4 continued

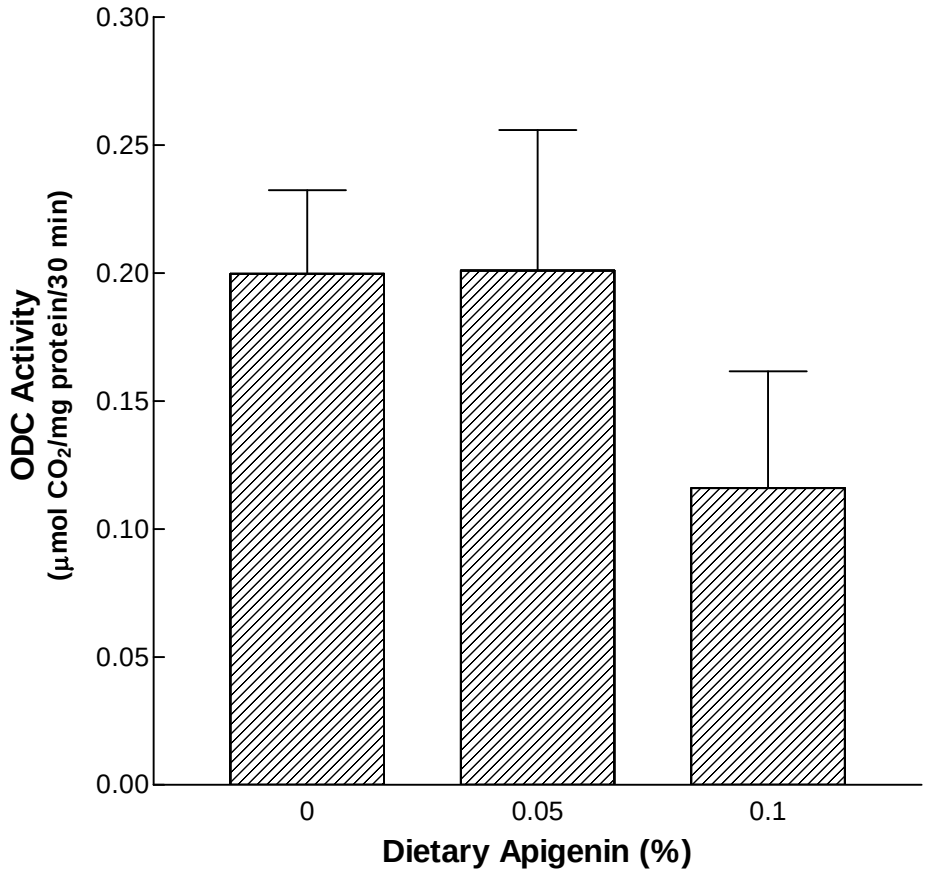
(Adenoma incidence) Total # mice assessed with adenomas / total # mice assessed (%)	Number of adenomas per colon^2
12/14 (86)	2.3±0.5
NA	NA
9/13 (69)	2.0±0.5
0/5 (0)	0
0/7 (0)	0
NA	NA
NA	NA
NA	NA
NA	NA
NA	NA

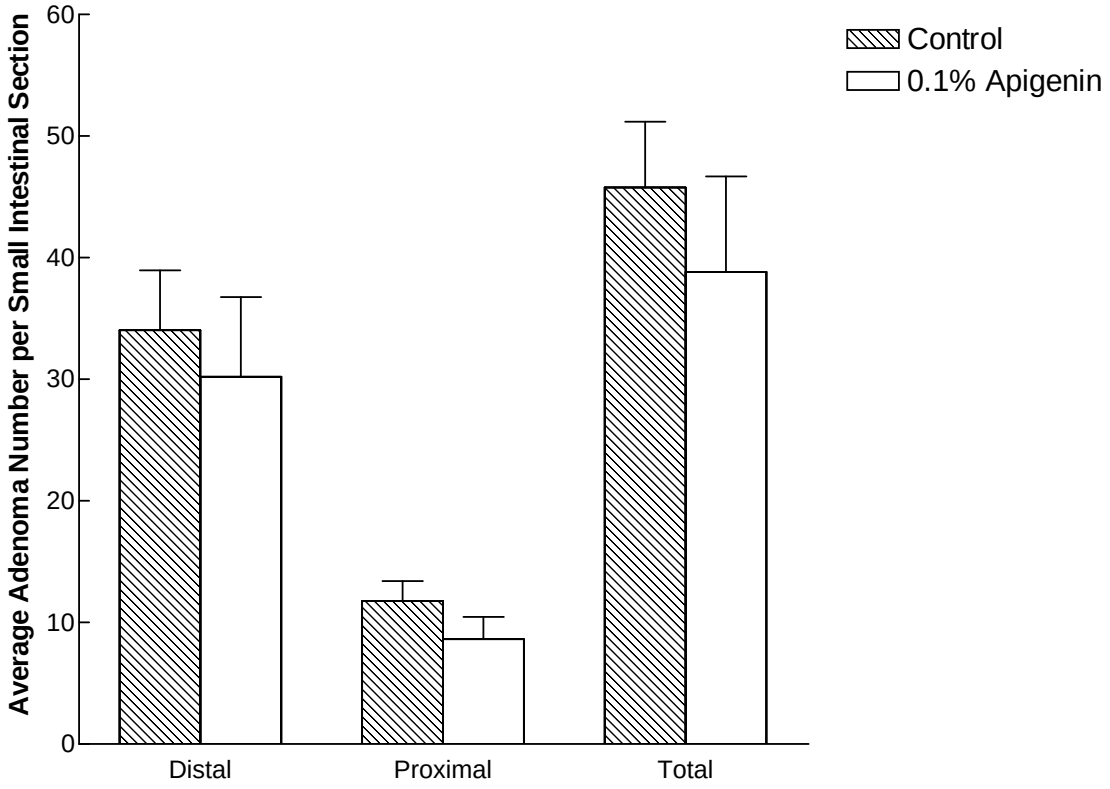
Figure 1. The effect of apigenin on ODC activity in Caco-2 cells treated with 0-30 μM apigenin concentration for 15-39 hr (n = 6). Data were expressed as mean ± SE. * represents significant difference at $P<0.05$ in comparison with the control using Student's t-test.

Figure 2. The effect of dietary apigenin on colonic ODC activity in CF-1 Mice treated with 5 mg/kg body weight. AOM and fed diets containing 0 - 0.1% apigenin for 6 weeks (n = 3). Data were expressed as mean ± SE. There were no significant differences in comparing apigenin treated mice with the control using Student's t-test.

Figure 3. The effect of dietary apigenin on small intestinal adenoma in Min mice fed diets for 10 weeks (n = 10). No significance difference was observed in comparing the apigenin-treated group with the control. Data were expressed as mean ± SE.


0.8
0.6
0.4
0.2
0.0
ODC Activity
(ηmol CO_2/mg protein/30 min)
*
*
0
10
30
Apigenin Concentration (μM)


0.30
0.25
0.20
0.15
0.10
0.05
0.00
ODC Activity
(µmol CO2/mg protein/30 min)
0
0.05
0.1
Dietary Apigenin (%)


Average Adenoma Number per Small Intestinal Section
60
50
40
30
20
10
0
Distal
Proximal
Total
Control
0.1% Apigenin

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