A Chemoprotective Fish Oil- and Pectin-Containing Diet Temporally Alters Gene Expression Profiles in Exfoliated Rat Colonocytes throughout Oncogenesis1–3

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Abstract

We have demonstrated that fish oil- and pectin-containing (FO/P) diets protect against colon cancer compared with corn oil and cellulose (CO/C) by upregulating apoptosis and suppressing proliferation. To elucidate the mechanisms whereby FO/P diets induce apoptosis and suppress proliferation during the tumorigenic process, we analyzed the temporal gene expression profiles from exfoliated rat colonocytes. Rats consumed diets containing FO/P or CO/C and were injected with azoxymethane (AOM; 2 times, 15 mg/kg body weight, subcutaneously). Feces collected at initiation (24 h after AOM injection) and at aberrant crypt foci (ACF) (7 wk postinjection) and tumor (28 wk postinjection) stages of colon cancer were used for poly (A)+ RNA extraction. Gene expression signatures were determined using Codelink arrays. Changes in phenotypes (ACF, apoptosis, proliferation, and tumor incidence) were measured to establish the regulatory controls contributing to the chemoprotective effects of FO/P. At initiation, FO/P downregulated the expression of 3 genes involved in cell adhesion and enhanced apoptosis compared with CO/C. At the ACF stage, the expression of genes involved in cell cycle regulation was modulated by FO/P and the zone of proliferation was reduced in FO/P rats compared with CO/C rats. FO/P also increased apoptosis and the expression of genes that promote apoptosis at the tumor endpoint compared with CO/C. We conclude that the effects of chemotherapeutic diets on epithelial cell gene expression can be monitored noninvasively throughout the tumorigenic process and that a FO/P diet is chemoprotective in part due to its ability to affect expression of genes involved in apoptosis and cell cycle regulation throughout all stages of tumorigenesis. J. Nutr. 141: 1029–1035, 2011.

Introduction

Colon cancer continues to be the second highest contributor to cancer deaths in the United States (1). It has been estimated that up to 80% of colon cancers may be preventable by dietary intervention (2). We have demonstrated that diets containing the combination of fish oil and pectin (FO/P)8 result in a lower tumor incidence than diets containing corn oil and cellulose (CO/C) (3). Fish oil is high in (n-3) fatty acids, whereas corn oil is high in (n-6) fatty acids. Pectin is a highly fermentable fiber that yields more butyrate upon microbial fermentation, whereas cellulose is poorly fermented. One of the mechanisms by which FO/P is protective against colon cancer is the induction of apoptosis, a programmed cell death that allows the removal of damaged cells (3–5). Tumor development depends not only on suppression of apoptosis but also on an increase in cell proliferation. We have reported that a FO/P diet also suppresses cell proliferation relative to a CO/C diet (3,6).

Cell cycle progression is mediated by cyclin dependent kinases (CDK) and cyclins, which are under both transcriptional and post-transcriptional regulation (7). We have demonstrated that fish oil with butyrate increases the expression of p21Waf1/Cip1, a CDK inhibitor (8). Apoptosis is regulated by multiple routes, including extrinsic and intrinsic pathways as well as the integrins, which control cellular adhesion (9,10). We have examined the effect of diet on apoptosis at various time points during...
carcinogenesis (4, 5, 11) and have reported that the expression of bcl2, one of the antiapoptotic factors in the intrinsic pathway, is downregulated in colon cells of rats fed a fish oil-rich diet (12). Therefore, it is important to identify the regulatory relationships among genes during the tumorigenic process to further elucidate the synergistic chemoprotective effects of fermentable fiber and fish oil.

We have developed a noninvasive technique in which intact eukaryotic mRNA can be successfully isolated from exfoliated colonocytes to monitor gene expression profiles (13–16). This novel technique facilitates the determination of changes in gene expression contributing to the regulation of apoptosis and cell proliferation during disease development. In this study, we are using this noninvasive methodology to monitor gene expression at 3 biologically important time points during colon tumorigenesis: initiation, aberrant crypt foci (ACF) formation, and tumor stage. The fecal gene expression results were compared with phenotypic data at the same time points to determine the mechanisms underlying the chemoprotective effects of a FO/P diet.

Materials and Methods

Rats and study. Male Sprague-Dawley rats (Harlan Teklad) were used to study the chemoprotective effect of FO/P at the initiation, ACF, and tumor stages of colon cancer. The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University and conformed to NIH guidelines. Rats were individually housed in a temperature- and humidity-controlled animal facility with a 12-h-light/12-h-dark cycle. After 1 wk of acclimation and 31 d of receiving the experimental diets, rats were injected with azoxymethane (AOM; Sigma; 15 mg/kg body weight). For the initiation stage analyses, 22 rats were killed 24 h after AOM injection. Fecal material was then collected and immediately homogenized in RNA isolation solution for microarray analysis and colon tissue samples were collected and processed as described below. Rats used for the ACF stage (n = 40) were maintained using the same diet and treatment conditions with the exception that animals received a second AOM injection 1 wk after the first injection. Seven weeks after the second AOM injection, rats were killed and colon tissue samples were collected. Rats for the tumor stage analyses (n = 80) were raised using the same diet and treatment conditions as the ACF stage rats, except animals were killed at 31 wk after the second AOM injection. Feces from the tumor stage rats were collected at 7 and 28 wk after the second AOM injection and colon tissue samples were collected at termination.

Diets. Rats were assigned to receive a diet containing either FO/P or CO/C as previously described (3). All diets contained oils at 15% by weight and 30% of energy. The 2 lipid sources differed in fatty acid composition; fish oil contained higher amounts of EPA [20:5 (n-3)] and DHA [22:6 (n-3)] than CO, which had higher amounts of linoleic acid [18:2 (n-6)]. The fish oil diet included 3.5 g corn oil/100 g diet to prevent essential fatty acid deficiency. The amount of fiber in the diet was 6% by weight, which is equivalent to 30 g/d for humans. Fiber sources had differences in fermentability; pectin is highly fermentable, whereas cellulose is poorly fermented. Citrus pectin was obtained from Danisco Cultor and cellulose was provided by Harlan Teklad. Corn oil and bulk vacuum-deodorized menhaden fish oil were obtained from Degussa. The antioxidant levels in the diets were balanced by including 15 mg d-α-tocopherol, 14 mg d-γ-tocopherol, and 5 mg tertiary butylhydroquinone/100 g diet in the FO/P diet and 19 mg tertiary butylhydroquinone/100 g diet in the CO/C diet. Rats were provided with fresh diet daily to prevent lipid oxidation and consumed food and water ad libum.

Tissue collection. Rats were killed by CO2 overdose and cervical dislocation. The colon was resected and 1 cm of the distal colon was fixed in 4% paraformaldehyde (PFA) and another 1 cm of distal colon was used for 70% ethanol fixation. At the ACF stage, the remaining colon was used for ACF scoring. Tissues from the tumor stage were evaluated for tumor incidence.

RNA isolation from fecal samples. To enrich the level of eukaryotic mRNA in the fecal samples, poly (A)+ RNA was isolated from total RNA using oligo(dT) cellulose micro spin columns and the mTRAP Maxi kit (Active Motif) (13). Fecal poly (A)+ RNA isolation was followed by DNase treatment and aliquots were analyzed on an Agilent Bioanalyzer 2100 to assess mRNA quality and quantity. The remaining sample was used for microarray analyses.

Microarray data acquisition. Fecal poly (A)+ RNA was used to monitor gene expression using CodeLink Rat Whole Genome Arrays (Applied Microarray) containing 35,129 gene probes. cRNA synthesis was performed using between 10 and 100 ng of fecal poly (A)+ RNA. Briefly, reverse transcriptase and a T7-oligo(dT) primer were used for first-strand cDNA and DNA polymerase was used for second-strand cDNA generation. After in vitro transcription incorporating biotinylated nucleotides, purified and fragmented cRNA was hybridized to a Rat Whole Genome Bioarray in an Innova 4080 shaking incubator (New Brunswick) at 300 rpm. After hybridization, the arrays were processed as previously described (11). Images of processed arrays were captured on an Axon GenePix Scanner.

High multiplicity ACF assay. To determine whether the FO/P diet was able to suppress formation of early preneoplastic lesions of colon cancer (aberrant crypts) compared with CO/C, we collected colon samples from rats 7 wk after the 2nd AOM injection. Colon sections were opened and placed flat within folded Whatman #1 paper, followed by fixation in 70% ethanol for 24 h. To identify aberrant crypts, tissue was stained in a 0.5% solution of methylene blue for 45 s. The total number of high multiplicity ACF (HM ACF) (foci containing 4 or more aberrant crypts) were counted using a 40X objective (17).

Colon cancer incidence. Colonos from rats killed at 31 wk after the second AOM injection were used to determine tumor incidence. Tumors were counted and tumor-bearing tissues were fixed in 4% PFA for 4 h and embedded in paraffin blocks for histological examination. Tumor sections (4 μm) were stained with hematoxylin and eosin and tumors were classified as adenomas or adenocarcinomas (3).

In situ apoptosis. Apoptosis was measured by terminal deoxynucleotidyl transferase mediated UTP-biotin nick end labeling of fragmented pieces of DNA using 4-μm sections of PFA-fixed, paraffin-embedded tissue. Apoptotic cells with condensed chromatin, apoptotic bodies, and intense brown staining were counted in 50 crypt columns for each rat. The apoptotic index was calculated as 100 × the mean number of apoptotic cells per crypt column divided by the total number of cells per crypt column (3).

Colonocyte proliferation. Cell proliferation was measured using the proliferating cell nuclear antigen assay. Sections (4 μm) of 70% ethanol-fixed, paraffin-embedded tissue were incubated with proliferating cell nuclear antigen monoclonal antibody (Signet Laboratories). Sections were incubated with biotinylated anti-mouse IgG (Vector Lab) and then stained with diaminobenzidine tetrahydrochloride (Sigma) and counterstained with hematoxylin. Twenty-five crypt columns were counted per rat. The number of cells per crypt column and the proportion of proliferating cells per crypt column were determined.

qRT-PCR confirmation of fecal microarray data. Four differentially expressed genes of known function and robust sample size from the microarray platform were selected for validation by qRT-PCR using an ABI 7900HT. These genes were B4galt1 for the 24-h time point, Musdhl and Pdgfa for the 7-wk time point, and Id3 for the 28-wk time point. In addition, we selected 3 other nondifferentially highly expressed genes with relevance to colon cancer (Ctsh, Tj3, and Tex1) (18–20). cDNA was synthesized from 2 ng fecal poly (A)+ RNA and amplified using Ovation PicoSL WTA RNA amplification System (NuGen Technologies). PCR was performed using SYBR Green PCR master mix (Applied
Biosystems). Primer sequences are shown in Supplemental Table 1 (Integrated DNA Technologies). Data are presented as the ratio of the expression level in FOP/P-fed rats to that of CO/C-fed rats.

**Statistical analyses.** Gene expression data for the fecal samples were normalized using the 2-stage, semiparametric normalization method of Liu et al. (21), which is specifically designed for data generated from partially degraded mRNA. Data were analyzed in SAS using a linear mixed model ANOVA procedure to evaluate the diet effect (FOP/P vs. CO/C) at each time point. To correct for multiple testing, a false discovery rate (22) was applied. All genes that were differentially expressed (false discovery rate, \( P < 0.05 \)) between diets at each time point were used for functional categorization and pathway analysis based on gene ontology (GO) (Database for Annotation, Visualization, and Integrated Discovery Bioinformatics Resources) (23). By importing the list of all differentially expressed genes, this program identified GO categories showing enrichment for genes in the list and the probability that the GO categories were being significantly affected by diet and time. We chose to study GO categories within the term “biological process” with a filter of enrichment \( P < 0.05 \).

Phenotypic data were analyzed using ANOVA to determine the effect of diet (FOP/P vs. CO/C) on apoptosis, proliferation, and HM ACF. Colon tumor incidence was analyzed by chi square analysis and reported as the percentage of rats bearing tumors. Values reported are LSmean ± SEM.

**Results**

The goal of this study was to monitor the protection provided by the combined FOP/P diet in terms of changes in global patterns of intestinal gene expression at the initiation, promotion, and tumor stages. Gene expression was monitored using microarray procedures and the resulting data were compared with phenotypic data at each of the 3 time points to determine whether the patterns of expression identified by GO analysis were predictive of changes in disease phenotypes detected in these rats.

**Initiation stage.** At the initiation stage of colon tumorigenesis, FOP/P resulted in higher levels of apoptosis in the colonic crypt compared with CO/C rats (\( P = 0.024 \); Table 1). Although there was no significant diet effect on the expression of genes involved in apoptosis 24 h after AOM injection, there was a lower expression of cell adhesion genes (B4galt1, Smo1c1, and Scarb2) in FOP/P rats compared with CO/C rats at this time point (Supplemental Table 2).

**ACF stage.** Rats receiving the FOP/P diet had fewer HM ACF than did rats receiving the CO/C diet (\( P = 0.0002 \)) (Table 1). In addition, the extent of the proliferative zone was lower in rats receiving the FOP/P diet compared with those fed the CO/C diet (\( P = 0.0001 \)) (Table 1). Relative to observations from rats consuming the CO/C diet, rats consuming the FOP/P diet had an elevated apoptotic index (\( P = 0.027 \)) (Table 1). The smaller number of cells in the crypt in FOP/P group (\( P = 0.0001 \)) (Table 1) likely was due to both suppression of cell proliferation and induction of apoptosis. In contrast to the initiation stage, at the HM ACF stage, there were 602 genes that were differentially (false discovery rate, \( P < 0.05 \)) expressed as a function of diet. Upon completion of GO analyses, 80 biological process categories were found to be significantly enriched (Supplemental Table 3). Among the 80 clusters, 5 were directly involved with cell cycle regulation (GO:0007049 cell cycle, GO:0022402 cell cycle process, GO:0000074 regulation of progression through cell cycle, GO:0051726 regulation of cell cycle, and GO:0000079 regulation of cyclin-dependent protein kinase activity). Because 4 of these categories are a subset of the parent category of cell cycle (GO:0007049), we focused on the parent category to include the maximum number of differentially expressed genes. The FOP/P diet yielded almost uniformly lower levels of expression of both cell cycle promoters and suppressors in this cell cycle category (Table 2).

**Tumor stage.** Similar to the reduction of early preneoplastic lesion numbers, colon tumor incidence evaluated 31 wk after the second AOM injection was lower in FOP/P rats than in CO/C rats (Table 1). Part of the protection against tumor formation may be attributable to the enhanced apoptotic index in the FOP/P rat colons compared with those from rats consuming CO/C (Table 1), which was elevated at all 3 stages of the tumorigenic process.

At the tumor stage, 81 genes were differentially expressed in response to diet and 13 biological processes were identified by GO analysis as being enriched. Of the 13 categories, 6 were associated with apoptosis (Supplemental Table 4). Among the remaining 75 differentially expressed genes, we identified 16 genes known to be involved in signal transduction and tumor development, progression, and invasion (Table 3).

**qRT-PCR confirmation of fecal microarray.** To validate the fecal microarray data, we performed qRT-PCR on select genes using the same fecal poly (A)+ RNA isolates. The regression between fecal microarray and qRT-PCR results demonstrate a reasonable degree of similarity in the pattern of expression for the 7 genes selected for this comparison (\( R^2 = 0.87 \)) (Fig. 1).

**Discussion**

Most chemoprevention studies are targeted to a single time point in the carcinogenic process. We used a noninvasive technique that permits the isolation of eukaryotic mRNA from exfoliated colonocytes in fecal material (13–16) to monitor temporal changes in gene expression. The purpose of the current study was to determine how diet influences the expression of genes at 3 discreet stages of tumorigenesis and if the differences were reflective of the changes in phenotypes measured at those time points.

At the initiation stage of colon tumorigenesis, there were only 3 annotated genes, which were differentially expressed as a function of diet. The low number of diet-induced differentially expressed genes at the initiation stage was not unexpected, because we have previously shown a relatively small number of...
At the HM ACF stage, we found that 602 genes were differentially expressed as a function of diet. This suggests that as carcinogenesis progresses to this stage, the ability of diet to affect gene expression and thereby provide a chemoprotective effect is enhanced, as reflected by the number of phenotypic changes that are detected (i.e. apoptosis, proliferative zone, and mutation of \( Pms2 \), which is documented to cause hereditary non-polyposis colorectal cancer (29). In the current study, we found that the FO/P diet may inhibit Wnt signaling. These findings suggest that the FO/P diet could suppress the uncontrolled cell proliferation that occurs in colon cancer cells at the ACF stage in part by modulating the expression of genes that are essential for cell cycle progression.

In addition to the suppression of cell proliferation, we previously reported on the cell-specific expression of \( O^6 \)-methylguanine DNA methyltransferase (\( MGMT \), DNA repair enzyme). The expression of \( MGMT \) in fish oil-fed rats was 4-fold higher than corn oil-fed rats in areas of colon crypts where apoptosis typically occurs (4). \( Pms2 \) is known to be involved in DNA mismatch repair systems and mutation of \( Pms2 \) is documented to cause hereditary non-polyposis colorectal cancer (29). In the current study, we found that the expression of \( Pms2_{predicted} \) was 3-fold higher in FO/P rats than in CO/C rats. We also observed that FO/P significantly enhanced apoptosis compared with CO/C (Table 1). Therefore, the FO/P diet may facilitate removal of DNA-damaged cells by increasing DNA repair and apoptosis.

At the tumor stage, the modulation of expression of several genes involved in apoptosis occurred in concert with the induction of apoptosis in the FO/P rats. Of the 6 differentially expressed genes involved in the apoptosis pathway, \( Mmp2 \) expression increased in the colon of CO/C tumor-bearing rats. This is noteworthy because \( Mmp2 \) has been implicated in colon carcinoma progression.
Tumor

Signal transduction

effective in promoting apoptosis than the CO/C diet. At molecular points, indicating at a level why the FO/P diet is more consistent with the apoptotic phenotype observed across all time rats compared with CO/C rats. This pattern of gene expression is consistent with the apoptotic phenotype observed across all time points, indicating at a molecular level why the FO/P diet is more effective in promoting apoptosis than the CO/C diet.

The expression of 11 genes (Slc8a1, Dupd1, Ppp1r7, Mfn1, Stx1a, Smoc1, Snip, Nnn1, Il23a, Il6ra, and Pthr2) involved in several signal transduction pathways was downregulated in FO/P rats compared with CO/C rats, suggesting that FO/P is capable of attenuating multiple signaling pathways at the tumor stage. For example, Mfn1, which is a transmembrane GTPase, is one of the genes downregulated by FO/P compared with CO/C. Mfn1 mediates mitochondrial fusion and elevated expression of Mfn1 has been demonstrated to increase the resistance of cells to death stimuli (34). With regard to this mechanism of action, 11 genes in the membrane category (GO:0016020) were differentially expressed at the tumor stage (indicated by “Y” in Table 3). This may be explained by the incorporation of DHA, a bioactive component of fish oil, into both plasma and mitochondrial membranes (35). Indeed, we and others have demonstrated that (n-3) PUFA promote an oxidation-reduction imbalance in the intestine (36–39). Recently, we demonstrated that DHA promotes mitochondrial oxidative stress and increases mitochondrial Ca2+ levels, which directly induce apoptosis in colonocytes (40,41).

Tumor-related genes involved in tumor formation, progression, and invasion were favorably modulated by FO/P consumption compared with CO/C. For example, the expression of Cyp2s1 was downregulated by FO/P compared with CO/C. This gene encodes for one of the cytochrome P450 superfamily members and plays a pivotal role in the oxidative metabolism of xenobiotics such as carcinogens. A study designed to identify markers of colon cancer prognosis demonstrated that the expression of Cyp2s1 was significantly higher in primary colon cancers than in normal colon tissue (42).

Existing studies with the AOM model of colon carcinogenesis have reported gene expression at discreet points in the tumor-
This makes it possible to determine the mechanisms whereby a
the fatty acid content of the diet.

divergent regulation of gene expression signatures in response to
with recent observations (47), indicating a clear time-dependent,
diet promoted apoptosis at all 3 time points, it differentially
protection against colon cancer. Interestingly, although the FO/P
discovered time-specific effects of diet that contribute to chemo-
depending upon the stage of tumorigenesis. Therefore, we have
expression of genes involved in cell adhesion (46), apoptosis, or
legenic process (46,11). Those studies also found changes in
expression of genes involved in cell adhesion (46), apoptosis, or
cell cycle (11). The unique contribution of the current study is
that we found that diet differentially affects apoptotic genes,
depending upon the stage of tumorigenesis. Therefore, we have
discovered time-specific effects of diet that contribute to chemoprotection against colon cancer. Interestingly, although the FO/P
diet promoted apoptosis at all 3 time points, it differentially
affected gene expression at each stage. These data are consistent with recent observations (47), indicating a clear time-dependent,
divergent regulation of gene expression signatures in response to
the fatty acid content of the diet.

We have demonstrated the feasibility of monitoring gene
expression over time using an mRNA-based noninvasive technique.
This makes it possible to determine the mechanisms whereby a
chemopreventive diet may inhibit colon carcinogenesis as well as
to monitor human disease progression and identify critical time points
for potential diet intervention. In this study, we identified differentially
expressed genes involved in apoptosis and/or cell proliferation at 3 time points during colon carcinogenesis. At the initiation stage, there were few differential effects of diet on gene expression. At the promotion stage, the expression of many more genes was affected by
diet, suggesting this stage is more susceptible to the FO/P dietary intervention. GO pathways enriched at this stage include cell proliferation. However, at the tumor stage, the main gene expression
pathway affected was principally associated with apoptosis. Conse-
sequently, the central mechanism by which FO/P produces a chemoprotective effect is through changes in gene expression that enhance
cell cycle regulation and apoptosis throughout tumorigenesis.

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paper; and J.R.L. had primary responsibility for final content.
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