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Dietary Fiber Differentially Alters Cellular Fatty Acid-Binding Protein Expression in Exfoliated Colonocytes During Tumor Development

Robert S. Chapkin, Amy E. Clark, Laurie A. Davidson, Friedhelm Schroeder, Debra L. Zoran, and Joanne R. Lupton

Abstract: We investigated the utility of noninvasive technology utilizing feces containing exfoliated colonocytes to determine whether changes in fecal fatty acid-binding proteins have predictive value in monitoring the neoplastic process. Ninety male Sprague-Dawley rats were randomly divided into four groups in a 2 × 2 factorial design, with two dietary fiber sources (wheat bran or oat bran) and two treatment groups (injection with a carcinogen, azoxymethane, or saline). Fresh fecal samples were collected at Week 16 postinjection, and tumor frequency was determined at Week 36 of the study. Semiquantitative "mimic" reverse transcriptase polymerase chain reaction was used to quantitate the expression of liver fatty acid-binding protein (L-FABP), intestinal fatty acid-binding protein (i-FABP), and acyl CoA-binding protein (ACBP) mRNA in fecal samples to establish their prognostic value. Rats fed wheat bran diets had a lower incidence of tumors ($p < 0.05$). There was no effect of carcinogen injection or tumor incidence on the expression of L-FABP, i-FABP, or ACBP mRNA. L-FABP and i-FABP mRNA expression were significantly higher ($p < 0.05$) in feces from animals fed a wheat bran diet than in feces from animals fed an oat bran diet. In contrast, the expression of ACBP mRNA was significantly lower ($p < 0.05$) in animals fed a wheat bran diet than in animals fed an oat bran diet. Wheat bran also increased ($p < 0.05$) the total excretion of L-FABP, i-FABP, and ACBP over a 48-hour period. These data suggest that exfoliated colonocyte fatty acid-binding protein mRNA status may provide insight into the mechanisms by which diet influences colonic physiology.

Introduction

Colon cancer is among the most common and lethal cancers in the world (1). The pathogenesis of colon cancer is a multistep process involving mutational activation of certain oncogenes and inactivation of tumor supressor genes, which results in uncontrolled growth and tumor formation (2). Despite recent advances in elucidating the molecular mechanisms that modulate the neoplastic process, the cure rate for colon cancer has not dramatically improved in the past 30 years (3,4). Therefore, early diagnosis of colon cancer is important, inasmuch as a large number of patients can be cured by surgical removal of the tumor if metastasis has not occurred (5). Tumor detection involves invasive procedures such as sigmoidoscopy and biopsy, both of which have drawbacks and risks (6). Therefore, development of highly sensitive noninvasive techniques for detection of colon cancer has received much interest. Current noninvasive methodologies to screen for fecal occult blood are subject to low sensitivity and specificity (5,7). For colorectal adenomas, the sensitivity is even lower, probably because of a fluctuation in, or absence of, occult blood loss (8). As a result, newer methods continue to be devised in an attempt to address these shortcomings and allow for home screening.

Approximately one-sixth to one-third of normal adult colonic epithelial cells are shed daily (9). Because the number of intact cells that can be isolated from fecal material is low (10), an enhanced detection system is required to amplify potential biomarkers of dietary and pharmacological risk assessment in exfoliated colonocytes (11). Therefore, the use of semiquantitative "mimic" reverse transcriptase (RT) polymerase chain reaction (PCR) has been optimized to detect the expression of genes with potential diagnostic value in the colon (11,12). This experimental approach provides a sensitive method for detection of mRNA isolated from feces containing exfoliated colonocytes and serves as a noninvasive means for monitoring changes in this population of cells (11).

A number of specific proteins, e.g., liver and intestinal fatty acid-binding proteins (L-FABP and i-FABP), acyl CoA-binding protein (ACBP), and sterol carrier protein-2, which can reversibly and noncovalently bind select intracellular lipids, have been identified (13). Although the physi-

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ological role of these intracellular binding proteins is thought to be regulation of intermediary metabolism (13), recent data show that these lipophilic molecules may influence tumor development (14–17). Specifically, tissue- and cell-specific patterns of expression of L-FABP and i-FABP have been linked temporally to events in intestinal differentiation in the colon (16). In addition, L-FABP, a putative carcinogen target protein, is capable of modulating cell proliferation (18). Therefore, we propose to use noninvasive methodology to determine whether changes in fatty acid-binding protein gene expression may have predictive value in terms of detecting the neoplastic process in the colon.

There is strong evidence that select dietary factors play a key role in the development of colon cancer (12,19,20). Poorly fermentable fibers, such as wheat bran, are protective against colon cancer, whereas readily fermented fibers, such as oat bran, may be promotive of tumor development (19). Dietary fiber can influence the levels of ligands for the fatty acid-binding proteins, which in turn may mediate colonic cell proliferation and differentiation (14,15,19). In addition, because the expression of fatty acid-binding proteins is altered during tumorigenesis (16), we determined the utility of a noninvasive technique to detect diet- and carcinogen-induced changes in fatty acid-binding protein expression. Specifically, the expression of L-FABP, i-FABP, and ACBP mRNA levels was quantitated in feces containing exfoliated colonocytes at an early stage in colon carcinogenesis, before overt neoplasia. Fecal samples were collected from rats injected with carcinogen or saline (vehicle control) and fed diets containing wheat bran or oat bran.

Materials and Methods

Animals and Diet Administration

All animals were treated in accordance with the guidelines published by the National Institutes of Health (21). Ninety weanling male Sprague-Dawley rats (Harlan Sprague Dawley, Houston, TX) weighing 40–60 g were randomly divided into four groups in a 2 × 2 factorial design: two diets differing only in the type of dietary fiber (wheat bran or oat bran) and two treatment groups [injection with carcinogen, azoxymethane (AOM), or saline]. Each carcinogen-injected group consisted of 34 rats; each saline-injected control group consisted of 11 rats. The rats were individually housed in hanging wire cages to minimize coprophagy and consumption of bedding. They were maintained in temperature- and humidity-controlled animal facilities with a 12:12-hour light-dark cycle for the duration of the study. Total fecal output was determined over a 48-hour period. Food and water were freely available throughout the study.

The composition of the diets is shown in Table 1. The diets were specifically designed to exploit the basic differences of the two types of dietary fibers present; in this model system, wheat bran produces concentrations of short-chain fatty acids in the distal colon of 33.9 mmol/g wet feces, whereas oat bran, a more fermentable fiber, produces concentrations of 52.5 mmol/g wet feces at the same site (19). The amount of fiber was chosen to approximate a moderate fiber intake, 6 g/100 g diet, which corresponds, on a body weight basis, to the recommended level for humans of 30 g fiber/day (4).

Table 1. Diet Ingredients and Nutrient Composition[a]

Item	Wheat Bran	Oat Bran
Diet ingredients[b]		
Dextrose	60.4	49.6
Casein	18.1	13.7
Corn oil	4.50	1.70
DL-Methionine	0.30	0.30
Mineral mix (AIN-93)	3.50	3.50
Vitamin mix (AIN-93)	1.00	1.00
Choline bitartrate	0.20	0.20
TBHQ (antioxidant)	0.01	0.01
Fiber supplement[c]	12.0	30.0
Macronutrient content		
Carbohydrate	62.0	60.0
Protein	20.0	20.0
Lipid	5.0	5.0
Fiber	6.0	6.0

a: Values are g/100 g.
b: Ingredients other than fiber supplements were mixed and supplied by Harlan Teklad (Madison, WI). TBHQ, *tert*-butylhydroquinone.
c: Fiber supplements were obtained from American Association of Cereal Chemists (AACC): AACC-certified hard red wheat bran (lot 195) containing 49.6% fiber, 13.3% carbohydrate, 16.0% protein, and 4.3% lipid and AACC-certified oat bran containing 20.0% fiber, 34.0% carbohydrate, 21.0% protein, and 10.9% lipid.

Carcinogen Administration

AOM (Sigma Chemical, St. Louis, MO) was diluted in sterile 0.9% sodium chloride, pH 7.2, and administered in two weekly subcutaneous injections at a dose of 15 mg/kg (19). The injections were given two and three weeks after the initiation of the feeding regimen. Each of the control rats was injected with an equal volume of the saline vehicle. The study was extended 36 weeks postinjection to allow for tumor development.

Fecal mRNA Isolation

Fecal samples were collected at 16 weeks from individual animals and weighed immediately after defecation. At this time, no tumors were detected (19). Poly(A)$^+$ RNA was isolated directly from feces after homogenization in lysis solution using an Ambion poly(A)$^+$ Pure Kit, as previously described (12). Poly(A)$^+$ was quantitated by slot blotting samples onto a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN). A biotinylated oligo(dT) probe (Promega, Madison, WI) was subsequently hybridized for detection. Dilutions of colonic mucosal total RNA of known concentration (as determined from the absorbance at 260 nm) were blotted to generate a standard curve. To assign

relative quantitative mRNA values to each sample, it was assumed that total RNA is comprised of approximately 3% poly(A)$^+$ RNA (22).

RT-PCR

Sample poly(A)$^+$ (100 ng) was reversed transcribed to generate first-strand cDNA using Superscript II RT (GIBCO-BRL, Gaithersburg, MD), as previously described (12). PCR was performed using PCR Supermix (GIBCO-BRL) containing 20 pmol of forward and reverse primer and 10 µl of RT reaction. A minus RT sample was run as a negative control. PCR was performed in a Gene Amp 2400 (Perkin-Elmer, Foster City, CA) with an amplification program consisting of 30 seconds of denaturation (94°C), 30 seconds of annealing (59, 50, and 50°C for L-FABP, i-FABP, and ACBP, respectively), and 45 seconds of extension (74°C), for 35, 40, or 42 cycles for L-FABP, i-FABP, and ACBP, respectively. PCR products were sequenced to ensure the fidelity of amplification.

PCR Primers and Product Sizes

For L-FABP, product size was 390 bp, forward primer was 5′-ggaaaggaaacctcattgccac-3′, and reverse primer was 5′-ctctcttgtagacgatgtcacccag-3′. For i-FABP, product size was 248 bp, forward primer was 5′-gcagatggaacagaactcactgg-3′, and reverse primer was 5′-cgagatggagaaaggaatccgac-3′. For ACPB, product size was 307 bp, forward primer was 5′-aaaactgggttgctgcctcttc-3′, and reverse primer was 5′-cctatgtggagaaggcagaagagc-3′.

Rapid Competitive RT-PCR

Because alterations in the expression of specific isoforms of fatty acid-binding proteins are temporally linked to the development of experimental colon cancer (16), semiquantitative "mimic" RT-PCR (23) was used to detect the expression of L-FABP, i-FABP, and ACPB in fecal samples to establish their prognostic value with regard to colon cancer. By using this method, relative gene expression was determined by coamplifying an exogenous DNA internal standard along with the target cDNA (23). Each internal standard differed in size with respect to the target cDNA but contained identical 5′ and 3′ ends. A known amount of internal standard (mimic; L-FABP = 0.30 pg, i-FABP = 7.49 pg, ACBP = 0.34 fg) was added to each PCR tube to quantitate the amplified product. The primer pair for the L-FABP internal standard was 336 bp, 5′-ggaaaggaaacctcattgccaccatgaaggcgatg-3′ (forward primer), and 5′-ctctcttgtagacgatgtcacccag-3′ (reverse primer); that for the i-FABP internal standard was 211 bp, 5′-gcagatggaacagaactcactggttcaaacgtgtag-3′ (forward primer), and 5′-cgagatggagaaaggaatccgac-3′ (reverse primer); and that for the ACBP internal standard was 280 bp, 5′-aaaactgggttgctgcctcttccaggtcacctcgcc-3′ (forward primer), and 5′-cctatgtggagaaggcagaagagc-3′ (reverse primer). RT-PCR was performed as stated above for L-FABP, i-FABP, and ACBP, except a known amount of internal standard was added to each PCR reaction. PCR products were separated on a 4% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME) and stained with ethidium bromide (Figure 1). Gels were scanned, and band intensities were quantitated with Intelligent Quantifier software (version 2.1, BioImage, Ann Arbor, MI). The relative amount of sample mRNA was calculated by dividing the sample band intensity by the internal standard band intensity. To ensure reproducibility of results, select samples were amplified in duplicate. A "positive control" consisting of mRNA from scraped rat colonic mucosa plus internal standard "mimic" was run on each agarose gel to standardize samples. Specific amplification of target cDNA was monitored by processing PCR negative controls consisting of tubes containing sample RNA without RT, reverse-transcribed sample without mimic, or mimic only. In addition, the fidelity of all PCR reactions was confirmed by DNA sequencing (12).

Fecal outputs, used for calculating total biological marker excretion per 48 hours, were determined during Week 4 of the study and then again one week before termination (Week 36). Feces from each animal were collected for 48 hours into preweighed vials. Because fecal output is constant in adult rats (8–36 wk postinjection) (19), Week 36 fecal outputs were utilized to calculate total excretion of fecal L-FABP, i-FABP, and ACBP mRNA.

Tumor Incidence

Animals were killed 36 weeks after the second AOM injection. After the large bowel was resected and opened longitudinally, the contents were rinsed with phosphate-buffered saline. The distance from the anus and the number and size of tumors were recorded. Tumor-bearing areas were dissected and fixed in 4% paraformaldehyde for four hours

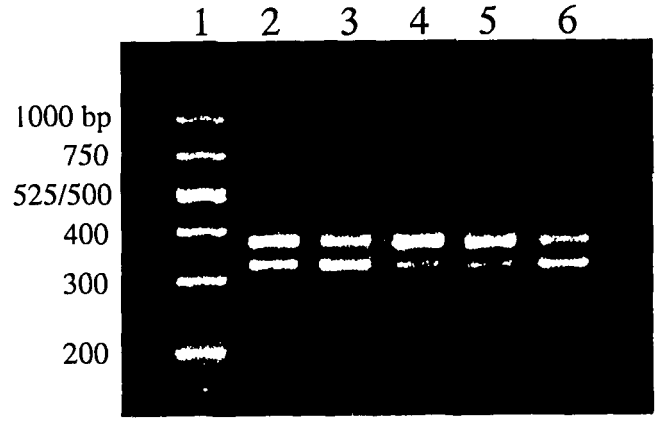

Figure 1. Representative agarose gel showing rapid competitive reverse-transcriptase polymerase chain reaction of liver fatty acid-binding protein. Lane 1, marker; Lane 2, rat colonic mucosa (standard); Lanes 3 and 6, animals fed wheat bran and treated with azoxymethane; Lanes 4 and 5, animals fed oat bran and treated with azoxymethane. Top band, amplified sample band (390 bp); bottom band, amplified internal standard (mimic, 336 bp).

(20). After fixation, tissues were embedded in paraffin blocks, and 4-μm sections were cut perpendicular to the surface of the lesions before staining with hematoxylin and eosin. Serial sections were made whenever necessary to expose the central part of a tumor or its stalk, when present. Serial sections were also taken in areas showing focal atypia, as characterized by pseudostratification of epithelial cells and their orientation along sinus basement membranes to identify microscopic tumor foci. Tumors were classified as adenomas or adenocarcinomas, as previously described (24).

Statistical Analysis

Data were analyzed to determine the effects of dietary fiber, carcinogen, presence of tumor, and fiber × carcinogen interaction using three-way analysis of variance. When $p < 0.05$ for the effects of fiber, tumor, or carcinogen but not for the interaction, total means were separated using a protected Fisher's least significant difference test.

Results

Fecal output was greater in rats consuming wheat bran ($p < 0.05$) than in those fed oat bran (Table 2). There were no differences between diet or treatment groups with respect to colon length or width.

Tumor Incidence

Tumor incidence was lower ($p < 0.03$) at 36 weeks in rats consuming wheat bran diets than in those consuming oat bran (19). Twenty-seven percent of rats consuming wheat bran developed tumors (9 of 33) compared with 52% of the oat bran-fed rats (17 of 33). There were no differences in the number of tumors that developed per rat. However, there was an effect of diet ($p < 0.05$) on the relative size of the tumors, with rats consuming oat bran diets having visible (macroscopic) tumors, whereas the rats consuming wheat bran had a greater number of microscopic tumors (i.e., the tumors were visible only by microscopic examination of the tissue, usually within Peyers patches) (19).

Dietary Fiber Alters Expression of Fecal L-FABP, i-FABP, and ACBP

No significant effect of carcinogen, tumor incidence, or interactions ($p > 0.05$) was observed. Dietary fiber source had a significant ($p < 0.05$) effect on fecal L-FABP, i-FABP, and ACBP mRNA expression (intensity of target band ÷ intensity of internal standard band × 100). Significant means are shown in Tables 3 and 4. As shown in Table 3, the expression of fecal L-FABP and i-FABP mRNA was significantly higher ($p < 0.05$) in rats fed a wheat bran diet than in those fed a diet containing oat bran. In contrast, the expression of fecal ACBP mRNA was significantly higher ($p < 0.05$) in rats fed an oat bran diet than in those fed wheat bran. Diet also had a significant ($p < 0.05$) effect on the total excretion of fecal L-FABP and ACBP mRNA over a 48-hour period (Table 4), calculated by multiplying the amount of marker excreted in the fecal wet weight used for poly(A)$^+$ isolation by total fecal wet weight excreted in 48 hours.

Discussion

Over the past decade, a revolution in molecular biology has provided powerful tools for elucidating mechanisms of cancer causation. Genetic alterations have been detected in serum, feces, and colonic effluent in colorectal and pancreatic cancer patients by noninvasive methodology based on the molecular pathogenesis of the disease (25–29). These protocols utilize DNA extraction procedures and detect genetic alterations using PCR. The major disadvantage of this methodology is that it will not detect alterations in gene expression and, therefore, greatly restricts the number of biomarkers that can be utilized to assess colon cancer risk. Recently, RT-PCR has been utilized to detect RNA viruses in stool specimens (30). In contrast, we have developed a noninvasive technol-

Table 2. Effect of Fiber on Food Intake, Body Weight, and Fecal Output in Rats Fed Experimental Diets[a,b]

	Wheat Bran		Oat Bran	
	AOM	Saline	AOM	Saline
24-h food intake, g				
Week 4	23.5 ± 0.4*	22.9 ± 0.7*	21.2 ± 0.4†	23.3 ± 0.7*
Week 36	20.4 ± 0.6*	21.9 ± 0.6*	20.6 ± 0.5*	21.6 ± 0.5*
Body wt, g				
Week 4	191.2 ± 3.0	200.00 ± 5.6	204.1 ± 2.8	210.7 ± 4.2
Week 16	410.4 ± 6.5*	416.4 ± 11.0*	438.5 ± 6.2†	445.7 ± 9.0†
Week 36	501.0 ± 8.0*	497.8 ± 16.9*	536.0 ± 9.2†	557.0 ± 14.8†
24-h fecal dry wt, g				
Week 4	1.83 ± 0.05*	1.57 ± 0.09‡	0.97 ± 0.04†	0.98 ± 0.04†
Week 36	1.79 ± 0.06*	1.81 ± 0.02*	1.23 ± 0.04†	1.23 ± 0.05†
Colon length, cm	15.8 ± 0.2*	16.1 ± 0.4*	16.2 ± 0.2*	16.6 ± 0.4*

a: Values are means ± SEM. AOM, azoxymethane.
b: Different symbols (*,†,‡) within rows indicate significant differences ($p < 0.05$).

Table 3. Dietary Fiber Influences Expression of Fecal L-FABP, i-FABP, and ACBP mRNA Expression[a,b]

	Wheat Bran	Oat Bran	*P* Value
L-FABP	1.62 ± 0.18*	0.82 ± 0.16†	0.0011
i-FABP	2.32 ± 0.30*	1.40 ± 0.28†	0.0230
ACBP	1.54 ± 0.44†	3.03 ± 1.54*	0.0163

a: Values are means ± SEM in arbitrary units from 28–33 rats/group. L-FABP, i-FABP, and ACBP, liver and intestinal fatty acid-binding protein and acyl CoA-binding protein mRNA in feces containing exfoliated colonocytes. Target mRNA expression was measured as integrated intensity [i.e., optical density × area (mm^2)] of sample band obtained from amplification of 100 ng of poly(A)$^+$ RNA divided by integrated intensity of internal standard (mimic band).

b: Means in a row not sharing a common symbol (*,†) are significantly different ($p < 0.05$).

Table 4. Dietary Fiber Influences Total Excretion of Fecal L-FABP and ACBP mRNA Over 48 Hours[a,b]

	Wheat Bran	Oat Bran	*P* Value
L-FABP	48.21 ± 3.93*	11.46 ± 4.31†	0.0001
i-FABP	45.47 ± 8.86*	50.16 ± 8.52*	0.9487
ACBP	73.34 ± 7.48*	24.31 ± 6.63†	0.0001

a: Values are means ± SEM in arbitrary units from 28–33 rats/group. See Table 3 footnote for details.

b: Means in a row not sharing a common symbol (*,†) are significantly different ($p < 0.05$).

ogy to detect changes in colonic gene expression. This methodology has the advantage of utilizing a fecal sample, which contains sloughed colonocytes (11). The procedure as described here represents a marked improvement over our earlier methodology (11,12), in that poly(A)$^+$ RNA is directly isolated from feces as opposed to first isolating total RNA. This results in an increased mRNA yield (up to 10-fold) and a reduction in the time to effect isolation. The extraction process results in a sufficient yield and stability of isolated fecal mRNA. This process is combined with rapid competitive PCR to detect and quantify diagnostic biomarkers.

We recently demonstrated that the expression of select fecal protein kinase C isozymes may serve as a noninvasive marker for development of colon tumors (12). However, because carcinogenesis is a multistep, multipath process, single biomarkers may be difficult to correlate to cancer. It is much more likely that batteries of risk biomarkers, particularly those representing the range of malignant transformation pathways, will prove more useful than a single biomarker in defining modulatable risks (31). In addition, because complex gene-environment interactions are likely to be risk determinants for most cancers (32), we are particularly interested in assessing the relation between the consumption of specific types of dietary fiber and colon cancer. Because the expression of fatty acid-binding proteins is responsive to changes in diet (14) and is altered during tumorigenesis (15,16), implicating their modulation in mitogenesis and tumor promotion (13,33,34), we determined the predictive potential of FABP mRNAs in feces at an intermediate time point when no macroscopic colonic tumors are present, i.e., at 16 weeks after AOM injection (35). Interestingly, no effects of carcinogen or tumor incidence were observed, indicating that FABPs are not early predictors of the malignant transformation process.

The increased consumption of fruits and vegetables containing dietary fiber has been linked to a reduced risk of colorectal cancer (4,36). The fibers chosen for this study, wheat bran and oat bran, are two important complex carbohydrate sources that have distinct physiochemical properties (19). Studying the effects of these fibers on exfoliated colonocyte gene expression may help elucidate the unresolved role of dietary fiber in colon cancer prevention. In this study, we demonstrated the ability of dietary fiber source to modulate steady-state fecal L-FABP, i-FABP, and ACBP mRNA levels. It is possible that these effects reflect changes in mucosal cell intermediary metabolism, since the ligands for these cytoplasmic and membrane-associated lipid-binding proteins, i.e., fatty acids, eicosanoids, and bile acids, are modulated by fiber (37–39). Interestingly, we have demonstrated that fiber source is capable of influencing the total number of exfoliated cells in a manner proportional to changes in colonic cell proliferation (40). This may be a mechanism by which diet influences total excretion of fecal mRNAs over a 48-hour period. With respect to the dietary lipid component, the amount of lipid in the two fiber-supplemented diets was identical, but the contribution of the lipid from the supplements was not. Of the 5 g/100 g of lipid in the diet, 0.52 g was contributed by lipids in wheat bran, whereas 3.3 g were contributed by lipids from oat bran. It is possible that the lipids contributed by the fiber supplements account in part for the observed effects on fatty acid-binding proteins, although this was not directly addressed in the present study.

In conclusion, dietary fiber is capable of eliciting significant changes in the expression of fecal L-FABP, i-FABP, and ACBP mRNA. This finding is noteworthy, because noninvasive detection of exfoliated cell mRNA could provide insight into the mechanisms by which diet influences colonic physiology, as we have previously shown for protein kinase C isozyme expression (12). Although fecal FABP mRNAs do not appear to be good predictors of colon cancer risk in an experimental carcinogenesis model, they may have potential as noninvasive diagnostic markers for dietary compliance. Additional experiments are required to determine the potential confounding effects of other dietary components on fatty acid-binding protein expression in exfoliated colonocytes.

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