**In Vivo Regulation of Colonic Cell Proliferation, Differentiation, Apoptosis, and P27Kip1 by Dietary Fish Oil and Butyrate in Rats**

Mee Young Hong1,2, Nancy D. Turner1, Mary E. Murphy3, Raymond J. Carroll3, Robert S. Chapkin1, and Joanne R. Lupton1

**Abstract**

We have shown that dietary fish oil is protective against experimentally induced colon cancer, and the protective effect is enhanced by coadministration of pectin. However, the underlying mechanisms have not been fully elucidated. We hypothesized that fish oil with butyrate, a pectin fermentation product, protects against colon cancer initiation by decreasing cell proliferation and increasing differentiation and apoptosis through a p27Kip1-mediated mechanism. Rats were provided diets of corn or fish oil, with/without butyrate, and terminated 12, 24, or 48 hours after azoxymethane (AOM) injection. Proliferation (Ki-67), differentiation (Dolichos Biflorus Agglutinin), apoptosis (TUNEL), and p27Kip1 (cell-cycle mediator) were measured in the same cell within crypts in order to examine the coordination of cell cycle as a function of diet. DNA damage (N7-methylguanine) was determined by quantitative IHC analysis. Dietary fish oil decreased DNA damage by 19% (P = 0.001) and proliferation by 50% (P = 0.003) and increased differentiation by 56% (P = 0.039) compared with corn oil. When combined with butyrate, fish oil enhanced apoptosis 24 hours after AOM injection compared with a corn oil/butyrate diet (P = 0.039). There was an inverse relationship between crypt height and apoptosis in the fish oil/butyrate group (r = –0.53, P = 0.040). The corn oil/butyrate group showed a positive correlation between p27Kip1 expression and proliferation (r = 0.61, P = 0.035). These results indicate the in vivo effect of butyrate on apoptosis and proliferation is dependent on dietary lipid source. These results demonstrate the presence of an early coordinated colonocyte response by which fish oil and butyrate protects against colon tumorigenesis.

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**Introduction**

There is accumulating epidemiologic, clinical, and experimental evidence showing that dietary modification is an important factor in the prevention of colon cancer (1). We have previously shown that, using an AOM-induced colon carcinogenesis model, a fish oil/pectin diet protects against colon cancer by lowering tumor incidence (2), and DNA damage at the initiation stage of colon carcinogenesis (3) compared with a corn oil/cellulose or corn oil/pectin diet.

Dietary fish oil, rich in n-3 polysaturated fatty acids (PUFA), that is, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), protects against colon cancer by altering cell kinetics (i.e., proliferation, differentiation, and apoptosis; refs. 3–8). Pectin is a soluble fiber and it is fermented by colonic microflora, which produces short chain fatty acids (e.g., acetate, propionate and butyrate), CO₂, methane, and other gases (9). Among the short chain fatty acids, butyrate is used as a major energy source by colonicocytes (10, 11). Even though some controversies remain concerning, the in vivo responses of colonocytes to butyrate exposure (9), butyrate has been shown to impact cell kinetics, for example, cell proliferation, differentiation, and apoptosis, which regulate development of colon carcinogenesis (12–16). Therefore, we hypothesized that the combination of fish oil with butyrate would protect against colon cancer initiation through an integrated pattern of changes in cell proliferation, differentiation, apoptosis, and p27Kip1 protein levels. This hypothesis was further supported by a very recent study (17) showing that pescevegetarians (vegetarians who consume fish and seafood products) have a much lower risk of developing colorectal cancer, indicating potential benefit from the interaction of n-3 PUFA and dietary fiber.

To date, a number of investigators have measured proliferation and apoptosis in the same preclinical model. Even though data generated at the animal target tissue level are informative, they show only an averaged overall trend. Measuring these variables in the same cell offers the potential to determine the coordination of physiological phenomena in a cell. Furthermore, even though p27Kip1 is a well-established inhibitor of cell proliferation, p27Kip1 may also regulate differentiation and apoptosis (18, 19). However, the connection of p27Kip1 with proliferation, differentiation, and apoptosis is not clear. By determining these four variables in the same cell, we were able to provide an opportunity to gain greater understanding of the regulation of cell kinetics in individual cells. To our knowledge, this study is the first to determine all four of these variables in the same cell.
Fish Oil and Butyrate Effects on Colon Cell Kinetics

Materials and Methods

Animals and study design

The animal use protocol was approved by the University Animal Care Committee of Texas A&M University (College Station, TX). Forty-eight male weanling Sprague–Dawley rats (100–120 g; Harlan) were individually housed and maintained in a temperature and humidity-controlled animal facility. This study was a 2 × 2 × 4 factorial design with two types of fat (corn oil or fish oil), with or without butyrate, and at four time points (0, 12, 24, or 48 hours) after carcinogen injection. Rats were provided with the defined diets for 3 weeks before carcinogen azoxymethane (AOM, 15 mg/kg of body weight, s.c.). Rats had free access to food and water at all times and 48-hour food intake was measured after 2 weeks of receiving diets. Body weights were recorded weekly throughout the study.

Diet

The four defined diets (Table 1) differed in lipid source (corn oil or fish oil) and the administration of butyrate pellets. The major differences between the fatty acid compositions of the two lipid sources were significantly higher amounts of EPA (20:5, n-3) and DHA (22:6, n-3) in the fish oil compared with corn oil diet, and higher amounts of 18:2 (n-6) in the corn oil diet. Diets were prepared based on the standard AIN-76A formulation with modification of 15% fat amount, corn oil or fish oil (Vacuum-deodorized Menhaden fish oil, OmegaPure; ref. 20). Diets contained equivalent amounts of antioxidants; 26 mg α-tocopherol, 14 mg γ-tocopherol, and 2 mg tertiary butylhydroquinone (TBHQ)/100g diet. Gastro-resistant slow-release butyrate pellets (S.A. Valpharma) designed to be primarily released in the colon (20) were supplemented (1.5 g/100g diet) into the diets. A higher butyrate concentration in the feces was reported in rats fed with these pellets (20).

Tissue acquisition

Rats were euthanized by CO₂ asphyxiation, and the entire colon was immediately resected. After removal of the rectum, 1 cm of the distal colon was fixed in 4% paraformaldehyde and another 1 cm was fixed in 70% ethanol. From the paraformaldehyde-fixed tissue section and serial sections were cut; one was used for colocalization of proliferation and p27Kip1, and the other for colocalization of differentiation and apoptosis. Since ethanol fixed tissue was used for quantitative localization of N²-methylguanine, N²-methylnitroguanine was not part of the colocalization protocol but was measured in a tissue section adjacent to that used for the paraformaldehyde-fixed sections.

Table 1. Composition of experimental diets

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<th>Ingredient</th>
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<th>FO (g)</th>
<th>FOB (g)</th>
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</table>

A mixture of antioxidants; 26 mg α-tocopherol, 14 mg γ-tocopherol, and 2 mg tertiary butylhydroquinone (TBHQ)/100g diet. The butyrate diets were supplemented with Gastro-resistant slow-release butyrate pellets (1.5 g/100 g diet, Valpharma, Serravalle, Italy).

Colocalization of cell proliferation and p27Kip1

Antigen was retrieved by microwave treatment in 0.1 mol/L sodium citrate buffer (pH 6.0). To block nonspecific background staining, tissue sections were incubated with normal rabbit serum (Jackson) in normal sheep serum (Jackson). A mixed solution of monoclonal anti–Ki-67 antibody (BD Biosciences) and rabbit anti-p27Kip1 antibody was used as the primary antibodies. Slides were incubated with biotinylated sheep anti-mouse IgG (Jackson) and Texas-Red Streptavidin (Vector laboratory Inc.). Subsequently, slides were incubated with fluorescein-labeled goat anti-rabbit IgG (Jackson). Finally, slides were counterstained and mounted with DAPI/antifade (Vector) and were stored at 4 °C before imaging. Omission of each or both primary antibodies was used as a negative control. At least 20 crypt columns per animal were chosen for quantitative analysis.

Imaging of colonic crypts were visualized using a Nikon Eclipse TE300 microscope (Nikon Inc.) equipped with an FITC filter (excitation 490–505 nm/emission 515–545 nm) and a Texas-Red filter (excitation 560–585 nm/emission 600–652 nm). Images were captured using a Micromax 5 MHz cooled digital CCD camera (Princeton Instruments) with a constant exposure time and 2 × 2 binning. The position of Ki-67–positive stained cell and the staining intensity of p27Kip1 were assessed by cell position within the crypt using a MetaMorph Imaging System (Version 6.6r3, Universal Imaging Corp.). For cell proliferation, the proliferation index was calculated as 100 times the number of labeled cells per crypt column divided by the total number of cells per crypt column (21). Proliferative zone was calculated as 100 times the position of the highest labeled cell divided by the number of cells per crypt column (21). For p27Kip1 quantification, nuclei on one side of a crypt column were outlined and the staining intensity was measured. Background staining intensity was subtracted from the staining intensity of the nuclei. Optimum offset and gain were determined by preanalysis of multiple darkly and lightly stained tissues to maximize the distribution of stain intensity so that small differences in staining were quantifiable. For accuracy and consistency purposes, once established, the settings remained constant for all images.

Colocalization of differentiation and apoptosis

Apoptosis was detected using ApopTag fluorescein in situ apoptosis kit (Intergen) and differentiation was determined using Dolichos Bilonos Agglutinin (DBA) glycoprotein staining. DBA has a carbohydrate specificity to α-N-acetylglactosamine (22). This carbohydrate residue is thought to increase with normal differentiation of colonic epithelial cells (23, 24).

Antigen sites in these tissue sections were retrieved by treatment in Proteinase K (5 μg/mL). Tissues were incubated with non-fat milk and then incubated with biotinylated DBA (10 μg/mL, Vector). Slides were incubated with Texas-Red conjugated streptavidin (Vector). Preincubation of 0.2 mol/L N-acetyl-D-galactosamine (Sigma) with DBA or omission of DBA was used as a negative control for DBA staining. TdT enzyme was applied to tissue sections and then sections were incubated in anti-digoxigenin-fluorescein in blocking solution. Omission of TdT enzyme was used as a negative control and DNase I-treated tissue section was used as a positive control for apoptosis. At least 20 crypt columns per animal were randomly chosen for quantitative analysis. The position of DAB-stained cells and apoptotic cells were recorded using a MetaMorph Image system. Images were captured using the same inverted...
fluorescent Nikon microscope as described above. The differentiation index and apoptosis index were calculated as previously described (24).

**In vivo measurement of N²-methylguanine DNA adducts**

DNA damage was measured by quantitative IHC using a rabbit polyclonal antibody to N²-methylguanine (gift from Dr. Geoff Margison, Paterson Institute for Cancer Research, Manchester, UK). Tissue sections were placed in prewarmed 50 mmol/L NaOH/40% ethanol at 55°C to denature DNA and neutralized with 5% acetic acid/40% ethanol. Sections were incubated with the primary antibody followed by biotinylated goat anti-rabbit IgG. The antibody–antigen complex was visualized using the DAKO Liquid DAB (diamino-benzidine tetrahydrochloride) Substrate-Chromagen System (DAKO). Liver N²-methylguanine DNA adducts in AOM-injected animals were used as a positive control. Omission of primary antibody was used as a negative control. Images of colonic crypts were visualized on a MICROSTAR IV, Reichert light microscope, captured by a digital camera (Sony DDC-970 MD, color 3CCD), and staining intensity (assessed by cell position) was analyzed using NIH Image software (NIH Image, version 1.61). Each nucleus on one side of a crypt column was outlined and the staining intensity was measured. Background staining intensity was subtracted from the staining intensity of the nuclei.

**Statistical analyses**

Proliferation, p27kip1, differentiation, apoptosis, and DNA damage data were analyzed using three-way ANOVA to determine the effect of fat, butyrate, and time. When \( P < 0.05 \) for the interactions, means of all diet groups were separated using Fisher Protected Least Significant Difference (LSD) test. When \( P < 0.05 \) for the effects of fat, fiber, or time but not for the interaction, overall means for fat, fiber, or time were separated using the Fisher LSD test. The correlations between variables were tested using Pearson correlations, and statistical significance was assessed using Fisher distribution, with calculations performed using PROC CORR in SAS (SAS Institute Inc.).

**Results**

There were no significant differences in food intake or body weight gain among any of the four treatment groups (data not shown).

**Images of colocalization of proliferation, p27kip1, differentiation, and apoptosis**

Figure 1A shows a representative image of colocalization of proliferation (shown in orange) and p27kip1 (shown in green). Proliferation was predominantly localized to the lower part of the crypts and p27kip1 (green) staining was localized in the nuclei of colonic cells within crypts. B, lectin binding (red) was primarily located in the upper part of the crypt and apoptotic cells (green) were found in the lower region of the crypt 9 hours after AOM injection. Blue staining represents DAPI counterstaining. A and B are derived from serial sections and crypts 1–5 in A are the same crypts shown in B. Arrows show the same cell labeled for cell proliferation and p27kip1 in A, and apoptosis in B.

**Carcinogen effects on DNA damage, proliferation, p27kip1, differentiation, and apoptosis**

N²-methylguanine DNA adducts, as an indicator of DNA damage, increased by 12 hours following AOM injection and then started to decrease by 48 hours after AOM injection (\( P < 0.001 \), Fig. 2A). Cell proliferation decreased at 12 hours after carcinogen injection and returned to basal levels at 48 hours after AOM injection (\( P < 0.001 \), Fig. 2B). In contrast, the level of p27kip1 was not changed by AOM injection (Fig. 2C). Differentiation was increased at 24 hours after AOM injection (\( P = 0.014 \), Fig. 2D) and apoptosis was maximized at the same time point (\( P < 0.001 \), Fig. 2E).

**Dietary fat and butyrate effects on DNA damage, proliferation, p27kip1, differentiation, and apoptosis**

Dietary fish oil resulted in lower DNA adduct levels compared with corn oil throughout all time points (\( P = 0.001 \), Fig. 3A). Even
though there was no butyrate effect on cell proliferation, there was an obvious main effect of dietary lipid after carcinogen injection. Dietary fish oil decreased cell proliferation index compared with the corn oil diet ($P = 0.003$, Fig. 3B main graph). At 48 hours after carcinogen injection, there was a compensatory increase in cell proliferation above that observed at 0 hour in corn oil-fed rats (10.2%–18.5%) but not in fish oil-fed rats (9%–8.41%; $P = 0.010$, Fig. 3B inset). The pattern of changes in proliferative zone over time, as a function of dietary fat type, was similar to proliferation index over time (data not shown). There was no significant effect of dietary fat or butyrate on p27$^{kip1}$. Dietary fish oil increased differentiation compared with the corn oil diet ($P = 0.039$, Fig. 3C), and butyrate treatment also increased differentiation ($P = 0.041$, Fig. 3C, inset). There was no main effect of dietary fat or butyrate on apoptosis. When the data were analyzed using a subplot analysis for the different time points, the fish oil/butyrate diet increased the apoptotic index compared with the other groups at 24 hours after carcinogen injection ($P = 0.039$, Fig. 3D).

Correlation among DNA adducts, proliferation, p27$^{kip1}$, differentiation, and apoptosis

After carcinogen injection, there was a decrease of proliferation and increase of apoptosis (Fig. 2B and 2E), which was associated with a decrease of crypt height (Fig. 4A). There was no significant correlation between proliferation and crypt height (data not shown).

Figure 2.
Carcinogen effects on DNA adduct level, cell proliferation, p27$^{kip1}$, differentiation, and apoptosis over time. A, DNA adduct levels were increased 12 hour after AOM injection and decreased by 48 hours after AOM injection ($P < 0.001$). B, cell proliferation decreased 12 hours after AOM injection and increased at 48 hour after AOM injection ($P < 0.001$). C, carcinogen injection did not affect p27$^{kip1}$ level. D, differentiation increased 24 hours after AOM injection ($P < 0.001$). Data, means ± SE. Means without a common letter are significantly different.

Figure 3.
Dietary fat effects on DNA adduct level, cell proliferation, p27$^{kip1}$, differentiation, and apoptosis. Dietary fish oil resulted in lower DNA damage (A, $P = 0.001$), cell proliferation (B, $P = 0.003$), and elevated differentiation (C, $P = 0.039$) compared with the corn oil diet. At 48 hours after carcinogen injection, there was a compensatory increase of cell proliferation beyond that observed at 0 hour in corn oil-fed rats but not in fish oil-fed rats ($P = 0.010$, B inset). Butyrate treatment increased differentiation ($P = 0.041$, C inset). Fish oil/butyrate diet increased apoptosis, relative to the other three groups at 24 hours after carcinogen injection (D, $P = 0.039$). CO, corn oil; CO/B, corn oil/butyrate; FO, fish oil; FO/B, fish oil/butyrate. Data, mean ± SE. Bars without a common letter are significantly different. * $P < 0.05$. 

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shown). There was an inverse relationship between crypt height and apoptosis in fish oil/butyrate-treated animals (coefficient = −0.52, P = 0.040; Fig. 4B). However, a significant inverse relationship was not detected in the corn oil/butyrate group (Fig. 4B).

As expected, DNA adduct levels were low in the saline-injected rats, and there was no correlation between adduct levels and proliferation (Fig. 4C). However, in carcinogen-injected animals, there was an inverse relationship between DNA adduct levels and cell proliferation (coefficient = −0.42, P = 0.010). In saline groups, there was an inverse relationship between DNA damage and apoptosis (coefficient = −0.70, P = 0.012; Fig. 4D). In contrast, there was a positive relationship between DNA damage and apoptosis after carcinogen injection (coefficient = 0.36, P = 0.033).

To further explore the interactive effects of dietary fat and butyrate treatment on p27\(^{kip1}\), a positive relationship between p27\(^{kip1}\) level and proliferation in the corn oil/butyrate group (coefficient = 0.61, P = 0.035) was observed (Fig. 5). In contrast, there was no significant correlation between p27\(^{kip1}\) level and proliferation in fish oil/butyrate diet (Fig. 5). Interestingly, at the same expression level of p27\(^{kip1}\), the corn/butyrate diet was associated with a higher level of proliferation compared with the fish oil/butyrate diet.

**Discussion**

The normal colon is a dynamic tissue dependent upon an equilibrium among cell proliferation, differentiation, and death (25). Colon tumors are initiated by nuclear damage leading to a disturbance of this steady state. In this study, the relationships among cell proliferation, differentiation, apoptosis, and a cell-cycle mediator p27\(^{kip1}\) were determined using a colocalization technique that allowed for the determination of all four variables in the same cell. To the best of our knowledge, this study is the first work using this approach to examine regulation of cell kinetics during cancer initiation and the response to diet. The associations between DNA damage and the four variables were also assessed.

Accumulating evidence indicates that dietary fish oil protects against colon cancer (2–8). The current data also strongly indicate that dietary fish oil decreased DNA damage and cell proliferation, and increased differentiation. At 48 hours after AOM injection, corn oil-fed animals had a colonocyte proliferation index that was elevated above the levels observed at 0 hour (control). In comparison, fish oil-fed rats exhibited restoration of colonocyte proliferation to a level similar to those at 0 hour, which may result in lower propagation of DNA-damaged cells in the fish oil-fed animals. The apoptotic index was increased, but only when rats received the combination of fish oil and butyrate.

Even though it is known that butyrate modulates cell-cycle kinetics, the effect of butyrate on colon cancer development is still debated (9). Results from some studies suggest that butyrate is chemopreventive by decreasing tumor growth via a reduction in cell proliferation and an increase in differentiation and apoptosis (13, 20, 26). The administration of 1% butyrate in drinking water has been reported to increase the percentage of rats with colonic tumors (27). Others report no benefit of butyrate administration.
The enhancement of apoptosis with fish oil feeding in combination with butyrate was also verified in experiments using ex vivo isolated epithelial cells. Cells from fish oil-fed rats incubated with butyrate induced apoptosis via alteration of mitochondrial function (decrease of mitochondrial membrane potential, cytochrome C release from mitochondria and increase of caspase-3 activity) compared with corn oil and butyrate incubated cells (40). In addition, it was reported that fish oil/pectin (butyrate generating fiber) diets decrease anti-apoptotic bcl-2 expression followed by increased apoptosis (41). The enhanced apoptosis was attributed to a reduction of bcl-2 via methylation of the bcl-2 promoter region in the fish oil/pectin group (42). In contrast, corn oil with butyrate treatment increased bcl-2 levels in mouse colonic cells (43). The proapoptotic effects of fish oil/pectin diet were also associated with the suppression of peroxisome proliferator-activated receptor (PPARγ) and PGE2, and increase of PGE2 (39) and modulation of miRNA and mRNA expression profiles (44).

In the present study, when butyrate was combined with a corn oil diet, there was a positive relationship between p27Kip1 and cell proliferation. The increase in p27Kip1 levels might compensate for the increase of cell proliferation induced by carcinogen administration because p27Kip1 is an inhibitor of cyclin-dependent kinase, which blocks progression of the cell cycle (18, 19). However, the increase of p27Kip1 was not sufficient to suppress the elevated cell proliferation associated with the corn oil/butyrate group because the cell proliferation was higher compared with the fish oil/butyrate group.

Butyrate may produce conflicting effects on the growth of normal versus cancerous colon cells through its impact on acetyl CoA/histone acetyltransferases (HAT) or as a histone deacetylase (HDAC) inhibitor (45). In normal colon, butyrate acts as a primary energy source and does not inhibit cell proliferation by epigenetic changes via activation of acetyl CoA/HATs. In contrast, in cancer cells butyrate slows cell proliferation but induces differentiation and apoptosis by functioning as a HDAC inhibitor. Further studies are needed to examine the interaction of butyrate with lipid sources with respect to histone acetylation and cell kinetics.

Collectively, data from our study indicate that an enhanced early coordinated response to carcinogen may be one mechanism by which fish oil and butyrate protect against colon tumorigenesis. Our data also suggest that the effects of butyrate may depend in part on the type of fat in the diet. This may partly explain the controversy and inconsistency of butyrate effects on cell-cycle kinetics and colon cancer development across in vivo and in vitro studies. Although our data do not address the cellular modifications in the colon that contribute to tumorigenesis, such as epigenetic modifications or stem cell-specific mutations, it does describe outcomes of those modifications and global changes in one form of DNA mutations. The ability of this combination of nutrients to alter global and gene-specific epigenetic states (20, 41, 14) at various stages of tumorigenesis and to modulate downstream events such as proliferation and apoptosis indicate the involvement of multiple mechanisms that contribute to risk reduction. However, further studies are needed to investigate the impacts of fish oil and butyrate on colon adult stem cell damage and epigenetic state.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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Authors’ Contributions
Conception and design: M.Y. Hong, N.D. Turner, R.S. Chapkin, J.R. Lupton
Development of methodology: M.Y. Hong, R.S. Chapkin, J.R. Lupton
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.Y. Hong, R.S. Chapkin, J.R. Lupton
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.Y. Hong, N.D. Turner, M.E. Murphy, R. Carroll, R.S. Chapkin, J.R. Lupton
Writing, review, and/or revision of the manuscript: M.Y. Hong, N.D. Turner, R.S. Chapkin, J.R. Lupton
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.R. Lupton
Study supervision: N. D. Turner, I.R. Lupton

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