Dietary Fat and Colon Cancer: Modulating Effect of Types and Amount of Dietary Fat on ras-p21 Function during Promotion and Progression Stages of Colon Cancer

Jagveer Singh, Rachid Hamid, and Bandaru S. Reddy

ABSTRACT

Although epidemiological and experimental studies have indicated a strong relationship between types and amount of dietary fat and colon tumorigenesis, the modulating effects of these nutritional factors at the molecular level have not been fully elucidated. Transforming proteins encoded by activated ras genes have been implicated in the etiology of many human malignancies, including colon cancer. It is now well established that the transforming ability of ras-p21 critically depends on its correct localization in plasma membrane. The posttranslational processing of the cytosolic precursor (pro-ras), as it is synthesized in the cytoplasm, and its proper anchorage to the cytoplasmic face of plasma membrane are determined by an important intermediate metabolite of dietary fat and an enzyme system that includes farnesyl protein transferase. To provide an understanding of the molecular basis of the relationship between the types and amount of dietary fat and the transforming function of ras, especially during the stages of promotion and progression of colon tumor development, we investigated the effect of various types and amount of dietary fat on the expression of ras-p21 during azoxymethane (AOM)-induced colon carcinogenesis. Male F344 rats were fed the semipurified American Institute of Nutrition-76A diet containing low-fat corn oil and were given s.c. injections of AOM dissolved in normal saline at a dose rate of 15 mg/kg body weight, once weekly, for 2 weeks. Control animals received s.c. injections of equal volumes of normal saline. Beginning 1 day after the second AOM or saline injection, groups of animals intended for the treatment with different types of high-fat dietary regimens were fed the semipurified American Institute of Nutrition-76A diets containing high levels of high-fat corn oil (HFCO) rich in omega-6 fatty acids or high levels of high-fat fish oil (HFFO) rich in omega-3 fatty acids; the remaining animals in experimental and control groups were continued on the low-fat corn oil diet until termination of the experiment. Groups of animals were sacrificed 1, 12, or 36 weeks after the last AOM or saline injection, and their colonic mucosa and grossly visible colon tumors from rats sacrificed 36 weeks after the last AOM injection were analyzed for the levels of expression of ras-p21. We found that AOM induced increasingly higher levels of ras-p21 expression with advancing stages of colon tumor development. The HFCO diet resulted in enhanced expression of AOM-induced ras-p21 as observed 36 weeks after the last AOM injection. In contrast, feeding the HFFO diet inhibited AOM-induced ras-p21 expression. These results correlate with increased incidence and multiplicity of grossly visible colon tumors in AOM-treated animals fed a HFCO diet versus decreased incidence and lower multiplicity of colon tumors in their counterparts on the HFFO diet. Further analysis of ras-p21 levels in cytosol and plasma membrane revealed that feeding a HFFO diet resulted in increasing accumulation of ras-p21 in cytoplasm with a concomitant decrease in membrane-bound ras-p21 levels as observed in animals sacrificed 12 and 36 weeks after the last AOM injection. Thus, the dietary HFCO may promote colon tumorigenesis by increasing ras-p21 expression, whereas HFFO appears to exert its antitumor activity by interfering with posttranslational modification and membrane localization of ras-p21.

INTRODUCTION

Colorectal cancer is the third most common malignant neoplasm worldwide (1) and the second leading cause of cancer deaths in the United States (2). Several epidemiological studies have indicated the influence of lifestyle factors, particularly nutritional factors, on the development of certain forms of cancers, including the colon carcinomas (3). Although Lucretius had articulated a possible role of overnutritio in the etiology of degenerative diseases as early as 50 B.C., it was not until the middle of this century that epidemiological surveys and studies in experimental animals stimulated tremendous interest in understanding the relationship between dietary fat and cancer (4, 5). In recent decades, several case-control, cohort, and experimental model studies of colon cancer have emerged, indicating a key role of the amount of dietary fat, especially animal fat or red meat intake, in the pathogenesis of this neoplasm (6–9). Such studies are consistent with findings that a high-fiber diet and appropriate consumption of fruits and vegetables are associated with lower rates of colon cancer in populations throughout the world (7, 10, 11). Studies in laboratory animals have also provided evidence incriminating the fatty acid composition of dietary fat as a major determinant of risk for colon cancer (12, 13). Recently, researchers have sought to identify the relative tumor-modulating capabilities of different types of dietary fat, such as fish oil and corn oil (14, 15). Corn oil, one of the important vegetable fats in the United States diet, contains high levels of omega-6 polyunsaturated fatty acids such as linoleic acid (c18:2, n-6) and has been shown to enhance colon tumorigenesis in rodents (16). In contrast, fish oil, which is rich in omega-3 polyunsaturated fatty acids such as eicosapentanoic acid (c20:5, n-3) or docosahexanoic acid (c22:6, n-3), displays no such enhancing effect (17). In a Phase II clinical trial of patients with colonic polyps, dietary fish oil supplements have been shown to inhibit cell proliferation in the colonic mucosa (18). Available data also indicate that consumption of dietary fish oil is associated with a suppressed inflammatory response in patients with rheumatoid arthritis (19) and psoriasis (20), and with improved survival of animals with endotoxin challenge or burn injury (21). However, the precise mechanism of fish oil and other omega-3 polyunsaturated fatty acid-induced anti-inflammatory, anti-tumor, and immunomodulating effects remains largely unknown.

High intake of dietary corn oil and other omega-6 polyunsaturated fatty acids exhibits dramatically different physiological and metabolic effects. These include: (a) changes in gut microflora (22); (b) increased concentrations of secondary bile acids in the lumen of the colon (23), which exert toxic effects on the colonic epithelium (9); (c) increased ornithine decarboxylase activity in the colonic mucosa resulting in enhanced epithelial polyamine levels, leading to increased colonic crypt cell proliferation; and (d) increased prostaglandin levels (24). Recent studies from our laboratory have shown that HFCO enhances activities of diverse enzymes, including protein kinases that

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3 The abbreviations used are: HFCO, high-fat corn oil; HFFO, high-fat fish oil; pro-ras, cytosolic precursor; FTTase, farnesyl protein transferase; HMG-CoA, hydroxymethylglutaryl CoA; AOM, azoxymethane; LFCO, low-fat corn oil; AIN, American Institute of Nutrition; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; TBST, Tris-buffered saline containing 0.2% Tween-20.
have been implicated directly or indirectly in colon tumor promotion and progression, whereas HFFO appears to suppress activities of these enzymes and reduce the chemically induced colon tumor burden (25-27). It is interesting that these kinases have been shown to participate in ras-mediated growth-promoting signal transduction pathways (28).

The ras-p21, a guanine nucleotide-binding 21-kDa protein product of ras genes that is anchored to the cytoplasmic face of plasma membrane, functions in the regulation of cell proliferation. Its normal function is subverted during neoplastic process. In fact, mutated versions of ras-p21 are implicated in the etiology of many human malignancies, including cancer of the colon (29, 30). It is now well established that the elements critical for ras-p21 function(s) is its interaction with the guanine nucleotides, guanosine 5'-triphosphate and guanosine 5'-diphosphate, and its association with plasma membrane (31). Although the interaction with guanine nucleotides clearly regulates the on/off state of ras-p21 as a biological switch for signal transduction pathways, the transforming activity of ras-p21 is critically dependent on its correct localization in the plasma membrane (32, 33). The ras-p21 is produced as a cytosolic precursor (pro-ras) that localizes to the plasma membrane after a series of posttranslational modifications (34). Willumsen et al. (35, 36) demonstrated that the cysteine residue of C-terminal CAAX sequence (where C is cysteine, A is an aliphatic residue, and X represents any residue) of the posttranslational modification of ras-p21 either through farnesol and 30% corn oil to alleviate any essential fatty acid deficiency. The composition of all experimental diets was adjusted so that the animals in all dietary groups would receive the same amount of calories, protein, vitamins, minerals, and fiber. The diets were prepared in our laboratory three times per week and were stored in airtight containers filled with liquid nitrogen in a dark, cold room at 4°C. Aliquots of the experimental diets were analyzed for their fatty acid composition. As expected, the corn oil diet contained high levels of omega-6 fatty acids such as linoleic acid, whereas the fish oil diet contained increased levels of docosahexaenoic acid and eicosapentaenoic acid. The contents of omega-6 fatty acid of HFCO and HFFO diets were 59 and 8%, respectively, and those of omega-3 fatty acid were 1.2 and 31% respectively. The levels of oleic acid (n-9) in the HFCO and HFFO diets were 24 and 16%, respectively, and the total saturated fatty acid levels were 14 and 28%, respectively.

**Experimental Procedure.** Beginning at 5 weeks of age, all rats were fed the modified AIN-76A (LFCO) diet. At 7 weeks of age, the animals intended for carcinogenesis were given AOM s.c. on a weekly basis for 2 weeks at a dose rate of 15 mg/kg body weight. The animals intended for vehicle treatment were s.c. injected with equal volumes of physiological saline. One day after the second AOM or saline injection, groups of animals designated for HFCO or HFFO diets were transferred to their respective dietary treatment, whereas the remaining animals treated with AOM or saline were continued on LFCO diet until termination of the experiment. Body weights of all the animals were recorded weekly until 16 weeks of age, and then every 4 weeks until the end of the study. Six animals from each group treated with AOM or saline and fed the control or experimental diets were sacrificed by CO2 euthanasia at weeks 1 and 12, and the remaining animals were killed 36 weeks after the last AOM or saline injection. The colons were rapidly removed and rinsed in ice-cold normal saline, slit open longitudinally, cleaned with ice-cold normal saline, and laid flat on a glass plate. Grossly visible colon tumors of larger than 0.3 cm in size from animals sacrificed 36 weeks after the last AOM injection were removed. Colonic mucosa that were free of tumors were scraped with a microscope slide. The snap-frozen samples of colonic mucosa and tumors were stored at -80°C until used for ras-p21 analysis.

**Measurement of ras-p21 Expression.** Quantitative estimation of total ras-p21 protein was carried out as described (41). For determination of cytosolic and membrane-bound ras-p21, the colonic mucosa and tumors were washed twice

<table>
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<th>Table 1 Percentage composition of experimental diet</th>
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<td><strong>Diet ingredients</strong></td>
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<tr>
<td><strong>Low fat</strong></td>
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<tr>
<td>Casein</td>
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<tr>
<td>taurine-methionine</td>
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<tr>
<td>Corn starch</td>
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*This was formulated based on the AIN standard reference diet with the modification of varying sources of carbohydrate (13).

*Corn oil and fish oil were added at the expense of starch. The composition of high-fat diets was adjusted so that animals in all dietary groups would consume approximately the same amounts of protein, minerals, vitamins, fiber, and calories (13).
in ice-cold PBS and resuspended in PBS-PMSF [10 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, and 0.2 mM PMSF] containing 0.1 mM leupeptin and 0.2 μg/ml aprotinin as protease inhibitors. Samples were homogenized on ice, and all the debris and nuclei were removed by centrifugation at 800 × g for 10 min at 4°C. The supernatants were further centrifuged at 40,000 × g for 30 min at 4°C. The resulting supernatants (cytosolic fractions) were saved, and the 40,000 × g pellets (membrane fractions) were solubilized in lysis buffer (10 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, 10 mM sodium deoxycholate, 1 mM PMSF, and 1% Triton X-100) containing 0.1 mM leupeptin and 0.2 μg/ml of aprotinin, and centrifuged at 40,000 × g for 30 min at 4°C. The clear supernatants representing solubilized membrane fractions were measured for their protein contents using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA) with BSA as standard.

**SDS-PAGE and Western Blotting.** SDS-PAGE and Western transfers were carried out essentially by the methods of Laemmli (42) and Towbin et al. (43), respectively. Cytosolic and membrane fractions corresponding to 300 μg of protein were solubilized in sample buffer [10% SDS, 600 mM Tris-HCl (pH 6.7), and 50% glycerol] containing B-mercaptoethanol and 50 μg/ml bromophenol blue. Samples were boiled for 2 min and resolved by extended electrophoresis on a 12.5% reduced polyacrylamide vertical slab gel with an overlay of 5% polyacrylamide, along with prestained SDS-PAGE molecular weight markers and the ras-p21 Western blot standards (Oncogene Science, Manhasset, NY). Electrophoretically resolved proteins were electrotransferred onto Hybond ECL membrane (Amersham Co., Arlington Heights, IL) in a Trans-blot Electroblotting transfer cell (Bio-Rad Laboratories).

**Immunodetection and Quantification of ras-p21 Using ECL.** After transblotting the electrophoretically resolved proteins, the blots were blocked with 5% nonfat dry milk dissolved in TBST. Blots were then incubated with pan-reactive anti-ras-p21 mouse monoclonal antibody (Ab-2; Oncogene Science) diluted in TBST containing 0.5% nonfat dry milk. This antibody is broadly reactive to p21 translational products of H-, K-, and N-ras genes. Blots were washed extensively in TBST and reincubated with peroxidase-linked secondary antibody (antimouse IgG; Amersham) diluted in TBST containing 0.5% nonfat dry milk. The blots were then thoroughly washed in excess TBST and probed with the ECL Western blot detection system (Amerham) using reflection autoradiography films (DuPont New England Nuclear, Boston, MA) according to the manufacturer’s instructions. The autoradiograms were scanned with an Image Master sharp laser densitometric scanner (PDI, Huntington, NY), and the peak areas representing ras-p21 bands of both the standards as well as the samples were integrated.

**Statistical Analysis.** Body weights and the expression levels of ras-p21 were compared between animals fed the control and experimental diets. The data were analyzed using unpaired t test and one-way ANOVA. Differences were considered statistically significant at P < 0.05.

**RESULTS**

**General Observations.** Table 2 presents the body weights of AOM- and vehicle-treated animals fed the control (LFCO) or experimental (HFCO and HFFO) diets. The body weights of animals fed the control or experimental diets and treated with AOM were comparable with the body weights of their saline-treated counterparts throughout the study. However, the body weights gained by animals fed the LFCO diet were approximately 10% below that of the animals fed the HFCO or HFFO diets in both the AOM- and saline-treated groups.

Because the objective of this study was to understand the mechanism by which different types and amounts of dietary fat modulate colon carcinogenesis during promotion and progression stages, the grossly visible colon tumors harvested 36 weeks after the last AOM injection were subjected to analysis of ras-p21 and were not processed for histopathological evaluation. The grossly visible colon tumor incidence (percentage of animals with tumors) in animals fed the LFCO, HFCO, and HFFO diets was 57, 76, and 40%, respectively, and the colon tumor multiplicity in terms of number of tumors/animal (expressed as the mean ± SD) was 0.73 ± 0.78, 1.38 ± 1.24, and 0.45 ± 0.6 respectively. No tumors were found in vehicle-treated animals.

**Western Blot Analysis and Differential Expression of ras-p21.** Fig. 1A and B, demonstrates the representative examples of Western blot analysis of total as well as cytosolic- and membrane-bound ras-p21. The majority of samples exhibited detectable levels of p21 with pan-reactive anti-ras (Ab-2) mouse monoclonal antibody. Ab-2, which broadly reacts with all the translational products of normal as well as mutated H-, K-, and N-ras genes, identified a duplet of ras-p21 species exhibiting differential electrophoretic mobilities. A standard curve of integrated absorbance from laser densitometric scans representing ras-p21 Western blot standards was plotted to determine the quantities of immunoreactive ras-p21 protein in samples (Fig. 2). Table 3 summarizes the results of Western blot analysis for total amounts, as well as the cytosolic- and membrane-bound ras-p21 levels in colon mucosa and in tumors of AOM-treated animals fed the LFCO, HFCO, and HFFO diets. Regardless of dietary regimen, very low (background) levels of ras-p21 were detected in saline (vehicle)-treated animals. AOM treatment induced increasingly higher levels of mucosal ras-p21 expression with advancing stages of colon carcinogenesis (1 versus 12 versus 36 weeks) in all dietary groups. Colon tumors exhibited higher levels of ras-p21 than the colon mucosa. The HFCO diet resulted in increasingly enhanced expression of the colon mucosal total ras-p21 in AOM-treated animals compared with that in their counterparts on LFCO or HFFO diets beginning 12 weeks after the last AOM injection. Similar observations were made in colon tumors. It is interesting that the levels of membrane-bound ras-p21 in colon mucosa, as well as in tumors of animals maintained on the HFFO diet, were significantly lower than those of animals fed the LFCO or HFCO diets. The levels of cytosolic ras-p21 in colon mucosa and in the tumors of animals fed the HFFO diet were correspondingly higher than those in tissues of animals fed the LFCO or HFCO diets. This effect was visible only 12 weeks after the last AOM injection. Animals fed the HFFO diet exhibited increasing levels of cytoplasmic ras-p21 (biologically inactive) and decreasing levels of membrane-bound (biologically active) ras-p21 in a time-dependent manner compared with those fed the LFCO or HFCO diets. This difference was more pronounced between the experimental (HFCO and HFFO) diets.
animals fed the HFFO and HFCO diets, which strongly correlated with the grossly visible colon tumor outcome. Although there were no significant differences in the expression levels of cytosolic ras-p21 between animals on the LFCCO and HFCO dietary regimens, the expression levels of membrane-bound ras-p21 in animals fed HFCO were significantly higher both in colon mucosa at 12 and 36 weeks after the last AOM injection and in colon tumors than in those animals fed the LFCCO diet.

DISCUSSION

The purpose of the present study was to investigate the modulating effect of types and amount of dietary fat on the expression levels of ras-p21 to provide an understanding of the molecular basis of the relationship between the dietary fat and the transforming function of oncogenic ras during postinitiation phases of colon carcinogenesis. Results of this experiment demonstrate that the omega-3 fatty acids present in fish oil inhibit the AOM-induced enhanced expression of ras-p21 and suppress colon tumor development, whereas omega-6 fatty acids present in corn oil enhance the levels of AOM-induced ras-p21 expression and increase the consequent colon tumor outcome. Previously, data from our laboratory have demonstrated that animals fed high amounts of dietary fat in the form of Menhaden oil (omega-3 fatty acids) had significantly lower incidence and lower multiplicity of AOM-induced colon adenocarcinomas compared with their counterparts ingesting an equivalent amount of corn oil containing omega-6 fatty acids (13). We have also shown earlier that chemopreventive agents, such as piroxicam and difluoromethylornithine, inhibit chemically induced colon cancer and suppress ras-p21 expression (44). Results of the present investigation corroborate our earlier findings. However, the most significant outcome of this study is that dietary HFFO, in contrast to HFCO, not only inhibits AOM-induced ras-p21 expression and the consequent tumor outcome, but also decreases the levels of membrane-bound (biologically active) ras-p21 while concomitantly increasing the levels of cytosolic precursor (pro-ras, biologically inactive) in a time-dependent manner. In addition, the HFCO diet enhanced the expression of AOM-induced total as well as membrane-bound ras-p21 in comparison to that with the LFCCO diet.

Although the role of ras in malignancy is not clear, it is well established that the association of ras-p21 to the inner surface of the plasma membrane is an absolute requirement for triggering ras oncogenicity (31). The trafficking of pro-ras from cytosol to plasma membrane is facilitated by a series of closely linked posttranslational modifications, i.e., farnesylation, proteolysis, and carboxymethylation, of which farnesylation, the first and obligatory step signalled by the consensus C-terminal CAAX motif present on all cytosolic precursors (pro-ras), requires transfer of farnesyl moiety from farnesyloprophosphate to Cys of CAAX motif in a reaction catalyzed by FPTase. Several studies have shown that inhibition of ras farnesylation blocks membrane association of ras-p21; prevents neoplastic transformation of cells; and causes accumulation of slower-migrating, unmodified cytosolic pro-ras, which display a dominant inhibitory phenotype and can antagonize the activity of membrane-associated oncogenic ras-p21 (32, 33, 45–47). Although the biochemical basis of this inhibitory activity is presently unknown, it is believed to result from the competition for regulatory and/or effector molecule(s) that are important for ras function. The cellular origin of farnesyl residues in the mevalonate pathway of cholesterol biosynthesis (37). Pharmacological inhibition by compactin and lovastatin of HMG-CoA reductase activity in a rate-limiting step in mevalonate biosynthesis has resulted in suppression of in vivo tumor growth of ras-transformed cells, reversal of ras-transformed cells to normal phenotype, and inhibition of ras-p21 membrane association in a dose-dependent manner (45, 46). Mevalonate is an important intermediate metabolite of dietary fat metabolism and is essential for the biosynthesis of all isoprenoid compounds, including farnesyl PP, a precursor of cholesterol (37). It is noteworthy that intake of specific types of fat alters
cholesterol biosynthesis (48, 49) and that this variation in the rate of cholesterol biosynthesis is associated with changes in the activity of HMG-CoA reductase (40, 49). It is interesting that dietary fish oil has been shown to significantly inhibit the activity of HMG-CoA reductase (48, 50). It is thus plausible that amount and types of lipids modulate production of farnesyl PP, through HMG-CoA reductase-catalyzed mevalonate biosynthetic pathway and affect farnesylation of pro-ras. Therefore, high dietary fat intake, such as effected by a HFCO diet and which increases tumor promotion, may lead to enhanced farnesylation, whereas a HIFFO diet, which decreases tumor promotion, may do so by inhibiting farnesylation of pro-ras, leading to a shift toward diminished membrane localization allowing accumulation of pro-ras in cytosol, and thereby reducing the function(s) of ras-p21. It is also possible that dietary fatty acids or their metabolites may have an impact on farnesylation of pro-ras by modulating the activity of FPTase, an enzyme that catalyzes the crucial transfer of farnesyl moiety to the cysteine residue of CAAAX motif of pro-ras from farnesyl PP, a cholesterol biosynthesis intermediate. Farnesyl PP, and pro-ras polypeptide containing the CAAAX motif are the substrates for FPTase function. Several analogues of farnesyl PP, and cell-active CAAAX peptidimetetics have been generated as potential inhibitors of posttranslational processing of pro-ras into ras-p21 (51). These FPTase inhibitors have demonstrated selective antiproliferative activity against ras-transformed cells both in cell culture and in laboratory animal studies (52). Inhibition of ras-p21 membrane association thus may be a viable molecular approach to cancer chemoprevention.

In conclusion, the present study has explored for the first time the modulation of posttranslational modification and membrane association of ras-p21 as a plausible molecular mechanism for the promotion of colon tumorigenesis by using different amounts of corn oil and fish oil in the diet. We have demonstrated that a high percentage of dietary corn oil promotes chemically induced colon carcinogenesis by facilitating the posttranslational processing of ras-p21, whereas increased levels of dietary fish oil suppress AOM-induced colon tumour development by interfering with posttranslational modification and membrane association of ras-p21, thus inhibiting the ras function.

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