Long-term feeding of various fat diets modulates azoxymethane-induced colon carcinogenesis through Wnt/β-catenin signaling in rats

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Submitted 19 June 2006; accepted in final form 25 December 2006

Fujise T, Iwakiri R, Kakimoto T, Shiraishi R, Sakata Y, Wu B, Tsunada S, Ootani A, Fujimoto K. Long-term feeding of various fat diets modulates azoxymethane-induced colon carcinogenesis through Wnt/β-catenin signaling in rats. Am J Physiol Gastrointest Liver Physiol 292: G1150–G1156, 2007. First published December 28, 2006; doi:10.1152/ajpgi.00269.2006.—The Wnt signaling pathway plays an essential role in carcinogenesis, and the amount of fat intake and composition of dietary fatty acids are crucial factors for colon carcinogenesis. We investigated whether various dietary fats affected the Wnt signaling pathway of colon tumorigenesis in azoxymethane (AOM)-treated rats. Male Sprague-Dawley rats were given intraperitoneal injections of AOM and supplemented with 10% corn, olive, beef, and fish oil for 44 wk. Aberrant crypt foci (ACF) and tumors were examined at 12 and 44 wk. Normal appearing colon mucosal proliferation and apoptosis were evaluated by 5-bromo-2′-deoxyuridine (BrdU) incorporation and percentages of fragmented DNA, respectively. Expressions of β-catenin, cyclin D1, Wnt2, Wnt3, and Wnt5a of normal appearing colon mucosa were analyzed by Western blot analysis. Long-term dietary corn oil and beef tallow increased ACF, tumor incidence, and tumor numbers in AOM-treated rats. In contrast, both olive and fish oil inhibited them. Dietary corn oil and beef tallow increased BrdU incorporation and the expression of cytosolic β-catenin and cyclin D1 and decreased apoptosis in the colon mucosa. Expressions of Wnt2 and Wnt3 in rats fed with beef tallow and Wnt5a in rats fed with corn oil increased with or without AOM-treatment. BrdU-incorporated cells were often observed at the tops of crypts in rats fed with beef tallow, whereas this was not observed in rats fed with the other diet. Long-term high intake of corn oil and beef tallow enhanced cell proliferation through Wnt signaling and modulated the distribution of proliferating cells, which might contribute to promoting effects in colon tumorigenesis.

THE ETIOLOGY of colorectal cancer is very complex, and both genetic and environmental factors are thought to be involved in this process. Among environmental factors, dietary habits play an important role. High consumption of meat and fat, together with low consumption of fruits, vegetables, vitamins, and fibers, has been suggested to increase risks of colorectal cancer (5, 13, 31, 37). Many epidemiological studies have demonstrated a positive relationship between dietary fat intake and colorectal cancer (37). Experimental studies have shown that a high-fat diet rich in n-6 polyunsaturated fatty acid (PUFA) and saturated fatty acids (SFA) promoted colon carcinogenesis, particularly in postinitiation or promotional phases and/or both (10, 26, 28, 39). On the other hand, diets rich in n-3 PUFA and n-9 monounsaturated fatty acid (MUFA) have been reported to reduce colon tumorigenesis in both the initiation and postinitiation phases (2, 26, 29), supporting epidemiological reports showing that an n-3 PUFA-rich diet suppressed the risk of colon cancer in humans (3, 7, 8). These experimental and epidemiological studies suggested that not only the amount of fat intake but also the composition of ingested dietary fatty acids are crucial factors for colon carcinogenesis.

The Wnt signaling pathway regulates a wide variety of processes in embryonic development and adult homeostasis including cell proliferation, morphology, motility, and cell fate at the cellular level (12, 30). The key molecule in this pathway is a multiprotein scaffold consisting of β-catenin, glycogen synthase kinase (GSK)-3β, and adenomatous polyposis coli (APC). The APC gene was first identified by genetic analysis in patients with a hereditary cancer syndrome termed familial adenomatous polyposis (13). Hereditary forms of colorectal cancer and ~85% of all sporadic colorectal cancers show loss of APC function (16). It is commonly accepted that the crucial tumor suppressor role of APC lies in its ability to destabilize cytoplasmic free β-catenin (35). Therefore, disrupted regulation of the Wnt signaling pathway plays a central role in the etiology of colon carcinogenesis (17, 19, 35).

Aberrant crypt foci (ACFs) were first identified in the colon mucosa of rodents exposed to carcinogens (4) and have also been confirmed to be present in the human colon (21). ACFs are regarded as preneoplastic or precancerous lesions in the colorectum of humans and rodents (4, 21). The number of crypts/foci was shown to increase with time following carcinogen treatment, and ACFs demonstrate increased cell proliferation in rodents (23, 24).

The present study aimed to investigate effects of dietary intake of diverse fatty acids on colon carcinogenesis in the azoxymethane (AOM)-induced rat colon cancer model and the effects of various fatty acids on the Wnt signaling pathway.

MATERIALS AND METHODS

Animals and experimental procedures. Male Sprague-Dawley rats were used in this study. Rats were divided into five dietary groups: standard chow, 10% corn oil, 10% olive oil, 10% beef tallow, and 10% fish oil. Each dietary group was then divided into AOM-treated and vehicle-treated subgroups. Rats were housed in plastic cages with filter tops in a holding room that was maintained under controlled conditions (illumination from 8:00 to 20:00). All rats had access to water and food ad libitum.

The experimental design is summarized in Fig. 1. Beginning at 6 wk of age, all rats were fed with standard chow. At 7 wk of age, rats intended for carcinogen treatment were intraperitoneally injected with AOM (Sigma, St. Louis, MO) dissolved in 1 ml physiological saline.
solution (PSS) once a week for 2 wk at a dose of 15 mg/kg body wt, whereas control rats were given an equal volume of PSS alone. One day after AOM or saline treatment, groups of rats designed for the corn oil, olive oil, beef tallow, and fish oil diets began to be fed with diets high in n-6 PUFA, high n-9 MUFA, high SFA, or high n-3 PUFA, whereas one group continued to be fed with standard chow. The fatty acid composition of standard chow consisted of 14.6% palmitic acid, 24.6% oleic acid, 46.6% linoleic acid, 3.8% α-linolenic acid, and 2.6% stearic acid. The energy of standard chow was 3.6 kcal/g and that of the 10% oil diet was 4.04 kcal/g. The corn oil diet included 10.5% palmitic acid, 29.0% oleic acid, 56.8% linoleic acid, and 1.9% stearic acid. The olive oil diet included 7.0% palmitic acid, 78.0% oleic acid, 12.0% linoleic acid, and 1.0% stearic acid. The beef tallow diet included 32.5% palmitic acid, 41.2% oleic acid, 2.7% linoleic acid, and 14.5% stearic acid. The fish oil diet included 12.0% docosahexaenoic acid and 18.6% eicosapentaenoic acid. Twelve weeks after the last injection of AOM or saline, some rats were killed, and colon ACF formation was analyzed. The remaining rats were killed at 44 wk, and the colon mucosa was collected for further analysis.

Collection of colon tissue samples. Under halothane anesthesia, rats were killed. After a laparotomy, the entire colon was carefully removed, flushed thoroughly with PSS, and opened longitudinally on its antimesenteric border to expose the luminal side. The mucosa and tumors were carefully harvested, respectively.

ACF analysis. For ACF analysis, 12 wk after the last injection of AOM or saline, rats were killed, and the colon was carefully removed. The colon was flushed with ice-cold PSS, opened longitudinally, placed with the flat mucosal side up between two pieces of wet filter papers, and fixed in 10% buffered formalin for ACF analysis. After a minimum of 24 h in buffered formalin, the colon was stained with 0.2% methylene blue dissolved in the same formalin solution for 5 min and rinsed with PSS. After being stained, the colon was placed with the mucosal side up on a slide, observed using a stereoscopic microscope, and assayed for numbers and multiplicity of crypts. ACFs were distinguished from the surrounding normal crypts by their features and defined as single or multiple crypts according to 1) whether they had altered luminal openings, 2) whether they exhibited thickened epithelia, and 3) whether they were larger than adjacent normal crypts, according to Bird (4). Crypt multiplicity corresponded to the number of crypts per foci and was categorized as containing <3 or ≥4 aberrant crypts/foci. Six rats were evaluated in each group.

5-Bromo-2′-deoxyuridine incorporation assay. We evaluated cell proliferation in the surrounding normal mucosa by 5-bromo-2′-deoxyuridine (BrdU) incorporation using the Cell Proliferation Kit (Amersham Pharmacia Biotech). Briefly, rats were intraperitoneally injected with the labeling reagent, which was supplied as a concentrated aqueous solution of BrdU and 5-fluoro-2′-deoxyuridine (10:1 ratio) at a dose of 10 ml/kg body wt at 44 wk after the last injection of AOM or saline. Two hours after the injection of the labeling reagent, rats were killed, and the colon was carefully removed. The colon was immediately fixed in 10% neutral buffered formalin. Samples were then embedded in paraffin and sectioned. Specimen were dewaxed and immersed in PBS containing 0.3% hydrogen peroxide for 10 min at room temperature. After specimens had been washed three times with PBS, sufficient reconstituted nuclease/anti-BrdU solution was applied directly onto the specimen. Colonos were incubated for 2 h at room temperature. After specimens had been washed three times with PBS, sufficient peroxidase-anti-mouse IgG2a solution was added onto specimens. Specimens were incubated for 1 h at room temperature. They were then soaked in PBS containing 0.02% diaminobenzidine and substrate/substrainer at a dose of 5 drops/50 ml diaminobenzidine solution for 1 min for color development. Finally, specimens were counterstained by an immersion in methyl green. A minimum of 50 crypts were randomly selected for the BrdU incorporation assay, and numbers of cells were assessed. Six rats were tested in each group.

DNA fragmentation assay. Forty-four weeks after the last injection of AOM or saline, colonic mucosa of all groups of rats were examined. The mucosal scraping was processed immediately after collection to minimize nonspecific DNA fragmentation. The amount of fragmented DNA was determined as previously described (1, 21) with some modifications. Briefly, mucosal scrapings of different intestinal segments were homogenized in 10 volumes of lysis buffer consisting of 5 mM Tris·HCl, 20 mM EDTA, and 0.5% (wt/vol) Triton X-100 (pH 8.0). A 1-ml aliquot of each sample was centrifuged for 20 min at 27,000 g to separate intact chromatin (pellet) from fragmented DNA (supernatant) (40). The supernatant was decanted and saved, and the pellet was resuspended in 1 ml Tris buffer (pH 8.0) with 10 mM Tris·HCl and 1 mM EDTA. Pellet and supernatant fractions were assayed for DNA content using a diphenylamine reaction as previously described (6). Results are expressed as percentages of fragmented DNA. Six rats were tested in each group.

Western blot analysis. Forty-four weeks after the last injection of AOM or saline, colonic mucosa of all groups of rats were examined. The mucosal scraping was immediately washed twice with ice-cold PBS (pH 7.4) and centrifuged at 1,000 g for 5 min at 4°C. The pellet was then resuspended with 2 volumes of buffer A and lysed at 4°C for
Results were statistically analyzed using the least-significant difference method. Colon cancer incidence was expressed as percentages of animals with cancer, and the results were evaluated by ANOVA in which multiple comparisons were performed using the Student’s t-test. Differences were considered significant if the probability of the difference occurring by chance was <0.05.

RESULTS

ACF formation at 12 wk. AOM-treated rats showed dilated irregular luminal openings, thicker epithelial linings, and protrusions toward the lumen. Twelve weeks from the start of the experiment series, few ACFs were found in rats without AOM treatment. In contrast, AOM-induced colonic ACFs developed in all rats treated with AOM (Fig. 2A). Among AOM-treated rats, rats fed with 10% corn oil and 10% beef tallow diets had significantly greater numbers of ACFs per colon compared with rats fed with standard chow (P < 0.05). On the other hand, numbers of ACFs per colon significantly decreased in AOM-treated rats fed with 10% olive oil and 10% fish oil diets compared with standard chow-fed rats (P < 0.05).

Crypt multiplicity of ACFs in rats treated with AOM is shown in Fig. 2B. Numbers of multicyclic ACFs, which were determined as containing up to 4 or more aberrant crypts per focus, were significantly higher in rats fed with 10% corn oil and 10% beef tallow diets than that of rats fed with standard chow (P < 0.05), whereas rats fed with 10% olive oil and 10% fish oil diets showed a significant decrease in numbers of multicyclic ACFs compared with standard chow-fed rats (P < 0.05).

Tumor incidence and tumor multiplicity at 44 wk. Results of tumor incidence and tumor multiplicity are summarized in Table 1. At 44 wk, no rat without AOM treatment developed colon adenoma or carcinoma. On the other hand, all rats fed with standard chow, 10% corn oil, and 10% beef tallow diets developed colon cancer 44 wk after the last injection of AOM. Two of six rats fed with 10% olive oil did not develop colon cancer, and four of six rats fed with 10% fish oil did not develop colon cancer. The numbers of colon tumors per rat were significantly higher in rats fed with 10% corn oil and 10% beef tallow than those of rats fed with standard chow (P < 0.05), whereas tumor multiplicity in colons of rats fed with 10% fish oil was significantly lower than that of rats fed with standard chow (P < 0.05). The number of tumors in rats fed with 10% olive oil was smaller than that of standard chow-fed rats, but there was no statistical significance between these two groups.

BrdU incorporation by the normal mucosal epithelium surrounding tumors. We evaluated BrdU incorporation of tumor tissues surrounding normally appearing mucosa to evaluate proliferation potential. The numbers of BrdU-incorporated epithelial cells per crypt significantly increased in AOM-treated rats compared with saline-treated rats at 44 wk (Fig. 3). In rats...
Table 1. Tumor incidence and tumor multiplicity in rats treated with AOM.

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<tr>
<th>Tumor Incidence, %</th>
<th>Tumor Multiplicity, Nos. of Colon Tumors/Rat</th>
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<tr>
<td></td>
<td>Without AOM</td>
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<tr>
<td>Standard chow</td>
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<tr>
<td>10% Corn oil</td>
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<td>10% Olive oil</td>
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<td>10% Beef tallow</td>
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<td>10% Fish oil</td>
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Values are means ± SE; n = nos. of rats (6 rats were studied in each group). No rat without azoxymethane (AOM) treatment showed colon tumors, including adenoma and carcinoma. All rats fed with standard chow, 10% corn oil, and 10% beef tallow developed colon cancer 44 wk after the last injection of AOM. Two of six rats fed with 10% olive oil and four of six rats fed with 10% fish oil did not develop colon cancer. Numbers of colon tumors per rat were significantly higher in rats fed with 10% corn oil and 10% beef tallow compared with rats fed with standard chow, whereas the tumor multiplicity of the colon in rats fed with 10% fish oil was significantly lower than that of rats fed with standard chow. *P < 0.05.

DISCUSSION

Many epidemiological and experimental studies have demonstrated that not only the amount but also the type of dietary fats differing in fatty acid composition were important in colon tumorigenesis (29, 32, 36), whereas controversies still exist regarding the influence of dietary fat on colon carcinogenesis. This study demonstrated that any type of high-fat diet did not result in the development of ACFs or colon tumors per se. With AOM treatment, both 10% corn oil and 10% beef tallow diets significantly enhanced the numbers of ACF and multiplicity of foci 12 wk after the start of experiments. In addition, all experimental rats fed with standard chow, 10% corn oil, and 10% beef tallow developed colon tumors 44 wk after AOM treatment, and the numbers of tumors significantly increased in rats fed with corn oil and beef tallow compared with standard chow-fed animals. These results indicated that the dietary intake of corn oil (rich in n-6 PUFA) and beef tallow (rich in SFA) promoted colon carcinogenesis in AOM-treated rats. In contrast to corn oil and beef tallow, olive oil (rich in n-9 MUFA) and fish oil (rich in n-3 PUFA) ameliorated AOM-induced ACF formation and colon carcinogenesis in this study. Our results were supported by those of previous studies showing that a high-fat diet rich in n-6 PUFA and SFA promoted colon carcinogenesis, particularly in the postinitiation and/or promotional phases in rodents (10, 28, 39).
whereas a high-fat diet rich in n-9 MUFA and n-3 PUFA inhibited colon tumorigenesis in both the initiation and postinitiation phases (2, 26, 29).

Most of the previous studies have examined cancerous tissues themselves to evaluate the effects of dietary fat (2, 10, 26, 28, 29, 39). This study focused on background characteristics of the colon mucosa after long-term dietary fat and evaluated proliferation states in normally appearing colon mucosa for carcinogenetic processes, because few studies have investigated the effects of dietary fat intake and fatty acid composition on normally appearing colon mucosa. BrdU incorporation of normally appearing colon mucosa surrounding colon tumors significantly increased in rats fed with 10% corn oil and 10% beef tallow but decreased in rats fed with 10% olive oil and 10% fish oil diet. These results indicated that long-term intake of 10% corn oil and 10% beef tallow accelerated the proliferation potential of the colon mucosa, which might promote colon carcinogenesis after AOM treatment. This study also demonstrated that the range of BrdU-positive cells spread to the upper portion of crypts in rats fed with corn oil and beef tallow. Two main hypotheses have been proposed for morphogenesis in a colon tumor: bottom-up morphogenesis and top-down morphogenesis. The development of human adenomatous polyps is believed to proceed through the top-down mechanism, in which genetically altered cells in the superficial portions of the mucosa spread laterally and downward to form new crypts that would connect to preexisting normal crypts and eventually replaced them (33, 38). Our observation of BrdU-positive cells in upper portions of the crypts indicated alterations in the distribution of proliferating cells in rats fed with corn oil and beef tallow, suggesting the involvement of top-down morphogenesis in carcinogenesis resulting from a high-fat diet.

Recent studies have indicated that the Wnt signaling pathway is required during stem cell homeostasis for normal progression of intestinal epithelial cells through the crypt-villous axis (25, 30), and dysregulation of the Wnt signaling pathway has been observed in many cancer tissues (25, 30). This study evaluated the expression of cyclin D1, a product gene in Wnt/β-catenin signaling activated through accumulation of β-catenin in the cytosol. The expression of cyclin D1 increased in rats fed with 10% corn oil and 10% beef tallow, and a significantly high accumulation of β-catenin in the cytosol was observed, suggesting that Wnt/β-catenin signaling was activated by dietary consumption of corn oil and beef tallow. Wnt expression, an upstream signal in the Wnt/β-
the reduction of apoptosis in the colon mucosa, and decreased apoptosis in the colon mucosa has been associated with the inhibition of the tumor suppressor gene p53-mediated mitochondria-dependent apoptotic pathway (39). This study showed a significant decrease in apoptosis in rats fed with corn oil and beef tallow, and a reduction of colon mucosal apoptosis by dietary corn oil and beef tallow might be the result of accelerated cell proliferation during the cancer promoting process. Many reports have suggested that the chemopreventive effect of n-3 PUFA was partly due to increased mucosal apoptosis (11). The present study indicated that there was an apparent suppression of colon carcinogenesis together with increased mucosal apoptosis in rats treated with AOM by dietary intake of olive oil rich in n-9 MUFA and fish oil rich in catenin signaling pathway, increased in rats fed with corn oil; and Wnt2 and Wnt3 increased in rats fed with beef tallow in rat colon mucosa regardless of AOM treatment. These results indicated that increased colon mucosal proliferation potential was, at least in part, attributed to the activation of Wnt/β-catenin signaling. Several studies in humans have indicated that expressions of Wnt genes were accelerated in colon carcinoma tissues compared with surrounding normally appearing mucosa using in situ hybridization (14, 18). In another study of human gastric cancer, coexistence of Wnt2 upregulation and β-catenin translocation was positively associated with lymph node metastasis (9). In this study, it is noteworthy that the upregulation of Wnt signaling in the normally appearing surrounding colon mucosa was observed in rats with long-term consumption of corn oil and beef tallow. In this study, Wnt2 and Wnt3 were upregulated in beef tallow-fed rats, and Wnt5a was upregulated in corn oil-fed rats. This kind of difference between these Wnt proteins has not been clearly demonstrated in previous publications, and this point warrants further exploration.

We previously demonstrated that a higher risk for colon cancer by dietary corn oil rich in n-6 PUFA was attributed to
n-3 PUFA. The increase in apoptosis might be one of the factors involved in tumor-inhibiting processes. In addition to the results of this study, other factors including ornithine decarboxylase activity (27), diacylglycerol (15), cyclooxygenase 2 (26, 34), and inducible nitric oxide synthase (20) may contribute to the reduced carcinogenesis together with n-3 PUFA and n-9 MUFA.

In conclusion, this study indicated that long-term intake of dietary SFA and n-6 PUFA accelerated colon carcinogenesis by increasing cell proliferation through the upregulation of the Wnt/β-catenin signaling pathway in AOM-treated rats. In contrast, n-9 MUFA and n-3 PUFA had suppressive effects on colon carcinogenesis. These results indicated that dietary fatty acid composition might be an important factor for the modulation of mucosal proliferation potential.

GRANTS

This work was supported in part by the president’s expenditure (research project expenditure) ofSaga University and Ministry of Education, Science and Culture in Japan Grants-In-Aid for Scientific Research 15590658 and project expenditure) of Saga University and Ministry of Education, Science,

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