Lack of Enhancing Effect of Lauric Acid on the Development of Aberrant Crypt Foci in Male ICR Mice Treated with Azoxymethane and Dextran Sodium Sulfate

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Abstract: The effect of lauric acid (LA), which is reported to induce cyclooxygenase (COX)-2 expression in macrophage cells (RAW 264.7) on the development of aberrant crypt foci (ACF), putative precursor lesions of colonic adenocarcinoma, was investigated in an inflammation-related mouse colon carcinogenesis model treated with azoxymethane (AOM) and dextran sulfate sodium (DSS). To induce ACF, male ICR mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight) and then followed by 1% DSS in drinking water for one week, starting one week after dosing of AOM (AOM/DSS group). The AOM/DSS/LA group was fed with a diet containing 1% LA for 7 weeks, starting one week after the cessation of DSS administration. Other groups included the AOM/LA group given AOM and 1% LA diet for 9 weeks, the DSS/LA group given DSS and the diet with 1% LA, the AOM group that received AOM alone, the DSS group given DSS alone in drinking water, the LA group fed with 1% LA-containing diet alone, and the untreated group. At week 10 (end of the study), the frequency of ACF did not significantly differ between the AOM/DSS group (7.4 ± 3.0) and the AOM/DSS/LA group (8.4 ± 5.0). The value was extremely low in the AOM/LA group (1.0 ± 1.0) and in the AOM alone group (2.4 ± 2.7). No ACF developed in other groups. Our findings suggest that dietary LA did not influence the occurrence of ACF in the AOM/DSS-induced mouse colon tumorigenesis, indicating a lack of LA enhancing effects on the early phase of inflammation-related mouse colon carcinogenesis. (J Toxicol Pathol 2007; 20: 93–100)

Key words: lauric acid, aberrant crypt foci, inflammation, azoxymethane, dextran sulfate sodium, mice

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the Western countries. Globally, CRC accounted for about 1 million new cases in 2002 (9.4% of the world) and the mortality rate is about one half that of incidence (about 529,000 deaths in 2002)³. The incidence of CRC is particularly high in Canada and Australia where there is high consumption of red and processed meat⁵. In contrast, Mediterranean countries have lower rates of CRC when compared with other Western countries⁵. The low incidence rates might be due to diet⁴, because the consumption of fruits, vegetables, fish and olive oil is quite high in Mediterranean countries. In 1969, Wynder et al.⁵ first suggested that patients with CRC have a high caloric intake in the form of fats, and that dietary fats may be involved in the pathogenesis of CRC development. Since their innovative case-control study, a number of epidemiological studies have implicated dietary fat in the etiology of CRC⁶. In most industrialized societies, CRC has a high incidence among both women and men. In Japan, CRC incidence has particularly increased since the end of World War II with an increase in dietary fat intake. Some of the inconsistencies in findings on dietary fats may relate to the fact that they are generally assessed in accordance with their quantity (total fat), origin (animal or vegetable) or type (saturated, monounsaturated or polyunsaturated)⁷–⁹. However, on the basis of results reported from a number of studies conducted in different countries, there is sufficient evidence to suggest that certain fatty acids play a role in CRC occurrence. Currently, some epidemiological studies have indicated that higher concentrations of butyric acid and eicosapentaenoic acid (EPA) provide protection against CRC¹⁰–¹². These fatty acids induce apoptosis in colonic cancer cells¹³,¹⁴. In contrast, specific fatty acids that increase
the risk of CRC are unclear. Recently, a high fat intake was reported to be associated with an increased risk for inflammatory bowel disease (IBD), such as ulcerative colitis (UC)\textsuperscript{15} and Crohn’s disease (CD)\textsuperscript{16}. CRC is one of the complications of both diseases\textsuperscript{17}.

Lauric acid (LA, C\textsubscript{12}H\textsubscript{24}O\textsubscript{2}, Fig. 1), also called n-dodecanoic acid is a medium chain fatty acid, which forms monolaurin in the human or animal body. The highest content of LA is found in a mother’s breast milk and coconut oil\textsuperscript{18}. LA occurs as the glyceride in many vegetable fats, especially coconut oil and laurel oil and is used chiefly in the manufacture of soaps, detergents, cosmetics and lauryl alcohol. It is also used in food additives and insecticides. LA was recently reported to have antiviral\textsuperscript{19} and antibacterial\textsuperscript{20,21} actions, and is able to destroy lipid-coated viruses including human immunodeficiency, herpes, cytomegalovirus and influenza viruses. More recently, interesting reports describing that LA induces the expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), both of which are involved in colon carcinogenesis\textsuperscript{22-25}, through toll-like receptor 4 in mouse macrophage 264.7 cells have been published\textsuperscript{26,27}. Over-expression of both enzymes was also immunohistochemically observed in the inflamed colon of a colitis-related mouse colon carcinogenesis model treated with azoxymethane (AOM) and dextran sodium sulfate (DSS)\textsuperscript{28}.

Recently, many studies have reported that several non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors, suppress the development of chemically-induced colon carcinomas in rats\textsuperscript{29-32}. In addition, clinical trials have demonstrated that a NSAID, sulindac, suppresses adenomas in patients with familial adenomatous polyposis\textsuperscript{33}. Nimesulide, a selective inhibitor of COX-2, suppresses the formation of aberrant crypt foci (ACF), a putative precancerous lesion of the colon cancer\textsuperscript{27,34-36}, induced by a colon carcinogen AOM in rats\textsuperscript{37}, and is able to inhibit the occurrence of colon adenocarcinoma induced by AOM/ DSS in mice\textsuperscript{38}. Similarly, iNOS, a generator of cellular nitric oxide is also overexpressed in colon tumors\textsuperscript{39,40}. These results suggest that COX-2 and iNOS play important role in the incidence and development of colon cancer.

In the current study, we investigated whether LA promotes the occurrence of ACF in an inflammation-related mouse colon carcinogenesis model\textsuperscript{41}. Also, we investigated whether LA-albumin complex induces COX-2 expression in RAW264.7 cells (a murine macrophage-like cell line) as previously described\textsuperscript{26}. RAW264.7 cells are known to be useful for examining the production of inflammatory mediators, including cytokines, prostaglandin E\textsubscript{2}, COX-2 and iNOS, after inflammatory stimuli\textsuperscript{42}.

Materials and Methods

Chemicals and reagents

A colonic carcinogen, AOM, was purchased from Sigma-Aldrich K.K. (Tokyo, Japan). DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH, USA). LA was obtained from Wako Pure Chemicals (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Bovine fetal serum albumin (BSA) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich K.K. Enhanced chemiluminescence western blot detection kits and reagents were purchased from Amersham Pharmacia Biotech. (Buckinghamshire, UK).

Animals, drinking water and diet

Male Crlj: CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo, Japan) aged 5 weeks were used in this study. They were maintained at the Animal Facility of Kanazawa Medical University according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (5 mice/cage) with free access to drinking water (tap water) and basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2°C). They were quarantined for the first 7 days after arrival, and then divided into experimental and control groups. DSS for induction of colitis was dissolved in water at a concentration of 1% (w/v) every day. Experimental diet containing LA (1%, w/w) was prepared every week by mixing with powdered basal diet CRF-1. The dose of LA was selected, based on a report by DeLany et al.\textsuperscript{43}

Experimental procedure

Thirty-six male ICR mice were divided into eight groups (Fig. 2). Groups 1 (n=5), 2 (n=5), 3 (n=5) and 5 (n=4) were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Group 1 was then given a one-week exposure of 1% DSS in drinking water, and was given no further treatment. Group 2 was given AOM and DSS, as per group 1. Then, mice of group 2 were fed a diet containing 1% LA for 7 weeks, starting one week after the cessation of DSS administration. Animals of group 3 were given a diet containing 1% LA for 9 weeks, starting one week after the AOM injection. Group 4 (n=4) was given 1% DSS in drinking water and a diet containing 1% LA alone for 7 weeks. Groups 5, 6 (n=4) and 7 (n=5) were treated with AOM alone, 1% DSS alone and 1% LA-containing diet alone, respectively. Group 8 (n=4) served as an untreated control. At week 10, all mice were sacrificed under ether
anesthesia to assess the occurrence of colonic ACF. They underwent careful necropsy, with emphasis on the colon, liver, kidney, lung and heart. All grossly abnormal lesions in any tissue, and the organs such as liver, kidney, lung and heart were fixed in 10% buffered formalin solution for histopathology and COX-2 immunohistochemistry.

**Determination of ACF**

The number of ACF per colon was determined according to the method described in our previous report\(^4\). At necropsy, the length (from the ileocecal junction to the anal verge) of the large bowel was measured. The colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. Colons were cut and fixed in 10% buffered formalin for at least 24 h. Fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 20 s, and placed on a microscope slide for counting ACF. After counting ACF, colons were routinely processed for histopathology.

**Histopathology and COX-2 immunohistochemistry**

Tissues, including colon, were examined on hematoxylin and eosin-stained sections. Colitis with or without ulceration (inflammation score) was also evaluated on hematoxylin and eosin-stained sections, according to the following grading system\(^5\): grade 0, normal colonic mucosa; grade 1, shortening and loss of the basal one third of the actual crypts with mild inflammation and edema in the mucosa; grade 2, loss of the basal two thirds of the crypts with moderate inflammation in the mucosa; grade 3, loss of entire crypts with severe inflammation in the mucosa, but with retention of the surface epithelium; and grade 4, loss of entire crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa.

Immunohistochemistry for COX-2 was carried out with 4-μm thick, paraffin-embedded sections. Anti-COX-2 mouse monoclonal antibody (diluted 1:200, Transduction Laboratories) was used as the primary antibody. To reduce the non-specific staining of mouse tissues by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Inc., Burlingame, CA, USA) was applied. Staining was performed using a LSAB KIT or DAKO EnVision kit (DAKO, Glostrup, Denmark) or Vectastain Elite ABC Kit (Vector Laboratories). At the last step, the sections were counter-stained with hematoxylin. As a negative control, the primary antibody was omitted.

**Cell culture**

RAW264.7 cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in DMEM containing 10% FBS, L-glutamine (330 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO\(_2\)/air environment. Cells (2×10\(^6\)) were plated in a 60-mm dish and cultured for an additional 18 h to allow the number of cells to approximately double. Cells were maintained in serum-poor (0.25% FBS) medium for another 18 h prior to the treatment with LA.

**Preparation of LA-albumin complexes**

LA was solubilized in ethanol or combined with fatty acid-free and low endotoxin BSA at a molar ratio of 10:1 (fatty acid : albumin) in serum-poor medium (0.25% FBS). Fatty acid-albumin complex solution was freshly prepared prior to each experiment.

**Western blotting**

For western blot analysis, 2×10\(^6\) cells were lysed in lysis buffer [protease inhibitor, phosphatase inhibitor, 10 mM Tris, 1% sodium dodecyl sulfate (SDS), 1 mM sodium vanadate (V)]. Protein concentration was determined using a DC protein assay (Bio-Rad Laboratories Ltd., Osaka, Japan), with γ-globulin used as the standard. Denatured proteins were separated using SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred to PVDF membranes. After blocking overnight at 4°C in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the membranes were incubated with the primary goat polyclonal antibody against COX-2 (SC-1745, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution and the primary goat polyclonal antibody against β-actin protein (SC-1615, Santa Cruz Biotechnology) at 1:1000 dilution. Then, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (AMI3404, Biosource International, Camarillo, CA, USA) at a dilution of 1:1000 for 2 h at room temperature. The blots were developed using an ECL western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band was analyzed using NIH Image.

**Statistical analysis**

Where applicable, data were analyzed using one-way ANOVA with the Bonferroni correction (GraphPad Instat...
version 3.05, GraphPad Software, San Diego, CA, USA) and $P<0.05$ as the criterion of significance.

**Results**

**General observation**

Bloody stool was observed in some mice in groups 1, 2, 4 and 6, when they were given DSS. There were no significant changes of weight gains of mice in any group during the study (data not shown). Mean body, liver and relative liver (g liver weight/100 g body weight) weights at sacrifice are shown in Figs. 3, 4 and 5, respectively. Although there were no significant differences in the body and liver weight among the groups (Figs. 3 and 4), differences of the relative liver weights between groups 4 and 7 ($P<0.05$) and groups 7 and 8 ($P<0.01$) were statistically significant (Fig. 5). No significant differences were noted among the groups in the length of the large bowel (Fig. 6).

**Frequency of ACF**

ACF (Fig. 7a and 7b) developed in mice of groups 1, 2, 3 and 5. They were mostly small ACF consisting of 1–3 aberrant crypts (Fig. 7a). Only a few large ACF consisting of 4 or more aberrant crypts (Fig. 7b) in groups 1 (2 large ACF) and 2 (1 large ACF) were seen. As summarized in Fig. 8, the numbers of ACF per colon in groups 1, 2, 3 and 5 were relatively low, with predominance in group 2. However, the mean numbers of ACF per colon did not significantly differ between groups 1 and 2. The value of group 3 was much lower than those of groups 1 ($P<0.01$) and 2 ($P<0.001$), and was smaller than that of group 5, but without statistical significance.

**Histopathology, colonic inflammation score and COX-2 immunohistochemistry**

There were no significant alterations in the histopathologies of the liver, kidney, lung, and heart among the groups. In the colon, inflammation with or without mucosal ulcer was observed in mice of groups 1, 2, 4, and 6. The order of inflammation score in the groups was as follows: group 2 (2.80 ± 0.84) > group 1 (2.60 ± 0.89) > group 4 (1.75 ± 0.50), group 6 (1.75 ± 0.96) > group 3 (0.40 ± 0.55) > group 5 (0.25 ± 0.50) > group 7 (0.20 ± 0.45) >
Fig. 7. (a) A small ACF consisting of 2 aberrant crypts and (b) a large ACF consisting of 5 aberrant crypts from a mouse that received AOM and 1% DSS. (a) Methylene blue stain. (b) Hematoxylin and eosin stain. Original magnification, (a) and (b) ×20.

Fig. 9. COX-2 immunohistochemistry of the non-lesional area of mouse colons from each of groups 1 (a), 2 (b), 4 (c), and 6 (d). Bars inserted are 60 μm.
Expression of COX-2 in RAW264.7 cells treated with LA-albumin complex

Since we did not observe a modifying effect of LA on AOM/DSS-induced colon carcinogenesis, we tested the effect of LA on the expression of COX-2 in RAW264.7 cells, under the conditions described by Lee et al.\(^\text{26}\) in which complexes with 10 µM LA-1 µM BSA, 50 µM LA-5 µM BSA or 100 µM LA-10 µM BSA induced COX-2 protein expression in RAW264.7 cells. We did not observe over-expression of COX-2 in RAW264.7 cells treated with complexes with 10 µM LA-1 µM BSA, 50 µM LA-5 µM BSA or 100 µM LA-10 µM BSA (data not shown), but the complexes with 500 µM LA + 50 µM BSA and 500 µM LA alone induced COX-2 expression (Fig. 10).

Discussion

In the current study, 1% LA feeding after exposure to AOM and DSS did not significantly enhance ACF formation, suggesting no synergistic effects of LA with DSS in inflammation-related mouse colon carcinogenesis. Since LA has been reported to induce expression of inflammatory marker gene products such as COX-2, inducible nitric oxide and interleukin (IL)-1β in mouse macrophage 264.7 cells, we expected treatment with LA to enhance ACF formation induced by AOM and DSS. However, our findings suggest no modifying effect of dietary LA in inflammation-related mouse colon carcinogenesis induced by AOM and DSS. Interestingly, dietary LA (group 3: AOM + LA) lowered ACF formation induced by AOM when compared to group 5 (AOM alone), but the difference between ACF formation in these two groups was not significant.

Lee et al.\(^\text{26}\) previously reported that even a low dose of LA (10 mM) and BSA (1 mM) complex can up-regulate COX-2 expression in RAW264.7 cells. In our in vitro study we were not able to reproduce their results, but a high dose of the complex, which contained 500 µM LA and 50 µM BSA, induced COX-2 expression. Moreover 500 µM LA alone also induced the expression. While in vitro experimental conditions are not relevant to in vivo experiments, data from our ACF assay may indicate that dietary LA at a dose of 1% does not influence the early phase of colon carcinogenesis, the inflammation score and the COX-2 immunohistochemical expression in the inflamed colon.

It is well known that patients with IBD, both UC and CD, are at increased risk of developing CRC. Furthermore, activated transcription factor NFκB is found in inflamed mucosal biopsies of patients with IBD\(^\text{16}\). NFκB can stimulate iNOS to generate NO and COX-2 to generate prostanoids that have proinflammatory and carcinogenic effects.\(^\text{47}\). COX-2 is over-expressed in about 90% of colorectal adenocarcinomas and in 40–90% of colorectal adenomas.\(^\text{48,49}\). COX-2 expression is thus observed at an early stage of colorectal tumor development and in most tumors, either benign or malignant. Animal experiments have been shown the direct evidence of the important contribution of COX-2 in colorectal tumor development. Indeed, treatment with selective COX-2 inhibitors in animal models of familial adenomatous polyposis (FAP) significantly reduced the number of polyps.\(^\text{50,51}\). Selective COX-2 inhibitors also reduce tumor incidence and multiplicity induced by AOM and DSS.\(^\text{18}\). These findings
confirm COX-2 plays an important role in colorectal carcinogenesis. LA was the most efficient at inducing COX-2 expression in mouse macrophage RAW264.7 cells among a group of saturated fatty acids tested (C8:0–C18:0)52. However, our results described here suggest that LA has no promoting effect on AOM/DSS- and AOM-induced ACF formation in mice. Also, LA feeding, at a dose of 1% in diet, did not cause severe inflammation and elevation of COX-2 expression in the mouse colon. A recent review article did not cause severe inflammation and elevation of COX-2 formation in mice. Also, LA feeding, at a dose of 1% in diet, did not cause severe inflammation and elevation of COX-2 expression in the mouse colon. In this study, we used 1% LA, a concentration that is 7 times greater than that used in their study43. To make certain our findings are in line with the review by Lu et al.52 further studies using different experimental models and conditions (different doses of LA) are warranted.

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References

Effect of Lauric Acid on ACF Formation


