Dietary effects of mead acid on *N*-methyl-*N*-nitrosourea-induced mammary cancers in female Sprague-Dawley rats

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Abstract. The effect of mead acid (MA; 5,8,11-eicosatrienoic acid) on the suppression of the development and growth of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary cancer in female Sprague-Dawley rats was examined. The MA diet (2.4% MA) or control (CTR) diet (0% MA) was started at 6 weeks of age, MNU was injected intraperitoneally at 7 weeks of age, and the rats were maintained on the respective diets for the whole experimental period (until 19 weeks of age). All induced mammary tumors were luminal A subtype carcinomas (estrogen and progesterone receptor positive and HER2/neu negative). The MA diet significantly suppressed the initiation and promotion phases of mammary carcinogenesis; MA suppressed the development (incidence, 61.5 vs. 100%; multiplicity, 2.1 vs. 4.5) and the growth (final tumor weight, 427.1 vs. 1,796.3 mg) of mammary cancers by suppressing cell proliferation, but not by accelerating cell death. There were evident changes in the major fatty acid composition of n-3, n-6, and n-9 fatty acids in the serum of the MA diet group; there was a significant increase in MA and significant decreases in oleic acid (OA), linoleic acid, arachidonic acid and docosahexaenoic acid. In non-tumorous mammary tissue, there was a significant increase in MA and a significant decrease in OA in the MA diet group. The n-6/n-3 ratios in serum and mammary tissue of the MA diet group were significantly decreased. The MA diet suppressed MNU-induced luminal A mammary cancer by lowering cancer cell proliferation. Therefore, MA may be a chemopreventive and chemotherapeutic agent. In addition to hormone therapy, MA supplementation may be a beneficial chemotherapeutic agent for the luminal A subtype of breast cancer.

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Introduction

Epidemiological investigation has shown the association between the risk of breast cancer and total fat intake (1). However, rather than total fat intake, subtypes of fatty acids are considered to have a more influential effect on breast cancer. Increased intake of n-3 fatty acids derived from marine products and decreased intake of n-6 fatty acids found in vegetable oils and processed foods results in a lower n-6/n-3 ratio and decreased breast cancer risk (2). Rat mammary cancer growth is suppressed by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (3,4) and accelerated by n-6 fatty acids, such as linoleic acid (LA) (5). An increased n-6/n-3 ratio is associated with rat mammary carcinogenesis (6). In agreement with rat mammary carcinogenesis studies, laboratory experiments have shown that n-3 fatty acids, such as EPA, suppress human breast cancer cell growth (7), while n-6 fatty acids, such as LA and arachidonic acid (AA), promote the growth of human breast cancer cells (7,8). Experimental evidence shows critical roles for n-3 and n-6 fatty acids in association with breast cancer growth.

In contrast to n-3 and n-6 fatty acids, the role of n-9 fatty acids in breast cancer has not been studied in detail. One study showed that <15% of breast cancers could be prevented if the populations of high-income countries shifted to the traditional Mediterranean diet (9). The Mediterranean diet contains high amounts of olive oil rich in n-9 oleic acid (OA), and the possible effect of OA in suppressing breast cancer has received attention. However, another study was inconclusive in showing that olive oil consumption lowers the breast cancer risk (10). In cell culture, although OA causes growth inhibition at higher concentrations, it produces growth acceleration of human breast cancer cells at lower concentrations (11). Thus, the effects of OA on breast cancer appear to be complex. Mead acid (MA) is an n-9 fatty acid produced from OA when essential n-3 and n-6 fatty acids are deficient; mammals elongate and desaturate OA to make the end product MA (12,13). Epidemiologically, MA was inversely associated with breast cancer risk as well as overall cancer risk (14). Experimentally, MA suppressed MCF-7 and KPL-1 human breast cancer cell growth in culture (15,16). MCF-7 and KPL-1 are luminal A

subtypes according to intrinsic subtype classification (17). MA also suppressed the growth and metastasis of KPL-1 cells transplanted in female athymic mice (16).

Mammary carcinogenesis is a multistep process in which normal breast epithelial cells experience DNA damage (initiation phase), followed by enhanced cell proliferation (promotion phase), and subsequently the acquisition of metastasis (progression phase) to acquire a malignant potential. According to *in vivo* and *in vitro* studies using breast cancer cell lines, MA suppressed the promotion/progression phase of carcinogenesis; however, the role of MA in the initiation phase remains to be elucidated. In contrast to using breast cancer cell lines, rodent mammary cancers induced by chemical carcinogens can be preferentially used to evaluate the initiation/promotion stage of mammary carcinogenesis. However, metastasis is hardly detectable in rodent systems. Therefore, the present study was designed to determine if MA can block the initiation/promotion stage of mammary carcinogenesis to explore the possible use of MA as a chemotherapeutic agent and a chemopreventive agent for mammary cancer. The intrinsic classification of breast cancer can recommend effective targeted therapy and predict responses to chemotherapy (18). Therefore, the intrinsic subtypes of chemically induced rat mammary cancers were determined to see the effects of MA in association with the intrinsic subtypes of induced mammary cancers.

Materials and methods

Diet. The experimental diets contained the same amount of nutrients but with different fatty acid compositions (Table I). In brief, the MA diet and control (CTR) diet were modifications of the AIN-76 diet. The MA diet contained 5% SUNTGM33 (Suntory Wellness, Tokyo, Japan), which contains 48.0% MA. SUNTGM33 is microbial oil obtained by fungal fermentation (19). The CTR diet contained 5% olive oil (Nacalai Tesque, Kyoto, Japan), which contains 74.7% OA; OA is a precursor of MA. The detailed fatty acid composition of SUNTGM33 and olive oil has been described previously; the MA diet contained 2.4% MA while the CTR diet contained 0% MA (16). Each experimental diet was formulated by Oriental Yeast (Tokyo, Japan).

Carcinogen. *N*-methyl-*N*-nitrosourea (MNU) in a powder form was obtained from Sigma (St. Louis, MO, USA) and was stored at 4˚C in the dark. Immediately prior to use, MNU was dissolved in physiological saline containing 0.1% acetic acid, and a 5 mg/ml solution was prepared. A single dose of 50 mg/kg body weight was administered intraperitoneally.

Animals and experimental procedures. The study protocol and animal procedures were approved by the Animal Care and Use Committee of Kansai Medical University (Hirakata, Osaka, Japan; permit no. 14-111). In brief, 52 6-week-old virgin female Sprague-Dawley rats [Crl:CD(SD)] were purchased from Charles River Japan (Hino, Japan). They were housed in groups of 4 or 5 in plastic cages with paper bedding (Paper Clean, SLC, Hamamatsu, Japan) in a specific pathogen-free environment maintained at 22±2˚C and 60±10% relative humidity with a 12-h light/dark cycle (lights on at 8:00 a.m. and lights off at 8:00 p.m.). The rats were randomly divided into four groups, which were the CTR diet group and the MA diet group with or without MNU (each n=13, Fig. 1). Fresh sterilized stocks of the pellet diet were provided to the animals twice a week starting at 6 weeks of age with any remaining diet being discarded to minimize the ingestion of oxidized fatty acid. Half of the animals received MNU at 7 weeks of age, and all animals remained on the same diets for the remainder of the experiment (until 19 weeks of age). Experimental diets and water were available freely. During the experiment, the dose of diet ingested, body weight and tumor volume were measured once a week. The tumor volume was calculated using the standard formula: $Width^2$ x length x 0.5. At sacrifice, blood was sampled by inferior vena cava puncture and subsequently the animals were sacrificed by exsanguination from aortic transection. At necropsy, all the organs were examined macroscopically, and macroscopically abnormal organs, mammary glands and mammary tumors were examined histologically. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE); blood samples and sections of the non-tumorous inguinal mammary tissues were used for fatty acid analysis. Throughout the experiments, animals were cared for in accordance with the Guidelines for Animal Experimentation of Kansai Medical University.

Cell kinetics and microvessel density. The cell kinetics (cell proliferation and cell death) in the 6 largest MNU-induced tumors (Groups 1 and 2) were evaluated. Cell proliferation was evaluated by anti-Ki-67 (Clone SP6, ready to use; Nichirei Biosciences, Tokyo, Japan). Cell death was evaluated by anti-phospho-histone H2A.X (γ-H2A.X) antibody (Clone Ser139, 1:100; Cell Signaling, Danvers, MA, USA), an immunomarker of the DNA damage response. Microvessel density was evaluated by anti-CD34 antiserum (polyclonal, 1:50; Aviva Systemic Biology, San Diego, CA, USA). Immunohistochemistry was performed with the Histofine MAX-PO for rats kit (Nichirei Biosciences) according to the manufacturer's protocol. Each slide was scanned with a high-resolution digital scanner (NanoZoomer 2.0 Digital Pathology; Hamamatsu Photonics, Hamamatsu, Japan) to prepare digital images. The NDPI image files were opened in color mode with NDP.view software (Hamamatsu Photonics). The images were changed to JPEG files at magnification, x40 in five randomly selected areas within each tumor that was used to analyze immunohistochemical staining (20,21). The Ki-67 and γ-H2A.X indexes were assessed by positive cells/1,000 cells as an index of cell kinetics, and CD34 was assessed by positive area/1 mm^2 as a parameter of tumor angiogenesis.

Immunohistochemistry-based surrogate intrinsic subtyping of MNU-induced mammary tumors. The intrinsic subtype of the MNU-induced tumors was evaluated using the largest mammary tumor in each rat (Group 1, n=13; Group 2, n=8). Estrogen receptor (ER) was visualized by anti-ER antibody (Clone 6F11, 1:40; Leica Biosystems Newcastle, Newcastle upon Tyne, UK), progesterone receptor (PgR) by anti-PgR antibody (Clone PR6, 1:100; Abcam, Cambridge, UK), and HER2/neu by anti-c-erbB2/Her2/neu antibody (Clone e2-4000+H3B5 antibody cocktail, 1:300; Thermo Scientific, Waltham, MA, USA). Positive status was evaluated according to previous studies (22-24). In brief, ER and PgR were

Table I. Composition of experimental diets.

Components	MA diet	CTR diet
Casein	20	20
DL-Methionine	0.3	0.3
Cornstarch	43	43
α-Cornstarch	12	12
Sucrose	10	10
Cellulose	5	5
AIN-76 mineral mix	3.5	3.5
AIN-76 vitamin mix	1	1
Choline bitartrate	0.2	0.2
SUNTGM33	5	0
Olive oil	0	5

Values are expressed in g/100 g diet. MA, mead acid; CTR, control.

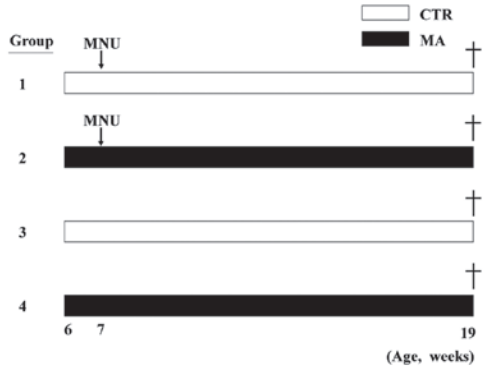

Figure 1. Schematic representation of the experimental protocol. CTR, control diet; MA, mead acid diet.

considered positive when there were ≥1% positive tumor nuclei in the slide, and HER2 was considered positive when there were ≥10% positive tumor cells with complete and intense circumferential membrane staining.

Fatty acid analysis of serum and mammary tissue. To determine fatty acid composition, blood samples and mammary tissues were collected. Samples from Groups 1 and 2 were collected from the 5 and 6 rats that had the largest mammary tumors, respectively. Samples from Group 3 and 4 were collected from 5 and 6 randomly selected rats, respectively. Sera were separated from whole blood that was cooled on ice by centrifugation for 10 min at 1,640 x g. Non-tumorous mammary tissues stored at -20˚C were thawed and homogenized. The fatty acid composition of the total phospholipid fraction of serum was determined. Total lipids were extracted by the method of Bligh and Dyer (25). The total phospholipid fraction was separated by thin-layer chromatography. For an internal standard, 1,2-diheptadecanoyl-sn-3-pfospfocholine (Avanti Polar Lipids, Inc., Alabaster, AL, USA) was added. Total phospholipid fractions were transmethylated with HCl-methanol, and subsequently the fatty acid composition was analyzed by gas chromatography (GC-2014, Shimadzu Corporation, Kyoto, Japan) with a capillary column DB-225 (0.25 mm x30 m x0.25 μm; J&M Scientific, Folsom, CA, USA). The entire system was controlled with gas chromatography software (GCsolution; Shimadzu Corporation). The fatty acid composition of the total lipid fraction of non-tumorous mammary tissues was determined. In brief, frozen tissues were thawed, minced and homogenized three times in 8 ml chloroform-methanol (2:1) by a polytron homogenizer (Kinematica, Lucerne, Switzerland) for 10 sec. The fatty acid analysis of total lipids in the mammary tissue was performed by the same method as previously mentioned (16).

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Body weight, tumor volume, tumor weight, cancer multiplicity, number of Ki-67 and γ-H2A.X-positive cells/1,000 cells, CD34-positive area/1 mm^2, ER and PgR-positive cells/1 mm^2, fatty acid composition, and the n-6/n-3 ratio among groups were analyzed by the t-test. The cancer incidence was analyzed with the χ^2 test.

Results

Host animals. During the experiment, the daily dose of food ingestion was compatible among groups, and although MNU treatment tends to decrease the body weight, the MA diet did not significantly influence the weight as compared with the CTR diet group. At necropsy, except for the development of mammary tumors, no organs or tissues were macroscopically abnormal.

Mammary carcinogenesis. All mammary tumors examined were histologically confirmed as mammary cancers. Therefore, the mammary tumors in the present study are referred to as mammary cancers. In MNU-treated rats, the MA diet (Group 2) tended to have a delayed development of palpable mammary cancer as compared to the CTR diet group (Group 1), and at the end of the experiment, the mammary cancer incidence was significantly lower in MA diet-fed rats (Group 2 vs. 1: 61.5 vs. 100%; P<0.05; Fig. 2A). At the end of the study, the MA diet group had a significantly decreased average cancer volume (data not shown) and final average cancer weight (Group 2 vs. 1: 427.1±106.9 vs. 1,796.3±378.8 mg; P<0.01; Fig. 2B) when MNU-induced macroscopic mammary cancers were compared. Including histologically detectable microscopic cancers, the MA diet significantly lowered cancer multiplicity (Group 2 vs. 1: 2.1±0.4 vs. 4.5±0.9; P<0.05; Fig. 2C). No mammary cancer was detected in the MNU-unexposed rats on either the CTR or MA diet (Groups 3 and 4, respectively).

Proliferation and apoptotic ratio of MNU-induced mammary cancer. To compare the cell kinetics of MNU-induced mammary cancers (cell proliferation and cell death), the number of Ki-67-positive and γ-H2A.X-positive cells/1,000 cancer cells from the CTR and MA diet groups was compared. The proliferation and apoptotic cell number are shown in Fig. 3A and B, respectively. The proliferation ratio in the CTR and MA diet group was 44.3±3.9 and 29.3±3.9, respectively (Group 2 vs. 1: P<0.05), while the apoptotic ratio in the CTR and MA diet group was 1.7±0.4 and 1.3±0.5 (P>0.05). MA significantly suppressed cancer cell proliferation but did not alter the cell death ratio.

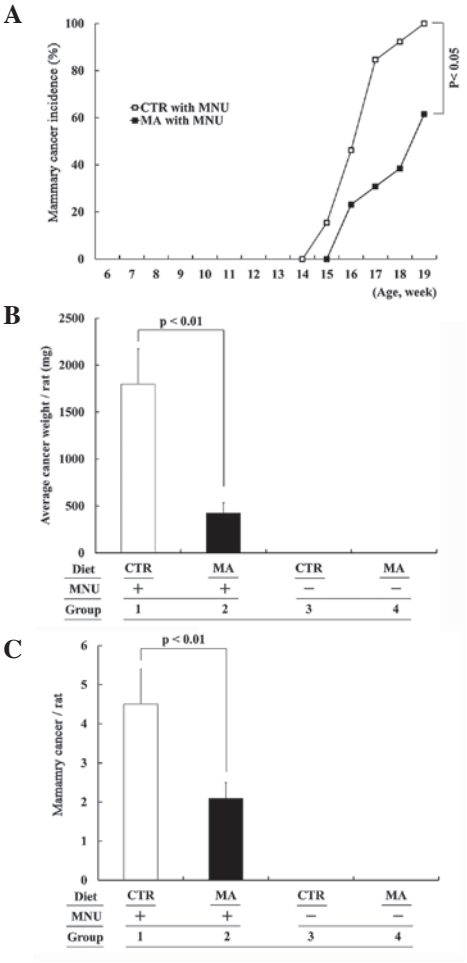

Figure 2. Effects of the mead acid (MA) diet on the *N*-methyl-*N*-nitrosourea (MNU)-induced mammary cancers in female Sprague-Dawley rats. (A) MA delayed and significantly suppressed the occurrence of MNU-induced palpable mammary cancer (Group 2 vs. 1). (B) MA significantly suppressed the final average weight of MNU-induced palpable mammary cancer. (C) When including microscopically detected tumors, MA significantly suppressed the multiplicity of mammary cancer. Mammary cancer was not detected in rats without MNU (Groups 3 and 4).

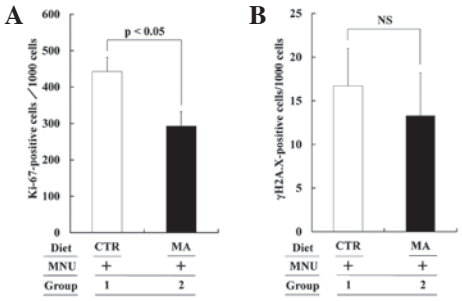

Figure 3. Effects of the mead acid (MA) diet on cell kinetics in *N*-methyl-*N*-nitrosourea (MNU)-induced mammary cancers. (A) The proliferation ratio was significantly suppressed in the mead acid (MA) diet group compared with the control (CTR) diet group (Group 2 vs. 1: P<0.05), while (B) the apoptotic ratio was not significantly different between the two groups (Group 2 vs. 1).

Microvessel density of MNU-induced mammary cancer. Although the cell growth was suppressed in the MA diet group, microvessel density of MNU-induced tumors, as evaluated by the CD34-positive area, was compatible between the

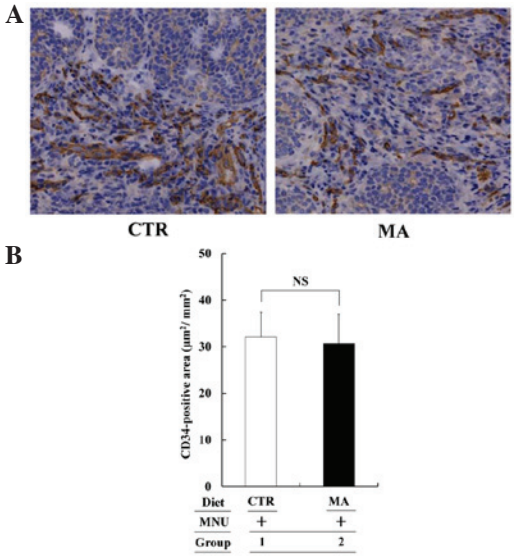

Figure 4. Effects of mead acid (MA) diet on angiogenesis in *N*-methyl-*N*-nitrosourea (MNU)-induced mammary cancers. (A) Representative staining of endothelial cells by CD34. (B) The MA diet did not affect angiogenesis in MNU-induced mammary cancers (Group 2 vs. 1).

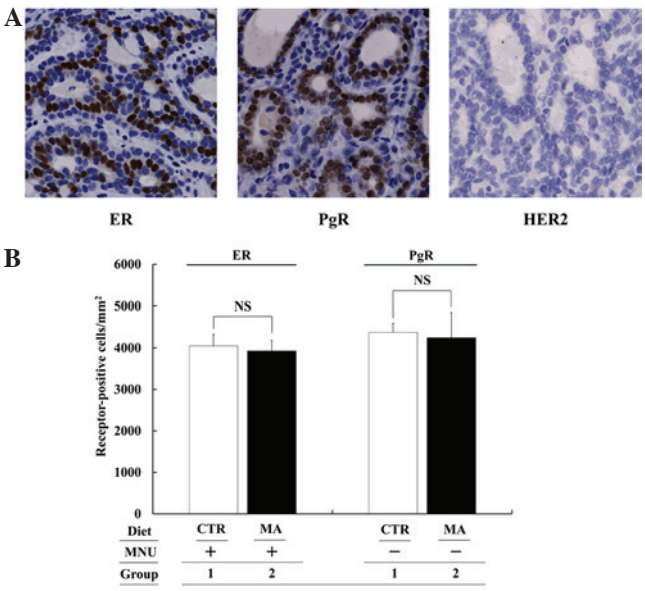

Figure 5. Estrogen and progesterone receptor (ER and PgR) and HER2/neu (HER2) expression on MNU-induced mammary cancers. (A) Immunohistochemistry revealed that all MNU-induced cancers were ER and PgR positive and HER2 negative. (B) Numbers of ER and PgR-positive cells per 1 mm^2 showed no difference between the CTR and MA diet groups (Group 2 vs. 1).

CTR and MA diet groups (Fig. 4). Therefore, the MA diet did not affect tumor angiogenesis in the MNU-induced mammary tumor system (Group 2 vs. 1: P>0.05).

Intrinsic subtype of MNU-induced mammary cancer. In the CTR and MA diet group (13 and 8 mammary cancers, respectively), all tumors were ER- and PgR-positive and HER2-negative (all luminal A subtype). Representative staining is shown in Fig. 5A. Therefore, MA effectively suppressed the growth and development of luminal A subtype

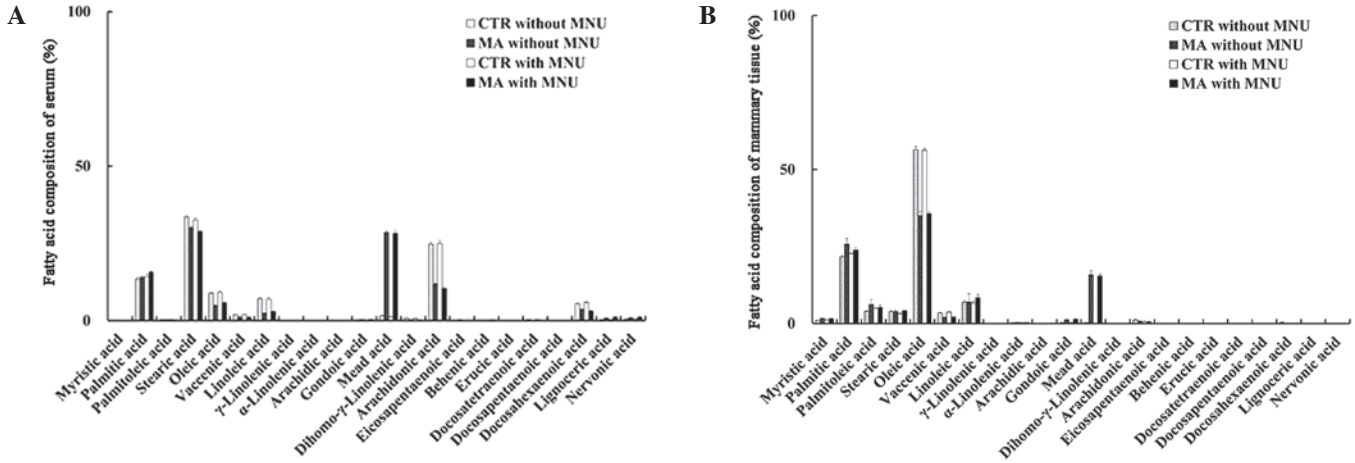

Figure 6. Comparison of fatty acid composition in female Sprague-Dawley rats fed either the mead acid (MA) diet or control (CTR) diet for 13 weeks and treated with or without *N*-methyl-*N*-nitrosourea (MNU) at 7 weeks of age. Fatty acid composition of (A) serum and (B) mammary tissue. Fatty acid composition of serum and mammary tissue reflected the difference in the contents of fatty acid in the diet.

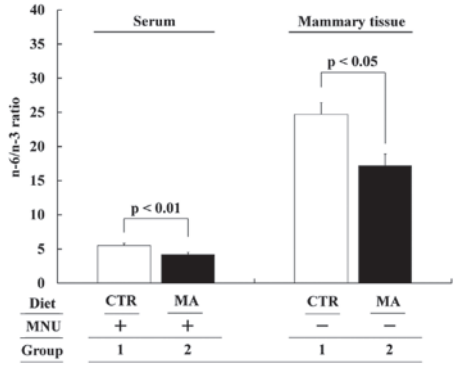

Figure 7. n-6/n-3 ratio in sera and mammary tissues after the mead acid (MA) diet or control (CTR) diet for 13 weeks. The MA diet significantly lowered the n-6/n-3 ratio in sera and mammary tissues (Group 2 vs. 1: P<0.01 and P<0.05, respectively).

tumors without affecting the number of ER- and PgR-positive cells/1 mm^2 (Group 2 vs. 1: not significant; Fig. 5B). However, the effect of MA against other intrinsic subtype tumors could not be evaluated from the MNU-induced mammary tumor system.

Fatty acid composition of serum and mammary tissue. The different diet groups had different fatty acid compositions of serum and mammary tissue that reflected the differences in the fatty acid composition of the respective diets. MNU treatment did not affect the fatty acid composition (Groups 1-4; Fig. 6A and B). Evident changes in the major n-3, n-6 and n-9 fatty acid composition of serum in the MA diet group were significant increase in MA concentration and significant decreases in OA, LA, AA and DHA, as compared to the CTR diet (Fig. 6A). Changes in the fatty acid composition of non-tumorous mammary tissues were a significant increase in MA and a significant decrease in OA (Fig. 6B). Changes in the fatty acid composition of serum and mammary tissue resulted in significantly decreased n-6/n-3 ratios in the sera and mammary tissues of the MA group (Group 2 vs. 1: P<0.01 and P<0.05, respectively; Fig. 7).

Discussion

The use of pharmacological or natural agents that inhibit the development of invasive breast cancer either by blocking the DNA damage that initiates cancer (blocking agent) or by arresting or reversing the progression of premalignant cells in which such damage has already occurred (suppressing agent) is desired (26,27). In the present study, 2.4% MA in the diet significantly suppressed MNU-induced mammary cancer incidence, multiplicity and average cancer volume and final cancer weight. These results indicate that MA suppressed the initiation and promotion phases of carcinogenesis. Our previous study on KPL-1 human breast cancer cells showed that 2.4% MA in the diet suppressed the promotion (growth) and progression phases (metastasis) (16). Taken together, 2.4% MA in the diet acted as a blocking and suppressing agent that suppressed all stages of mammary carcinogenesis; thus, MA is a candidate molecule to be used as a chemotherapeutic and chemopreventive agent.

The effects of n-9 OA on breast cancer remain controversial (11). By contrast, epidemiological results and experimental data show the beneficial effects of n-9 MA against breast cancer (14-16); MA suppressed the growth of MCF-7 and KPL-1 human breast cancer cells in culture. In the present study, in agreement with a study of KPL-1 cells transplanted into female athymic mice (16), MA suppressed the development and growth of MNU-induced mammary cancer. In these two experiments, the mechanism of growth suppression was decreased cell proliferation, not accelerated apoptosis. Studies of the effects of MA on breast cancer in humans and animals are limited (14-16). MA dose-dependently inhibits vascular endothelial growth factor (VEGF)-stimulated angiogenesis (28). The VEGF pathway may act indirectly via endothelial cells and may be involved in tumor angiogenesis; or the VEGF pathway may act directly via the VEGF receptor in cancer cells and participate in the growth modification of breast cancer cells (16). The VEGF-VEGFR interaction on cancer cells may partially explain the actions of MA in modifying cancer cell growth. However, the microvessel density in MNU-induced tumors was not affected by MA, which is consistent with findings from athymic mice implanted with KPL-1 cells (16). In contrast to the lack of detailed studies

on n-9 fatty acids, the growth inhibitory action of n-3 fatty acids and the growth stimulatory action of n-6 fatty acids have been studied in detail (26). Changes in the n-6/n-3 ratio appear to be noteworthy (6,29). The present study showed that MA decreased the n-6/n-3 ratio in serum and in mammary tissue, which may have been indirectly involved in suppressing cell proliferation of MNU-induced mammary cancers. However, more precise mechanisms of the MA-mediated decrease in cell proliferation should be determined.

Detailed molecular characterization of individual cancers will enable cancer patients to receive tailored targeted therapies that improve outcomes and decrease therapy toxicity. Immunohistochemistry-based surrogate intrinsic subtyping is convenient when specimens for gene analysis are not available (18). Immunohistochemical staining for ER, PgR, and HER2 can differentiate breast cancer into luminal A (ER^+ and/or PgR^+, $HER2^-$), luminal B (ER^+ and/or PgR^+, $HER2^+$), HER2 (ER^- and PgR^-, $HER2^+$), and triple negative (ER^- and PgR^-, $HER2^-$) subtypes (18,30). Endocrine therapy for luminal A subtype, endocrine therapy and trastuzumab plus chemotherapy for luminal B subtype, trastuzumab plus chemotherapy for HER2 subtype, and chemotherapy for triple negative cases are recommended (18,31). MCF-7, KPL-1 (17), and all the MNU-induced mammary cancers were luminal A, which means that MA supplementation in addition to endocrine therapy may further improve the outcome of luminal A breast cancer. However, assessments of the efficacy of MA against other breast cancer subtypes are required.

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