Mead acid inhibits the growth of KPL-1 human breast cancer cells *in vitro* and *in vivo*

YUICHI KINOSHITA[1], KATSUHIKO YOSHIZAWA[1], KEI HAMAZAKI[2], YUKO EMOTO[1], TAKASHI YURI[1], MICHIKO YUKI[1], NOBUAKI SHIKATA[3], HIROSHI KAWASHIMA[4] and AIRO TSUBURA[1]

[1]Department of Pathology II, Kansai Medical University, Hirakata, Osaka 573-1010; [2]Department of Public Health, Faculty of Medicine, University of Toyama, Sugitani, Toyama 930-0194; [3]Division of Diagnostic Cytopathology and Histopathology, Kansai Medical University Takii Hospital, Moriguchi, Osaka 570-8507; [4]Institute for Health Care Science, Suntory Wellness Ltd., Shimamoto, Osaka 618-8503, Japan

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Abstract. The effects of mead acid (MA; 5,8,11-eicosatrienoic acid) on the suppression of breast cancer cell growth and metastasis were examined *in vitro* and *in vivo* by using the KPL-1 human breast cancer cell line. MA suppressed KPL-1 cell growth in culture with an IC_{50} value of 214.2 μM (65.7 μg/ml) for 72 h, and MA significantly suppressed transplanted KPL-1 tumor growth (tumor volume and tumor weight: 872±103 mm^3 and 1,000±116 mg vs. 376±66 mm^3 and 517±84 mg) and regional (axillary) lymph node metastasis (67%, 10/15 vs. 10%, 1/10) in female athymic mice fed an MA-rich diet for 8 weeks. Tumor suppression was due to the suppression of cell proliferation. In ELISA, although vascular endothelial growth factor (VEGF) levels were unchanged, VEGF receptor (VEGFR)1 and VEGFR2 levels were significantly decreased after treatment with a 214.2-μM dose of MA for 72 h; E-cadherin levels were unchanged. As VEGF, VEGFR1 and VEGFR2 expression was co-localized in KPL-1 cells, the mechanism leading to cell growth suppression was VEGF signaling directly to KPL-1 cells by an autocrine process. In contrast, MA did not influence angiogenesis. The mechanisms of action were through VEGF signaling directly to cancer cells.

Introduction

Breast cancer is one of the most common types of cancer in industrial countries. Epidemiological studies indicate that the incidence and mortality of breast cancer is up to 5-fold higher in Western countries than in some Asian countries, and Asian migrants to the USA eventually acquire the breast cancer incidence of their host country, suggesting the importance of environmental factors (1). Among the environmental factors, it is clear that dietary exposure influences breast cancer risk, and particularly dietary intake of fat may play an important role in the genesis of breast cancer (2-5). Not only the amount of fat consumption, but also the type of dietary fat may have different effects on breast carcinogenesis. Long-chain polyunsaturated fatty acids (PUFAs) have different effects on mammary tumorigenesis based on the double bond position (6-8). The first double bond located at the 3rd, 6th or 9th carbon from the terminal methyl group of a fatty acid is called an n-3, n-6 or n-9 series fatty acid, respectively. Epidemiological studies and preclinical data indicate a positive association between dietary n-6 PUFA and breast cancer risk, while n-3 PUFA possesses chemopreventive properties (9,10). n-3 PUFAs such as eicosapentaenoic acid (EPA; 20:5n-3) suppress human breast cancer cell growth (11). Among n-3 PUFAs, docosahexaenoic acid (DHA; 22:6n-2) suppresses mammary carcinogenesis more effectively than EPA (7). In contrast, n-6 PUFAs such as linoleic acid (LA; 18:2n-6) and arachidonic acid (AA; 20:4n-6) promote the growth of human breast cancer cells (11,12). The typical American high-fat diet contains high levels of LA (13). Olive oil rich in oleic acid (OA; 18:1n-9) suppresses mammary carcinogenesis (14). However, it is difficult to determine if the effects of OA on experimental carcinogenesis are due to specific biochemical properties of OA or simply due to the substitution for n-6 PUFA (15). The role of n-9 PUFA in relation to breast cancer has not been studied in detail.

Mead acid (MA; also referred to as 5,8,11-eicosatrienoic acid), which was first characterized by James F. Mead, is a carboxylic acid with a 20-carbon chain and three methylene-interrupted *cis* double bonds, in which the first double bond is located at the ninth carbon from the terminal methyl group of a fatty acid (20:3n-9; Fig. 1). MA is a minor constituent of plasma and tissue in adult mammals. Elongation and desaturation of OA take place to form MA when n-6 and n-3 essential fatty acids, particularly LA, are deficient. Therefore, MA elevation in the blood is an indication of essential fatty acid deficiency. MA is found in large quantities in cartilage; MA decreases osteoblastic activity for the maintenance of cartilage to prevent ossification and suppresses angiogenesis

Correspondence to: Professor Airo Tsubura, Department of Pathology II, Kansai Medical University, Hirakata, Osaka 573-1010, Japan
E-mail: tsubura@hirakata.kmu.ac.jp

Key words: breast cancer, KPL-1 cells, mead acid, VEGF, VEGF receptors, E-cadherin

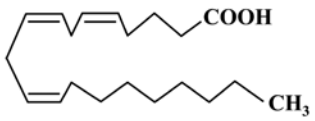

Figure 1. Chemical structure of mead acid (5,8,11-eicosatrienoic acid; 18:3n-9). The number before the colon indicates the number of carbon atoms, and the number after the colon indicates the number of double bonds. The conformation of the double bond is cis in configuration, and the first double bond is located at the 9th carbon from the terminal methyl group.

to maintain avascular status (16,17). Angiogenesis plays an important role in the growth of breast cancer (18), and anti-angiogenic agents [inhibitors of vascular endothelial growth factor (VEGF)] and the VEGF receptor (VEGFR) may be promising targets for breast cancer control (19,20). The loss of cell adhesion is related to cancer invasion and metastasis. MA is related to the expression of the cell-cell adhesion molecule E-cadherin in human cancer cell lines including breast cancer cells (21,22). E-cadherin-mediated signaling can influence invasive and metastatic behavior (23,24). VEGF and/or E-cadherin signaling may modulate breast cancer growth at the primary site, and invasion and metastasis in the MA-rich condition. In addition, leukotriene B_4 (LTB_4) enhances tumor growth in human cancer cells including breast cancer cells (25,26). Dietary supplementation with MA suppresses LTB_4 in rats (27,28). Moreover, a nested case-control study revealed an inverse association between MA and breast cancer risk as well as overall cancer risk (29); in the present study, neither the n-6/n-3 ratio nor AA intake correlated with breast cancer risk. Collectively, MA may exert cancer preventive properties. However, MA shows different effects on different types of cancer cells (21,22). The present study was designed to explore the effect of MA on KPL-1 human breast cancer cell growth and metastasis. The mechanisms of action were investigated based on VEGF and E-cadherin signaling and the modulation of fatty acid composition.

Materials and methods

Cell line. KPL-1 is a human breast cancer cell line established from the malignant effusion of a breast cancer patient (30). This cell line was derived from a patient with recurrent breast cancer that appeared during postsurgical adjuvant chemoendocrine therapy including tamoxifen and medroxy-progesterone acetate. The KPL-1 cell line is estrogen receptor (ER)-positive, progesterone receptor (PgR)-negative, and human epidermal growth factor receptor-2 (HER2)-negative (luminal A subtype) (31). Xenograft athymic mice injected with KPL-1 cells often develop lymph node metastasis (30). KPL-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) in 5% CO_2/95% humidified air at 37˚C.

MA preparation. MA used for *in vitro* experiments was purchased from Sigma and dissolved to 5 mg/500 μl (32.6 mM) in ethanol and stored at -30˚C. MA was diluted in DMEM containing 10% FBS to achieve final concentrations of 16.25, 32.5, 75, 150 and 300 μM.

Table I. Composition of experimental diets.

	MA diet	Control 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.

MTT assay. KPL-1 cells were seeded at $2x10^3$ cells/well in 96-well plates in DMEM with 10% FBS. The cells were treated with ethanol (final concentration, 0.9%) or the indicated concentration of MA for up to 72 h. Cell proliferation was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (32), and IC_{50} value was calculated.

Animal experiments. Animals were housed in groups of four or five in plastic cages with paper bedding (Paper Clean, SLC, Hamamatsu, Japan) in a specific pathogen-free room maintained at 22±2˚C and 60±10% relative humidity with a 12-h light/dark cycle (lights on at 8:00 AM and lights off at 8:00 PM). The mice were randomly divided into two groups, the control diet group (n=15) and the MA diet group (n=10). Both experimental diets were modifications of the AIN-76 diet (33) and contained the same amount of nutrients but had different fatty acid compositions (Table I). The MA diet contained 5% SUNTGM33 (a kind gift from Suntory Wellness, Tokyo, Japan), which contains 48.0% MA (Table II). SUNTGM33 is a microbial oil obtained by fungal fermentation (34). Olive oil purchased from Nacalai Tesque (Kyoto, Japan) was used for the control diet. The fatty acid compositions of SUNTGM33 and olive oil are listed in Table II. Each experimental diet was formulated by Oriental Yeast (Tokyo, Japan). Four-week old female athymic BALB/c mice purchased from Charles River Japan (Kyoto, Japan) were fed either a control or MA diet starting at 4 weeks of age. In comparison to the control diet, the MA diet for 8 days starting at 4 weeks of age did not cause weight gain. Therefore, the MA diet group was switched to the control diet for 4 successive days to accelerate weight gain; thereafter, the MA diet group was treated with a cyclic feeding regime of 5 days on the MA diet followed by 2 days on the control diet throughout the experiment. The control group was treated with the control diet throughout the experiment. Schematic representation of the *in vivo* experiment is shown in Fig. 2. At 6 weeks of age, both groups were inoculated with $2.5x10^5$ KPL-1 cells in 100 μl DMEM supplemented with 10% FBS into the thoracic mammary fat pad (MA diet group was on the third day of MA diet). During the experiment, the dose of

Table II. Fatty acid composition of SUNTGM33 and olive oil.

Fatty acid composition (%)	SUNTGM33	Olive oil
Myristic acid (14:0)	0.12	0.00
Palmitic acid (16:0)	3.35	10.88
Palmitoleic (16:1n-7)	0.00	0.85
Stearic acid (18:0)	4.96	3.32
Oleic acid (18:1n-9)	25.10	74.71
Vaccenic acid (18:1n-7)	0.14	1.89
Linoleic acid (18:2n-6)	0.42	6.90
α-Linolenic acid (18:3n-3)	0.00	0.68
Arachidic acid (20:0)	0.00	0.46
Gondoic acid (20:1n-9)	4.95	0.31
Mead acid (20:3n-9)	48.02	0.00
Arachidonic acid (20:4n-6)	1.23	0.00
Eicosapentaenoic acid (20:5n-3)	0.47	0.00
Behenic acid (22:0)	2.15	0.00
Lignoceric acid (24:0)	5.34	0.00
Nervonic acid (24:1n-9)	2.42	0.00
Total n-9	80.49	75.02
Total n-3	0.47	0.68
Total n-6	1.65	6.90
n-6/n-3	3.53	10.19
Total SFA	15.91	14.66
Total MUFA	32.61	77.76
Total PUFA	50.15	7.58

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

diet ingested, body weight and locally growing tumor volumes were measured once per week. Tumor volume was calculated by using the standard formula: width^2 x length x 0.5. Thirty-seven days after tumor cell inoculation (fifth day on MA diet), body weight was measured, and bromodeoxyuridine (BrdU; Invitrogen, Camarillo, CA, USA) was injected (50 mg/kg animal body weight) via the abdominal cavity, blood was sampled and then animals were sacrificed by exsanguination from aortic transection. At autopsy, all organs were examined macroscopically, and the primary tumors and local axillary lymph nodes were examined histologically; half of the primary tumor was snap frozen and used for fatty acid analysis. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The study protocol and animal procedures were approved by the Animal Care and Use Committee of Kansai Medical University (permit no. 13-060). Throughout the experiments, animals were cared for in accordance with the Guidelines for Animal Experimentation of Kansai Medical University.

Fatty acid analysis. The fatty acid composition of the total phospholipid fraction of serum was determined. Total lipids were extracted by the method of Bligh and Dyer (35). The total phospholipid fraction was separated by thin-layer chromatography. For an internal standard, 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL, USA) was added. Total phospholipid fractions were transmethylated with HCl-methanol and then the fatty acid composition was analyzed by gas chromatography (GC-2014; Shimadzu Corporation, Kyoto, Japan) with a capillary column DB-225 (0.25 mm x 30 m x 0.25 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 KPL-1 tumors was determined. In brief, frozen tumor 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 tumor was performed by the same method as previously mentioned (35,36).

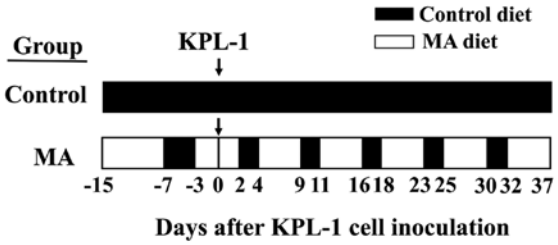

Figure 2. Schematic representation of the *in vivo* experimental protocol.

Microvessel density and cell kinetics. Microvessel density of primary KPL-1 tumors was evaluated by anti-CD34 antiserum (Abcam, Cambridge, UK). The cell kinetics (cell proliferation and cell death) in primary KPL-1 tumors were evaluated. Cell proliferation was evaluated by anti-BrdU antibody (clone B44, 1:50; Becton-Dickinson, Franklin Lakes, NJ, USA) by using an LSAB staining kit (Dako, Glostrup, Denmark). Cell death was evaluated by anti-phospho-histone H2A.X (γ-H2AX) antibody (Ser139, 1:100; Cell Signaling, Danvers, MA, USA), an immunomarker of the DNA damage response. Each slide was scanned with a high-resolution digital slide 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 x40 magnification in five randomly selected areas within each tumor that were used to analyze immunohistochemical staining (37-39).

CD34, VEGF, VEGFR1, VEGFR2 and E-cadherin immunohistochemistry. Cell blocks were prepared from KPL-1 cells cultured with or without 214.2 μM MA for 72 h (IC_{50} value for 72 h). Cell blocks were prepared from cultured KPL-1 cells; cells were centrifuged at 1,000 rpm for 5 min, and cell pellets were fixed in 10% neutral buffer formalin and embedded in paraffin. Antisera used to detect CD34, VEGF, VEGFR1, VEGFR2 and E-cadherin were anti-CD34 antiserum (1:20; Abcam), anti-VEGF antiserum (1:50), anti-VEGFR1 antiserum (1:500) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-VEGFR2 antiserum (1:400) (Cell Signaling Technology, Danvers, MA, USA), respectively, by using an LSAB staining kit (Dako), and anti-E-cadherin antibody (NCH38 ready to use) by using Histofine MAX-PO (both from Nichirei Biosciences, Tokyo, Japan) according to

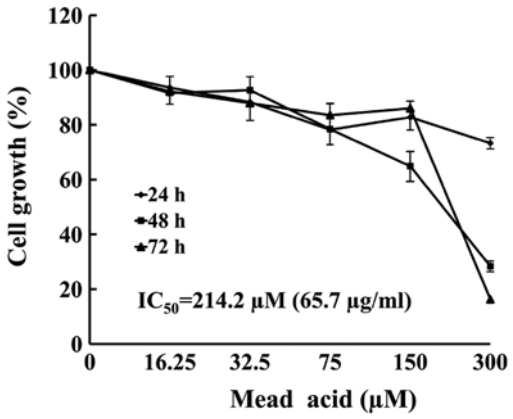

Figure 3. Growth inhibition curves of KPL-1 cells *in vitro* caused by mead acid (MA). MA was diluted in 10% ethanol, stored at 4˚C, and diluted in DMEM with 10% FBS to the final concentration immediately before use. KPL-1 cells were plated on 96-well plates at $2x10^3$ cells/well, and cell proliferation was determined by MTT assay. The IC_{50} value was 214.2 μM. Data are means ± SEM of 3 independent experiments.

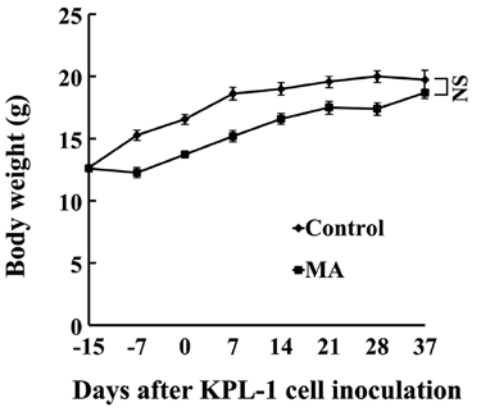

Figure 4. Body weight change in female athymic BALB/c mice fed control and mead acid (MA) diet for 8 weeks. Body weight was lighter in the MA diet group during the experiment, but the final body weight difference between the groups was not significant at the end of the experiment.

the manufacturer's instructions. The reaction products were visualized with 3-3'-diaminobenzidine tetrahydrochloride. CD34 immunohistochemistry was applied for angiogenesis evaluation in KPL-1 tumor grown in athymic mice, and VEGF, VEGFR1, VEGFR2 and E-cadherin immunohistochemistry, respectively, were applied in cultured KPL-1 cells in cell pellets.

Quantitation of VEGF, VEGFR1, VEGFR2 and E-cadherin. KPL-1 cells cultured with or without 214.2 μM MA for 72 h (IC_{50} value for 72 h) were sampled. VEGF was quantified in the culture supernatant, and VEGFR1, VEGFR2 and E-cadherin were measured in the cell lysate. For cell lysate preparation, after washing cells with PBS (-), cell pellets were homogenized with RIPA buffer (Wako, Osaka, Japan), and the cell lysate was centrifuged at 14,000 rpm for 15 min at 4˚C to obtain supernatant containing cell lysate. Protein concentrations were measured by the DC protein assay kit (Bio-Rad, Hercules, CA, USA). VEGF, VEGFR1, VEGFR2 and E-cadherin levels were measured by enzyme-linked immunoabsorbent assay (ELISA) with a human VEGF assay kit (IL-226; IBL, Fujioka, Japan), VEGFR1 human ELISA kit (ab119613), VEGFR2 human ELISA kit (ab100665) (both from Abcam), and E-cadherin human ELISA kit (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturers' protocols. Each protein standard or protein purified from each group was incubated overnight at 4˚C against VEGF or E-cadherin; 90 min at 37˚C against VEGFR1; or 150 min at room temperature against VEGFR2. After the final colors were developed with the addition of each color reagent, absorbance was measured at 450 nm. Cell sample concentration was calculated from a standard curve and corrected for protein concentration. The limits for detection for VEGF, VEGFR1, VEGFR2 and E-cadherin were 8, 156, 70 and 39 pg/ml, respectively.

Statistical analysis. Values are expressed as the means ± standard error of the mean (SEM). Body weight, tumor volume, tumor weight, fatty acid composition, CD34-positive area and the number of BrdU- and γ-H2AX- positive cells per 1 mm^2 among the groups were analyzed by t-test. The incidence of metastasis was analyzed with the χ^2 test.

Results

KPL-1 cell growth inhibition in vitro. The KPL-1 cells were treated with 5 concentrations (16.25-300 μM) of MA for up to 72 h. The MTT assay revealed that MA induced growth inhibition in a dose- and time-dependent manner (Fig. 3). The IC_{50} value of MA against KPL-1 cells was 214.2 μM (65.7 μg/ml) for a 72-h treatment.

Host animals. During the experiment, the daily dose of MA ingestion was 63-80 mg/mouse/day (mean, 74 mg/mouse/day). Body weight gain in the MA group was significantly smaller during the experiment but at the end of the experiment (eating the respective diet for 8 weeks), the difference in body weight between the MA and control diet group was not statistically significant (Fig. 4). No organs or tissues were macroscopically abnormal. Throughout the experiment, the MA diet group ingested less food than the control diet group (average, 92.1%).

Primary KPL-1 tumor growth and metastasis. Locally growing KPL-1 tumors in the MA diet group grew slower than in the control diet group. The final tumor volume was significantly smaller (Fig. 5A) and the final tumor weight was significantly lighter (Fig. 5B) in the MA diet group as compared to the control diet group ($p<0.01$ and $p<0.05$, respectively). Tumor volume two days before the termination of the experiment was 872±103 and 376±66 mm^3, and the final tumor weight was 1,000±116 and 517±84 mg in the control and MA group, respectively. Regional (axillary) lymph node metastasis was found in both groups (Fig. 5C). Similarly, as compared with the control diet group, metastasis was significantly suppressed in the MA diet group (66.7%: 10/15 vs. 10%: 1/10).

Morphology and angiogenesis of KPL-1 tumor. Although the growth of KPL-1 tumors was suppressed in the MA diet group, the morphology was comparable in both groups (data

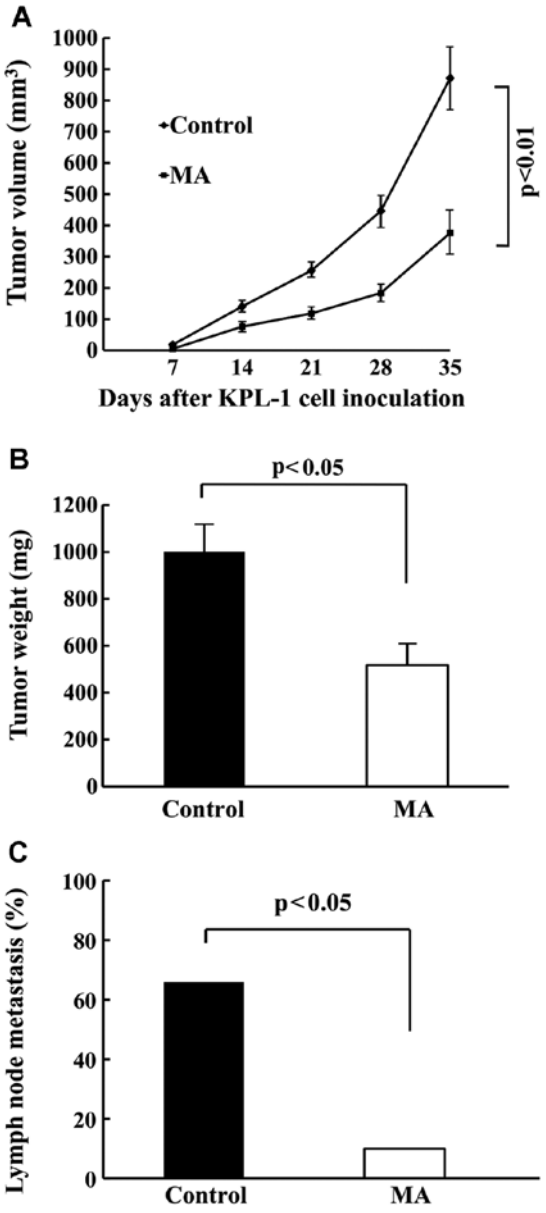

Figure 5. Effects of mead acid (MA) diet on the KPL-1 primary tumor growth at the inoculation site and axillary lymph node metastasis in female BALB/c mice. (A) MA suppressed KPL-1 tumor growth in that the primary tumor volume was significantly smaller ($p<0.01$); (B) the final primary tumor weight was significantly lighter ($p<0.05$); and (C) metastasis was significantly decreased ($p<0.05$).

not shown). The angiogenesis of KPL-1 tumors at the primary site (Fig. 6A) as well as the CD34-stained area was compatible between the control and MA diet groups (Fig. 6B).

Proliferation and apoptotic ratio of KPL-1 tumor. To compare the cell kinetics of KPL-1 tumors (cell proliferation and cell death), the number of BrdU-positive cells and γ-H2AX-positive cells per 1,000 cells in primary tumors from the control and MA diet groups of mice was compared. The proliferation and apoptotic ratios are shown in Fig. 7A and B, respectively. The proliferation ratio in the control and MA diet groups was 11.8±0.7 and 8.7±0.9%, respectively ($p<0.05$), while the apoptotic ratio in the control and MA diet groups was 8.7±1.3 and 9.6±1.2% (not statistically significant).

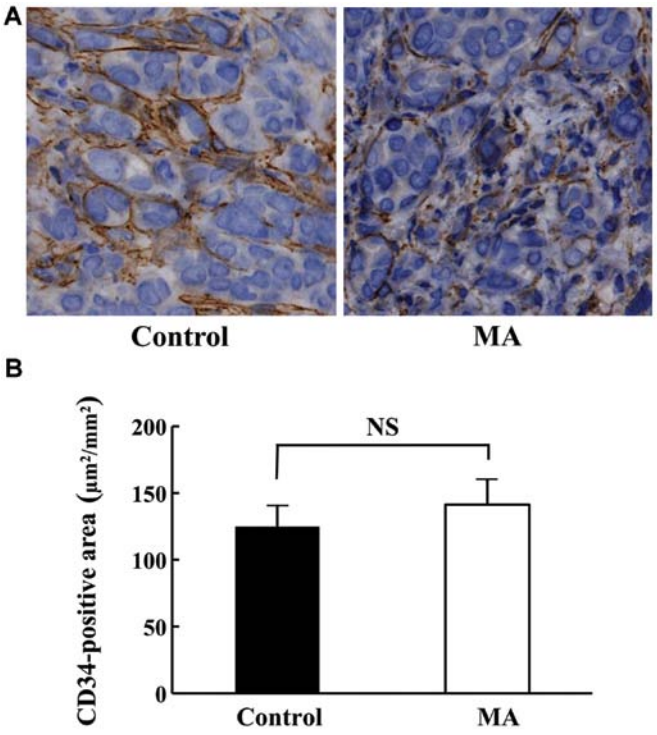

Figure 6. Vascularity of KPL-1 tumors at primary sites in the mead acid (MA) and control diet groups. (A) Vascularity and (B) microvessel density was compatible between groups (CD34 staining, respectively).

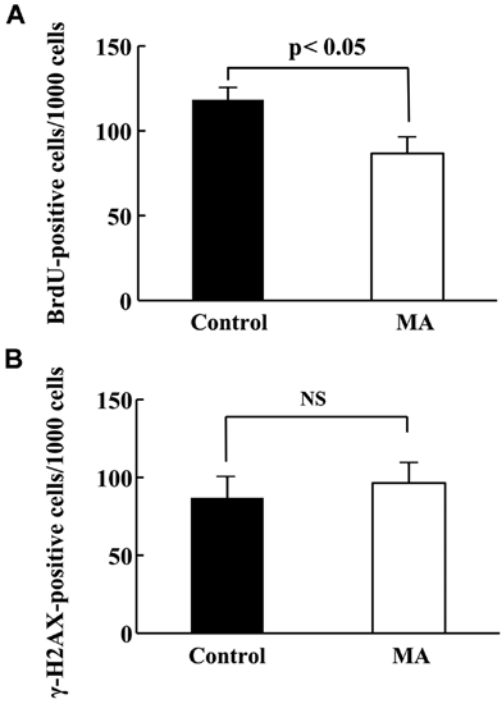

Figure 7. Effects of mead acid (MA) diet on cell kinetics in KPL-1 tumors in female athymic BALB/c mice. (A) The proliferation ratio was significantly different ($p<0.05$) between the MA diet and control diet groups, while (B) the apoptotic ratio was not significantly different between the two groups.

Fatty acid analysis. Fatty acid composition of sera and KPL-1 tumors in the control and MA diet group is shown in Fig. 8A and B, respectively. In the sera, the concentration of

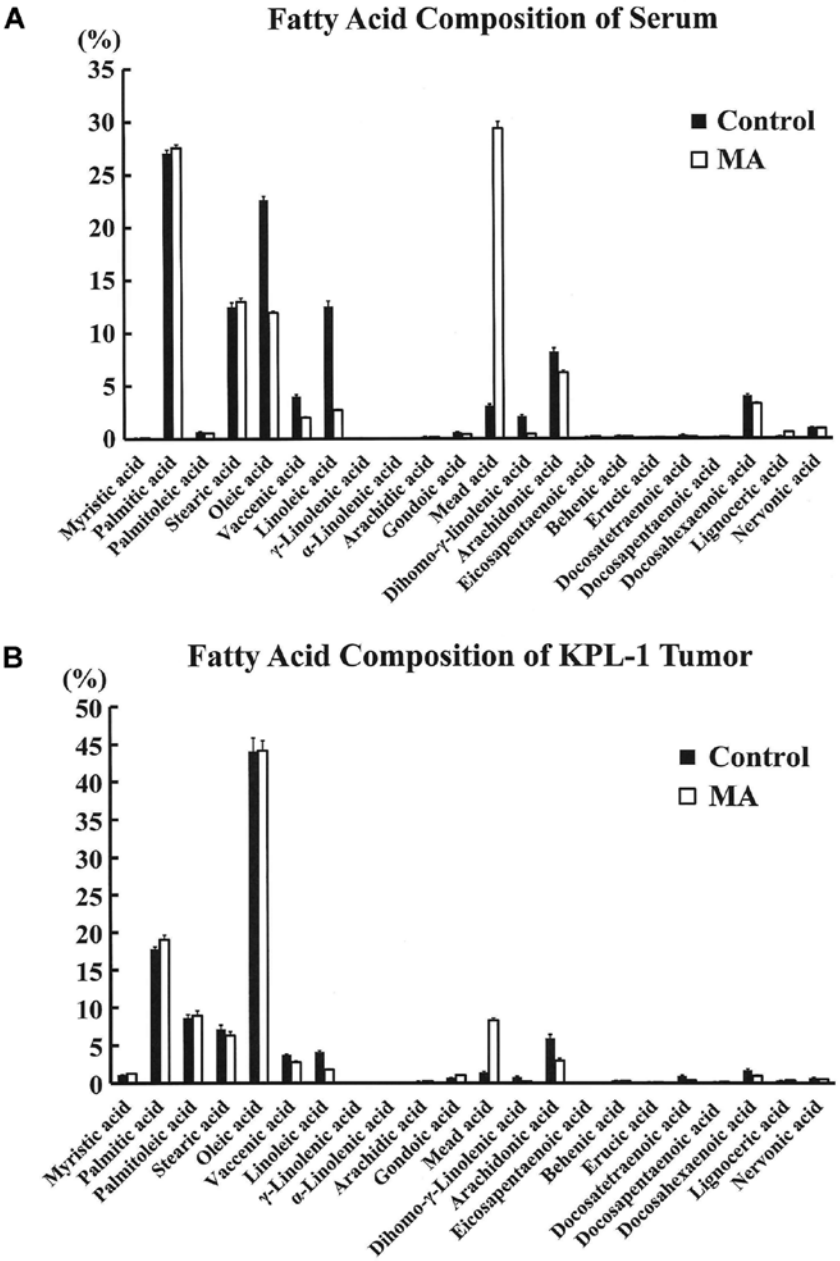

Figure 8. Comparison of fatty acid composition in female athymic BALB/c mice inoculated with KPL-1 cells and fed a mead acid (MA) diet for 8 weeks. (A) Total phospholipid fraction of the serum and (B) total lipid fraction of the KPL-1 tumor.

MA was significantly higher in the MA diet group as compared to controls (237.2±17.6 vs. 23.8±1.1 μg/ml). In contrast, LA and AA were significantly lower in the MA diet group as compared with controls. In the KPL-1 tumor, the fatty acid profile was generally similar to that of the fatty acid composition in the serum. However, the serum OA composition was significantly higher in the control diet group, as MA was replaced by OA in this diet, while the OA concentration in KPL-1 tumors was comparable. The MA diet significantly decreased the n-6/n-3 ratio in the sera and the tumors (Fig. 9).

Expression of VEGF, VEGFR1, VEGFR2 and E-cadherin in KPL-1 cells in culture. KPL-1 cells strongly expressed VEGF, VEGFR1 and VEGFR2 in the cytoplasm and E-cadherin at the cell surface. Although VEGF expression was unchanged,

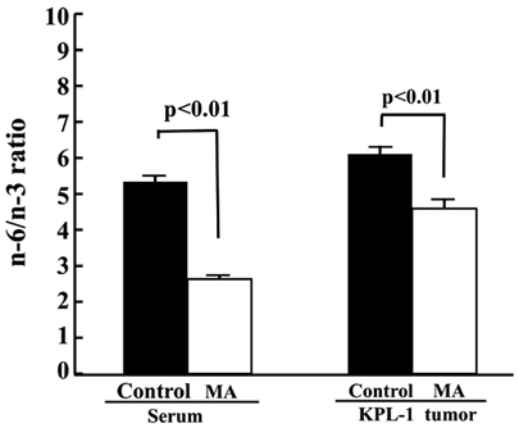

Figure 9. n-6/n-3 ratio in sera and KPL-1 tumors after mead acid (MA) diet. In sera and tumors, the MA diet significantly lowered the n-6/n-3 ratio.

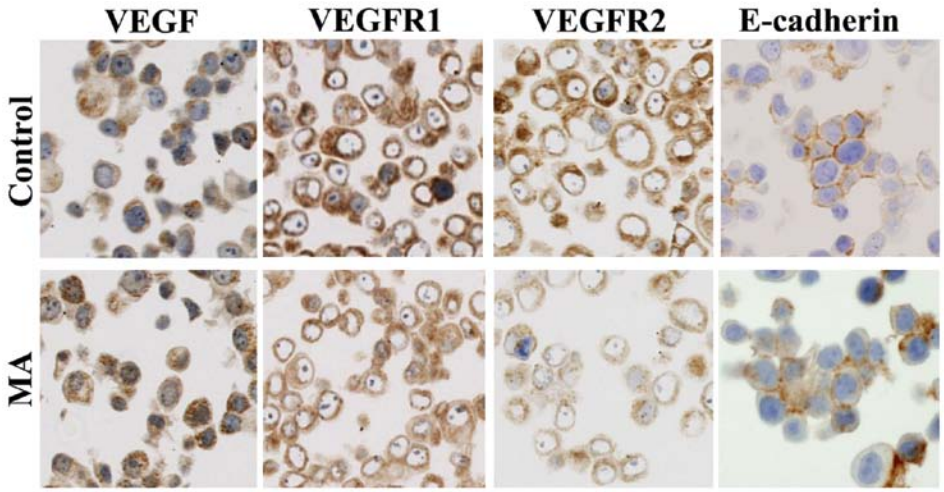

Figure 10. Immunohistochemical expression of VEGF, VEGFR1, VRGFR2 and E-cadherin in cultured KPL-1 cells cultured with or without the IC_{50} dose of mead acid (MA; 214.2 μM) for 72 h. KPL-1 cells strongly expressed all four molecules examined and MA treatment tended to alter the reactivity.

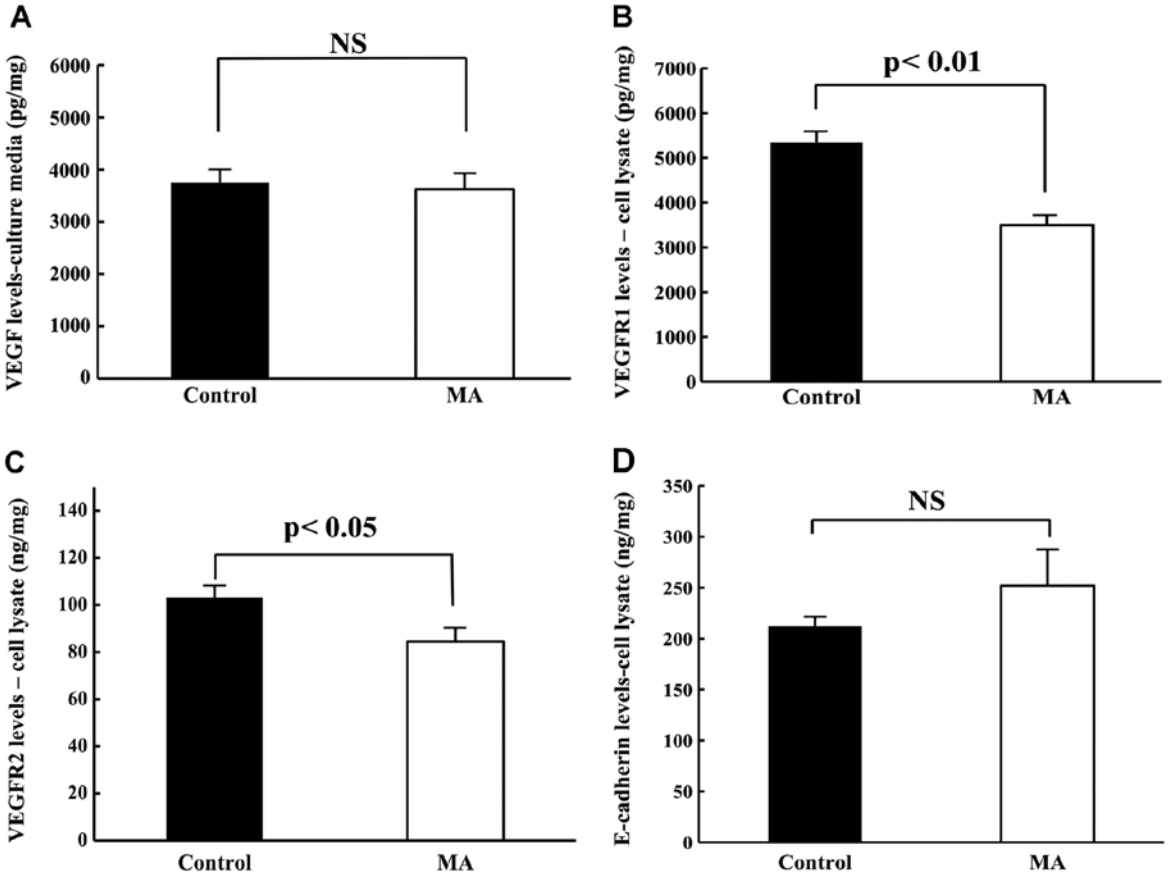

Figure 11. Effects of IC_{50} dose of mead acid (MA, 214.2 μM) for 72 h on changes in levels of (A) VEGF, (B) VEGFR1, (C) VEGFR2 and (D) E-cadherin in cultured KPL-1 cells. Levels were quantified by enzyme-linked immunoabsorbent assay (ELISA). Data are the means ± SEM of 3 independent experiments. MA significantly lowered VEGFR1 and VEGFR2 levels, while VEGF and E-cadherin levels were similar. Data are the means ± SEM of 3 independent experiments.

VEGFR1 and VEGFR2 expression tended to diminish and E-cadherin expression tended to increase in KPL-1 cells treated with the IC_{50} dose of MA for 72 h (214.2 μM) (Fig. 10).

Quantification of VEGF, VEGFR1, VEGFR2 and E-cadherin levels in KPL-1 cells in culture. Levels of VEGF, VEGFR1, VEGFR2 and E-cadherin in KPL-1 cells cultured with or without the IC_{50} dose of MA for 72 h were compared. Although the VEGF levels in KPL-1 tumor cells were similar (Fig. 11A), MA treatment significantly decreased VEGFR1 and VEGFR2 levels and tended to increase E-cadherin levels (Fig. 11B-D).

Discussion

In the present study, MA suppressed KPL-1 human breast cancer cell growth in culture with an IC_{50} value of 214.2 μM (65.7 μg/ml) for 72 h and significantly suppressed KPL-1 tumor growth and regional (axillary) lymph node metastasis in female athymic mice. Tumor suppression was due to decreased cell proliferation. The body weight of the MA diet group, although it was similar to the control diet-fed mice at the termination of the present study, was significantly lighter during the experimental period; the dose of food intake was less in the MA-fed mice. Thus, the lighter body weight in the MA diet group may be due to consuming a low amount of the MA diet. The serum MA level in MA diet-fed mice was 237.2 μg/ml, which is higher than the IC_{50} value. Thus, the IC_{50} dose of MA used in the *in vitro* experiments is achievable and may not cause serious side-effects in female mice. In contrast to n-3 PUFA, which invariably exerts antiproliferation action on human tumor cell lines, the n-9 series of MA causes different actions on different human tumor cells, depending on their origin and cellular types (21,22). MA may function in a cell-specific manner. However, in agreement with MCF-7 human breast cancer cells, the MA diet suppressed KPL-1 tumor growth and metastasis in female athymic mice. Tumor growth is a balance between cell proliferation and cell death. Mammary tumors of the VEGF knockout mice exhibit cell-cycle arrest and induction of apoptosis (37). With the present dose of MA, the MA diet suppressed KPL-1 tumor growth cytostatically by diminishing cell proliferation; however, it was below the dose needed to cause apoptosis.

Tumor angiogenesis is closely related to the growth of breast cancer, and VEGF and its receptors are essential for breast cancer growth (18-20). Cartilage is an avascular tissue and contains high levels of MA; MA dose-dependently inhibits VEGF-stimulated angiogenesis (17). However, MA did not alter angiogenesis as evaluated by microvessel density in KPL-1 tumors in athymic female mice. Although VEGF is well known for its key roles in blood vessel growth, it also promotes a range of other functions, such as cell adhesion, survival, migration and invasion (40). VEGF, VEGFR1 and VEGFR2 were immunohistochemically detected in KPL-1 cells. The presence of VEGFR1 and VEGFR2 as well as VEGF on KPL-1 cells may raise the possibility that VEGF may promote tumor growth not only by inducing angiogenesis but directly through the activation of VEGFR1 and VEGFR2 (41-43). In the present study, VEGF levels in cultured KPL-1 cells treated with MA were comparable to the levels in KPL-1 cells without MA treatment. However, VEGFR1 and VEGFR2 levels were significantly decreased in KPL-1 cells treated with MA. As VEGF, VEGFR1 and VEGFR2 were co-localized in KPL-1 cells, the present results suggest that VEGF signaling did not modulate angiogenesis, however it directly modulated the growth of VEGFR-positive tumor cells via an autocrine process, an endothelial cell-independent pathway (44). Expression of VEGF and its receptors (VEGF1 and VEGFR2) is associated with poor outcome in breast cancer patients (45). VEGF knockout directly decreases the proliferation of breast cancer cells (37).

Downregulation of E-cadherin is associated with loss of cellular adhesiveness and initiates metastatic outgrowth resulting in poor prognosis (46,47). Downregulation of E-cadherin is required to initiate metastatic outgrowth of breast cancer (47). VEGF increases transcription factor Snail, which is associated with breast cancer metastasis and reduces E-cadherin expression in breast cancer cells (48). Although MA tended to increase E-cadherin levels, the difference did not reach statistical significance. VEGF increases the cellular invasion of T-47D breast cancer cells on Matrigel/fibronectin-coated transwell membranes (41). Therefore, VEGF, but not E-cadherin, may contribute to the mechanisms of suppression of invasion and metastasis in the KPL-1 tumor system.

In addition to affecting the VEGF pathway, MA may function via other unknown mechanisms. In a human case-control study of breast fat tissues, increased n-6/n-3 PUFA ratios and decreased n-3 PUFA levels were found in breast cancer patients as compared with controls (49,50). Alteration in the n-6/n-3 PUFA ratio appears to be important (51) and n-3 PUFA, namely EPA and DHA (4) or n-6 PUFA, such as LA and AA (11,12), is important. The MA diet-fed mice had significantly decreased LA and AA levels, and the n-6/n-3 PUFA ratio was significantly decreased in both the sera and the KPL-1 tumors, which may lead to growth suppression of KPL-1 cells. The essential fatty acid LA cannot be synthesized in the body and must be derived from the diet. Dietary LA shows a negative effect on the incorporation of dietary MA into the plasma lipid fraction and membrane phospholipids (52). LA is converted via intermediates to dihomo-γ-linolenic acid and to AA. Thus, an inverse relationship between n-9 PUFA MA and n-6 PUFA LA and AA may exist. In the present MA diet, decreased expression of n-6 PUFA, namely LA and AA, in the MA-fed mice, may suppress breast cancer cell proliferation and invasion (4). However, n-6 content in the control diet was considerably below 4%; n-6 PUFA (LA) at a dose of ~4% is required for maximal acceleration of mammary tumorigenesis (53).

In conclusion, MA effectively suppressed the growth and metastasis of KPL-1 human breast cancer cells via VEGF signaling.

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