Nutrition and Cancer

Carp Oil or Oleic Acid, but Not Linoleic Acid or Linolenic Acid, Inhibits Tumor Growth and Metastasis in Lewis Lung Carcinoma–Bearing Mice[1]

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ABSTRACT I examined the effects of carp oil, oleic acid, linoleic acid and linolenic acid on tumor growth and metastasis to the liver in mice implanted intrasplenically with highly metastatic Lewis lung carcinoma (LLC) tumors. Carp oil (0.1 or 0.2 mL per mouse) significantly reduced tumor growth and metastasis to the liver. Carp oil at 100 or 1000 mg/L inhibited the DNA synthesis in LLC cells, the capillary-like tube formation of human dermal microvascular endothelial cells (HMVEC) at 1000 mg/L and the adherence of LLC cells to HMVEC at 10 to 1000 mg/L (in vitro). Carp oil (0.2 mL per mouse) inhibited the angiogenesis induced by Matrigel supplemented with vascular endothelial growth factor (VEGF) and heparin (in vivo). Antitumor and antimetastatic actions of carp oil might be partly attributable to the inhibition of DNA synthesis in LLC cells and angiogenesis through the inhibition of the adherence of LLC cells to the microvascular endothelium. Oleic acid (0.1 or 0.2 mL per mouse) significantly inhibited the metastasis to the liver, but it had no effect on the primary solid-tumor growth. Oleic acid inhibited the angiogenesis in both in vitro and in vivo models. Oleic acid at 1000 μmol/L inhibited the DNA synthesis in LLC cells but did not affect the DNA synthesis in HMVEC. These inhibitory actions of oleic acid may be attributable to the inhibition of angiogenesis induced by the tumor. Linoleic acid and linolenic acid had no effect on tumor growth or metastasis to the liver. J. Nutr. 132: 2069–2075, 2002.

KEY WORDS: • *carp oil* • *antitumor activity* • *antimetastatic activity* • *angiogenesis* • *oleic acid*

The freshwater carp (*Cyprinus carpio* L.) has long been used in Korea, China, and Japan as a health food. In ancient Chinese medicine, carp was eaten as a diuretic and used as a remedy for eye fatigue. In Japan, carp meat and blood have traditionally been eaten as a tonic. Although it has recently been thought that carp extract prevents recurrence of tumors and metastasis after stopping radiation and/or cancer chemotherapy, the basis for this is unclear. Dietary oils such as those from herbs, seeds and fish are widely used for the prevention of such conditions as thrombosis, hyperlipidemia and arteriosclerosis. Oleic, linoleic, linolenic, eicosapentaenoic (EPA[3]) and docosahexaenoic acids (DHA) are contained in vegetable, herb, seed and fish oils. There are a number of reports that fatty acids have pharmacological actions (1–3). It was previously reported that dietary oils prevent tumor growth in both in vivo and in vitro studies, and conversely, that dietary oils accelerated tumor growth (4–8). Antitumor and antimetastatic actions by dietary oils and various fatty acids need to be clarified.

Dietary fats are absorbed from the small intestine and metabolized in the liver. Therefore, in the present study we examined the effects of carp oil, oleic acid, linoleic acid and linolenic acid on tumor growth and metastasis to the liver in intrasplenic LLC-implanted mice.

MATERIALS AND METHODS

Materials. Carp oil was supplied by Carp Food (Tottori, Japan). Oleic, linoleic and linolenic acids were purchased from Wako Pure Chemicals (Osaka, Japan). Matrigel® basement membrane (with or without growth factor) was obtained from Becton Dickinson Labware (Bedford, MA). [Methyl-^{3}H]thymidine (specific activity, 740 GBq/mmol) was purchased from NEN Life Science Products (Boston, MA). Dulbecco's modified Eagle's medium (DMEM) and microvascular endothelial base medium (HEBM) were obtained from Nissui Pharmaceutical (Tokyo, Japan) and Clonetics (San Diego, CA), respectively. CS-C medium kits were purchased from Cell Systems (Kirkland, WA). The medium kits consist of a mixture of DMEM and Ham's F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Auckland, New Zealand), 15 mmol/L Hepes and CS-C growth factor. Antibiotic and antimycotic solutions (100×) containing 10^7 U of penicillin, 10 g of streptomycin and 25 mg of amphoptericin B/L in 9 g/L NaCl were purchased from Sigma Chemical (St. Louis, MO). Six-, 12-, 24- and 48-well plates were purchased from Corning Glass Works (New York, NY). Collagen (Type I)-coated 6- and 24-well plates were purchased from Sumitomo Bakelite (Tokyo, Japan). Vascular endothelial growth factor (VEGF) was purchased from Wako Pure Chemicals. The laboratory diet of mice

[1] Supported by research grants from Carp Food Co. (Tottori, Japan).
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[3] Abbreviations used: BCECF, 3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxylfluorescein; BCECF-AM, 3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxylfluorescein acetoxymethylester; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; HEBM, human microvascular endothelial base medium; HMEGM, human microvascular endothelial basal medium supplemented growth factor; HMVEC, human adult dermal microvascular endothelial cells; LLC, Lewis lung carcinoma; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; VEGF, vascular endothelial growth factor.

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was purchased from Oriental Yeast (Osaka, Japan). Other chemicals were of reagent grade.

Fatty acids in carp oil. The fatty acid composition was determined according to the method of Nelson et al. (9). Briefly, the total lipid extracts were transmethylated at 90°C for 2 h by adding 70 mL HCl/L in methanol (5 mL). The fatty acid methyl esters (FAME) were then extracted with *n*-hexane and analyzed by gas liquid chromatography (GC-14b; Shimadzu, Kyoto, Japan) under the following conditions: column, 0.25 mm I.D. × 25 m length (capillary column; Shinwa Chemical Industries, Tokyo, Japan); carrier gas flow rate, 1.5 mL helium/min; column temperature, 250°C; injection and flame-ionization detector temperature, 200°C. The composition of the fatty acids is shown in **Table 1**.

Cells. The highly metastatic, drug-resistant mouse LLC cells were obtained from Riken Gene Bank (Tukuba, Japan) and maintained in DMEM supplemented with 100 mL FBS/L, penicillin (1×10^5 U/L), streptomycin (100 mg/L) and amphotericin B (0.25 mg/L). Human adult dermal microvascular endothelial cells (HMVEC) were purchased from Cell Systems, seeded onto collagen (Type I)-coated 24- or 6-well plates and maintained in Clonetics microvascular endothelial basal medium supplemented with growth factor (MEGM) or in CS-C media.

Animals. Female 5-wk-old C57BL/6 strain mice were obtained from Clea Japan (Osaka, Japan). They were housed for 1 wk in a room maintained at 25 ± 1°C with 60% relative humidity and given free access to a nonpurified diet [per 100 g of diet: water, 8 g; crude carbohydrate, 51.3 g; crude protein, 24.6 g; crude lipid, 5.6 g; crude fiber, 3.1 g; mineral mixture, 6.4 g; vitamin mixture, 1 g (Oriental Yeast)] and water. The room was illuminated for 12 h/d starting at 0700 h. Mice were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University. The Animal Studies Committee of Ehime University approved the experimental protocol.

Antitumor and antimetastatic activities in intrasplenic LLC-implanted mice. Cultured LLC cells were harvested by trypsinization, washed and suspended at 7.5×10^8 cells/L in DMEM supplemented with 100 mL FBS/L and 1 g Matrigel/L (minus growth factor). Matrigel (minus growth factor) was used to prevent the cell suspension from leaking out of the spleen. LLC-bearing mice (control) were prepared by intrasplenic implantation of 1.5×10^5 cells (0.2 mL) into the spleen of C57BL/6 female mice on d 0, and sham-operated mice (normal) were injected with PBS alone into the spleen. Carp oil (expt. 1), oleic acid (expt. 2), linoleic acid (expt. 3) or linolenic acid (expt. 3) (0.1 or 0.2 mL per mouse) was administered orally once at 0700 h daily for 20 consecutive days, starting 12 h after implantation of the tumor cells. After intrasplenically transplanting LLC cells or injecting PBS, the body weight of each mouse was reduced about 1 g; the body weight was regained 2 d after treatment.

The consumption of dietary fats by Japanese people is more than 65 g per day. In this study, the excess doses of fivefold (0.1 mL per mouse per day) and 10-fold (0.2 mL per mouse per day) carp oil, oleic acid, linoleic acid or linolenic acid relative to the consumption of dietary fats were used to evaluate their pharmacological actions. Sham-operated mice and LLC-implanted mice were given distilled water alone (0.2 mL per mouse per day) on the same schedule. On d 21, blood was obtained by venipuncture from mice under pentobarbital anesthesia, and then the spleen, thymus, lung and liver were removed and weighed for evaluation of the antitumor and antimetastatic activities and side effects. Blood samples were chilled in test tubes containing heparin, and the numbers of red cells and leukocytes were measured using a Coulter Counter (Japan Scientific Instruments, Tokyo, Japan). The hemoglobin content in the blood was determined using Hemoglobin-Test kits (Wako Pure Chemicals). The number of tumor colonies in the liver was counted manually.

TABLE 1

The fatty acid components of carp oil

Fatty acid component	g/kg carp oil
Myristic acid, 14:0	20
Palmitic acid, 16:0	238
Palmitoleic acid, 16:1(n-7)	78
Stearic acid, 18:0	33
Oleic acid, 18:1(n-9)	366
Linoleic acid, 18:2(n-6)	182
EPA, 20:5(n-3)	9
DHA, 22:6(n-3)	23
Others	51

Histological examination. All liver tissues were fixed in 100 mL buffered formalin/L for at least 24 h, progressively dehydrated in solutions containing an increasing percentage of ethyl alcohol (700, 800, 950 and 1000 mL/L), cleared in Histoclear, embedded in paraffin under vacuum, sectioned at 5-μm thickness, deparaffinized and stained with Harris hemotoxylin and eosin. Four different phase-contrast microscopic fields (×20 magnification) per plate were photographed.

DNA synthesis in LLC cells. LLC cells were placed in DMEM supplemented with 100 mL FBS/L at 1×10^4 cells/well in 24-well culture plates. After the cells were cultured overnight, the medium was changed to fresh DMEM supplemented with 100 mL FBS/L and carp oil (expt. 4) or oleic acid (expt. 5) for 20 h. The medium was replaced with [^{3}H]thymidine (18.5 kBq = 0.5 μCi/well) in DMEM supplemented with 100 mL FBS/L. The fatty acid concentrations in serum are 0.3 to 0.9 mmol/L in normal humans; therefore, the concentrations of carp oil or various fatty acids used were of 0.1 to 1000 mg/L for carp oil (0.35 to 3500 μmol fatty acids/L) or of 0.1 to 1000 μmol/L for various fatty acids. After further incubation for 4 h, the cells were washed twice with cold phosphate-buffered saline (PBS), immersed in 1 mL of 50 g trichloroacetic acid (TCA)/L for 1 h at 4°C, washed twice with 50 g TCA/L and solubilized with 100 μL of 0.2 mmol NaOH/L containing 2.5 g Triton X-100/L. Thymidine incorporation into the cells was determined by liquid scintillation counting.

DNA synthesis in HMVEC. HMVEC (1×10^4 cells per well) were seeded onto collagen (Type I)-coated 24-well culture plates in CS-C medium. After the cells were cultured overnight, the medium was replaced by fresh medium, and the cells were exposed to the indicated amounts of carp oil or oleic acid for 20 h; thymidine incorporation was measured by the methods described above.

Preparation of BCECF-labeled LLC cells. Loading of BCECF into the LLC cells was carried out by the modified methods described previously (10,11). Briefly, 3 μmol BCECF-AM/L was added to the LLC cell suspension (2×10^8 cells/L) in DMEM supplemented with 100 mL FBS/L and 1 mmol EDTA/L, and the cells were incubated for 30 min at 37°C with gentle agitation in a water bath. After the incubation period, the reaction mixture was centrifuged at 410 × *g* to remove the medium containing BCECF-AM. The cells were then washed twice with DMEM supplemented with 100 mL FBS/L and suspended at a final concentration of 1×10^8 cells/L in DMEM supplemented with 100 mL FBS/L.

LLC cell adhesion to HMVEC. BCECF-labeled LLC cells (1×10^4 cells per well) were seeded onto HMVEC monolayers (third passage) grown on collagen-coated 24-well culture plates and cultured in DMEM containing 5 mmol Hepes/L and 25 g BSA/L with the indicated amounts of carp oil (expt. 4) or oleic acid (expt. 5) for 2 h at 37°C in a humidified chamber containing 5% CO_2. After the incubation period, the HMVEC were gently washed twice with the above medium to remove nonadherent LLC cells. The LLC cells that adhered to HMVEC were solubilized by adding 2.5 g Triton X-100/L in 0.1 mmol NaOH (1 mL)/L, and the number of LLC cells was measured by fluorimetry (FP-777; Jasco, Tokyo, Japan) with excitation at 500 nm and emission at 540 nm. The various numbers (1×10^6, 3×10^6, 5×10^6 and 1×10^7 cells/L) of BCECF-labeled LLC cells were prepared, solubilized by adding 2.5 g Triton X-100 in 0.1 mmol NaOH (1 mL)/L. The fluorescence intensity of various numbers of BCECF-labeled LLC cells was used to prepare a standard curve.

Matrigel-induced tubelike network formation of HMVEC (in vitro). Matrigel-induced tubelike formation of HMVEC was assayed according to the methods described in previous reports (11–13).

Briefly, Matrigel supplemented with growth factor (150 μL) was placed into each well of a 48-well culture plate at 4°C and allowed to polymerize by incubation for 1 h at 37°C. HMVEC (second passage, 2×10^4 cells) were seeded onto the Matrigel in 270 μL of DMEM supplemented with 200 mL FBS/L and incubated with the indicated amounts of carp oil (expt. 4) or oleic acid (expt. 5) at 37°C for 24 h in a humidified 5% CO_2 atmosphere. Three different phase-contrast microscopic fields (×40 and ×100 magnification) per well were photographed, and the light micrograph images were stored in a computer. The total length of tube structures in each photograph (×40 magnification) was measured using Adobe Photoshop software (Adobe, Tokyo, Japan).

Matrigel-induced neovascularization (in vivo). For in vivo experiments, Matrigel (minus growth factor)-induced neovascularization was assayed according to the methods described in a previous report (10). Briefly, female C57BL/6 mice were injected subcutaneously with 0.5 mL of Matrigel containing 20 μg of VEGF and 32 $\times 10^3$ U heparin/L on d 0. Carp oil (expt. 6) or oleic acid (expt. 7) (0.1 or 0.2 mL per mouse) was orally administered from d 1 to d 5 to mice implanted with Matrigel containing VEGF and heparin. The mice were killed on d 6 with an overdose of pentobarbital, and the gels were removed and weighed. The hemoglobin content in the gels was determined using Hemoglobin-Test kits (Wako Pure Chemicals).

Statistical analysis. All values are expressed as means ± SEM. Data were analyzed by one-way ANOVA, and then differences among means were analyzed using Fisher's protected least-significant difference (LSD) multiple-comparison test. Differences were considered significant at $P < 0.05$.

RESULTS

Antitumor and antimetastatic activities. The spleen weights of mice with intrasplenically implanted LLC cells were significantly greater than those of sham-operated mice (**Figs. 1, 2**). In LLC-bearing mice, the increase in spleen weight was significantly inhibited by oral administration of carp oil at doses of 0.1 or 0.2 mL per mouse (expt. 1) (Figs. 1, 2). Oleic acid (expt. 2), linoleic acid (expt. 3) and linolenic acid (expt. 3) at a dose of 0.1 or 0.2 mL per mouse did not inhibit tumor growth in the spleen (**Table 2**). LLC-bearing mice had tumor metastasis to the liver, with about 50 to 70 tumor colonies per mouse (Figs. 1, 2, Table 2). Carp oil (0.2 mL per mouse) (expt. 1) and oleic acid (0.1 or 0.2 mL per mouse) (expt. 2) significantly reduced the number of tumor cell colonies that metastasized to the liver compared with the number in intrasplenic LLC-bearing mice (Figs. 1, 2, Table 2). On the other hand, neither linoleic acid nor linolenic acid affected metastasis to the liver in LLC-bearing mice (expt. 3) (Table 2). The growth of metastatic tumors in the livers of LLC-bearing mice was also inhibited by orally administered carp oil (expt. 1) or oleic acid (expt. 2) at 0.1 or 0.2 mL per mouse (**Fig. 3**).

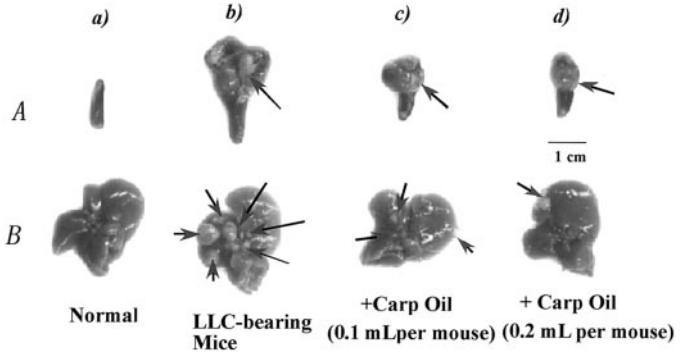

FIGURE 1 Photographs of the inhibition of intrasplenic tumor growth (*A*) and LLC tumor metastasis to the liver (*B*) on d 21 by carp oil in Lewis lung carcinoma (LLC)-bearing mice (expt. 1). Solid-type LLC was prepared by intrasplenic implantation of 1.5×10^5 cells (0.2 mL) containing 1 g/L Matrigel (with reduced growth factor) into the spleens of C57BL/6 female mice on d 0. Sham-operated mice (*a*) and LLC-implanted mice (control, *b*) were given distilled water alone for 20 d. Other LLC-implanted mice were administered 0.1 mL (*c*) or 0.2 mL (*d*) of carp oil per mouse orally once daily for 20 d. Tumors are indicated with arrows.

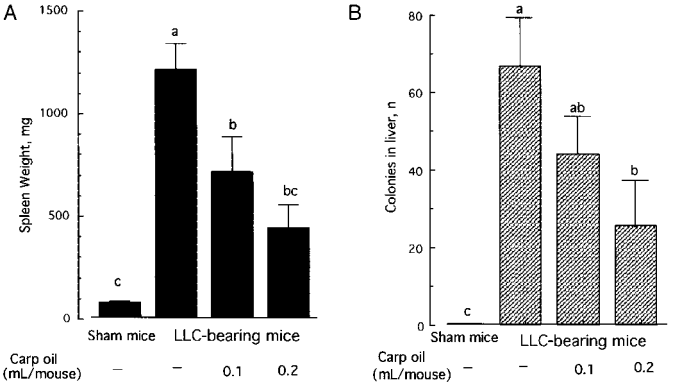

FIGURE 2 Effects of carp oil on final primary solid tumor growth in the spleen (*A*) and the number of colonies of LLC cells metastasizing to the liver (*B*) on d 21 in LLC-bearing mice (expt. 1). Values are means ± SEM; $n = 5–8$. The sham-operated group consisted of five mice; the LLC-bearing group (control) and carp oil–treated groups (0.1 or 0.2 mL per mouse) consisted of eight mice per group. Columns not sharing a letter are significantly different, $P < 0.05$.

Food intake, body and tissue weights, and blood count. Food intake did not differ among groups [values expressed in g (means ± SEM): sham-operated (normal) groups, 2.57 ± 0.11; the control group, 2.46 ± 0.16; carp oil, 2.70 ± 0.13 (0.1 mL per mouse), 2.64 ± 0.13 (0.2 mL per mouse); oleic acid, 2.75 ± 0.16 (0.1 mL per mouse), 2.65 ± 0.16 (0.2 mL per mouse); linoleic acid, 2.63 ± 0.31 (0.1 mL per mouse), 2.73 ± 0.28 (0.2 mL per mouse); linolenic acid, 2.65 ± 0.11 (0.1 mL per mouse), 2.51 ± 0.14 (0.2 mL per mouse)]. The total energy intakes of carp oil–, oleic acid–, linoleic acid– and linolenic acid–treated groups were greater than those of sham or LLC-bearing mice. Carp oil (expt. 1) and oleic acid (expt. 2) did not affect the final body, liver and lung weights of LLC-bearing mice compared with the values in sham-operated mice (expts. 1 and 2) (**Table 3**). Linoleic acid and linolenic acid also did not affect the liver and lung weights of LLC-bearing mice compared with those of sham-operated mice (expt. 3) (Table 3). Linoleic acid (0.1 mL per mouse) or linolenic acid (0.1 or 0.2 mL per mouse) significantly increased the final body weight compared to that of LLC-bearing mice (expt. 3) (Table 3). In LLC-bearing mice, the thymus weight was significantly lower than that in sham-operated mice. The number of leukocytes in LLC-bearing mice was significantly greater than that in sham-operated mice. In contrast the number of red cells and the hemoglobin concentration in LLC-bearing mice were significantly lower than those in normal mice (Table 3), that is, the intrasplenic implantation of LLC cells caused anemia. The increase in the leukocyte number in LLC-bearing mice was inhibited by orally administered carp oil (0.1 or 0.2 mL per mouse), oleic acid (0.1 or 0.2 mL per mouse) or linoleic acid (0.2 mL per mouse) (Table 3). Moreover, oleic acid at 0.1 or 0.2 mL per mouse inhibited the reduction of red cell number and hemoglobin concentration in LLC-bearing mice (Table 3). However, neither linoleic acid nor linolenic acid prevented the reduction of red cell number or hemoglobin

TABLE 2

Effects of oleic acid, linoleic acid and linolenic acid on the spleen weight and the number of metastatic tumor colonies in the livers of Lewis lung carcinoma (LLC)–bearing mice[1]

Treatment	*n*	Spleen weight	Metastatic tumor colonies to liver
		mg	*n*
Experiment 2			
Sham-operated mice (Normal)	5	79.1 ± 7.08^{b}	0.0 ± 0.0^{c}
LLC-bearing mice (Control)	6	504.9 ± 97.6^{a}	47.2 ± 12.4^{a}
+ Oleic acid (0.1 mL/mouse)	6	334.4 ± 100.4^{ab}	14.8 ± 6.0^{bc}
(0.2 mL/mouse)	6	541.3 ± 172.1^{a}	23.5 ± 4.2^{b}
Experiment 3			
Sham-operated mice (Normal)	5	57.6 ± 2.62^{b}	0.0 ± 0.0^{c}
LLC-bearing mice (Control)	6	889.4 ± 170.3^{a}	53.4 ± 10.3^{ab}
+ Linoleic acid (0.1 mL/mouse)	6	929.6 ± 149.0^{a}	55.6 ± 12.5^{ab}
(0.2 mL/mouse)	6	767.9 ± 126.3^{a}	67.0 ± 5.2^{a}
+ Linolenic acid (0.1 mL/mouse)	6	926.4 ± 139.0^{a}	54.9 ± 6.4^{ab}
(0.2 mL/mouse)	6	680.9 ± 86.9^{a}	43.7 ± 3.2^{b}

[1] Values are means ± SEM. Values for an experiment not sharing a letter differ, $P < 0.05$.

concentration. Rather, the oral administration of linolenic acid at 0.1 mL per mouse further reduced the hemoglobin concentration in LLC-bearing mice (Table 3).

DNA synthesis, angiogenesis and LLC cell adherence (in vitro). Carp oil inhibited DNA synthesis in LLC cells at 100 or 1000 mg/L, but not at 0.1 to 10 mg/L (expt. 4) (**Table 4**). Oleic acid inhibited DNA synthesis in LLC cells at 1000 μmol/L (expt. 5) (Table 4). Carp oil (100 or 1000 mg/L) or oleic acid (1000 μmol/L) reduced the amount of LLC cell protein in each well as well as DNA synthesis in LLC cells (data not shown). Carp oil (0.1 to 1000 mg/L) or oleic acid (0.1 to 1000 μmol/L), did not affect DNA synthesis in HMVEC (data not shown). Carp oil inhibited the Matrigel-induced tubelike network formation of HMVEC at 1000 mg/L, but not at 0.1 to 100 mg/L (expt. 4) (Table 4). Oleic acid also significantly inhibited the Matrigel-induced formation of a tubelike network of HMVEC at 10 to 1000 μmol/L, but not at 0.1 or 1 μmol/L (expt. 5) (Table 4). Carp oil inhibited the adherence of LLC cells to HMVEC at 10 to 1000 mg/L, but not at 0.1 and 1 mg/L (expt. 4) (Table 4). On the other hand, oleic acid at 1 or 10 μmol/L slightly increased ($P < 0.04$) the adherence of LLC cells to HMVEC, but at 0.1, 100 or 1000 μmol/L did not have an effect (expt. 5) (Table 4). The total protein amounts in this assay were also reduced by carp oil at 10, 100 or 1000 mg/L ($P < 0.0341$), but oleic acid had no effect (data not shown).

Matrigel (minus growth factor)-induced angiogenesis (in vivo). Matrigel (minus growth factor) supplemented with 20 μg of VEGF and 32×10^3 U heparin/L increased the weight of the gel and the hemoglobin concentration in the gels 6 d after implantation compared with Matrigel alone ($P < 0.002$) (**Table 5**). Orally administered carp oil (0.2 mL per mouse × 5 d) (expt. 6) or oleic acid (0.1 or 0.2 mL per mouse × 5 d) (expt. 7) inhibited the increases in the weight and hemoglobin content of the gels induced by the Matrigel/VEGF/heparin mixture (Table 5).

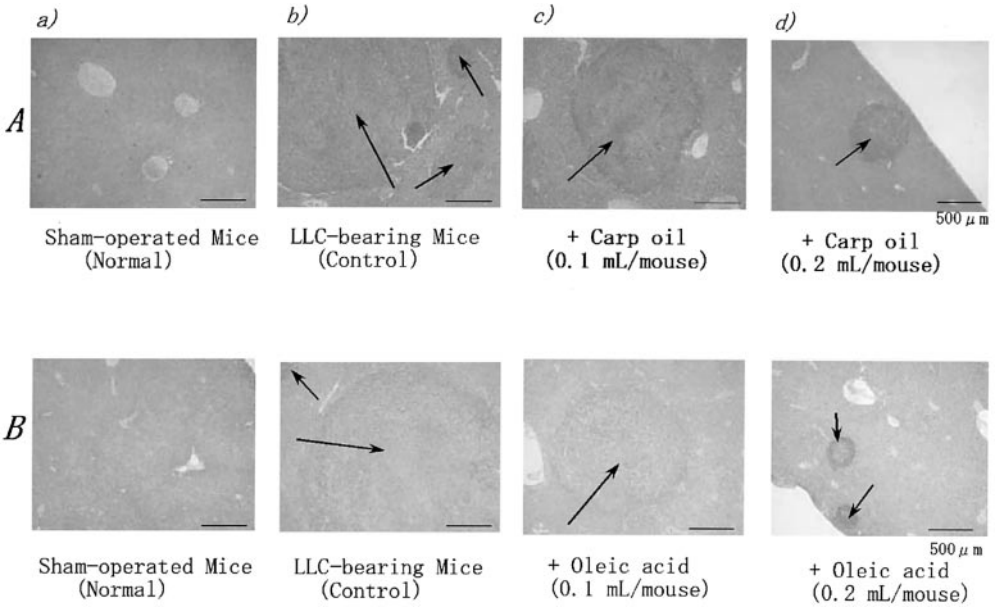

FIGURE 3 Histology of the inhibition of LLC tumor growth metastasizing to the liver on d 21 by carp oil (*A*) or oleic acid (*B*) in LLC-bearing mice. Photographs of livers from sham-operated mice (*a*), LLC-implanted mice (*b*) and LLC-implanted mice administered 0.1 mL (*c*) or 0.2 mL (*d*) of carp oil (A) or oleic acid (B) per mouse orally for 20 d. Tumors are indicated with arrows.

TABLE 3

Effects of carp oil, oleic, linoleic and linolenic acids on the weights of body, lung, and thymus, the numbers of leukocytes, red cells, and the hemoglobin concentration in Lewis lung carcinoma (LLC)–bearing mice[1]

Treatment	*n*	Initial body	Final body	Liver	Lung	Thymus	Leukocytes	Red cells	Hemoglobin
		g			*mg*		*×10³/μL*	*×10⁴/μL*	*g/L*
Experiment 1									
Sham-operated mice (Normal)	5	17.8 ± 0.29	19.8 ± 0.33	1.27 ± 0.06	140.3 ± 6.05	78.49 ± 5.72^{a}	2.48 ± 0.23^{b}	786.6 ± 8.23^{a}	121 ± 1.2^{a}
LLC-bearing mice (Control)	8	17.1 ± 0.16	20.2 ± 0.23	1.45 ± 0.10	133.4 ± 6.47	45.58 ± 3.51^{b}	7.08 ± 1.83^{a}	579.1 ± 52.9^{b}	91 ± 7.9^{b}
+ Carp oil (0.1 mL/mouse)	8	17.3 ± 0.20	20.0 ± 0.23	1.44 ± 0.06	150.8 ± 10.1	50.60 ± 5.91^{ab}	3.80 ± 0.22^{b}	572.6 ± 61.7^{b}	89 ± 9.6^{b}
(0.2 mL/mouse)	8	17.6 ± 0.33	20.3 ± 0.21	1.37 ± 0.06	152.0 ± 16.8	57.88 ± 5.92^{ab}	3.40 ± 0.34^{b}	653.6 ± 45.0^{ab}	103 ± 7.3^{ab}
Experiment 2									
Normal	5	17.5 ± 0.12	21.1 ± 0.86	1.25 ± 0.07	157.7 ± 6.69	51.83 ± 2.20^{a}	3.44 ± 0.89^{b}	770.6 ± 17.6^{a}	118 ± 1.9^{a}
Control	6	17.3 ± 0.21	19.0 ± 0.23	1.28 ± 0.09	130.7 ± 7.13	30.25 ± 6.31^{b}	6.22 ± 1.25^{a}	303.2 ± 64.4^{c}	46 ± 10.0^{c}
+ Oleic acid (0.1 mL/mouse)	6	17.5 ± 0.18	21.4 ± 0.60	1.29 ± 0.08	132.3 ± 7.42	37.12 ± 4.62^{ab}	3.26 ± 0.14^{b}	487.4 ± 45.9^{b}	74 ± 7.2^{b}
(0.2 mL/mouse)	6	17.7 ± 0.13	19.7 ± 0.34	1.25 ± 0.07	121.5 ± 23.9	43.52 ± 7.79^{ab}	3.78 ± 0.49^{b}	567.3 ± 59.4^{b}	89 ± 9.6^{b}
Experiment 3									
Normal	5	16.3 ± 0.26	18.2 ± 0.20^{bc}	1.22 ± 0.04	159.1 ± 7.23	49.80 ± 3.37^{a}	2.86 ± 0.44^{b}	764.8 ± 14.8^{a}	118 ± 1.6^{a}
Control	6	16.0 ± 0.22	17.8 ± 0.54^{c}	1.27 ± 0.28	157.1 ± 7.42	31.20 ± 3.19^{b}	6.67 ± 1.26^{a}	493.0 ± 53.5^{b}	73 ± 8.0^{b}
+ Linoleic acid (0.1 mL/mouse)	6	17.0 ± 0.32	19.7 ± 0.40^{a}	1.44 ± 0.26	146.8 ± 5.80	31.70 ± 4.82^{b}	6.95 ± 0.78^{a}	418.3 ± 59.3^{b}	63 ± 8.9^{b}
(0.2 mL/mouse)	6	16.5 ± 0.28	18.3 ± 0.45^{bc}	1.43 ± 0.19	134.7 ± 9.13	23.46 ± 4.54^{b}	3.78 ± 0.59^{b}	503.7 ± 14.4^{ab}	73 ± 3.1^{b}
+ Linolenic acid (0.1 mL/mouse)	6	17.1 ± 0.33	19.3 ± 0.32^{ab}	1.28 ± 0.17	167.6 ± 18.5	33.39 ± 3.74^{b}	5.57 ± 1.03^{ab}	342.2 ± 43.8^{b}	50 ± 7.5^{c}
(0.2 mL/mouse)	6	17.1 ± 0.30	19.1 ± 0.26^{ab}	1.16 ± 0.14	154.6 ± 14.5	29.75 ± 4.30^{b}	4.82 ± 0.76^{ab}	396.7 ± 50.9^{b}	60 ± 7.9^{b}

[1] Values are means ± SEM. Values for an experiment not sharing a letter differ, $P < 0.05$.

DISCUSSION

After the removal of malignant tumors by surgical operation, radiation therapy and/or adjuvant therapy with cancer chemotherapy drugs may be curative. However, cancer chemotherapy can cause severe gastrointestinal toxicity with diarrhea and mucosis and hematologic toxicity with leukopenia and immunosuppression, adverse effects that are dose limiting. In a series of studies of the preventive effects of natural products on tumor growth, I previously showed that carp extract and fish oils inhibited adverse reactions such as gastrointestinal toxicity, myelotoxicity and immunosuppression induced by 5-fluorouracil (5-FU) without affecting the anti-

TABLE 4

Effects of carp oil and oleic acid on DNA synthesis in LLC cells, Matrigel-induced capillary-like tube network formation from HMVEC and the adherence of LLC cells to HMVEC (in vitro)[1]

Treatment	[^{3}H]Thymidine incorporation into DNA of LLC cells	Capillary-like tube formation	Adherence of LLC cells to HMVEC
	×10³ dpm/well (%)	*mm length/field (%)*	*×10³ LLC cells/well (%)*
Experiment 4			
Carp oil, *mg/L*			
0	8.31 ± 1.92^{a} (100)	58.92 ± 3.06^{a} (100)	4.19 ± 0.124^{a} (100)
0.1	6.33 ± 1.55^{ab} (76.2)	56.10 ± 4.61^{a} (95.2)	4.04 ± 0.165^{a} (92.1)
1	6.11 ± 1.39^{ab} (73.5)	61.90 ± 5.19^{a} (105.1)	3.86 ± 0.131^{a} (96.4)
10	6.48 ± 1.84^{ab} (78.0)	61.17 ± 5.08^{a} (103.8)	3.51 ± 0.099^{b} (83.8)
100	4.61 ± 0.81^{b} (55.4)	56.00 ± 3.81^{a} (95.0)	2.69 ± 0.142^{c} (64.2)
1000	4.76 ± 0.91^{b} (57.2)	32.24 ± 4.83^{b} (54.7)	2.43 ± 0.077^{c} (58.0)
Experiment 5			
Oleic acid, *μmol/L*			
0	6.56 ± 0.72^{ab} (100)	63.65 ± 1.83^{a} (100)	3.78 ± 0.113^{b} (100)
0.1	6.34 ± 0.64^{ab} (96.6)	62.41 ± 1.38^{a} (98.1)	3.94 ± 0.104^{b} (104.2)
1	6.29 ± 0.84^{ab} (95.9)	60.67 ± 0.52^{a} (95.3)	5.06 ± 0.453^{a} (133.9)
10	5.58 ± 1.13^{bc} (85.1)	54.44 ± 1.86^{b} (85.5)	4.48 ± 0.182^{ab} (118.5)
100	9.82 ± 2.87^{a} (149.7)	53.80 ± 1.25^{b} (84.5)	4.18 ± 0.098^{b} (110.6)
1000	1.61 ± 0.40^{c} (24.5)	48.11 ± 1.45^{b} (75.6)	4.09 ± 0.124^{b} (108.2)

[1] Values are means ± SEM, $n = 4$. Values for an experiment not sharing a letter differ, $P < 0.05$.

TABLE 5

Effects of carp oil and oleic acid on Matrigel-induced angiogenesis (in vivo)[1]

Treatment	*n*	Matrigel weight	Hemoglobin content
		mg	*mg/Matrigel*
Experiment 6			
Matrigel alone	5	77.0 ± 5.82[c]	7.0 ± 0.71[c]
Matrigel + VEGF (20 μg/L) + heparin (32 × 10^3 U/L) (Control)	5	397.2 ± 48.3[a]	24.4 ± 4.09[a]
Matrigel, VEGF, heparin + Carp oil (0.1 mL/mouse)	5	405.8 ± 78.2[a]	32.0 ± 3.61[a]
Matrigel, VEGF, heparin + Carp oil (0.2 mL/mouse)	5	163.6 ± 29.3[b]	11.8 ± 1.59[b]
Experiment 7			
Matrigel alone	4	74.2 ± 2.12[b]	15.0 ± 2.55[b]
Matrigel + VEGF (20 μg/L) + heparin (32 × 10^3 U/L) (Control)	4	330.4 ± 41.1[a]	34.8 ± 5.17[a]
Matrigel, VEGF, heparin + oleic acid (0.1 mL/mouse)	4	105.8 ± 23.0[b]	18.8 ± 1.11[b]
Matrigel, VEGF, heparin + oleic acid (0.2 mL/mouse)	4	106.6 ± 5.39[b]	19.7 ± 0.88[b]

[1] Values are means ± SEM. Values for an experiment not sharing a letter differ, $P < 0.05$.

tumor activity of 5-FU (14,15). Surgery to excise breast carcinoma, colon carcinoma and osteogenic sarcoma may be followed by the rapid growth of distant metastases to the lung, liver, large intestine and so forth. It has been reported that subcutaneous LLC implantation in the foot pad or back in C57BL/6 mice resulted in lung metastasis in addition to tumor growth (16–19).

In the present study, I found that the intrasplenic implantation of LLC cells resulted in tumor metastasis to the liver and caused anemia with the reduction of red cell number and hemoglobin concentration in the blood. Primary solid-tumor growth in the spleen and liver metastasis were inhibited by the oral administration of carp oil in LLC-bearing C57BL/6 mice. On the other hand, the oral administration of oleic, linoleic and linolenic acids did not affect tumor growth in the spleen, whereas oleic acid inhibited metastasis to the liver and metastatic tumor growth in the livers. In contrast, linoleic and linolenic acids did not affect metastasis to the liver. Carp oil at 100 or 1000 mg/L significantly inhibited DNA synthesis in LLC cells. Oleic acid inhibited DNA synthesis in LLC cells at 1000 μmol/L. Angiogenesis is the growth of new capillary blood vessels from preexisting capillaries and postcapillary venules. Solid tumors cause neovascularization, and the resultant angiogenesis from solid tumors stimulates growth and metastasis (18,20–26). Carp oil or oleic acid inhibited Matrigel-induced angiogenesis (both in vitro and in vivo). Carp oil and oleic acid did not affect DNA synthesis in HMVEC. Tumor cell interactions with platelets, endothelial cells and the subendothelial matrix are essential intermediate steps in the metastatic cascade of tumors (20,23,24,27–29).

To test the antitumor and/or antimetastatic activities of carp oil or oleic acid, I next examined the effects of carp oil or oleic acid on the interactions of LLC cells with HMVEC. Carp oil (10 to 1000 mg/L) inhibited the adherence of LLC cells to monolayer HMVEC, whereas oleic acid (1 or 10 μmol/L) significantly increased. Therefore, these findings suggest that the mechanism of antitumor and antimetastatic actions of carp oil might involve the inhibition of DNA synthesis in LLC cells and the inhibition of angiogenesis through the inhibition of LLC cells' adherence to the microvascular endothelium. On the other hand, the inhibitory actions of oleic acid on metastasis to the liver and metastatic tumor growth in the liver cannot be explained by effects on DNA synthesis in LLC cells or microvascular endothelial cells or the adherence of LLC cells to the microvascular endothelium; rather, these inhibitory actions by oleic acid are partly attributable to the inhibition of the angiogenesis induced by tumors.

In conclusion, it seems likely that the antitumor and antimetastatic activities of carp oil may be partly ascribed to a fatty acid, oleic acid, as an active substance; however, the antitumor and antimetastatic effects of carp oil are insufficient by themselves to explain the action of oleic acid. Further work will be needed to identify the active substance(s) in carp oil.

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