# Effects of carp extract on tumor growth in spleen and number of colonies of Lewis lung carcinoma (LLC) cells metastasizing to liver in intrasplenic LLC-implanted mice

Yoshiyuki KIMURA*

*Second Department of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan.*

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**Abstract**

Carp has been used in Korea, China and Japan as a health food source. In ancient Chinese medicine, carp was eaten as a diuretic, and used as a remedy for eye fatigue. Though it has recently been thought that carp extract has antitumor action against primary solid tumors and antimetastatic action toward the liver, the basis for this hearsay is unclear. In the present study, the effects of carp extract on tumor growth and the number of colonies of tumor cells metastasizing to the liver in Lewis lung carcinoma (LLC)-bearing mice were examined. It was found that the intrasplenic implantation of LLC cells resulted in tumor metastasis to the liver. Tumor growth in the spleen and the number of LLC metastasizing to the liver were inhibited by the oral administration of carp extract for 20 consecutive days at a dose of 250 or 500 mg per kg body weight in LLC-bearing mice. On the other hand, carp extract had no cytotoxic effect against LLC cells (*in vitro*). Since the contents of lipids and unsaturated fatty acids in carp extract are a small amount, the antitumor activity and the reduction in the number of metastatic nodules to the liver by carp extract could not be explained by lipids or unsaturated fatty acids. Further work is needed to identify the active substance(s) in carp extract.

**Key words** carp extract, antitumor activity, metastatic LLC colonies, mice.

## Introduction

Carp (*Cyprinus carpio* L.) has been used in Korea, China and Japan as a health food source. 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. In the previous report, it was shown that carp extract prevented the occurrence of myelotoxicity (the reduction of leukocyte number), and of gastrointestinal toxicity (the reduction of small intestinal weight) induced by 5-fluorouracil (5-FU) without loss of the antitumor activity of 5-FU in sarcoma 180-bearing mice.[1] Recently, it was reported that carp oil and oleic acid in carp oil had antitumor and antimetastatic activities in mice with intrasplenical Lewis lung carcinoma (LLC).[2] Though it has recently been thought that carp extract has antitumor action against primary solid tumors and antimetastatic action toward the liver, the basis for this hearsay is unclear. Therefore, to clarify whether carp extract inhibits the tumor growth and the number of colonies of tumor cells metastasizing to the liver, these effects of carp extract on intrasplenic LLC-implanted mice were examined.

## Materials and Methods

*Materials* : Carp extracts (Lot. No. CA13322) prepared by extraction with Japanese wine were supplied by Carp Food Co. Ltd. (Tottori, Japan). Briefly, the whole body of the carp (male, 1 kg) was extracted with Japanese wine (1.8 L) for 24 h at 90℃, and then further extracted by the addition of Japanese wine (1.8 L) for 72 h. The extract was concentrated to 400 mL by boiling, and then freeze-dried. Yield 139 g. Carp extract was suspended in distilled water and used in this study. Matrigel® basement membrane (without growth factor) was obtained from Becton Dickinson Labware (Bedford,

*To whom correspondence should be addressed. e-mail : yokim@m.ehime-u.ac.jp

MA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Ltd. (Tokyo, Japan) and used as culture medium. Antibiotic and antimycotic solutions were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum (FBS) was purchased from ICN Biochemicals (Aurora, OH). Culture plates were purchased from Corning Glass Works (NY). [Methyl-$^3$H] thymidine (specific activity; 740 GBq/mmol) was purchased from NEN Life Science Products (Boston, MA). Other chemicals were of reagent grade.

*Cells* : The highly metastatic, drug-resistant mouse Lewis lung carcinoma (LLC) cells were obtained from Riken Gene BANK (Tsukuba, Japan) and maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 $\mu$g/ml) and amphotericin B (0.25 $\mu$g/ml).

*Animals* : Female C57BL/6 mice (5 weeks old) were obtained from Clea Japan Co. (Osaka, Japan). They were housed for 1 week in a room maintained at 25 $\pm$ 1℃ with 60% relative humidity and given free access to 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 1 g and vitamin mixture 1 g: Oriental Yeast Co. (Osaka, Japan)]. The room was illuminated for 12 h per day 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.

*Measurement of tumor growth and the number of colonies of tumor cells metastasizing to the liver in LLC-bearing mice* : Cultured LLC cells were harvested by trypsinization, washed and suspended at 5 x $10^5$ cells/ml in DMEM supplemented with 10% FBS containing 1 mg/ml Matrigel. Matrigel was used to prevent the cell suspension from leaking out of the spleen. Solid-type LLC was prepared by intrasplenic implantation of 1 x $10^5$ cells (0.2 mL) into the spleens of C57BL/6 female mice on day 0. Carp extract (250 or 500 mg/kg) was administered orally once using gastric tube at 0700h daily for 20 consecutive days, starting 12 h after implantation of the tumor cells. Sham-operated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule. On day 21, blood was obtained via venipuncture in mice under pentobarbital anesthesia, and then the spleen, thymus, lung and liver were removed and weighed for evaluation of antitumor activity, the number of colonies of LLC cells metastasizing to the liver and side effects. The blood samples were chilled in test tubes containing heparin, and the number of red cells and leukocytes and hemoglobin content were measured using a Coulter Counter (Japan Scientific Instruments Ltd., Tokyo, Japan). The number of tumor colonies in the liver was counted manually. The sham-operated group (normal) consisted of 5 mice; the LLC-bearing group (control) and carp extract (250 or 500 mg/kg)-treated group consisted of 8 mice per group.

*Measurement of DNA synthesis in LLC cells* : LLC cells were placed in DMEM supplemented with 10% FBS at 1 x $10^4$ cells per well in 24-well culture plates. After the cells were cultured overnight, the medium was changed to fresh DMEM with 10% FBS and the cells were exposed to the indicated amounts of carp extract (5 to 100 mg/ml) for 20 h; the medium was then replaced with [$^3$H] thymidine (18.5 kBq=0.5 $\mu$Ci per well) in DMEM with 10% FBS. After further incubation for 4 h, the cells were washed twice with phosphate buffered saline (PBS), immersed in 1 mL of 5 % trichloroacetic acid (TCA) for 1 h at 4℃, washed twice 5 % TCA and solubilized with 1 mL of 0.2 mM NaOH containing 0.25 % Triton X-100. Thymidine incorporation into the cells was determined by liquid scintillation counting.

*Analysis of amino acids and fatty acids in carp extract* : The amino acids contained in carp extract were analyzed by the Japan Food Analysis Center Foundation (Tokyo, Japan). The fatty acid composition was determined according to the method of Nelson *et al.*[3] Briefly, the total lipid extracts were transmethylated at 90℃ for 2 h by adding 7 % methanol-HCl (5 ml). The fatty acid methyl esters were then extracted with n-hexane and analyzed by gas liquid chromatography (GC-14b, Shimadzu Co., Kyoto, Japan) under the following conditions: column: 0.25 mm id x 25 m length (capillary column, Shinwa Chemical Industries Ltd., Tokyo, Japan); carrier gas flow rate: 1.5 ml/min (helium), column temperature: 250℃; injection and flame-ionization detector temperature: 200℃. The composition of amino acids and fatty acids are shown in Table I.

*Data and statistical analyses* : All values are expressed as means $\pm$ S.E.M. Data were analyzed by one-way ANOVA, and then differences in means among

Table I The amino acid and fatty acid components of carp extract (g)

| Amino acid components and total fatty acids mg per g | | | | Fatty acid components g per 100 g fatty acid | |
|---|---|---|---|---|---|
| Hypro | 0.46 | Met | 3.8 | Myristic acid (14:0) | 2.0 |
| Asp | 36.8 | Ileu | 20.1 | Palmitic acid (16:0) | 23.8 |
| Thr | 22.2 | Leu | 27.6 | Palmitoleic acid (16:1 n-7) | 7.8 |
| Ser | 19.9 | Tyr | 20.4 | Stearic acid (18:0) | 3.3 |
| Glu | 22.6 | Phe | 17.4 | Oleic acid (18:1, n-9) | 36.6 |
| Pro | 30.9 | His | 23.4 | Linoleic acid (18:2, n-6) | 18.2 |
| Gly | 39.6 | Lys | 17.1 | EPA (20:5, n-3) | 0.9 |
| Ala | 49.7 | Arg | 10.0 | DHA (22:6, n-3) | 2.3 |
| Cys | 1.9 | Taurine | 1.34 | Others | 5.1 |
| Val | 20.5 | Fatty acid | 2.85 | | |

groups were analyzed using Fisher's protected LSD multiple comparison test (significantly different at $p<0.05$).

## Results and Discussions

The spleen weights of mice with intrasplenic implantation of LLC cells were significantly greater than those of sham-operated mice (normal mice) (Figs. 1 and 2). In intrasplenic implantation of LLC, the increase of spleen weight was significantly attenuated by orally administered carp extract at doses of 250 and 500 mg/kg (Figs. 1 and 2), but carp extract at a dose of 125 mg/kg had no effect (data not shown). Carp extract (500 mg/kg) significantly reduced the number of tumor cell colonies that metastasized to the liver compared with the number in control intrasplenic LLC-bearing mice (Figs. 3 and 4). Carp extract had no effect on the final body, liver or lung weight in LLC-bearing mice compared with the respective values in sham-operated mice (normal mice) (Table II). In LLC-bearing mice, the thymus weight was significantly lower than that in normal mice. Carp extract at a dose of 250 mg/kg significantly pre-

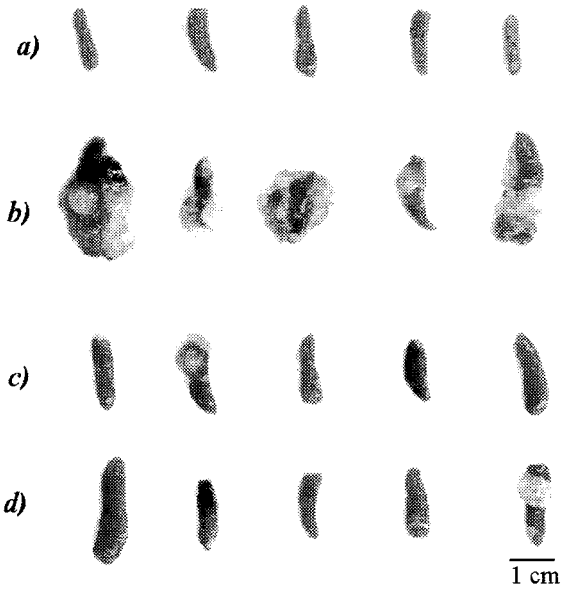


Fig. 1 Photographs of spleen 21 days after intrasplenic implantation of LLC in C57BL/6 mice.
Sham-operated mice (normal, a) and mice with intrasplenic implantation of LLC (control, b) were administered distilled water orally for 20 days. Two hundred fifty mg (c) or 500 mg/kg body weight of carp extract (d) was administered orally for 20 days to mice with intrasplenically implanted LLC.

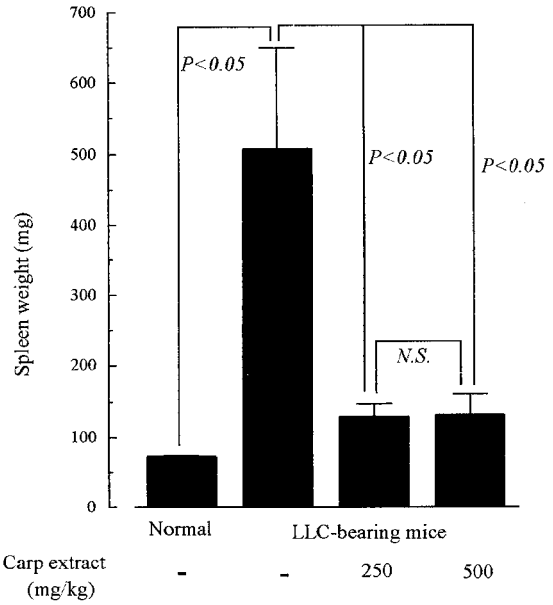


Fig. 2 Effects of carp extract on final spleen weight on day 21 in C57BL/6 mice with intrasplenically implanted LLC cells.
Solid-type LLC was prepared by intrasplenic implantation of $1x10^5$ cells (0.2 mL) into the spleen of C57BL/6 female mice on day 0. Carp extract (250 or 500 mg/kg) was administered orally once at 0700h daily for 20 consecutive days, starting 12 h after implantation of the tumor cells. Sham-operated mice (normal) and LLC-implanted mice (control) were given distilled water alone on the same schedule.
Values are means ± S.E.M., $n$ = 5-8.

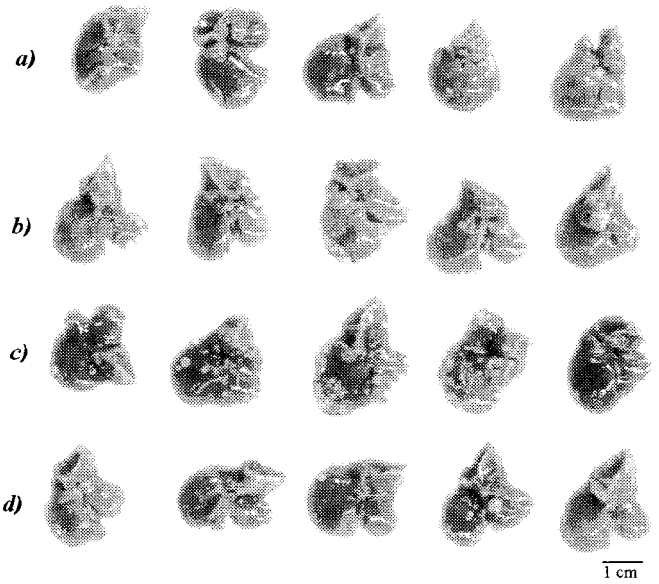


Fig. 3 Photographs showing the inhibition of metastasis to the liver by orally administered carp extract in C57BL/6 mice intrasplenically implanted with LLC cells.
Sham-operated mice (normal, a) and mice with intrasplenically implanted LLC (control, b) were administered distilled water orally for 20 days. Two hundred fifty mg (c) or 500 mg/kg body weight (d) of carp extract was administered orally for 20 days to mice intrasplenically implanted with LLC.

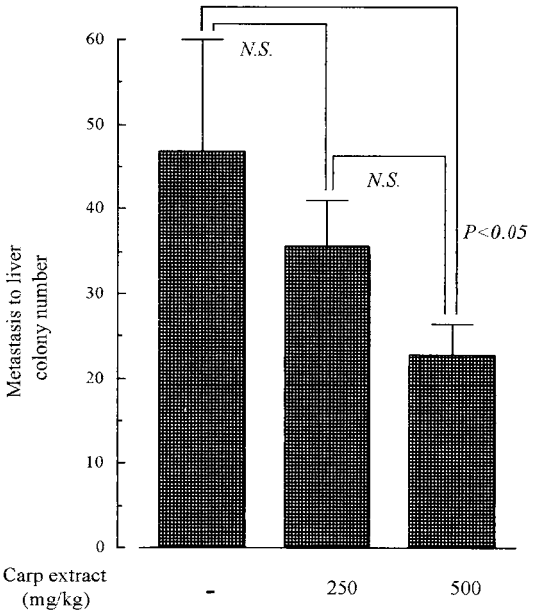


Fig. 4 Effects of carp extract on the number of colonies of LLC cells metastasizing to the liver on day 21 in mice intrasplenically implanted with LLC cells.
Values are means ± S.E.M., $n$ = 5-8.

Table II Effects of carp extract on the weights of body, lung and thymus in Lewis lung carcinoma (LLC) - bearing mice[1]

| | $n$ | Intial body *(g)* | Final body *(g)* | Liver *(g)* | Lung *(mg)* | Thymus *(mg)* |
|---|---|---|---|---|---|---|
| Normal | 5 | 17.0±0.13 | 19.0±0.33 | 1.12±0.056 | 150.4±6.58 | 70.6±3.80* |
| LLC-bearing mice (Control) | 8 | 16.9±0.10 | 19.0±0.23 | 1.26±0.033 | 142.5±7.94 | 53.2±3.21 |
| + Carp extract | | | | | | |
| (250 mg/kg) | 8 | 17.2±0.26 | 19.3±0.38 | 1.29±0.092 | 140.1±4.17 | 66.2±2.30* |
| (500 mg/kg) | 8 | 17.0±0.20 | 18.8±0.20 | 1.12±0.050 | 143.3±9.37 | 61.0±5.57 |

[1]Values are means ± S.E.M. *Significantly different from LLC-bearing mice (Control) : $p<0.05$.

Table III Effects of carp extract on the numbers of leukocytes and red cells and the hemoglobin content in blood of Lewis lung carcinoma (LLC) - bearing mice[1]

| | $n$ | Leukocytes *(μl)* | Red cells *(x $10^4$/μl)* | Hemoglobin *(g / 100 ml)* |
|---|---|---|---|---|
| Normal | 5 | 2220.0±307.2* | 769.6±16.0* | 11.88±0.24* |
| LLC-bearing mice (Control) | 8 | 4300.0±549.7 | 682.0±28.3 | 10.41±0.44 |
| + Carp extract | | | | |
| (250 mg/kg) | 8 | 4800.0±406.2 | 726.4±8.12 | 11.08±0.15 |
| (500 mg/kg) | 8 | 3612.5±740.3 | 719.9±27.6 | 10.94±0.50 |

[1]Values are means ± S.E.M. *Significantly different from LLC-bearing mice (Control) : $p<0.05$.

vented the reduction of the thymus weight in LLC-bearing mice, but at a dose of 500 mg/kg, carp extract had no effect on the reduction of the thymus weight. The number of leukocytes in LLC-bearing mice was significantly greater than that in normal mice (Table III). In contrast, the number of red cells and the hemoglobin content in LLC-bearing mice were significantly lower than those in normal mice. Thus, it was found that the intrasplenic implantation of LLC cells caused anemia. The number of leukocytes was not affected by the oral administration of carp extract. On the other hand, carp extract (250 or 500 mg/kg) tended to prevent the reduction of red cell number and hemoglobin content in blood of LLC-bearing mice, although the reductive effects were not significant (Table III). Carp extract had no effect on DNA synthesis of LLC cells at concentrations of 5 to 100 $\mu$g/ml (data not shown).

After the removal of malignant tumors by surgical operation, radiation therapy and/or adjuvant therapy with cancer chemotherapy drugs may be curative. However, treatment with cancer chemotherapy drugs causes severe gastrointestinal toxicity with diarrhea and mucosis, and hematologic toxicity with leucopoenia and immunosuppression, and these side effects are dose-limiting factors. It has already been reported that carp extract had no effect on the survival day and rate in sarcoma 180 ascites-bearing ICR mice, but that carp extract inhibited side effects such as gastrointestinal toxicity and myelotoxicity induced by 5-FU.[1] On the other hand, the removal of certain cancers, for example, breast carcinoma, colon carcinoma and osteogenic sarcoma, may be followed by the rapid growth of distant metastases to the lung, liver, *etc*. It has been reported that subcutaneous LLC-implantation in the footpad or back in C57BL/6 mice resulted in lung metastasis in addition to the tumor growth.[4-6] Hasegawa and Saiki reported that intrasplenical implantation of a highly metastatic melanoma B16-F-10 cells in C57BL/6 mice caused liver metastasis.[7] In the present study, it was found that the intrasplenic implantation of LLC cells resulted in tumor metastasis to the liver. The tumor growth in the spleen and the number of colonies of tumor cells metastasizing to the liver were inhibited by the oral administration of carp extract for 20 consecutive days at a dose of 250 or 500 mg/kg in LLC-bearing mice. On the other hand, carp extract had no cytotoxic effect against LLC cells *in vitro* (data not shown). There have been a number of reports that oleic acid, linoleic acid and eicasapentaenoic acid (EPA) have antitumor activity in tumor-bearing animals.[8-10] Zhu *et al.*[11] reported that oleic acid (200 and 400 mg/kg) or linoleic acid (200 and 400 mg/kg) significantly prolonged the life span of Ehrlich ascites carcinoma-bearing mice and inhibited the growth of Ehrlich solid carcinoma in mice compared with the findings in untreated control mice. Recently, it has been reported that carp oil and oleic acid (0.1 or 0.2 mL per mouse; 4.75 g/kg or 9.5 g/kg) in carp oil have antitumor and/or antimetastatic activities in mice with intrasplenically implanted LLC.[2] Though the carp extract contains oleic acid, linoleic acid, EPA and docosahexaenoic acid (DHA) as unsaturated fatty acids, the contents of unsaturated fatty acids containing in carp extract is 0.19% (w/w). The content of oleic acid in carp extract is about 0.10% (w/w) and is a very small amount. Thus, the doses of carp extract (250 and 500 mg/kg) used in this study corresponded to the doses of 0.25 and 0.5 mg/kg of oleic acid. Therefore, the antitumor activity and the reduction in number of metastastic nodules to the liver by carp extract could not be explained by the unsaturated fatty acids in carp extract. Rather, in the present study, it was elucidated that the contents of amino acids and unidentified substances in carp extract were greater than those of fatty acids. Further work is needed to identify which of the amino acids or the other substances in carp extract is active and to clarify the mechanism of antitumor and antimetastatic activities.

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## 和文抄録

鯉は韓国，中国および日本において，健康食素材として使用されている。中国の医方書には，鯉は利尿薬，眼精疲労として食されていることが記載されている。鯉エキスには原発腫瘍や肝臓への転移癌に対して効果があると言われているが，その伝聞は明らかでない。現在の研究において，ルイス肺癌細胞移植マウスにおける鯉エキスの抗腫瘍および抗転移効果を検討した。脾臓内に移植した癌細胞の増殖および肝臓への癌転移は鯉エキス

250mg/kg および 500mg/kg の 20 日間の連続経口投与によって抑制された。一方，鯉エキスはルイス肺癌細胞に対する直接的な細胞毒性を示さなかった。鯉エキス中には脂質および不飽和脂肪酸含有量が少ないことから，鯉エキスの抗腫瘍および肝臓への LLC 細胞の転移コロニー数に対する抑制効果は，脂質や不飽和脂肪酸で説明することが出来ない。さらに，鯉エキスの活性成分の同定が必要である。

*〒791-0295 愛媛県温泉郡重信町大字志津川
愛媛大学医学部医化学第二教室 木村善行

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