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# Choline-deficient-diet Decreases Fibroblasts in the Circulating Tumor Cell (CTC) Microenvironment

KANA MATSUURA[1], ATSUSHI SUETSUGU[1,2,3], TOMOYUKI SATAKE[1], MIKI NAKAMURA[1], TAKAHIRO KUNISADA[1], MASAHITO SHIMIZU[1] and ROBERT M. HOFFMAN[2,3]

[1]*Gifu University Graduate School of Medicine, Gifu, Japan;*
[2]*AntiCancer, Inc., San Diego, CA, U.S.A.;*
[3]*Department of Surgery, University of California, San Diego, CA, U.S.A.*

**Abstract.** *Background/Aim: Circulating tumor cells (CTCs) may have an important role in metastasis. CTC clusters, which contain fibroblasts, indicate poor prognosis. In the present study, we used our malignant lymphoma metastatic mouse model to compare the effect of a choline-deficient-diet (CDD) and the control diet (CD) on fibroblasts in CTCs. Materials and Methods: We compared the number and morphology of CTCs in CDD and CD mice using color-coded imaging with fluorescent proteins. Malignant lymphoma EL4 cells expressing RFP were injected in the spleen of transgenic C57B/6-GFP mice, which were fed a CDD or CD. Two weeks later, we harvested and observed the number of CTCs and fibroblast-like cells both in heart blood and portal blood. Imaging of CTC morphology was performed with smeared glass slides and in culture. Results and Conclusion: There was no significant difference in the number of CTCs between CDD and CD mice. The number of fibroblast-like cells in the CTC microenvironment in CD mouse portal blood was significantly larger than in CDD mouse portal blood. These differences may be caused by deficiency in choline that leads to less metastasis in choline-deficient-diet-induced fatty liver.*

Circulating tumor cells (CTCs) were discovered in the 19th century (1). CTCs have an important role in metastasis and indicate poor cancer prognosis (2). Large numbers of CTCs indicate extremely poor prognosis. CTC clusters which comprise multiple cell types such as fibroblasts may have higher metastatic potential (3-7). We have investigated the mechanism of liver metastasis in fatty liver, and showed that fewer metastases and cancer-associated fibroblasts (CAFs) occurred in choline-deficient-diet-induced fatty liver (8).

We have previously developed a syngeneic mouse model of malignant lymphoma using murine EL-4 lymphoma cells expressing red fluorescent protein (RFP) implanted in transgenic mice expressing green fluorescent protein (GFP), and described the color-coded imaging of the CTC microenvironment (8-12).

In the present study, using the EL4 mouse model of metastatic lymphoma, the fibroblasts present in the CTC microenvironment were compared between mice fed a choline-deficient-diet (CDD) and control diet (CD), and analysed by color-coded imaging.

## Materials and Methods

*Cell line and culture conditions*. EL4, mouse malignant lymphoma cells, were engineered to stably express red fluorescent protein (RFP) as previously reported (9-11). The cells were maintained in RPMI 1640 medium (Gibco-BRL, Grand island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin (Gibco-BRL). The cells were cultured in a humidified atmosphere containing 5% $CO_2$ at 37˚C.

*GFP transgenic mice*. Transgenic C57B/6-GFP mice were obtained from the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan). The C57B/6 mice expressed the *Aequorea victoria* GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer (13). Eight to ten-week-old C57B/6-GFP transgenic mice were used as the host. Fatty liver was generated in mice (n=9) on a choline-deficient-diet (CDD) for 4-12 weeks. Another group of mice (n=9) were provided with a control diet (CD) for 4-12 weeks until the end of the experiment. Diets were purchased from Oriental Yeast Co. Ltd., Tokyo, Japan.

*Mice*. In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were



*Correspondence to:* Atsushi Suetsugu, MD, Ph.D., Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu, Japan, 1-1 Yanagido, Gifu 501-1194, Japan. Tel: +81 582306308, Fax: +81 582306310, e-mail: asue@gifu-u.ac.jp; Robert M. Hoffman, Ph.D., AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111, U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, e-mail: all@anticancer.com

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anesthetized by subcutaneous injection of a 0.02 ml solution of 20 mg/kg ketamine. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. Animals were housed in a barrier facility on a high efficiency particulate arrestance (HEPA)-filtered rack under standard conditions of 12-h light/dark cycles (12).

*Malignant lymphoma metastasis models*. Murine malignant lymphoma cancer cells ($2.0\times10^6$) were injected in the spleen of mice on a CDD or CD. All mice were sacrificed on day 14, and analysed for primary tumor and liver metastasis (8-10, 12).

*CTC collection and culture (Table I, Figure 1)*. Blood (0.2 ml) was obtained from C57B/6-GFP mice from the portal circulation and heart puncture, two weeks after the injection of EL4 lymphoma cells. A drop of blood was smeared on a glass slide and covered with a cover slip. CTCs were detected by confocal microscopy. Cells in the blood were cultured on plastic dishes with the above described method.

*Tumor imaging*. The SZX7 microscope and FV1000 confocal microscope (Olympus Corp. Tokyo, Japan) were used for imaging.

*Study approval*. All experiments were conducted in accordance with the Institutional Guidelines of Gifu University and were approved by the Animal Research Committee and the Committee on Living Modified Organisms of Gifu University.

## Results and Discussion

RFP expressing EL4 lymphoma cells were injected in the spleen of CD and CDD mice. Two weeks later, we performed laparotomy to obtain portal blood (0.2 ml), and also withdrew heart blood by cardiac puncture (0.2 ml). After that mice were sacrificed and the spleen and liver were examined for the presence of primary tumor and metastases, respectively. There was a tendency for CD mice to have more liver metastases than CDD mice, but there was no significant difference. The primary tumor in the spleen of CD and CDD mice had no difference in size (Figure 2).

CTCs and CTC clusters were observed in blood smears on glass slides shortly after sacrifice and also incubated in culture for several days. There was no significant difference in the number of CTCs between CD and CDD mice (Table I). However, there was a significant difference between CD and CDD mice in the number of fibroblast-like cells in the CTC microenvironment from portal blood culture (Table I, Figures 1, 3). In heart blood, few fibroblast-like cells were observed in the CTC microenvironment.

As previously reported, CTC clusters, which contain fibroblasts, may enhance metastasis and the fibroblasts may confer vascular specificity (2-7). Because choline is a precursor of methionine, lower methionine levels in CDD mice may inhibit methionine metabolism which is necessary for metastasis, and may also disturb migration of fibroblasts (14-19).

Table I. *CTC and fibroblast-like cell counts from heart blood and portal blood, which were harvested from CD and CDD mice. The median of red fluorescent protein (RFP)-expressing cells (CTCs) in 10 μl blood from heart or portal circulation and total green fluorescent protein (GFP)-expressing fibroblast-like cells remaining on the dishes after a 7-day blood culture of EL4 lymphoma-bearing CD and CDD mice are shown.*

A

| | Number of RFP expressing cells (CTCs) |
|---|---|
| CD heart blood | 308 |
| CDD heart blood | 208 |
| CD portal blood | 634 |
| CDD portal blood | 450 |
| | ($\times10^4$ cell/10μl blood) |

B

| | Number of GFP fibroblast-like cells/field |
|---|---|
| CD heart blood | 2.0 |
| CDD heart blood | 2.25 |
| CD portal blood | 12.04 |
| CDD portal blood | 7.44 |

CD: Control diet; CDD: choline deficient diet.

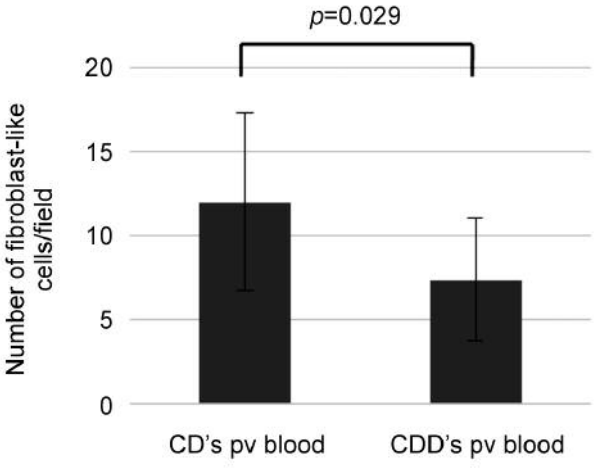


Figure 1. *Fibroblast-like cells in the CTC microenvironmant in portal vein blood. CD: Control diet; CDD: choline deficient diet.*

## Conclusion

In our previous study, the fewest metastases and cancer-associated fibroblasts were observed in mice with EL4 lymphoma fed choline-deficient-diet which induced fatty liver (8). The present study showed that portal blood of CDD mice had the fewest fibroblast-like cells in the CTC microenvironment. This may be caused by re-programmed

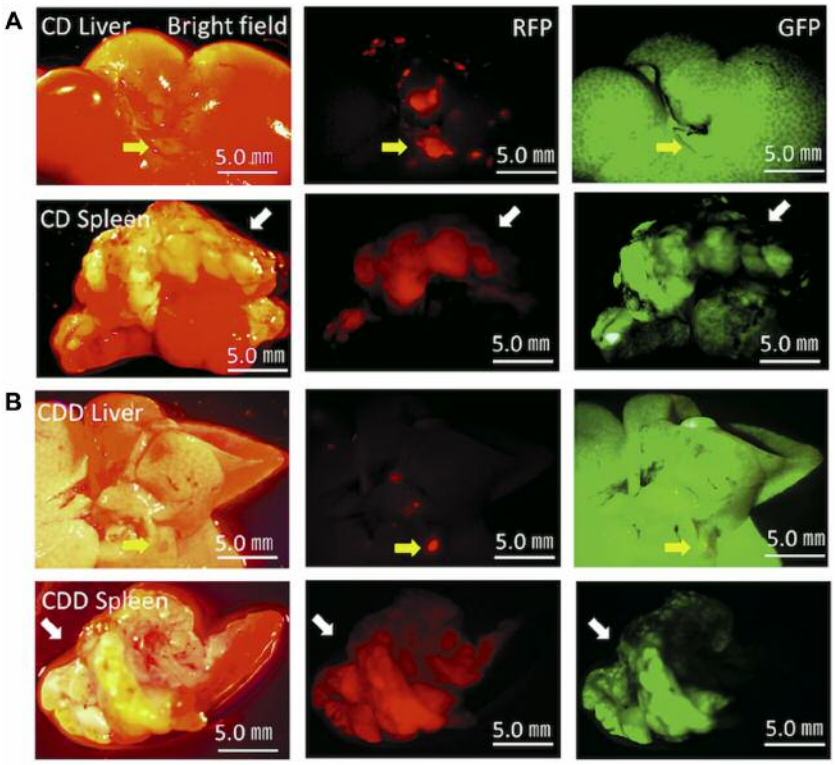


Figure 2. *Bright-field and fluorescence images of liver and spleen. RFP-expressing EL4 metastases were observed in the liver and spleen of CD and CDD mice. Yellow arrows indicate liver metastases. White arrow indicates primary tumor in the spleen. Images were obtained with the SZX7 microscope. (Bar=5 mm). CD: Control diet; CDD: choline deficient diet.*

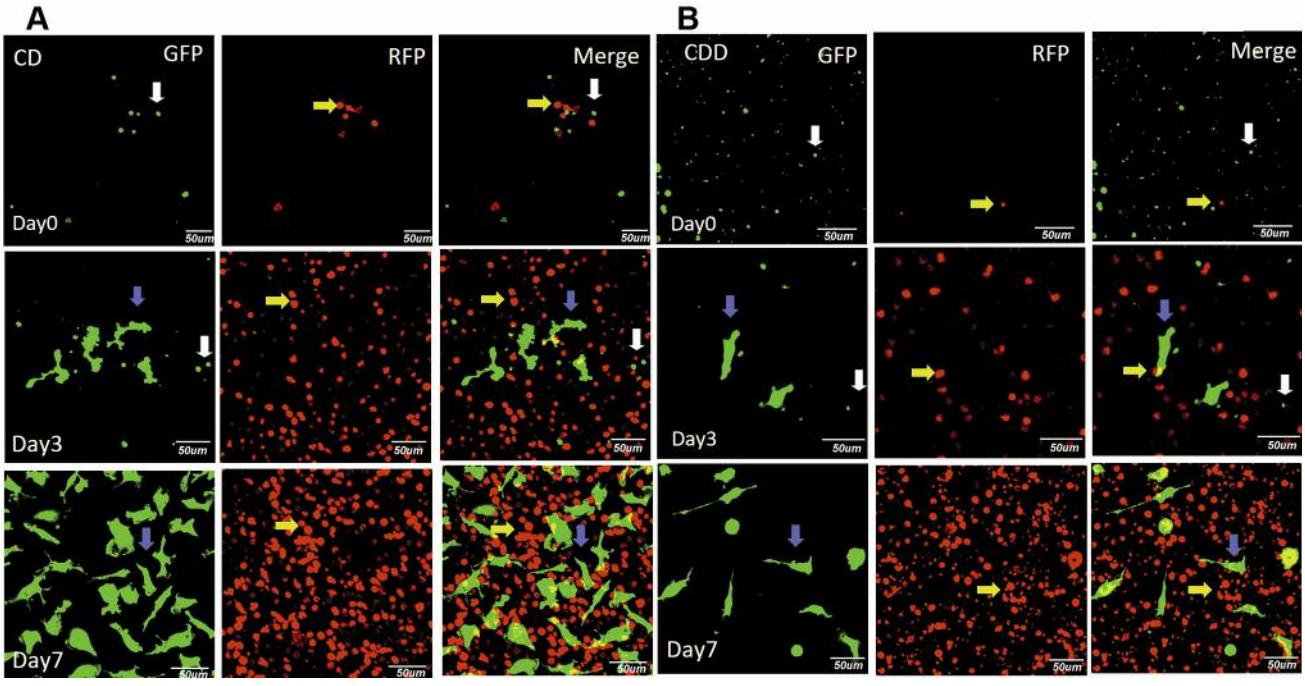


Figure 3. *Representative images of cultured portal blood cells harvested from CD and CDD mice. (A) High-magnification images of cells cultured from portal blood from CD mice. Yellow arrows indicate EL4 cancer cells. White arrows indicate blood cells. Blue arrows indicate fibroblasts in the CTC TME. (Bar=50 μm). (B) High-magnification images of cells cultured from portal blood from CDD mice. Yellow arrows indicate EL4 cancer cells. White arrows indicate blood cells. Blue arrows indicate fibroblasts in the CTC TME. (Bar=50 μm). CD: Control diet; CDD: choline deficient diet.*

methionine metabolism, which may have been the result of deficiency in choline (14-19).

## Conflicts of Interest

None of the Authors have any conflict of interest in regard to this study.

## Authors' Contributions

Kana Matsuura and Atsushi Suetsugu designed the study. Kana Matsuura, Tomoyuki Satake and Miki Nakamura participated in the animal experiments and collected data. Kana Matsuura wrote the initial draft of the manuscript. Atsushi Suetsugu and Takahiro Kunisada contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. Masahito Shimizu and Robert M. Hoffman critically reviewed the manuscript. All Authors approved the final version of the manuscript.

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