Fatty Acids Induce Stemness in the Stromal Cells of a CT26 Mouse Tumor Model

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Keywords

Fatty acid · Cancer stroma · Stromal stem cell

Abstract

The potential effects of 2 types of fatty acids on colorectal cancer (CRC) were assessed using cancer stromal cells. Linoleic acid (LA; C-18, n-6 unsaturated fatty acid) and elaidic acid (EA; C-18, *trans* acid), both known to affect colon carcinogenesis and cancer progression, were administered by gavage to BALB/c mice, which were inoculated with CT26 syngeneic colon cancer cells in the back. Both EA and LA treatments enhanced tumor growth and metastasis. EA and LA also increased the number of CD133-positive stromal cells in the tumor capsule. Importantly, those cancer cells at the tumor periphery, physically attached to CD133-positive stromal cells, also expressed CD133. These findings suggest that EA and LA might induce stemness in cancer cells through physical association and promote cancer metastasis.

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Introduction

Cancer stroma is consistent with several types of stromal cells and extracellular matrices, which are associated with cancer cells. Fibroblasts, myofibroblasts, and mesenchymal stem cells (MSC) are a type of cancer stromal cells. Especially, MSC have been known to play an important role in cancer progression. MSC activated by TGF- β transdifferentiate to carcinoma-associated fibroblasts, which secrete chemokine-like bioactive substances that enhance metastasis [1]. MSC induce stemness in cancer cells via secretion of CXCL12 or SDF-1 [2–4]. MSC also inhibit the proliferation of T lymphocytes [5] and induce regulatory T lymphocytes [6–8] to suppress antitumor immunity. Interaction with MSC causes an increase in the metastatic potential of cancer cells. Thus, the number of MSC in cancer stroma is closely associated with the malignant phenotype of cancer cells.

Fatty acids possess a physiological activity that affects cancer cells. Linoleic acid (LA) is an n-6 unsaturated fatty acid with an 18-carbon chain. The procarcinogenic effects of LA on colorectal cancer (CRC) are well known. These include induction of chronic inflammation by the production of prostaglandin E2 [9] or secretion of high-

Fig. 1. Effect of elaidic acid (EA) and linoleic acid (LA) on the growth of CT26 subcutaneous (s.c.) tumors. **a** BALB/c mice were treated with either EA or LA (1 mg/mouse) or vehicle (V; 70% ethanol) once a week by gavage administration. CT26 cells were inoculated into the subscapular subcutaneous tissue. Body weight (**b**), tumor weight (**c**), and number of metastatic nodes in the neck (per mouse) (**d**) were examined 4 weeks after inoculation. * $p < 0.05$.

mobility group box-1 [10]. LA is also reported to inhibit peritoneal metastasis and in vitro transformation [11]. Persistent treatment with LA induces dormancy of subcutaneous tumors in mice, which is thought to be associated with a low proliferation of cancer stem cells (CSC) [10, 12].

Trans fatty acids (TFA) are mainly generated by industrial hydrogenation and they are used as food constituents, especially in margarine and shortening [13]. TFA consumption is a well-known dietary risk factor for cardiovascular diseases such as atherosclerosis and myocardial infarction [14, 15], since it increases the levels of low-density lipoprotein-cholesterol and decreases the levels of high-density lipoprotein-cholesterol [16]. In 2015, the FDA issued a final decision that there is no consensus that industrially produced TFA are generally recognized as safe for any use in human food, and that *trans* fat must be removed from prepared foods within 3 years from this decision, i.e., by June 2018 [17]. TFA are associated with carcinogenesis [18–20]. Moreover, cancer metastasis [21, 22] and drug resistance [23] are promoted by elaidic acid (EA), a major component of dietary TFA [24], through the enhancement of stemness and epithelial-mesenchymal transition [22, 23].

In the present study, we aimed to assess the effect of EA on cancer stromal cells in a mouse subcutaneous tumor model.

Materials and Methods

Cells and Reagents

The CT26 mouse colon cancer cell line was a kind gift from Prof. I.J. Fidler (MD Anderson Cancer Center, Houston, TX, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA).

Animals

Four-week-old male BALB/c mice were purchased from SLC Japan (Shizuoka, Japan). The animals were maintained in accordance with institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University, following the current regulations and standards of the Japanese Ministry of Health, Labor, and Welfare (approval No. 9559).

Animal Models

To establish a subcutaneous tumor model, CT26 cancer cells (1×10^7) were inoculated into the scapular subcutaneous tissue of BALB/c mice. Mice were observed for 4 weeks following inoculation. Mice were fed a CE-2 diet (containing 5% crude fat

Fig. 2. Effect of elaidic acid (EA) and linoleic acid (LA) on the stemness of CT26 tumors. The expression of nucleostemin (NS) and CD133 in the tumors was examined by immunohistochemistry. Scale bar, 50 μ m. **b, c** Number of NS-positive and CD133-positive cells, respectively, out of 500 cells counted. **d** Relative mRNA levels of NS in mice ($n = 3$). Error bars represent SD. * $p < 0.05$. V, vehicle; ig., intragastric gavage administration.

derived mainly from soybean oil; CLEA Japan, Inc., Tokyo, Japan). EA (1 mg/mouse; Sigma) or LA (1 mg/mouse; Wako) diluted with vehicle (70% ethanol) was administered by gavage every 7 days.

Immunohistochemistry

Consecutive 4- μ m sections were immunohistochemically stained using the immunoperoxidase technique, as described previously [25]. Antibodies against mouse CD133 (Proteintech, Rosemont, IL, USA), and nucleostemin (NS; Santa-Cruz Biotechnology, Inc., Dallas, TX, USA) were used at 0.5 μ g/mL. Color development was achieved using 3-3'-diaminobenzidine (DAKO,

Carpinteria, CA, USA). Specimens were counterstained with Meyer's hematoxylin stain (Sigma) to visualize the nuclei. After immunostaining, the number of positively stained nuclei was counted under a microscope on all slides.

For immunofluorescent staining, primary antibodies were labeled with fluorescent dye by using the specific labeling kit for each dye (ThermoFisher Scientific, Yokohama, Japan). Antibodies for CD133 (Proteintech), CD73 (Abnova Corp., Taipei City, Taiwan), and AE3 (Santa-Cruz) were labeled with Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 350, respectively. DAPI (Santa-Cruz) was used for nuclear staining. The images were captured using an all-in-one microscope (Keyence, Osaka, Japan).

Fig. 3. Effect of elaidic acid (EA) and linoleic acid (LA) on the interaction between stromal and cancer cells. **a** The expression of CD133 at the tumor periphery was examined by immunohistochemistry. Scale bar, 50 μ m. EV, enlarged view. Scale bar, 20 μ m. FI, immunohistochemistry with CD133 (stem cell marker; Alexa Fluor 488, green), CD73 (bone marrow mesenchymal cell marker; Alexa Fluor 568, red), AE3 (epithelial cell marker; Alexa Fluor 350, blue), and DAPI (nuclear marker). **b** Number of CD133-positive stromal cells out of 200 cells counted. **c** Number of CD133-positive cells at the tumor periphery out of 200 cells counted. * $p < 0.05$. HPF, high-power field.

Reverse Transcription-Polymerase Chain Reaction

Total RNA (1 μ g) was synthesized using a PAX gene Blood RNA Kit (Qiagen, Valencia, CA, USA). Blood was collected by cardiocentesis after anesthesia from 3 mice in each group. Polymerase chain reaction (PCR) products were confirmed on 2% agarose gel and visualized with ethidium bromide. The following primer sets (Sigma Genosys, Ishikari, Japan) were used for amplification: mouse NS (*Gln3*, accession No. AY181025.1): forward 5'-CAG GAT GCT GAC GAT CAA GA-3', reverse 5'-TTG ATT GCT CAG GTG ACA GC-3', and mouse β -actin (*ActB*, accession No. NM_007393.4): forward 5'-AGC CAT GTA CGT AGC CAT CC-3', reverse 5'-CTC TCA GCT GTG GTG GTG AA-3'. PCR conditions were set in accordance with the manufacturer's instructions.

The quantitative reverse transcription (RT)-PCR was performed using QuantiTect Primer Assays (Qiagen) and the ready-designed primers following the manufacturer's instructions. The assay is based on the detection of RT-PCR products by SYBR green.

Statistical Analysis

Statistical significance was calculated using unpaired Student *t* tests with InStat software (GraphPad, Los Angeles, CA, USA). Statistical significance was defined as a 2-sided *p* value < 0.05 .

Results

Effect of EA and LA on the Growth of CT26 Subcutaneous Tumors

CT26 cells were inoculated into the subscapular subcutaneous tissue of syngeneic BALB/c mice, which were also administered either EA or LA (Fig. 1a). No statistical difference was observed in the weight of mice among the LA, EA, and control groups (Fig. 1b). Tumor weight and the number of nodal metastases were highest in the EA group, followed by the LA group (Fig. 1c, d).

Effect of EA and LA on the Stemness of CT26 Tumors

The effects of LA and EA on the stemness of CT26 cells were assessed by examining NS and CD133 expression (Fig. 2a–c). The number of NS-positive cells was highest in the EA group, followed by the LA group. In contrast, the number of CD133-positive cells was highest in the LA group, followed by the EA group.

NS mRNA was detected by RT-PCR in the blood of mice treated with EA as well as in that of mice treated with LA. EA treatment increased NS mRNA in the blood 12 h

after gavage; however, administration of LA or vehicle did not alter NS mRNA levels in the blood.

Effect of EA and LA on the Interaction between Stromal and Cancer Cells

Immunohistochemistry of the tumors showed that CD133-positive stromal cells surrounded the tumor periphery (Fig. 3a). The number of CD133-positive stromal cells was highest in the LA group, followed by the EA group (Fig. 3b). Interestingly, both the LA and EA groups showed CD133-positive stromal cells attached to cancer cells expressing CD133 at the tumor periphery (Fig. 3a, enlarged view). Most of the stromal cells expressed CD73, a bone marrow mesenchymal cell marker. Multifluorescent immunohistochemistry showed that CD133+/CD73+ stromal cells increased with an increase in CD133+/AE3+ cancer cells via treatment with LA or EA. In contrast, a physical association between cancer and stromal cells was not found in the vehicle group. The number of CD133-positive cancer cells at the tumor periphery was higher in the LA and EA groups than in the vehicle group (Fig. 3c).

Discussion

In the present study, we examined the effects of LA and EA, which possess reactivity to CSC, on tumor stromal cells. The most important finding is that both LA and EA enhanced the stemness of stromal cells as well as the physical attachment between cancer cells and stromal cells.

We showed that treatment with LA and EA promoted tumor growth and nodal metastasis. The prometastatic effect of LA and EA could be explained by the enhancement of stemness in cancer cells. Moreover, our data showed that LA and EA possessed reactivity to cancer stromal cells. LA and EA treatment increased the number of CD133-positive stromal cells in the tumors. These cells are compatible with MSC, which might have originated from bone marrow-derived MSC or induced topical MSC.

CT26 mouse colon cancer cells express NS and CD133; however, each positivity is different. NS is expressed in many cells, whereas CD133 is expressed in a few cells. NS expression is closely associated with CD133 in breast cancer cells [26]. The difference in positivity between NS and CD133 might be reflected by their recognition of the range of stemness. Our previous data suggest that NS recognized CSC, progenitor cells, and proliferating cells, whereas CD133 recognized CSC [21]. The increase in NS-positive cancer cells might be associated with proliferative stem cells, which are suspected to possess a high met-

astatic potential. Moreover, LA upregulates CD133, whereas growth inhibition is provided from the activation of PPAR γ [27]. LA metabolites act as ligands of PPAR γ . These differences between LA and EA affect their capacity to promote metastability.

Cell-to-cell attachment between stromal cells and cancer cells at the tumor periphery was observed in mice treated with LA and EA. This interaction is known to promote the metastatic ability of cancer cells by causing an increase in cancer stemness [2–4], acquisition of the epithelial-mesenchymal transition phenotype via secretion of CXCR4, SDF-1, or TGF- β from stromal stem cells [28], induction of regulatory T cells by stromal stem cells [2–4], fusion of cancer cells with stromal stem cells [29], and multicellular sphere formation of cancer cells and stromal cells [30].

The findings of the current study are consistent with those of our previous studies, which showed that metastatic potential is located at the tumor periphery [31, 32]. We previously demonstrated that cancer cells at the tumor periphery express metastasis-associated genes, such as EGFR and MMP-2/-9, and repress E-cadherin [31, 32], which might be triggered by CSC induction at the tumor periphery.

In this study, we showed that fatty acids affect mesenchymal cells in cancer stroma; however, our data suggest that fatty acids exert direct bioactive effects on cancer stromal cells, which might be considered a CSC niche, in which interactions between cancer cells and stromal cells increase stemness in both types of cells. The precise mechanism underlying the enhancement of stemness through the cancer-stroma physical interaction is a subject of further study.

Acknowledgements

The authors thank Ms. Tomomi Masutani for her expert assistance with the preparation of this paper. This work was supported by MEXT KAKENHI grant No. 16675788.

Disclosure Statement

We declare that there is not any financial support or relationships which may pose a conflict of interest in the contents of the submitted manuscript. All authors have approved the comments.

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