Abstract. Trans fatty acids (TFAs) are risk factors of cardiovascular disorders, and a few studies have reported the cancer-promoting effects of TFAs. In the present study, we examined the effects and signaling of elaidic acid (EA), a TFA, in colorectal cancer (CRC) cells. Oral intake of EA increased the metastasis of CT26 mouse CRC cells by inducing the expression of stemness markers nucleostemin (NS) and CD133. Mechanisms underlying EA-induced signaling were confirmed by determining the binding of EA to G-protein coupled receptor 40 (GPR40) and GPR120 by performing surface protein internalization assay. We found that c-SRC mediated EGFR transactivation was induced by the binding of EA to G-protein coupled receptor 40 (GPR40) and GPR120. Moreover, EGFR signaling upregulated NS and Snail expression and downregulated E-cadherin expression in wild-type APC-containing CT26 cells, and upregulated NS, Wnt5a and CD44 expression in APC-null HT29 cells. These results indicate that EA enhances the stemness and epithelial-mesenchymal transition of CRC cells. These results also indicate the prominent metastatic potential of EA-treated cancer cells and highlight the important implications of EA on public health.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in Japan (1). The number of patients with CRC and the number of deaths due to CRC have increased in Japan because of the popularity of the western lifestyle (2,3). Some dietary ingredients, such as fatty acids, are risk factors for CRC (4). Linoleic acid (LA) increases the risk of colorectal carcinogenesis by generating prostaglandin E2, which induces chronic persistent inflammation of the mucosa (5). LA also induces dormancy in CRC cells (6).

Trans fatty acids (TFAs) have been evaluated as a possible CRC risk for decades (7); however, recent studies have clearly demonstrated that TFAs are associated with colon carcinogenesis. Patients who do not use non-steroidal anti-inflammatory drugs and postmenopausal women showed higher CRC risks from TFAs (8). Compared with patients in the lowest quartile of TFA consumption, those in the highest quartile showed an increased odds ratio of 1.86 for the prevalence of colorectal adenomas (9). Moreover, elaidic acid (EA), the major TFA (10), is increased in the plasma of colon adenoma patients (11). Additionally, EA promotes colon carcinogenesis in Min mice (12) and induces the proliferation of Ehrlich ascites sarcoma cells (13). Thus, TFAs, particularly EA, possess carcinogenic properties; however, the related intracellular signals have not been fully determined.

Long-chain fatty acids (LCFAs) bind to specific membrane-bound receptors, namely G-protein coupled receptor 40 (GPR40) and GPR120 (14). GPR40 and GPR120 bind to various LCFAs. EA is an LCFA containing 18 carbon chain with a trans configuration and is a structural isomer of oleic acid (OA). OA also binds to GPR40 and 120, which is coupled with Gq, to stimulate calcium ion influx and inositol phosphate synthesis in smooth muscle cells (15), inducing smooth muscle contraction. OA inhibits c-Jun N-terminal kinase and nuclear factor κB signaling (16), which suppress tumor necrosis factor α-induced insulin resistance and inflammation.

However, no information is available regarding EA receptors. EA is a well-known dietary risk factor of cardiovascular disorders such as atherosclerosis and myocardial infarction (17,18). OA is present at low concentrations in CRC tissues (19), whereas EA is increased in the plasma of colon adenoma patients (11). OA exerts protective effects against...
cardiovascular disorders by decreasing low-density lipoprotein cholesterol levels (20). In contrast, EA exerts proinflammatory effects (16,21), suggesting the presence of different intracellular signaling pathways for EA and OA.

In the present study, we examined the effect of EA on the metastasis of CRC cells and determined the receptors and signaling pathways of EA to explain its role in CRC.

Materials and methods

Cell culture and reagents. Mouse colon cancer cell line CT26 was kindly provided by Dr I. J. Fidler (MD Anderson Cancer Center, Houston, TX, USA) and human colon cancer cell line HT29 was purchased from Dainihon Pharmacy, Co., Ltd., (Tokyo, Japan). Cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) in 5% CO2 at 37°C. Cell morphology was evaluated daily by performing microscopic examination. Each cell line was routinely tested for mycoplasma contamination by performing genomic PCR. Before manuscript submission, viability of each cell line was tested by performing trypan blue exclusion assay.

EA (CAS no. 112-79-8; Wako Pure Chemical Industries Ltd., Osaka, Japan), OA (CAS no. 112-80-1; Wako Pure Chemical Industries), methyl-β-cyclodextrin (MbCD; Sigma-Aldrich), human epidermal growth factor (EGF; Peprotech EC Ltd., London, UK), PPI (SRC inhibitor; Sigma-Aldrich), Wort inhibitor (IWP3; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) (22), ERK inhibitor (3-[2-aminoethoxyphenyl]-5-[(4-ethoxyphenyl)methylene]-2,4-thiazolidinedione; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p38 inhibitor (SB 203580; Abcam, Cambridge, MA, USA) were purchased.

Assessment of cell growth and apoptosis. The cells (1x10^7/well) were seeded in a 12-well dish. Cell growth was assessed by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or by counting the number of cells with autocytometer (Sysmex, Kobe, Japan) after 48 h, as previously described (23). Apoptosis and necrosis were assessed using an apoptosis/necrosis detection kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). Annexin V-EnzoGold and 7-AAD present in the kit fluorescently label apoptotic and necrotic cells, respectively. Apoptosis or necrosis was determined by examining 2000 cells under a fluorescent microscope (Nikon, Tokyo, Japan). PCR products obtained were electrophoresed on a 2% agarose gel and stained with ethidium bromide. β-actin (ACTB) mRNA (GenBank accession no. NM_001101) was amplified for use as an internal control.

Immunohistochemical analysis. Cell spheres formed were fixed in 10% buffered formalin for 12 h at 4°C and were embedded in 1% agar. The agar specimens were processed into a paraffin-embedded block and 4-µm-thick sections were cut using a microtome. Consecutive tissue sections were immunohistochemically stained by following the immunoperoxidase technique, as previously described (24). Immunoblotting analysis was performed using 0.2 µg/ml secondary antibodies (Medical and Biological Laboratories, Co., Ltd., Nagoya, Japan). Color development was performed using diamine benzidine hydrochloride (Dako) and counterstaining was performed using Mayer’s hematoxylin (Sigma-Aldrich). The number of NS- or MIB1-positive nuclei were counted and expressed as the mean number of positive nuclei from 50 high-power fields of tumor specimens.

Animal models. BALB/c male mice (4-weeks old) were purchased from Japan SLC (Shizuoka, Japan). The mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University and the current regulations and standards established by the Ministry of Health, Labor and Welfare. Each experimental group included five mice.

A subcutaneous tumor model was established by inoculating CT26 cells (1x10^5 cells) suspended in Hank’s balanced salt solution (Sigma-Aldrich) into the scapular subcutaneous tissue of the mice, which were fed with standard diet (CE-2; CLEA Japan, Tokyo, Japan). The fatty acid composition did not contain TFAs). For analyzing cancer metastasis to the liver, lungs and peritoneum, cancer cells (1x10^5 cells) were inoculated into the spleen, tail vain and peritoneal cavity, respectively. EA or OA (10 mg/mouse) was administered by gavage every 7 days, and metastatic status was assessed after 4 weeks. The dosage of 10 mg/mouse/week (equivalent to 0.5 g/kg body weight/week) is approximately equal to 0.53 g/kg body weight/week, which is according to the Nutrition Facts label of foods 4.6 g per day (25). Peritoneal metastasis was determined by measuring the weight of the mesenterium with tumors, which was separated from the intestinal tube. The liver and lung metastases were determined by counting the number of tumors on organ surface.

Western blot analysis. Whole-cell lysates were prepared as previously described (24). Cell fractions were extracted...
by processing the cells with Cell Fractionation kit (Abcam), according to the manufacturer's instruction.

Proteins (50 µg) present in cell lysates were separated by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% gels and were electrotransferred onto nitrocellulose membranes for immunoblotting analysis. The membranes were incubated with primary antibodies, followed by incubation with peroxidase-conjugated IgG antibodies (MBL). Tubulin antibody (Proteintech Group, Inc., Rosemont, IL, USA) was used to measure the amount of protein loaded per lane. Immune complexes were visualized using CSA system (Dako). Antibodies against GPR40 and GPR120 (Abnova Corp., Taipei City, Taiwan); EGFR, E-cadherin (ECD) and β-catenin (Transduction Laboratories, Lexington, KY, USA); phosphorylated EGFR, NS, phosphorylated ERK1/2 (pThr202), phosphorylated SRC (pTyr530), Notch1, APC, Wat5α and CD44 (Santa Cruz Biotechnology); phosphorylated p38 (pThr180/pTyr182; Cusabio, College Park, MD, USA); CD33 and Snail1 (pSer246) (Biorbyt, Cambridge, UK); and phosphorylated β-catenin (pSer33/37; LifeSpan Biosciences, Inc., Seattle WA, USA) were used as primary antibodies.

Small interfering RNA. Stealth Select RNAi oligonucleotides (siRNAs) against human and mouse GPR40 and GPR120 and against mouse EGFR were purchased from Santa Cruz Biotechnology. AllStars Negative Control siRNA was used as a control (Qiagen, Valencia, CA, USA). Cells were transfected with 20 nM siRNA by using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's recommendations.

Surface expression assay. Surface expression assay was performed to determine the effect of agonist-mediated GPR internalization. Briefly, monolayer-cultured CT26 cells in a 96-well dish were preincubated with peroxidase-labeled antibody against GPR40 or GPR120, followed by treatment with EA, OA (35 µM) or vehicle. At each time-point, the cells were counted by performing the MTT assay and were fixed in 4% paraformaldehyde for 15 min at room temperature. Surface levels of GPRs were measured by performing tetramethylbenzene reaction, followed by treatment with H₂SO₄ and measurement of absorbance at 450 nm.

Statistical analysis. Statistical analyses of experimental data were performed by unpaired Student's t-test and one-way ANOVA with post hoc test. Normal distribution of the data samples was confirmed by the Kolmogorov-Smirnov normality test. A two-sided P<0.05 was considered statistically significant.

Results

EA enhances metastasis. As shown in Fig. 1, CT26 mouse CRC cells were inoculated into the subcutaneous tissue (Fig. 1A), spleen (liver metastasis) (Fig. 1B), tail vein (lung metastasis) (Fig. 1C) and peritoneum (Fig. 1D) of BALB/c mice by gavage administration with EA, OA, or control vehicle. EA-treated mice showed higher growth of subcutaneous tumors and metastases to the liver, lungs and peritoneum than control mice. In contrast, OA-treated mice only showed increased peritoneal metastasis compared with the control mice. Tumors of EA-treated mice showed increased mRNA expression of CD133 and NS (Fig. 1E). Moreover, the number of NS-positive tumor cells increased in the tumors of EA-treated mice but not...
Figure 2. Intracellular signaling of EA. (A) EGFR, phosphorylated EGFR, ERK1/2 and p38 phosphorylation in EA-treated CT26 cells. (B-D) Effect of EA or OA (70 µM) on EGF-induced EGFR, ERK1/2, p38 and SRC phosphorylation in CT26 and HT29 cells was examined. EGF (10 ng/ml) was added to increase the basal phosphorylation levels of EGFR. (B) Western blot analysis. Tubulin served for an internal control. (C and D) Semi-quantification of the results of western blot analysis by performing densitometric scanning. Each bar represents mean ± SD of three independent experiments. *Statistical difference from EGF alone was calculated by the unpaired Student’s t-test.

Figure 3. Receptors of EA. (A) Protein expression of long chain fatty acid receptors GPR40 and GPR120 in CRC cells transfected with control siRNA (Csi) or siRNA against the gene encoding GPR40 or GPR120. (B) Effect of the knockdown of genes encoding GPR40 and/or GPR120 on the number of sphere-forming cells. Error bar, SD of three independent experiments. *Statistical difference between GPR40 and GPR120 was calculated by the unpaired Student’s t-test. (C and D) Surface expression assay for assessing the internalization of GPR40 and GPR120 in CRC cells treated with EA or OA (70 µM). Error bar, SD. *Statistical difference from control was calculated by the unpaired Student’s t-test. (E and F) Effect of SRC inhibitor (PP1, 0.05 µM) and knockdown of the gene encoding GPR40 (si40) or GPR120 (si120) on EA-induced EGFR phosphorylation (70 µM) in CRC cells. EGF (10 ng/ml) was added for increasing the basal phosphorylation levels of EGFR. Tubulin was used as an internal control.
in the tumors of control or OA-treated mice (Fig. 1F and G). These results suggest that EA increases the stemness of CT26 cells to enhance their metastatic potential.

**Intracellular signaling pathways of EA.** Next, we examined the intracellular signals of EA (Fig. 2). EA treatment induced the phosphorylation of EGFR, ERK1/2 but not p38 (Fig. 2A). The effects of EA and OA on EGFR activation were compared (Fig. 2B-D). Phosphorylation of EGFR and ERK1/2 induced by OA was less pronounced than that induced by EA in both CRC cells. In contrast, phosphorylation of p38 induced by OA was more pronounced than that induced by EA. Importantly, EA treatment induced SRC phosphorylation at higher levels than those by OA treatment in both cell types.

**Receptor of EA.** GPR40 and GPR120 function as receptors of long chain fatty acids (26). We observed that CRC cells expressed different levels of GPR40 and GPR120 (Fig. 3A) and that CT26 cells showed higher GPR120:GPR40 ratio (5.3) than HT29 cells (1.2).

We knocked down genes encoding GPR40 and/or GPR120 and examined the effects of gene knockdown on the number of sphere-forming cells (Fig. 3B). Knockdown of the gene encoding GPR120 decreased the number of sphere-forming cells more profoundly than the knockdown of the gene encoding GPR40. Concurrent knockdown of both GPR40- and GPR120-encoding genes blocked EA-induced increase in the number of sphere-forming cells.

Binding affinity of EA to GPR40 or GPR120 was confirmed by examining the cell surface expression of these GPRs. Cell surface expression of both GPR40 and GPR120 was down-regulated by internalization for 2 h after EA treatment, after which it recovered (Fig. 3C). Results of the surface expression assay indicate that unlike EA, OA may preferentially bind to GPR40 (Fig. 3D).

**EGFR transactivation by EA.** Proto-oncogene tyrosine protein kinase (SRC) plays an important role in OA-induced EGFR transactivation (27). Therefore, we examined the role of SRC in EA-induced signal transduction. We observed that...
SRC inhibition suppressed the phosphorylation of EGFR and ERK1/2 in CT26 cells after concurrent treatment with EA and EGF (Fig. 3E). Moreover, knockdown of the gene encoding GPR40 or GPR120 decreased the phosphorylation of EGFR and ERK1/2 (Fig. 3E). Knockdown of the gene encoding GPR120 inhibited the phosphorylation of EGFR and ERK1/2 more profoundly than the knockdown of the gene encoding GPR40. We also observed these effects in HT29 cells (Fig. 3F). These findings suggest that EA activates GPR120 and (to a lesser extent) GPR40 in CRC cells for transactivating EGFR through SRC.

Effect of EA on cancer stem cell-associated signals. Next, we examined sphere formation as a feature of cancer stem cells (Fig. 4A and B). We observed that the number of spheres and MIB1-positive proliferating cells increased after EA treatment and decreased after knocking down the genes encoding GPR40 and GPR120. Treatment of CT26 cells with EA altered stem cell marker expression, i.e., it increased NS expression and decreased CD133 expression (Fig. 4C). Moreover, EA treatment decreased Notch and NICD expression and induced β-catenin nuclear translocation associated with EMT, as indicated by the upregulation of Snail expression and downregulation of E-cadherin expression. Thus, EA signaling inhibited Notch signaling and activated canonical Wnt pathway in CT26 cells. In contrast, EA treatment of HT29 cells harboring truncated APC (28), which showed constitutively activated canonical Wnt signaling, increased NS expression and decreased CD133 expression (Fig. 4D). In addition, EA treatment of HT29 cells decreased Notch and NICD expression and increased Wnt5a and CD44 expression. These results indicate that EA signaling inhibits Notch signaling and activates non-canonical Wnt signaling in HT29 cells.

**EA signaling and stemness.** To confirm that EA induced the stemness and metastatic ability of CRC cells, we examined the effects of Wnt and ERK1/2 inhibitors by performing the sphere assay (Fig. 5A). Treatment with Wnt and ERK1/2 inhibitors completely abolished EA-induced increase in sphere number and liver metastasis. To confirm EA signaling pathway, NS expression in CT26 cells was examined at the ligand (Fig. 5B), receptor (Fig. 5C), and post-receptor signaling levels (Fig. 5D). At the ligand level, EA treatment increased NS expression to a similar level as that after EGF treatment, suggesting that EGFR transactivation induced by EA was as effective as that induced by EGF. At the receptor level, inhibition of the crosstalk linker SRC and knockdown of GPR40 and GPR120 suppressed EA-induced NS expression, which was as effective as that induced by the knockdown of EGFR. At the post-receptor signaling level, inhibition of ERK1/2, p38 and Wnt suppressed EA-induced NS expression.

**Discussion**

The results of the present study indicate that EA strongly enhances cancer metastasis by increasing the stemness and the epithelial-mesenchymal transition (EMT) of colon cancer cells.

GPRs function as receptors of LCFAs. Particularly, GPR40 and GPR120 are receptors for unsaturated LCFAs such as OA or LA (26). In the present study, the binding of EA to GPR40 and GPR120 was confirmed in surface protein internalization.
assays. Knockdown of the genes encoding these two receptors did not completely suppress the EA-induced increase in the proliferation of cancer spheres. However, further studies are needed to examine other potential receptors that effectively prevent EA-induced proliferation of cancer spheres.

**GPR40 and GPR120 perform different functions under different conditions.** In islet β cells, GPR40 activation increases insulin resistance, whereas GPR120 activation increases insulin sensitivity (29). The motility and invasion of melanoma, lung and pancreatic cancer cell lines are enhanced by GPR40, but suppressed by GPR120 (30-32).

The results of the present study indicate that GPR40 and GPR120 are cooperatively involved in EA-induced EGFR transactivation. In OA-treated MCF7 cells, GPR40 and GPR120 induce EGFR transactivation (27). Transactivation may involve the participation of both GPR40 and GPR120. OA-activated EGFR induces activator protein 1 to enhance cell proliferation and matrix metalloproteinase expression (27). We observed that EA crosstalk from GPRs to EGFR was mediated by SRC. A previous study reported that the crosstalk between GPRs and EGFR activated phospholipases C, D and A2, which subsequently activated protein kinase C, extracellular receptor kinase and Akt (33). Moreover, our data showed that EA-activated EGFR induced Wnt to enhance stemness and EMT. The causes of the underlying differences downstream of transactivated EGFR by EA and OA are unclear. Therefore, further studies are necessary to explore other receptors or signaling pathways involved downstream of EGFR.

In wild-type APC-containing CT26 cells, EA signaling through EGFR induced EMT and increased stemness by activating the canonical Wnt pathway. In contrast, in APC-null HT29 cells, EA-EGFR signaling increased stemness by activating the non-canonical Wnt pathway. This is consistent with our previous findings that EGFR increased stemness by activating Wnt signaling through the nuclear translocation of β-catenin (22). In the present study, we found that EA, GPRs, SRC-EGFR signaling, and phosphorylation of extracellular receptor kinase 1/2 and p38 were involved in EA-induced Wnt activation. Thus, our results indicate that EMT and stemness enhance the metastatic potential of CRC cells. Notably, EA treatment upregulated NS, but not CD133 and increased MIB1-positive stem cells in the sphere. These findings suggest that cancer stem cells activated by EA are heterogeneous and show a preference for proliferative stem cells. Moreover, our results suggest that inhibition of EA signaling is a relevant therapeutic strategy for inhibiting EA-induced metastasis of CRC cells.

**EA have been reported as EA incorporated into mitochondria membrane reducing early closing and refractoriness of voltage-dependent anion channels (34). EA affects global DNA methylation by inducing a pro-inflammatory transcriptional profile different from that induced by OA in vitro. Moreover, maternal EA administration induces DNA hypermethylation in mouse progeny (35). The macrophages from rats fed a diet containing hydrogenated fat showed upregulation of cytosolic phospholipase A2, nuclear factor-κB p65 and Toll-like receptor-2 and -4, and downregulation of peroxisome proliferator activated receptor-γ and adiponectin receptor-1 and -2, enhancing pro-inflammatory phenotypes (36). In human, administration of an EA-rich diet to healthy volunteers resulted in decreased mitogen-induced CD69 expression on CD8+ T cells and phagocytic activity on neutrophils (37). Such epigenetic, pro-inflammatory and anti-cytotoxic immune properties may affect carcinogenic and pro-metastatic potential of EA in addition to enhancing the stemness of cancer cells.

Gavage administration of EA had a marked pro-metastatic effect in the syngeneic tumor model using CT26 CRC cells and BALB/c mice. Because the mice were fed with standard diet containing no TFAs, the enhanced metastatic ability is thought to be associated with EA administration. EA is a major component of dietary TFAs (10), and the dosage used in the experiments was in accordance with the recommendations by the Nutritional Label in America in 2003 (25). Indeed, our data showed that EA strongly promotes cancer metastasis. Metastasis is a major problem in cancer treatment and makes it difficult to cure cancer (38). Prevention of metastasis should be emphasized in the prevention of carcinogenesis. The ubiquitous usage of TFAs in western-style diets suggests that TFAs play a major role in the carcinogenesis and metastasis of CRC. The control of TFA-associated cancer development and progression is urgently needed.

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