Conjugated linoleic acid inhibits peritoneal metastasis in human gastrointestinal cancer cells

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The effect of conjugated linoleic acid (CLA) on peritoneal metastasis was examined by in vitro treatment of cancer cells and mouse peritoneal metastasis models. First, cell growth of MKN28 human gastric cancer cells and Colo320 human colon cancer cells was suppressed by CLA in a dose-dependent manner with an increment in apoptosis. CLA significantly inhibited invasion into type IV collagen-coated membrane of MKN28 and Colo320 cells (p < 0.05). CLA-induced growth inhibition was recovered by the exposure to anti-PPARγ antibody. Cyclooxygenase (COX)-2 metabolizes LA to its eicosanoids. In contrast, linoleic acid (LA) has a carcinogenic property. LA, an ω-6 polyunsaturated fatty acid, is a steroisomer of CLA. Cyclooxygenase (COX)-2 metabolizes LA to prostaglandin (PG) E2, which plays roles in carcinogenesis due to its properties of proinflammation, proliferatation and immuno-suppression.1,2 Animal models of colon carcinogenesis revealed relevant effects on suppression of metastasis.13,14 Thus, CLA might act as anti-metastatic agent as well as an anti-carcinogenic agent.

Conjugated linoleic acid (CLA) is composed of positional- and stereo-isomers of octadecadienoate (18:2). Chemoprotective properties of CLA are reported in experimental cancer models and in vitro examinations.2,3 In contrast, linoleic acid (LA) has a carcinogenic property. LA, an ω-6 polyunsaturated fatty acid, is a stereoisomer of CLA. Cyclooxygenase (COX)-2 metabolizes LA to prostaglandin (PG) E2, which plays roles in carcinogenesis due to its properties of proinflammation, proliferatation and immuno-suppression.2,3 Animal models of colon carcinogenesis revealed the carcinogenic property of LA.4–6 Differently from LA, CLA is not a precursor of PGE2. CLA activates peroxisome proliferator-activated receptor (PPARγ)-α as a ligand.7,10,11 PPARγ is a nuclear hormone receptor superfamily of ligand-activated transcription factors, which initiate transcription of genes associated with energy homeostasis.11 PPARγ is activated by endogenous secreted prostaglandins and fatty acids; 15-deoxy-Δ6(12,14)-prostaglandin J2 is a strong endogenous ligand of PPARγ. PPARγ possesses an anti-carcinogenic effect in colon cancer. Synthesized PPARγ agonists including troglitazon have been shown to be effective chemopreventive agents in a rat model of carcinogenesis and in AOM-induced colon cancer in mice.12 Moreover, a decrease in PPARγ expression is associated with cancer metastasis.13,14 Thus, CLA might act as anti-metastatic agent as well as an anti-carcinogenic agent.

In our study, we examined the anti-metastatic effect of CLA on peritoneal dissemination. Peritoneal metastasis of cancer causes poor quality in patients’ lives.15–17 We tested intraperitoneal administration of CLA in a mouse peritoneal metastasis model, and revealed relevant effects on suppression of metastasis.

Material and methods

Cell culture

A human gastric cancer cell line, MKN28, was kindly provided by Dr. W. Yasui (Hiroshima University Graduate School, Hiroshima, Japan). A human colon cancer cell line, Colo320 was provided by American Tissue and Cell Culture. The cells were routinely maintained in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (FBS, Sigma Chemical Co.) under the condition of 5% CO2 in air at 37°C. CLA (Calbiochem, Darmstadt, Germany) was dissolved with 70% ethanol (50 μg/μl). For negative control, the same amount of 70% ethanol was used.

Cell growth

The cells were harvested from 80% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% EDTA. BALB/c nu/nu mice were inoculated with MKN28 and Colo320 cells into their peritoneal cavity, and administrated with CLA intraperitoneally (weekly, 4 times). CLA treatment did not affect food intake or weight gain of mice. CLA treatment significantly decreased metastatic foci of both cells in the peritoneal cavity (p < 0.005). Survival rate in mice inoculated with MKN28 or Colo320 cells was significantly recovered by CLA treatment (p = 0.0025 and 0.0052, respectively). Protein production in MKN28 and Colo320 cells treated with CLA showed a decrease in epidermal growth factor receptor and transforming growth factor-α and an increase in Bax. These findings suggest that CLA inhibits metastasis of human gastric and colon cancer cells.

Key words: peritoneal metastasis; conjugated linoleic acid; PPARγ; gastrointestinal cancer

Antisense phosphorothioate (S)-oligodeoxynucleotide assay

The 18-mer S-oligodeoxynucleotide (ODN) for the antisense sequence from the 1st to the 18th nucleotide of PPARγ cDNA coding region were synthesized and purified by reverse-phase high-performance liquid chromatography (Sigma Genosys, Ishikari, Japan). The sequence of PPARγ was 5 ’ -CCT GGG GTA AGG TGC-3 ’. The sense sequences 18-mer was for negative control. The cells were pretreated with 3 μM antisense or sense S-ODN for 6 days, with medium exchanges and additions of anti-sense or sense S-ODN every 2 days. After that, the cells were used for further experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

PPARγ mRNA expression was assessed by RT-PCR with 0.5 μg of total RNA extracted by an RNeasy kit (Qiagen, Hilden, Germany). Primer sets for PPARγ were as follows: upper 5 ’ -GAC ATC GTC TTC CAG-3 ’ and lower 5 ’ -CCC CAT CTT TAT TCA TCA-3 ’. PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. β-actin mRNA was also amplified for internal control. The experiment was repeated twice.

Animal model

BALB/c nu/nu athymic mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were maintained according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, the Institutional Animal Care and Use Committee at the Hiroshima University Graduate School of Medicine, and in accordance with the Animal Welfare Act.
the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University, in accordance with the current regulations and standards of the Ministry of Health, Labour and Welfare. The mice were used according to the institutional guidelines when they were 5 weeks old. Cells were briefly trypsinized and washed with Hanks balanced saline solution (HBSS), 3 times. The cells suspended by HBSS were injected into the peritoneal cavity by $1 \times 10^7$ in each mouse. 10 mice were injected for each group. After the inoculation, CLA [100 pmol in 1 ml phosphate-buffered saline (PBS)] was weekly injected into the peritoneal cavity 4 times. The mice were sacrificed to count the number of metastatic foci in the peritoneal cavity. In another experiment set, mouse survival was observed until 16 weeks after the inoculation.

**Immunoblot analysis**

Whole-cell lysates were prepared as described previously. Fifty microgram lysates were subjected to immunoblot analysis in 12.5% sodium dodecyl sulfate-polyacrylamide gels followed by electrotransfer onto nitrocellulose filters. The filters were incubated with primary antibodies and then with peroxidase-conjugated IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan). An α-tubulin antibody was used to assess the levels of protein loaded per lane (Oncogene Research Products, Cambridge, MA). The immune complex was visualized by CSA system (DAKO Corp, Carpinteria, CA). Anti-epidermal growth factor receptor (EGFR) and anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as primary antibodies.

**Enzyme-linked immunosorbant assay (ELISA)**

TGF-α secretion into cultured medium was detected by TGF-α ELISA kit (Calbiochem-Novabiochem International, Darmstadt, Germany) according to its provider’s instruction. Cultured medium was filtered with a 0.2 μm filter (Becton-Dickinson Labware, Bedford, MA) before the usage.

**Statistical analysis**

Statistical significance was examined by the 2-tailed Fisher’s exact test, the 2-tailed chi-square test and the 2-tailed, unpaired Mann-Whitney test by using InStat software (Graphpad Software, Los Angiles, CA). Survival curves were calculated by Kaplan-Meier model (Statview 4.5, Abacus Concepts, Inc., Berkeley, CA). Difference of survivals was calculated by Cox proportional hazard model (Statview 4.5). Statistical significance was defined as a 2-sided $p$-value of less than 0.05.

**Results**

**Effect of CLA on growth, apoptosis, and invasion in MKN28 and Colo320 cells**

We first examined the growth inhibitory effect on cancer cells by CLA in vitro (Fig. 1a). The CLA treatment showed growth inhibition in MKN28 and Colo320 cells in a dose-dependent manner. MKN28 cells showed 98% and 99% growth inhibition by treatment with 1 and 2 nM CLA, respectively. Colo320 cells showed 82% and 99% growth inhibition by treatment with 1 and 2 nM CLA, respectively. Colo320 cells showed 82% and 99% growth inhibition by treatment with 1 and 2 nM CLA, respectively.

**Figure 1** – Effect of CLA on growth, apoptosis and invasion in MKN28 and Colo320 cells. (a) Cell growth inhibition by CLA. Cell growth inhibition in cells treated with 1 and 2 nM CLA is significantly higher than that in cells treated with 0.2 or 0.5 nM CLA. (b) Percentage of apoptotic cells induced by CLA. Apoptotic cells were counted from 1,000 cells examined by Giemsa staining. Apoptotic cells were significantly increased in CLA-treated cells compared to nontreated cells. (c) In vitro invasion assay of CLA-treated cells. Invading cells were significantly decreased compared to nontreated cells. (a–c) Bar, mean of 3 independent experiment. Error bar, SD.
2 nM CLA respectively. CLA induces apoptosis on MKN28 and Colo320 cells (Fig. 1b). Treatment with 1 nM CLA induced apoptosis in MKN28 and Colo320 cells to 47% and 32%, respectively. The induction of apoptosis by CLA showed a dose-dependent manner. MKN28 and Colo320 cells were examined invasive capacity by in vitro invasion assay using culture inserts with type IV collagen-coated membrane (Fig. 1c). Treatment with 1 nM CLA decreased invading cell numbers in MKN28 and Colo320 cells to 2% and 8%, respectively, compared to those in nontreated cells. CLA-induced inhibition of invasion showed a dose-dependent manner.

**Effect of antisense S-ODN on PPAR\(\gamma\) growth inhibition by CLA**

Constitutive expression of PPAR\(\gamma\) was repressed by exposure to antisense S-ODNs of PPAR\(\gamma\) in MKN28 and Colo320 cells (Fig. 2a). MKN28 and Colo320 cells were treated with CLA under exposure to sense or antisense S-ODNs of PPAR\(\gamma\) (Fig. 2b,c). Exposure to antisense S-ODNs for PPAR\(\gamma\) also significantly reduced CLA-induced growth inhibition in MKN28 and Colo320 cells.

**Effect of CLA on peritoneal colonization of MKN28 and Colo320 cells**

We next examined effect of CLA on metastasis of MKN28 and Colo320 cells using nude mice models. MKN28 and Colo320 cells were inoculated by 1×10⁷ in the peritoneal cavity of BALB/c nude mice. After the inoculation, the mice were intra peritoneally administrated with CLA (100 pmol in 1 ml PBS), weekly 4 times. Treatment with CLA did not affect body weight of the mice (Fig. 3a). At 6 weeks after the inoculation, the number of the colonized foci of cancer cells was counted (Fig. 3b). In MKN28 cells, 14±1 per mouse of colonized foci were observed in untreated mice, whereas its coloniziation was decreased to 2±1.5 foci per mouse by treatment with CLA. In Colo320 cells, 21.5±2.5 per mouse of colonized foci were observed in untreated mice, whereas its colonization was reduced to 2±2 foci per mouse by treatment with CLA.

We also examined survival rate of mice inoculated MKN28 or Colo320 cells using the same model (Fig. 3c,d). After 12 weeks from the inoculation, all nontreated mice died marking peritoneal dissemination of MKN28 or Colo320 tumors. In contrast, 8 (80%) and 7 (70%) mice were surviving in CLA-treated mice inoculated with MKN28 and Colo320 cells. Kaplan-Meier analysis showed significantly worse survivals of nontreated mice than that of CLA-treated mice inoculated with MKN28 and Colo320 cells (\(p = 0.0025\) and \(p = 0.0052\), respectively). Thus, CLA-treated mice showed better survivals than mice without CLA treatment in both cell lines.

**Effect of CLA on production of EGFR and TGF-\(\alpha\) proteins in MKN28 and Colo320 cells**

Finally, we examined protein levels of EGFR and Bax and secretion of TGF-\(\alpha\) in CLA-treated MKN28 and Colo320 cells (Fig. 4a,b). In CLA-treated MKN28 and Colo320 cells, EGFR protein and TGF-\(\alpha\) secretion were significantly decreased. EGFR protein levels in CLA-treated MKN28 and Colo320 cells were decreased to 21% and 29%, respectively, compared to those of nontreated cells. In contrast, Bax protein levels in CLA-treated
MKN28 and Colo320 cells were increased to 168% and 1027%, respectively, compared to those of nontreated cells. TGF-α secreted by CLA-treated MKN28 and Colo320 cells were decreased to 17% and 13%, respectively, compared to those of nontreated cells.

Discussion

In our study, we examined the direct effects of CLA on growth, invasion, apoptosis and metastasis of gastrointestinal cancer cells in vitro and in vivo. Our results showed that CLA inhibits cell growth and invasion, and induces apoptosis in cancer cells. Moreover, CLA inhibited cancer cell colonization in the peritoneal cavity and decreased death of cancer-burdened mice. CLA treatment decreased EGFR production and TGF-α secretion. These results indicate that CLA has inhibitory effect on peritoneal metastasis.

CLA is 1 of the ligands of PPARγ on activation in normal adipose tissue and tumors.9,10 We confirmed that effects of CLA depended on PPARγ in terms of specific antisense S-ODN to PPARγ. The results showed that CLA-induced growth inhibition in cancer cell lines was almost abrogated by repression of PPARγ. Although a small degree of growth inhibition was still found in both cell lines exposed to PPARγ antisense S-ODN, this inhibition disappeared by concurrent exposure to antisense S-ODNs for PPARα and PPARα. It suggests that CLA has some ligand activity PPARα, which might contribute to growth inhibitory effect by CLA.

PPARγ is a multifunctional nuclear receptor. PPARγ is dimerized with retinoic X receptor and binds specific responsive element within promoter DNA sequence to regulate gene expression transcriptionally.19 Especially, PPARγ is responsible for regulation of lipid metabolizing factors and proinflammatory cytokines.11,20,21 Association of PPARγ to cancer metastasis is reported in non-small cell lung cancer,22 colon cancer,23 thyroid cancer24 and breast cancer.25 As to molecular basis of anti-metastatic effects of PPARγ, some reports show that downregulation of EGFR and upregulation of Bax are associated with anti-proliferative and proapoptotic effects,26–28 which is associated with growth inhibition and apoptosis in cancer cells. In our study, we confirmed that CLA treatment decreased EGFR and TGF-α expression and increased pro-apoptotic Bax expression in both MKN28 and Colo320 cells. Yoshizumi et al.23 reported that metastasis of HT29 human colon cancer cells is inhibited by the synthetic PPARγ agonist, thiazolidinedione through induction of terminal differentiation. We examined alteration of different status of MKN28 and Colo320 cells by CLA testing protein levels of p21waf1. However, no remarkable change was detected (data not shown).

CLA is a group of polyunsaturated fatty acids and positional octadecadienoate and stereoisomers of octadecadienoate (18:2), which is 1 of essential fatty acids. Linoleic acid, a stereoisomer of CLA, is a precursor of PPARγ ligand, but possesses only 1/10,000 activities to induce growth inhibition, apoptosis and invasion,


24. Galusca B, Dumollard JM, Chambonniere ML, Germain N, Prades JM, Peoc’h M, Estour B. Peroxisome proliferator activated receptor γ compared to CLA (data not shown). CLA is a natural content of some food, such as beef, lamb, and also in vegetable oils. In our rodent model, CLA administration showed no obvious side effects, such as retarded weight gain, disorder of social behaviors, which suggests that CLA might be administered in safe and provide anti-cancer and anti-metastatic effect in cancer-burdened individuals. Although we used CLA with nondietary delivery, it is important to realize that anti-tumor activity in such an artificial setting (ip injection in mice) has been seen with numerous agents that were not proven to be useful in patients. Thus, it is necessary to test suppression of metastasis by dietary CLA in adequate animal models. The implications of our study for human therapy studies are of great interest in control of cancer metastases.

In our study, we showed that inhibitory effect of PPARγ on peritoneal metastasis, and activation of PPARγ by CLA. The results suggest that PPARγ is a key target of metastasis therapeutics.

Acknowledgements

We thank Mrs. K. Isoe for excellent assistance to prepare the manuscript.

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