Linoleic-Acid-Induced Growth Suppression Induces Quiescent Cancer Cell Nests in Nude Mice

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Key Words

Linoleic acid • Cancer growth • Quiescence

Abstract

We examined the effect of linoleic acid (LA) on tumor formation. Cell growth was suppressed by LA in a dose-dependent manner in MKN28 and Colo320 cells. Continuous treatment with LA provided growth arrest in both cells at 5–7 weeks after the treatment. LA-pretreated MKN28 and Colo320 cells showed higher tumorigenicity (9/10 and 10/10, respectively) than nontreated cells (2/10 and 3/10, respectively; $p < 0.01$) in nude mice. In contrast, LA-pretreated MKN28 and Colo320 cells showed more suppressed tumor growth than nontreated cells ($p < 0.01$). LA-pretreated MKN28 and Colo320 cells with LA administration after the inoculation did not form macroscopic tumors. Histological examination revealed small cancer cell aggregations, which showed no proliferative activity. In LA-treated MKN28 and Colo320 cells, protein production of Bcl-2 was increased, whereas Bak, EGFR and VEGF levels were decreased. These findings suggest that LA might induce quiescence and subsequent dormancy in cancer cells.

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Introduction

Linoleic acid (LA), one of the essential fatty acids, possesses opposite effects on tumor cells. One of its effects is a protumoral effect as a precursor of prostaglandin E_2 via metabolism by cyclooxygenases 2, which plays important roles in cell-proliferative and chronic inflammatory conditions associated with carcinogenesis, especially colon cancer development [1–7]. Another is an antitumoral effect as a precursor of peroxisome proliferator-activated receptor (PPAR)- γ . LA metabolites by 15-lipoxygenase 1, such as 9-hydroxyoctadecadienoic acid, 13-hydroxyoctadecadienoic acid and 13-oxooctadecadienoic acid, have biological effects as PPAR- γ ligands [8]. PPAR- γ is evidently thought as an anti-carcinogenic protein by stimulation with conjugated LA (CLA) [9, 10] and troglitazone (TGZ) [11]. We confirmed inhibitory effects of LA on azoxymethane-induced colon carcinogenesis and peritoneal metastasis of human gastric and colon cancer cells in nude mice, which were abrogated by exposure to PPAR- γ antisense S-oligodeoxynucleotide.

Cancer cell tumorigenicity in nude mice depends on several factors in cancer cells themselves and their host. To form macroscopic tumors, cancer cells must survive in their host tissue against host immunity, proliferate and

use host endothelial cells to make tumor vasculature. These steps are similar to those in metastasized cancer cells on the metastasis sites [12]. If cancer cells do not endure the attacks by host immunity, they cannot form tumor. If they survive but do not proliferate, or if they survive and proliferate but do not make tumor vasculature, cancer cells stay as microscopic cell aggregation. These two conditions are thought to be equivalent to tumor dormancy [13].

In this study, we examined the effects of LA on tumor cancer cells and on tumorigenicity of LA-treated quiescent cells to elucidate factors affecting tumor formation or dormancy.

Materials and Methods

Cell Culture

MKN28 and MKN45 human gastric cancer cell lines were kindly provided by Dr. Wataru Yasui (Hiroshima University, Hiroshima, Japan). Colo320 and WiDr human colon cancer cell lines were purchased from Dainihon Pharmaceutical Co. Ltd. (Tokyo, Japan). The cells were routinely maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo., USA) containing 10% fetal bovine serum (Sigma Chemical Co.) under the conditions of 5% CO₂ in air at 37°C. LA and CLA (Calbiochem, Darmstadt, Germany) were dissolved with 70% ethanol (50 µg/µl). For negative control, the same amount of 70% ethanol was used. TGZ was purchased from Calbiochem.

Cell Growth

The cells were harvested from 80% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% EDTA (Sigma Chemical Co.). The cells were seeded at a density of 1×10^4 or 2×10^4 cells/well of 24-well tissue culture plates and treated under the conditions mentioned in the Results section. The number of the cells was counted by an autocytoometer (Sysmecs, Kobe, Japan) at 48 h after the treatment. The experiment was repeated 3 times. Cells were also cultured with weekly reseeding by 1×10^5 to examine the effects of long-term treatment of LA. The experiment was repeated 3 times.

Animal Model

Forty Balb/c *nu-nu* athymic mice (4 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were maintained according to 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, Labor and Welfare. The mice were used according to the institutional guidelines when they were 5 weeks old. To access the effect of LA on tumorigenicity of MKN28 and Colo320 cells, the cells were pretreated with LA (100 µg/ml) for 5 weeks, with medium exchanged and addition of LA every 7 days. After that, 1×10^7 cells were injected subcutaneously into the backs of the mice in each group. Tumor growth at the inoculation site was weekly monitored for 12 weeks after the inoculation.

Immunohistochemistry

Sections (4 µm thick) were immunostained by antiproliferating cell nuclear antigen (PCNA) monoclonal antibody (Dako Corp., Carpinteria, Calif., USA). Immunohistochemical staining was performed by the immunoperoxidase technique following antigen retrieval; microwave treatment (500 W, 5 min, 3 times) in citrate buffer was used. After peroxidase block by 3% H₂O₂-methanol for 10 min, the specimens were incubated with PCNA (diluted to 1:20 by PBA) for 2 h at room temperature. The specimens were briefly washed by PBS and incubated with secondary antibody conjugated with peroxidase (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). The specimens were then washed by PBS and color-developed by stable diaminobenzidine solution (Dako Corp.). The specimens were counterstained with Meyer-hematoxylin (Sigma Chemical Co.).

Immunoblot Analysis

Whole-cell lysates were prepared as described previously [14]. 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 & Biological Laboratories). Anti-tubulin antibody was used to assess the levels of protein loaded per lane (Sigma Chemical Co.). The immune complex was visualized by a CSA system (Dako Corp.). Anti-epidermal-growth-factor-receptor (EGFR), anti-vascular-endothelial-growth-factor (VEGF), anti-Bax (Santa Cruz Biotechnology) and anti-Bak (Oncogene Research Products, Cambridge, Mass., USA) antibodies were used as primary antibodies.

Statistical Analysis

Statistical significance was examined by the two-tailed Fisher's exact test, the two-tailed χ^2 test, and the two-tailed, unpaired Mann-Whitney test by using Instat software (Graphpad Software, Los Angeles, Calif., USA). The statistical significance was defined as a two-sided *p* value of less than 0.05.

Results

Effect of LA on Cell Growth of Four Human Gastrointestinal Cancer Cells

Growth of 4 human gastrointestinal cancer cells treated with various concentrations of LA is shown in figure 1a. In all cell lines, cell number was significantly decreased by LA treatment at concentrations of 200 and 400 µg/ml. Four cell lines showed a more than 70% decrease in cell number.

MKN28 and Colo320 cells were treated with LA (100 µg/ml) for 10 weeks (fig. 1b, c). Continuous treatment with LA (100 µg/ml) provided growth arrest from 4 to 6 weeks after the treatment. By repetitive reseeding of cells (1×10^5 cells/well), the regrowing activity of these cells was gradually reduced. After the 4th splitting in MKN28 cells, and the 6th splitting in Colo320 cells, these cells did

Fig. 1. Effect of LA on the growth of human cancer cells. **a** Cell growth of 4 human gastrointestinal cancer cells treated with LA (48 h) showing growth inhibition in a dose-dependent manner. Error bar = Standard deviation. **b, c** Effect of LA on growth inhibition in human cancer cells by long-term treatment. Cells were

continuously treated with LA (100 µg/ml) for the indicated period with weekly reseeding by 1×10^5 cells/well. If cells were less than 1×10^5 cells/well, all cells were reseeded. The standard deviation of each cell number was less than 10% of the value.

Fig. 2. Effect of various concentrations of LA on growth inhibition in MKN28 and Colo320 cells by long-term treatment. MKN28 cells (**a-d**) and Colo320 cells (**e-h**) were continuously treated with LA (0, 50, 100 and 200 µg/ml) for the indicated period with week-

ly reseeding by 1×10^5 cells/well. If cells were less than 1×10^5 cells/well, all cells were reseeded. The standard deviation of each cell number was less than 10% of the value.

not regrow but decreased. Moreover, cells did not recover from growth arrest until cessation of LA treatment. Thus, continuous LA treatment provided cells a quiescence status.

We confirmed the growth-inhibitory effect of LA using various LA concentrations in MKN28 and Colo320 cells (fig. 2). In comparison with the cells treated with 100 µg/ml LA (fig. 2c, g), cells treated with 50 µg/ml LA were

Fig. 3. Effect of LA on tumor formation of MKN28 and Colo320 cells in the subcutis of nude mice. **a** Tumorigenicity of MKN28 and Colo320 cells 12 weeks after inoculation. **b, c** Growth of subcutaneous tumors in nude mice. Tumor diameter was examined. MKN28 – pre and Colo320 – pre: cells were pretreated by LA (100 μ g/ml) for 5 weeks before inoculation. MKN28 – pre + post and Colo320 – pre + post: nude mice inoculated with the LA-pretreated cells were treated with LA (100 μ g/ml, i.p., weekly). Error bar = Standard deviation. **a–c** None = No treatment; pre = cells were treated with LA (100 μ g/ml) for 5 weeks before inoculation; post = mice were administered LA (300 μ g/mouse, i.p., weekly) after inoculation. **d** Colo320 cell aggregate in the subcutis of nude mice treated with LA after inoculation. PCNA: the Colo320 cell aggregate was negative for PCNA in immunohistochemistry.

provided quiescence status with longer treatment periods (fig. 2b, f). In contrast, MKN28 and Colo320 cells treated with 200 μ g/ml LA showed marked apoptosis and finally disappeared by 4 or 5 weeks after treatment, respectively (fig. 2d, h).

Tumorigenicity of LA-Treated MKN28 and Colo320 Cells in Nude Mice

We next examined the effects of LA on tumorigenicity of MKN28 and Colo320 cells using the nude mouse model. Cells treated with or without LA (100 μ g/ml) for 5 weeks to induce quiescence were inoculated subcutaneously into the back of nude mice. For 12 weeks after the inoculation, tumors at the inoculation site were monitored. Untreated MKN28 and Colo320 cells formed tumors in 2 and 3 mice, respectively, out of 10 mice, whereas LA-pretreated MKN28 and Colo320 cells formed tumors in 9 and 10 mice, respectively, out of 10 ($p = 0.0055$ and $p = 0.0031$, respectively; fig. 3a). Thus, tumorigenicity was more pronounced in LA-treated cells than untreated cells. In contrast, tumor growth was significantly slower in LA-treated cells than that in untreated cells (fig. 3b, c; $p < 0.01$ at 12 weeks). In both MKN28 and

Colo320, cells treated with LA showed tumor sizes by 10% smaller than those in untreated cells.

We confirmed the effect of LA in nude mice on tumorigenicity of LA-induced quiescent cells (fig. 3b–d). Administration of LA into mice after inoculation of LA-pretreated cells completely suppressed macroscopic tumor formation (fig. 3b, c, pre + post groups). However, histological examination revealed that small cell aggregates (up to 500 μ m in diameter) were confirmed in the subcutaneous tissues at the inoculation sites of each mouse (fig. 3d). Histologically the cell aggregates did not involve any blood vessels and host inflammatory cell infiltration. The cancer cells in the aggregates lacked proliferative activity as examined by PCNA immunohistochemistry.

Effect of LA on Protein Production in MKN28 and Colo320 Cells

Finally, we examined the alteration of protein levels in 5-week LA-treated MKN28 and Colo320 cells (fig. 4a). In comparison with protein levels in untreated cells, anti-apoptotic Bcl-2 was increased, whereas proapoptotic Bak was decreased in LA-treated MKN28 and Colo320 cells.

Fig. 4. Effect of LA on protein production in MKN28 and Colo320 cells. Protein levels of Bcl-2, Bak, EGFR and VEGF were assessed by immunoblot analysis. **a** Effect of LA and LA withdrawal was examined. None = Untreated cells; LA = cells treated with LA (100 μ g/ml, 5 weeks); revert = cells cultured for 7 weeks in LA- regular

medium after 5 weeks of LA treatment. β -Actin served as a loading control. **b** PPAR- γ ligands CLA and TRZ provided alteration of protein levels, which is similar to that of LA in LA-withdrawn cells.

EGFR and VEGF were decreased in both LA-treated cells. The cells withdrawn from LA for 7 weeks ('revert' in fig. 4a) showed recovery in Bcl-2 levels compared to those in untreated cells. Levels of Bak, EGFR and VEGF were partially recovered by LA withdrawal.

Our previous studies show that the antitumoral effect of LA depends on PPAR- γ activation by LA metabolites, which have PPAR- γ ligand activity [15]. In the final set of experiments, we treated 5-week LA-treated MKN28 and Colo320 cells with PPAR- γ ligands, CLA and TGZ in an LA-free condition (fig. 4b). Cells treated with CLA or TGZ maintained the increase in Bcl-2, and decrease in Bak, EGFR and VEGF, which were caused by LA treatment.

Discussion

In the present study, continuous LA treatment induced growth arrest in gastric and colon cancer cells with a decrease in EGFR and VEGF. Quiescence is defined as an unproliferative cell status. Quiescent cells show arrested cell cycling with alteration of protein levels and/or phosphorylation levels in cell-cycle-related proteins and growth factors. In the present study, we detected the loss of proliferative activity and confirmed the alteration of protein levels in the LA-treated cells. These quiescent cells formed aggregations after inoculation into the subcutis of nude mice. In contrast, cessation of LA treatment after inoculation (LA withdrawal) induced regrowth of

the cells to form macroscopic tumors. Interestingly, LA-withdrawn cells formed smaller but more numerous tumors than untreated cells. LA-treated cells showed cell growth arrest but subsequent regrowth after LA withdrawal. That is different from arrests by aging or senescence, which are irreversible, and senescent cells are refractory to growth factors [16]. Quiescent cell aggregation in nude mice showed no host immune cell infiltration and no tumor vascularity. These features of LA-induced quiescence resemble to those of tumor dormancy.

Tumor dormancy is recognized as a time delay between primary treatment and metastatic recurrence in clinical situations [17, 18]. Tumor dormancy means several conditions: arrested solitary cell, tumor cell nest without proliferative activity, small tumor with balanced cell growth and death, and proangiogenic tumors [19–21]. We confirmed proliferative activity in these cells by bromodeoxyuridine (BrdU). BrdU administration in drinking water for 3 weeks after inoculation did not show any positive cells in the nests (data not shown). The findings suggest that BrdU was not incorporated into DNA in the nest cells for 3 weeks. Thus, the LA-induced dormancy-like state is thought to be compatible with the true quiescent state, in which conditioned tumor cells are kept in the G_0 phase. In further examination, details of cell turnover should be examined in the tumor cell nests to prove that the LA-induced quiescence is the dormancy status.

Dormant cells are resistant to chemotherapeutic agents and considered to be the source of disease recur-

rence and metastasis of unknown origin [22–26]. Reactivated dormant cancer cells provide the same disease progression in spite of the period of tumor dormancy [18]. Dormant cell formation is associated with poor clinical outcome of breast cancer [27]. In an animal model, highly metastasizing tumor is associated with more pronounced early cell death after inoculation and faster growth, whereas slowly metastasizing tumor is associated with less pronounced early cell death and slow growth [13]. Those findings are supported by our previous data that highly metastatic KM12SM colon cancer cells form larger necrotic areas in the early phase after inoculation and more developed tumor vasculature than slowly metastatic KM12C cells [28]. Thus, the word ‘highly metastatic’ means rapid tumor formation, whereas ‘slowly metastatic’ is associated with tumor dormancy. In the present study, LA-induced quiescent tumor cells form smaller tumors with higher tumorigenicity after LA withdrawal than untreated cells. In this context, it is noticeable that ‘slowly metastatic’ tumors possess a high metastatic potential but are not activated to grow. In our model, LA withdrawal made a trigger for the dormant cells to grow. Our presented data suggest that intake of dietary active substances such as PPAR- γ ligand, LA or CLA should be referred to metastasis and recurrence in cancer patients.

The findings that LA-induced quiescent cells regrown by LA withdrawal form smaller tumors than untreated cells with high tumorigenicity resemble the clinical features of dormancy, which is associated with delayed metastasis. In the peritoneal dissemination model, regrowth of MKN28 and Colo320 tumors after LA treatment was more pronounced than growth of untreated tumors (data not shown). Moreover, the findings suggest the mechanisms to retain dormancy and to regrow from dormancy. We previously revealed that an antitumor effect of LA is responsible for PPAR- γ [29]. 15-Lipoxygenase is known as an anti-inflammatory and anticarcinogenic factor [30, 31]. The oxidative metabolites of LA by 15-lipoxygenase, in particular 9-hydroxyoctadecadienoic acid, 13-hydroxyoctadecadienoic acid and 13-oxooctadecadienoic acid, have biological effects as a PPAR- γ ligand [8]. We confirmed the involvement of PPAR- γ in LA-induced quiescence by exposing PPAR- γ antisense S-oligodeoxynucleotide (data not shown). Exposure to PPAR- γ antisense S-oligodeoxynucleotide reversed the LA-induced quiescence in both MKN28 and Colo320 cells. PPAR- γ antisense treatment reversed LA antitumor effects in azoxymethane-induced carcinogenesis and peritoneal metastasis [15, 29]. In the present study, LA-treated cells

showed the same alteration of protein levels to those in cells treated with PPAR- γ ligands, TGZ and CLA. CLA is a stereoisomer of LA and possesses strong activity as a PPAR- γ ligand. TGZ is a synthesized PPAR- γ ligand for antidiabetic agents. Both CLA and TGZ induced quiescence at the lower concentration than that which induces apoptosis (data not shown). These findings suggest that PPAR- γ activation is associated closely with cell quiescence.

PPAR- γ , a multifunctional nuclear receptor belonging to the hormone receptor family, induces alteration of various protein productions [32, 33]. Activation of PPAR- γ affects expression of EGFR, cyclin D₁ [34] and Bax [35, 36]. We previously reported that LA and CLA decrease the expression of EGFR and transforming growth factor α , and increase Bax expression [15, 29, 37]. These alterations might be associated with cell growth inhibition and induction of apoptosis. In the present study, long treatment with LA induced a decrease in EGFR, VEGF and Bak, and an increase in Bcl-2 in both MKN28 and Colo320 cells. These alterations might be associated with inhibition of cell growth, angiogenesis and apoptosis, which well explained the characteristics of LA-treated cancer cell aggregations. The aggregation showed no PCNA-positive cells and no tumor vasculature.

Although dormant cells are refractory to cancer treatment, tumor-dormancy-targeting antimetastasis treatment is important to control cancer metastasis [19, 38]. Our present data suggest the mechanism of tumor dormancy. Further examination to reveal biological and molecular knowledge of the mechanisms of dormancy could provide great benefits for cancer patients.

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