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Conjugated Linoleic Acid Reduction of Vascular Endothelial Growth Factor Expression in Murine Mammary Tumor Cells through Alteration of Prostaglandin E_2

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Abstract

Conjugated linoleic acid (CLA) is a group of positional and geometric isomers of linoleic acid that have been used to reduce the incidence, growth and metastasis of breast, colon, prostate and gastric cancer in animals. CLA could reduce tumor growth by altering angiogenesis; a process requiring associated angiogenic factors such as vascular endothelial growth factor (VEGF). In this study, we determined whether CLA could modulate the expression of VEGF in murine mammary tumor cells and adipocytes. The *c*9, *t*11-CLA isomer reduced VEGF transcripts and protein when mammary tumor cells were stimulated with PMA. That isomer also reduced VEGF expression in unstimulated mouse 3T3-L1 adipocytes. Since VEGF can be regulated by cyclooxygenase-2 (COX-2), we determined whether CLA could alter COX-2 enzyme expression and PGE_2 production. The *c*9, *t*11-CLA isomer reduced not only COX-2 enzyme expression but also PGE_2 production. Thus, *c*9, *t*11-CLA could modulate neovascularization by alteration of VEGF expression from mammary tumor cells and adipocytes by reducing COX-2 metabolites.

Key words: conjugated linoleic acid, vascular endothelial growth factor, cyclooxygenase, prostaglandin E_2

INTRODUCTION

Since breast cancer develops over a long period of time, that malignancy can provide a unique opportunity for chemopreventive intervention. Numerous investigators have used dietary components as possible chemopreventive agents and have found that conjugated linoleic acid (CLA) may be a good candidate. CLA is composed of a family of geometric and positional isomers of octadecadienoic acid and is a minor dietary fatty acid present in meat and dairy products from ruminants. Synthetic preparations of CLA isomers also exist and have been used in many animal studies of tumorigenesis. CLA has previously been shown to inhibit mammary carcinogenesis and to modify the initiation, promotion and progression stages (1). Moreover, CLA can decrease the metastasis of breast tumor cells (2,3). In addition to its effects on mammary tumorigenesis, CLA has been shown to affect adipose tissue and more specifically, the proliferation, differentiation and apoptosis of adipocytes (4).

The mechanism of action of CLA is not fully understood but may involve effects on angiogenesis. Indeed, CLA was shown to inhibit angiogenesis in vivo (5). Generally, when tumors reach a size of 2 mm^3, they start to require a blood supply to provide sufficient nutrients and oxygen (6). To form new blood vessels, not only tumor cells but also host cells, such as infiltrating immune cells and adipocytes, secrete angiogenic factors such as vascular endothelial growth factor (VEGF) and leptin (7-9). VEGF is a multifunctional cytokine which can stimulate vascular permeability, endothelial cell proliferation, migration, and tube formation (10). While VEGF expression can be regulated by hypoxia and inflammatory mediators (11,12), fatty acids have been shown to regulate it as well (5,13,14). CLA-associated decreases in new blood vessel formation in breast tumors has been correlated with serum VEGF and VEGF receptor, Flk-1 concentration in mice (5). In addition, CLA has been shown to reduce serum VEGF and leptin concentration and to induce apoptosis of preexisting blood vessels in mice (15).

VEGF can be regulated by prostaglandin E_2 (PGE_2) (13,16,17) since dietary fatty acids can modify eicosanoids, several investigators have proposed that one means of regulating VEGF expression by fatty acids could be through alteration of COX-2 expression (13,18). Specific fatty acids have been shown to not only alter COX-2 expression but also PGE_2 secretion (18-20). For example, CLA has been shown to modulate COX-2

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expression and PGE_2 production in mouse macrophages, human endothelial cells and colon cancer tumors (21-23). Those results indicated that CLA could modulate angiogenesis by alteration of VEGF expression and thus alteration of tumor growth and metastasis. Therefore, in this study we sought to determine whether the observed CLA-altered angiogenesis could be through changes in VEGF expression in murine mammary tumor cells as well as adipocytes.

MATERIALS AND METHODS

Reagents

Linoleic acid as well as *c*9, *t*11- and *t*10, *c*12-CLA were purchased from Cayman Chemical Co. (Ann Arbor, MI). MEM, vitamin mixture, non-essential amino acid, and sodium pyruvate solution were purchased from Gibco (Carlsbad, CA) and fatty acid-free bovine serum albumin, forskolin, phorbol 12-myristate 13-acetate (PMA), dexamethasome and isobutylmethylxanthine were from Sigma (St. Louis, MO).

Cell culture

Mouse mammary tumor cell line 4526 was grown in MEM supplemented with 5% bovine calf serum, L-glutamine, vitamin mixture, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 0.2 mg/mL gentamicin. Cells were washed with phosphate-buffered saline (PBS) and fatty acids in ethanol added as described below. Ethanol (<0.05%) alone was used as a vehicle control.

3T3-L1 cells were cultured in DMEM containing 10% bovine calf serum. To induce pre-adipocyte differentiation of 3T3-L1 cells, 2-day post-confluent cells were treated with 1 μM dexamethasome and 0.5 μM methylisobutylxanthine for 48 hours. After the induction period, cells were switched to differentiation medium (DMEM with 10% bovine calf serum) and maintained for 5～7 days.

Kinetic quantitative PCR

Tumor cells were plated at a density of 1×10^6 per 60 mm^2 culture dishes and incubated with 5 μM of *c*9, *t*11-CLA, *t*10, *c*12-CLA, or linoleic acid (LA) and EtOH as a vehicle for 8 or 24 hr. RNA was isolated using the Total RNA Isolation kit (Ambion, TX). RNA concentration was determined by RiboGreen as described by the manufacturer (Molecular Probes, Carlsbad, CA). Isolated RNA was converted to cDNA by SuperScript first-strand synthesis system for RT-PCR (Invitrogen, CA) according to manufacturer's instructions. Equal amounts of cDNA from the RT reactions and specific oligonucleotide primers for VEGF, leptin, COX-1, and COX-2 were added to the PCR reactions according to manufacturer's instructions for quantitative PCR (Light-Cycler, Roche, IN). Initial denaturation occurred at 90°C for 30 s, followed by 40 cycles of denaturation at 90°C for 1 s, annealing at 54°C for 7 s, and extension at 70°C.

Each PCR reaction mixture was then subjected to electrophoresis for confirmation with a 2% agarose gel followed by staining with ethidium bromide. The primer sequences were designed by using primer premier; VEGF (forward), 5′-TTACTGCTGTACCTCCACC-3′; VEGF (reverse), 5′-ACAGGACGGCTTGAAGATG-3′; COX-1 (forward), 5′-GCATGTGGCTGTGGATGTCATCAA-3′; COX-1 (reverse), 5′-ACTAAGACAGACCCGTCATC-TCCA-3′; COX-2 (forward), 5′-AAAGACACTCAGG-TAGAGATGATC-3′; COX-2 (reverse), 5′-GAATGAC-TCAACAAACTGAGTGAG-3′; β-actin (forward), 5′-TCACCCACACTGTGCCCATCTAC-3′ and β-actin (reverse), 5′-GAGTACTTGCGCTCAGGAGGAGC-3′. Expression of VEGF, COX-1, and COX-2, were normalized to β-actin expression.

Quantitation of secreted VEGF

Mouse mammary tumor cells were incubated with fatty acids for 4, 8, and 24 hours and their supernatants were tested for VEGF with a commercial enzyme-linked immunosorbent assay ELISA kit according to the manufacturer's instructions (R&D, Minneapolis, MN).

Assessment of PGE_2 secretion

Mouse mammary tumor cells were incubated with CLA isomers for 16 hours then stimulated with or without 100 nM PMA for 4 hours. Supernatants were tested for PGE_2 secretion with a commercial PGE_2 EIA kit according to the manufacturer's instructions (Cayman, Ann Arbor, MI).

RESULTS

Effect of CLA isomers on VEGF expression

Initially we sought to determine whether CLA could alter in vitro VEGF expression in murine mammary tumor cells. None of the fatty acids tested altered the constitutive level of VEGF transcripts but after stimulation with PMA, *c*9, *t*11-CLA reduced ($p<0.05$) VEGF mRNA expression by 30% while *t*10, *c*12-CLA and LA had no effect (Fig. 1). VEGF protein levels were also measured after CLA treatment with or without PMA stimulation (Fig. 2). *c*9, *t*11-CLA did not alter constitutive VEGF secretion but reduced ($p<0.05$) PMA-stimulated VEGF protein secretion after 24 hr. The *t*10, *c*12-CLA isomer significantly ($p<0.05$) increased both constitutive and PMA-stimulated VEGF protein while LA had no effect.

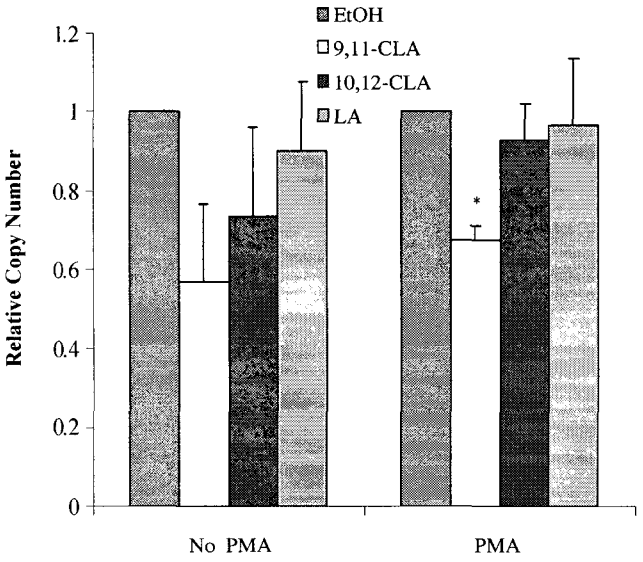

Fig. 1. CLA alteration of VEGF mRNA expression in PMA-stimulated murine mammary tumor cells. Line 4526 cells were incubated with 5 μM fatty acid for 24 hr (unstimulated) or 16 hr then stimulated with 100 nM PMA for 8 hr. Values are means±SEM of 2 separate experiments. *Significantly ($p<0.05$) less compared to control.

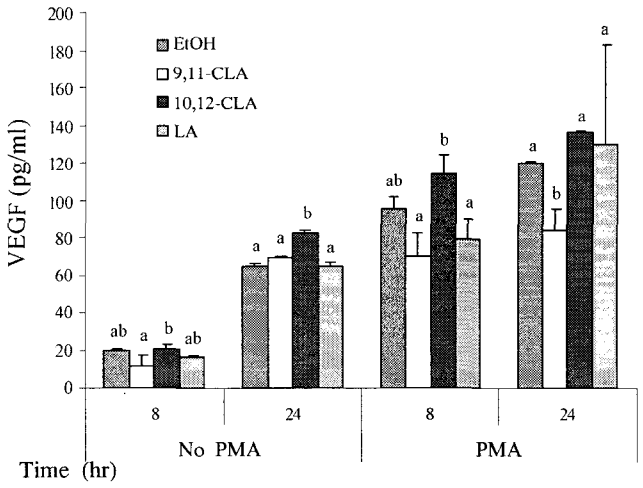

Fig. 2. Effect of CLA isomers on PMA-stimulated VEGF secretion in mammary tumor cells. Line 4526 cells were incubated with 5 μM fatty acids for 16 hr, then treated with or without 1 μM PMA for 8 or 24 hr. VEGF protein levels were measured by ELISA. Values are means±SEM of 3 separate experiments. Values not sharing the same letter within the treatment groups were significantly ($p<0.05$) different.

Effect of CLA on VEGF expression in adipocytes

Breast tumors are surrounded by adipose tissues and adipocytes have been shown to produce several angiogenic factors such as VEGF and leptin. In addition, CLA has been shown to modulate adipocyte metabolism by alteration of fat storage, and lipoprotein lipase (24,25). Therefore, CLA may alter angiogenic factors secreted from adipocytes. Thus, we sought to determine whether

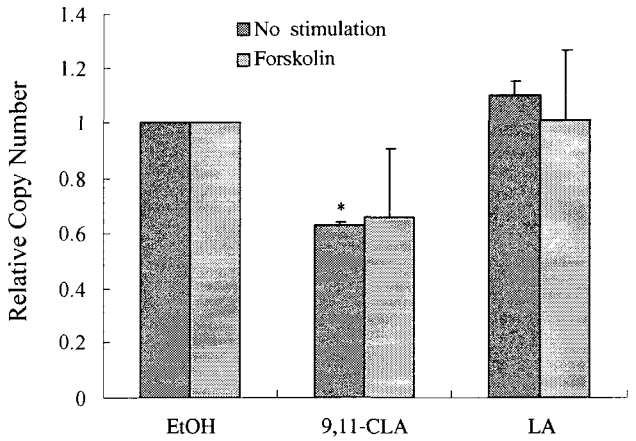

Fig. 3. Effect of CLA on adipocyte VEGF mRNA expression after stimulation with forskolin. 3T3-L1 cells were incubated with 5 μM fatty acids for 16 hr then stimulated with or without 10 μM forskolin for 8 hr. Values are means±SEM of 2 separate experiments. *Significantly ($p<0.05$) less than control.

CLA isomers could modulate VEGF mRNA from mouse adipocyte 3T3-L1 cells. We have previously shown that *c*9, *t*11-CLA was more effective than *t*10, *c*12-CLA in suppressing VEGF. The *c*9, *t*11-CLA isomer reduced ($p<0.05$) VEGF transcripts in 3T3-L1 adipocytes in the absence of forskolin but not with forskolin stimulation (Fig. 3). LA and *t*10, *c*12-CLA (data not shown) did not alter VEGF mRNA expression when adipocytes were stimulated with or without forskolin.

Effect of CLA on COX expression and PGE_2 production

Other investigators have shown that VEGF expression was directly correlated with COX-2 expression and PGE_2 production (13,17). In addition, several studies have shown that fatty acids altered COX expression (14,18). Therefore, CLA isomers may alter VEGF expression through COX mRNA expression and PGE_2 production. Initially we assessed whether CLA isomers could modulate COX-1 and COX-2 expression in murine mammary tumor cells. *c*9, *t*11-CLA reduced ($p<0.05$) COX-2 expression but did not alter COX-1. The *t*10, *c*12-CLA isomer had no effect on COX-1 or COX-2 transcripts (Fig. 4). We also assessed whether *c*9, *t*11-CLA could modulate PGE_2 secretion from tumor cells. Both *c*9, *t*11-CLA and *t*10, *c*12-CLA reduced ($p<0.05$) PGE_2 secretion by 50% independent of PMA stimulation and when compared to the effect of LA (Fig. 5).

DISCUSSION

We have previously shown that isomers of CLA can alter mouse mammary tumor cell metastasis (2,3,26). Angiogenesis which can be altered by factors such as

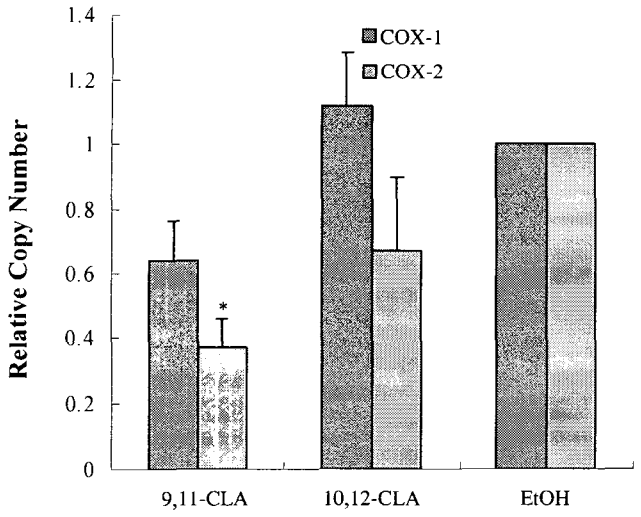

Fig. 4. CLA alteration of COX-1 and COX-2 mRNA expression in mouse mammary tumor cells. Cells were incubated with CLA for 16 hr. Values are means ± SEM of 2 separate experiments. *Significantly ($p<0.05$) less than control.

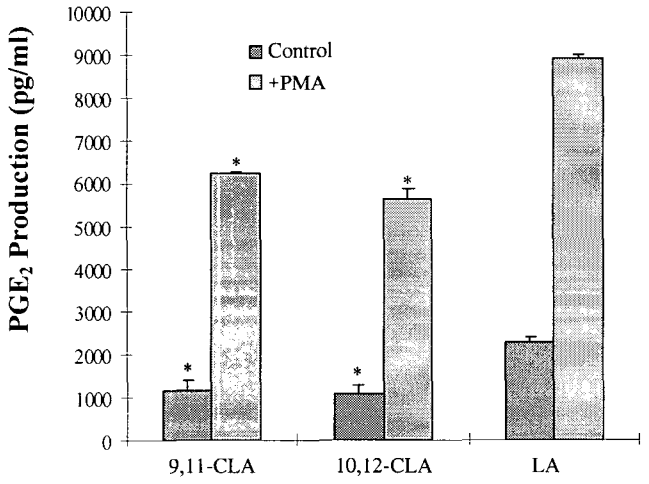

Fig. 5. Effect of CLA on PGE_2 production by mouse mammary tumor cells after PMA stimulation. Line 4526 cells were incubated with CLA for 16 hr then treated with or without 100 nM PMA for 4 hr. Values are means ± SEM. *Significantly ($p<0.05$) less than control.

VEGF is a critical step for tumor growth and metastasis because it can influence nutrient and oxygen support for tumor growth. In this study, we have shown that *c*9, *t*11-CLA reduced VEGF mRNA and protein secretion by murine mammary tumor cells and 3T3-L1 mouse adipocytes.

VEGF production can be regulated by several factors such as hypoxia, as well as by inflammatory mediators such as TNF-α and PGE_2 (11,12). Several studies have shown that fatty acids could modulate tumor growth by alteration of vascularization (13,27). For example, fish oil has been shown to reduce not only VEGF expression but also VEGF receptor expression on endothelial cells (27). Dietary CLA has been shown to reduce VEGF and VEGF receptor expression in a dose-dependent manner in the total mouse mammary gland (5). Dietary CLA also has been shown to modulate blood vessel formation in mice and reduced leptin levels (15). In this study, we showed that *c*9, *t*11-CLA reduced VEGF mRNA and protein expression from PMA-stimulated murine mammary tumor cells.

VEGF expression in tumors can be regulated by several factors, one of which is PGE_2 (16,28-30). Moreover, COX-1 and COX-2 expression can influence VEGF mRNA expression in tumors, while PGE_2 can stimulate VEGF secretion from macrophages, a cell often found in tumors (13). These findings suggest that alteration of COX-2 expression and/or PGE_2 production could influence VEGF expression and thus alter neovascularization.

CLA has been shown to alter COX-2 expression and PGE_2 production in mouse macrophages and human endothelial cells (21,22,31). We have shown that the *c*9, *t*11-CLA isomer reduced COX-2 expression in murine mammary tumor cells as well as the production of PGE_2. This is important because expression of COX-2 is often increased in malignant tumors compared to normal tissues and had been shown to regulate not only tumor growth but also neovascularization. Therefore, *c*9, *t*11-CLA could be a possible therapeutic agent to reduce tumorigenesis through alteration of PGE_2. There are several possible ways in which CLA could be altering PGE_2. One way of course could be alteration of the COX-2 enzyme. Another possible way could be similar to fish oil fatty acids whereby 20-carbon substrates for COX-2 could be altered. CLA may alter substrate availability in the cell membrane and thus a decrease in PGE_2 levels.

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