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Effects of Human Breast Stromal Cells on Conjugated Linoleic Acid (CLA) Modulated Vascular Endothelial Growth Factor-A (VEGF-A) Expression in MCF-7 Cells

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Abstract. *Background: Conjugated linoleic acid (CLA), a naturally occurring compound found in ruminant dairy and beef products, has been shown to possess anti-cancer ability in vivo and in vitro. There are several CLA isomers in ruminant-produced food products, among which t10,c12-CLA and c9,t11-CLA are most potent. Vascular endothelial growth factor-A (VEGF-A) has been implicated as an angiogenesis-activating cytokine. Our previous results indicated that CLA induced suppression of VEGF-A in MCF-7 cells, which may be one of CLA's anticancer mechanisms. Materials and Methods: The effects of t10,c12-CLA and c9,t11-CLA on VEGF-A mRNA and protein expression in MCF-7 cells, which were co-cultured with human breast stromal cells isolated from breast tissues of surgical specimens of mammoplasty and breast cancer patients, were detected by RT-PCR and Western blot analysis. Results: VEGF-A mRNA and protein expressions were significantly ($p<0.05$) elevated in co-cultured MCF-7 cells in comparison with cultured MCF-7 cells alone. Normal human breast stromal cells contribute greater effects in increasing VEGF-A protein expression in MCF-7 cells. Both t10,c12-CLA and c9,t11-CLA significantly ($p<0.05$) decreased VEGF-A mRNA and protein levels in co-cultured MCF-7 cells. t10,c12-CLA appeared to be the more active isomer than c9,t11-CLA. Conclusion: The results indicate that dietary CLA might serve as a chemo-therapeutic agent in human breast cancers by down-regulating VEGF-A expression.*

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CLA is produced by rumen fermentation of linoleic acid and is deposited in the subcutaneous fat (sub-Q) and intramuscular fat (IM or marbling) layer in beef cattle, and is also present in dairy milk fat (1). There are several CLA isomers in ruminant-produced foods, among which *c*9,*t*11-CLA and *t*10,*c*12-CLA are most potent (2). CLA has been found to possess anti-carcinogenic, anti-diabetic, anti-atherogenic and anti-adipogenic activities in mouse and human cell lines, and in *in vivo* animal studies using mice (2). Human studies of CLA and breast cancer revealed that a diet composed of CLA-rich foods, particularly cheese, may protect against breast cancer in postmenopausal women (3). It was recently reported, in a large epidemiological study, that CLA dietary intake was associated with the regulation of estrogen receptor (ER) expression in breast cancer patients (4). CLA has been found to possess the ability to reduce the risk of having an ER-negative tumor in premenopausal breast cancer, which may lead to a better therapeutic outcome for breast cancer patients as their cancer will probably respond to anti-estrogen therapy (4). CLA studies in our laboratory (5, 6) also demonstrated the anti-mammary tumor effects of CLA on (I) anti-angiogenesis by: suppressing the predominant vascular endothelial growth factor (VEGF)-A isomers, VEGF-A^{121} and VEGF-A^{165}, mRNA expression in MCF-7 cells; (II) decreasing estrogenic agent-induced breast cancer cell proliferation; and (III) regulating ERα and ERβ in epithelial-stromal cell interactions in human breast cancer.

Angiogenesis is the process of forming new blood vessels from the existing vascular network. Cancer cells growing into tiny tumors need to link up to the organ's blood vessels (7). Tumor-associated angiogenesis is considered to go through two phases and is believed to be separated by the "angiogenic switch" (8). The first is the so-called avascular phase and tumor diameters were not more than 1-2 mm.

This state of tumors is balanced between proliferation and apoptosis. The second is the so-called vascular phase referring to the potential tumor growth stage (8). The angiogenic switch is believed to correlate with the balance change between activating angiogenesis molecules, such as VEGF (9-10), matrix metalloproteinase (MMP)-9 (9), cyclooxygenase-2 (11) and hypoxia inducible factor 1 alpha (HIF-1α) (12), and inhibiting angiogenesis molecules, such as thrombospondin-1 (TSP1) (13-14), MMP2 (15) and Eph receptor A2 (EphA2) (16-17). If angiogenesis was blocked, tumor growth stopped at a diameter of about 1-2 mm, implicating the therapeutic values of angiogenesis inhibitors in the treatment of human cancers (7, 18). Molecules involved in the angionenesis process are mainly secreted by cancer epithelial cells and act on surrounding normal host tissue (19). This signaling activates certain genes in the host tissue that, in turn, produce proteins to encourage growth of the tumor microvasculature, which is a critical early step in tumor stroma generation (19). One of the most potent angiogenesis-activating molecules is the VEGF family discovered as a tumor-secreted protein. Unbalanced secretion of VEGF family members induces abnormal vessels in tumors (8, 19). The VEGF family, including VEGF-A, -B, -C, -D and -E, is associated with angiogenesis and lymph-angiogenesis, and acts by binding to VEGF receptors, the tyrosine kinase receptor, mainly expressed on the surface of vascular endothelial cells (18). Current interest is focused on VEGF-A, which is a well-studied VEGF family member, because of its important and unique properties (8, 19). VEGF-A is a member of the dimeric glycoprotein family and belongs to the platelet-derived growth factor (PDGF) superfamily (19). The VEGF-A gene is located on chromosome 6p21.3. Based on differential exon splicing, four VEGF-A isomers, containing 121, 165, 189 or 206 amino acids, represented as VEGF-A^{121}, VEGF-A^{165}, VEGF-A^{189} and VEGF-A^{206}, respectively, are formed which contain different primary structures. In human cells, isomers VEGF-A^{121}, VEGF-A^{165} and VEGF-A^{189} are the three major VEGF-A isomers (corresponding murine proteins are one amino acid shorter) (8, 19, 21). In a pancreatic-islet cancer model, the angiogenesis switch was proved to be VEGF-A-related (10). It has been demonstrated that ~90% of primary breast carcinoma with high microvessel density overexpressed VEGF-A (20). VEGF-A is overexpressed in most malignant tumors, inducing endothelial cell migration and proliferation and protecting against endothelial cell apoptosis and senescence (19). Another important feature of VEGF-A is that the hypoxic state of the growing tumor triggers expression of hypoxia-inducible factor, which binds to the VEGF-A promoter to stimulate VEGF-A production (21). Regulation of VEGF-A expression by hormones in rodent and human uterus models has been shown that estrogen- and progestins-induced VEGF-A expression can be blocked by anti-estrogen and anti-progestin, respectively. In addition, progestin also up-regulated VEGF-A in a hormone-responsive breast cancer cell line (22). Studies from breast cancer patients showed that the VEGF-A level in plasma and serum collected from breast cancer patients is linked with estrogen receptor status (23).

The local microenvironment of cancer is thought to be crucial for cancer progression, because cancer epithelial cells are surrounded by variable types of stromal cells (24). From experimental cancer models, the extracellular microenvironment has been demonstrated to influence tumor formation, the rate of cellular proliferation, the ability of the cancer cells to metastasize and the extent of invasiveness. In cancers, the influences of the microenvironment are mediated, in part, by paracrine signaling between epithelial cancer cells and the surrounding stromal cells (25, 26). It has been suggested that stromal cells may serve as a local reservoir for CLA and, thus, may inhibit breast cancer cell progression (27). Although VEGF-A is mainly expressed in malignant epithelial cells, lesser amounts of VEGF-A can also be detected in stromal cells and vascular endothelial cells, which may imply both autocrine and paracrine signals of this cytokine in the tumor microenvironment (19). Our current study investigated whether CLA-modulated breast stromal cells involved the microenvironment and whether these effects are capable of suppressing the VEGF-A angiogenesis biomarker in the hormone-responsive breast cancer cell line, MCF-7, which may lead to suppression of human breast neoplasms.

Materials and Methods

+98% purity *t*10,*c*12-CLA and *c*9,*t*11-CLA were purchased from Matreya, Inc. (PA, USA) and the CLA stock solution was prepared based on the paper published by Dr. Ip's group (28). The main difference in our CLA stock is that dextran-coated charcoal (DCC, Dextran T-70; Pharmacia; activated charcoal; Sigma)-treated fetal bovine serum (FBS, GibcoBRL, Bethesda, MD, USA) was added to pure *t*10,*c*12-CLA and *c*9,*t*11-CLA to form a CLA-serum protein complex.

Immortalized cell line. MCF-7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were planted in 75-cm^2 culture flasks in a humidified incubator (5% CO_2, 95% air, 37˚C) and cultured in phenolred-free high-calcium Dulbecco's Modified Eagle's Medium and Ham's F12 Medium (DMEM/F12, 1.05 mM $CaCl_2$) supplemented with 5% FBS. The medium was renewed every two days.

Isolation of stromal cells from human breast tissues. Normal and cancerous human breast tissues were obtained through the Tissue Procurement Program at The Ohio State University Hospital in Columbus, Ohio, USA. Tissues were placed in DMEM/F12 and stored at 4˚C. The isolation of stromal cells from human breast

tissues and culture condition have been described in detail previously (29). Briefly, the tissues were minced and digested in 0.1% collagenase I (GibcoBRL) supplemented with 5% FBS and antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 mg/ml amphotericin B) (GibcoBRL) in a 37˚C humidified incubator (5% CO_2 : 95% air) overnight. The digested mixture was centrifuged at 200 xg for 5 min at 25˚C. The cell pellet was re-suspended and allowed to settle by gravity 3 times. Stromal cells in the supernatant were then centrifuged at 200 xg for 5 min at 25˚C and the pelleted stromal cells were re-suspended in phenol red-free high-calcium DMEM/F12 (1.05 mM $CaCl_2$) supplemented with 5% FBS. Using this method to separate primary cultured stromal cells and primary cultured epithelial cells from human breast tissues has been shown to produce a high purity of stromal cells, as described previously in our laboratory (29). Although the primary cultured epithelial cell type could also grow in the high-calcium medium, stromal cells grew much faster than epithelial cells during the first week of culture, thus leading to stromal cell dominance in our culture conditions. In the current study, stromal cells were isolated and co-cultured with MCF-7 cells.

Co-culture system and treatments. Treatments and total RNA and protein extractions were performed on the primary cultured human breast stromal cells not propagated beyond the third passage, and the viabilities of the MCF-7 cells and breast stromal cells were greater than 95%, as determined by the trypan blue dye exclusion method (30). Co-cultures of MCF-7 cells and breast stromal cells were performed by using flat-bottomed cell culture plates. Stromal cells ($0.5x10^6$ cells/well) were seeded on the nucleopore polycarbonate membrane (0.4 μm pore size) of the cell culture inserts (upper chamber). MCF-7 cells ($1.0x10^6$ cells/well) were seeded on the bottom plates (lower chamber). Because of the difference in the cell sizes of MCF-7 (~ 5 μm) and stromal cells (~ 10 μm), the MCF-7 cell number seeded on the bottom chamber was twice that of the stromal cells. The seeded MCF-7 and stromal cells were not over 90% confluence on the day of harvest. Since MCF-7 and stromal cells were originally both cultured in phenolred-free high-calcium DMEM/F12 (1.05 mM $CaCl_2$), in the co-culture of these two cell types, phenolred-free high-calcium DMEM/F12 (1.05 mM $CaCl_2$) supplemented with 5% DCC-treated FBS was used. After 2 days, the cells were treated with 40 μM *t*10,*c*12-CLA, 40 μM *c*9,*t*11-CLA or vehicle as control in the same medium for 3 days.

Reverse transcription-polymerase chain reaction (RT-PCR). At the end of the treatment, breast stromal cells on cell culture inserts were removed and discarded. Total RNA from MCF-7 cells on the bottom plates was isolated in 1 ml TRIZOL® Reagent (GibcoBRL), according to the manufacturer's instructions. RT-PCR was used to produce cDNA and performed in a gradient mastercycler (Eppendorf®) as described in detail previously (29). The PCR conditions for VEGF-A^{121} and VEGF-A^{165} were optimized for $MgCl_2$ concentration, annealing temperature and cycle number. The newly synthesized cDNAs were used as templates for PCR after adjusting the reagent concentrations to 1.25 mM $MgCl_2$, 0.24 μM primers, 2.5 μl 10X PCR Buffer (GibcoBRL) and 1 U Platinum® Taq DNA polymerase (GibcoBRL). The reactant was incubated at 95˚C for 5 min. Then, 32 cycles of amplification were performed with each cycle consisting of denaturation at 95˚C for 1 min, annealing at 70˚C for 30 sec and extension at 72˚C for 1 min. For 36B4 (loading control), the PCR conditions were as described before (30, 31) and only modified in terms of $MgCl_2$ concentration: 1.25 mM $MgCl_2$.

The primer sequences for VEGF-A^{121} and VEGF-A^{165} were 5'-ATC TTC AAG CCG TCC TGT GTG-3' (sense) and 5'-TCA CCG CCT CGG CTT GTC ACA-3' (antisense). The primer sequences for 36B4 were 5'-AAA CTG CTG CCT CAT ATC CG-3' (sense) and 5'-TTG ATG ATA GAA TGG GGT ACT GAT G-3' (antisense). The final PCR products (10 μl), mixed with 1 μl 10x loading buffer, were separated on a 1.5% agarose gel containing ethidium bromide. The lengths of the PCR products were 231 bp for VEGF-A^{121}, 363 bp for VEGF-A^{165}, and 563 bp for 36B4. The specific bands were quantified by ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA). The results are presented as the ratio of VEGF-A^{121} or VEGF-A^{165} to 36B4.

Western blot analysis. At the end of the treatment, breast stromal cells on the cell culture inserts were removed and discarded. MCF-7 cells on the bottom plates were washed with ice-cold PBS and then lysed with extraction reagent (Pierce, Rockford, IL, USA) and protease inhibitor (Pierce) in ice. Cell lysates were separated by centrifugation at 15,000 rpm in a cool room (4˚C) for 30 min. An equivalent amount of protein (50 μg) from each supernatant with sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.004% bromophenol blue and 5% β-mercaptoethanol) was boiled for 5 min and resolved in a 4-15% SDS-polyacrylamide ready gel (Bio-Rad, Hercules, CA, USA). After electrophoreses, the proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) by semi-dry transfer system (Bio-Rad) at room temperature. The transblotted membrane was washed twice with phosphate-buffered saline containing 0.1% Tween 20 (PBST). After blocking with PBST containing 10 % non-fat milk for 1 h at room temperature, the membrane for VEGF-A was incubated with the diluted (1:500) VEGF-A mouse monoclonal antibody (Santa Cruz, CA, USA, SC-7269) and the membrane for β-actin was incubated with the diluted (1:1000) β-actin goat polyclonal antibody (Santa Cruz) in PBST, 5% non-fat milk at 4˚C overnight. After that, the membrane for VEGF-A was incubated with diluted (1:3000) anti-mouse secondary antibody (Amersham, Piscataway, NJ, USA) and the membrane for β-actin was incubated with diluted (1:5000) donkey anti-goat secondary antibody (Amersham) in PBST, 5% non-fat milk for 1 h at room temperature. The immunoblots were enhanced by ECL Plus™ Western Blotting Detection reagent (Amersham, Buckinghamshire, UK) and visualized by the Fuji image system (FUJIFILM Medical Systems U.S.A., Inc., Stamford, CT, USA).

Statistics. The data, from 3 replicated wells as one cell group, was presented as the mean±standard deviation (SD) and was analyzed using StatView® (SAS Institute Inc. Cary, NC, USA) ANOVA unpaired *t*-test. A *p*-value less than 0.05 was considered to be statistically significant.

Results

c9,t11-CLA and t10,c12-CLA inhibited cell proliferation of co-cultured MCF-7 cells. Based on the CLA concentration in normal physiological human serum (10-70 μM) and in humans who take CLA long-term supplementation (50-350 μM), the CLA concentration applied in *in vitro* studies has ranged

from 12.5-250 μM, the mid-normal to supraphysiological-pharmacological levels (31-32). CLA's biological activities on breast cancer cells regarding the ER status have demonstrated that CLA (25-200 μM) appeared to inhibit ERα-positive breast cancer cell growth but not ERα-negative breast cancer cells (31). Our preliminary studies showed that: i) the effective dose range in inhibiting human breast cancer epithelial and stromal cell proliferation is *t*10,*c*12-CLA at 10-80 μM and *c*9,*t*11-CLA at 40-160 μM for 3-day treatment; and ii) these experimental data also indicated different biological effects of t10,c12-CLA and c9,t11-CLA; finally iii) CLA was more effective on the estrogen-responsive breast cancer cell line, MCF-7, (unpublished data) than the estrogen-non-responsive breast cancer cell line, MDA-MB-231. Therefore, we chose the relatively lower but effective dose, 40 μM, of these two CLA isomers to investigate the effects of breast stromal cells in CLA-modulated VEGF-A expression in estrogen-responsive MCF-7 cells.

The purity of the human breast stromal cells used for the current study were examined by morphology and confirmed by immunohistochemical staining previously reported from our laboratory (29). We have been able to show that breast stromal cells exhibited typical spindle-shaped morphology and that the majority of breast stromal cells (>95%) were immunopositive for the presence of vimentin, while no expression of cytokeratin was detected, which confirmed the fibroblastic nature of the breast stromal cells.

The effects of the CLA isomers, *c*9,*t*11-CLA and *t*10,*c*12-CLA, on the proliferation of MCF-7 cells, which were either cultured alone, or co-cultured with breast cancer stromal cells (referred to hereafter as CASC), or co-cultured with normal breast stromal cells (referred to hereafter as NSC), were detected.

The proliferation rate of co-cultured MCF-7 cells was significantly ($p<0.05$) higher in comparison with MCF-7 cells cultured alone (Figure 1). *c*9,*t*11-CLA and *t*10,*c*12-CLA both decreased cell proliferation in cultured alone MCF-7 cells in a similar way. However, only *t*10,*c*12-CLA inhibited cell proliferation in co-cultured MCF-7 cells. Our results suggested that: i) NSC and CASC both contribute similar effects in increasing MCF-7 cell proliferation; ii) *t*10,*c*12-CLA is more potent in inhibiting MCF-7 growth.

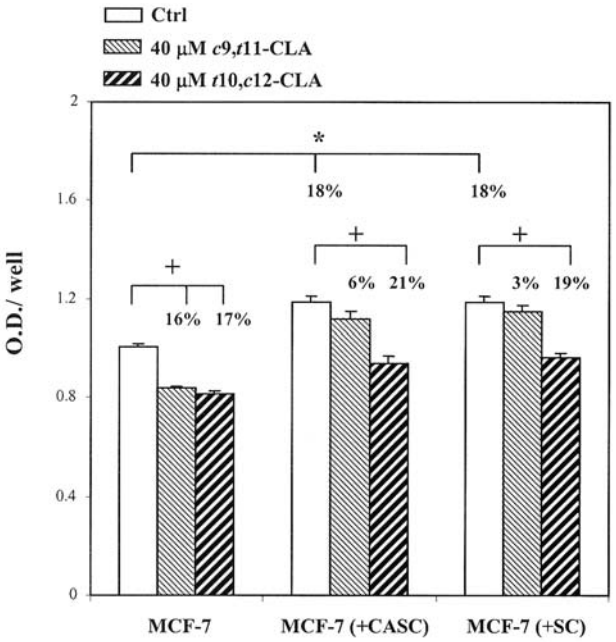

Figure 1. *CLA inhibited proliferation of co-cultured MCF-7 cells. MCF-7, MCF-7 (+CASC), and MCF-7 (+SC) represent MCF-7 cells were cultured alone, MCF-7 cells were co-cultured with breast cancer stromal cells (CASC), and MCF-7 cells were co-cultured with normal breast stromal cells (SC), respectively. Cells were treated by two CLA isoforms and proliferation rate of MCF-7 cells was quantified by using CellTiter™ 96 AQueous assay and optical density was read at 490nm (OD 490 nm) in 96-well plates by an ELISA plate reader for each group separately. Bars represent mean±SD, n=3. *p<0.05 stands for control group in MCF-7 (+CASC) and MCF-7 (+SC) versus control group in MCF-7 cells cultured alone; +p<0.05 for control versus 40 μM c9,t11-CLA and 40 μM t10,c12-CLA.*

c9,t11-CLA and t10,c12-CLA decreased VEGF-A^{121} and VEGF-A^{165} mRNA expressions in co-cultured MCF-7 cells. In human cells, differential exon splicing of VEGF-A formed VEGF-A isomers which contain 121, 165, 189 and 206 amino acids, represented as VEGF-A^{121}, VEGF-A^{165}, VEGF-A^{189} and VEGF-A^{206}, respectively. The vascular permeability properties of VEGF-A^{121} and VEGF-A^{165} angiogenesis cytokines have been suggested to be the initial and crucial step in tumor angiogenesis (19, 33-34). To compare the effects of *t*10,*c*12-CLA and *c*9,*t*11-CLA on either VEGF-A^{121} or VEGF-A^{165} mRNA expressions in MCF-7 cells, MCF-7 cells were cultured alone, or co-cultured with CASC, or co-cultured with NSC. VEGF-A^{121} and VEGF-A^{165} mRNA expression levels in MCF-7 cells were determined by RT-PCR after the cells had been treated by both CLA isomers, *c*9,*t*11-CLA and *t*10,*c*12-CLA, for 3 days and the results are shown in Figures 2 and 3, respectively.

The experimental data demonstrated that: i) VEGF-A^{121} mRNA expression of co-cultured MCF-7 cells was significantly ($p<0.05$) higher in comparison to MCF-7 cells cultured alone. Similarly, CASC and NSC contributed to up-regulate VEGF-A^{165} mRNA expression in MCF-7 cells; ii) *c*9,*t*11-CLA and *t*10,*c*12-CLA both down-regulated VEGF-A^{121} and VEGF-A^{165} mRNA expressions in cultured alone MCF-7 cells; iii) *t*10,*c*12-CLA decreased both VEGF-A^{121} and VEGF-A^{165} mRNA expressions in MCF-7 cells which were either co-cultured with CASC or with NSC; iv) in

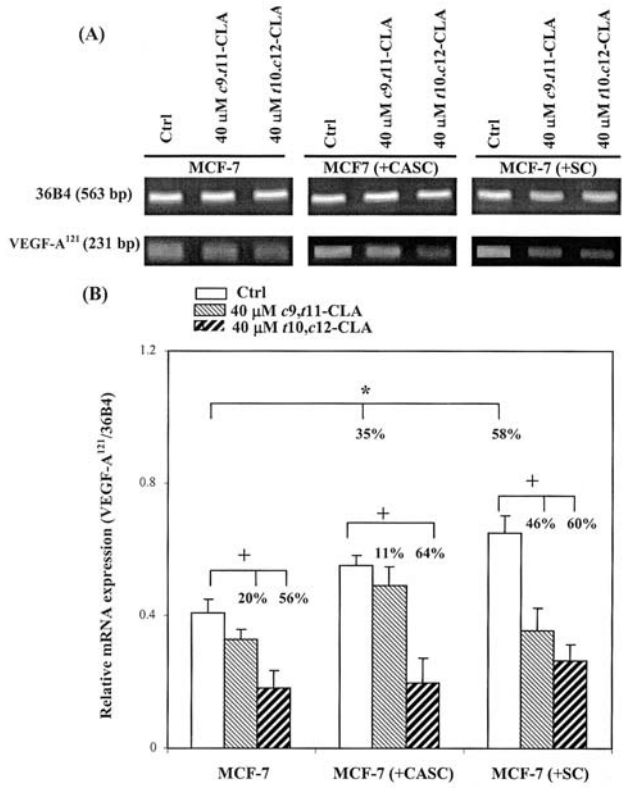

Figure 2. *CLA down-regulated VEGF-A^{121} mRNA expression in co-cultured MCF-7 cells. (A) Ethidium bromide-stained PCR products separated in a 1.5% agarose gel. MCF-7, MCF-7 (+CASC) and MCF-7 (+SC) represent MCF-7 cells cultured alone, MCF-7 cells co-cultured with breast cancer stromal cells (CASC), and MCF-7 cells co-cultured with normal breast stromal cells (SC), respectively. The cells were treated by two CLA isoforms and total RNA from MCF-7 cells was isolated from each group separately. 36B4 was used as loading control. (B) The mRNA ratio of VEGF-A^{121} to 36B4 was measured by densitometry. Bars represent mean±SD, n=3. *p<0.05 stands for control group in MCF-7 (+CASC) and MCF-7 (+SC) versus control group in MCF-7 cells cultured alone; +p<0.05 for control versus 40 μM c9,t11-CLA and 40 μM t10,c12-CLA.*

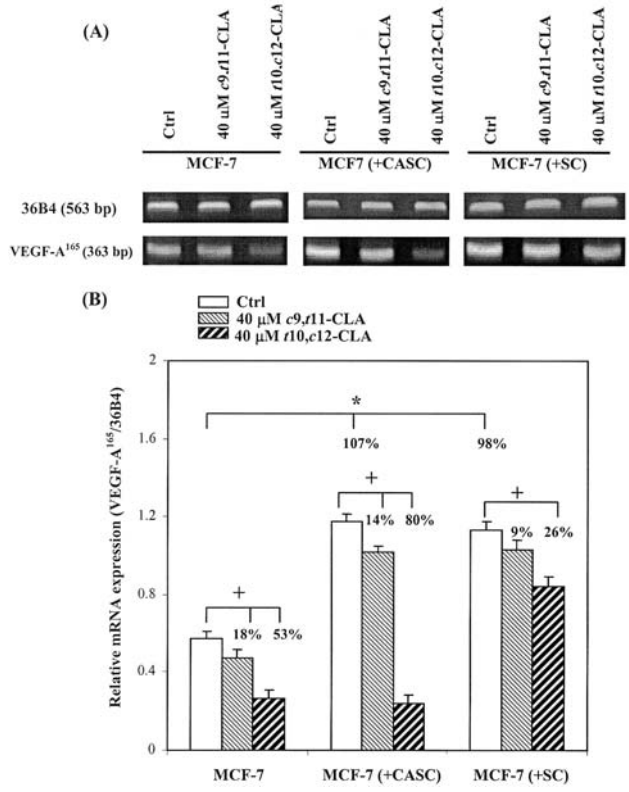

Figure 3. *CLA down-regulated VEGF-A^{165} mRNA expression in co-cultured MCF-7 cells. (A) Ethidium bromide-stained PCR products separated in a 1.5% agarose gel. MCF-7, MCF-7 (+CASC) and MCF-7 (+SC) represent MCF-7 cells cultured alone, MCF-7 cells co-cultured with breast cancer stromal cells (CASC) and MCF-7 cells co-cultured with normal breast stromal cells (SC), respectively. The cells were treated by two CLA isoforms and total RNA from MCF-7 cells was isolated from each group separately. 36B4 was used as loading control. (B) The mRNA ratio of VEGF-A^{165} to 36B4 was measured by densitometry. Bars represent mean±SD, n=3. *p<0.05 stands for control group in MCF-7 (+CASC) and MCF-7 (+SC) versus control group in MCF-7 cells cultured alone; +p<0.05 for control versus 40 μM c9,t11-CLA and 40 μM t10,c12-CLA.*

contrast, *c*9,*t*11-CLA-regulated VEGF-A^{121} and VEGF-A^{165} mRNA expressions was more complicated because *c*9, *t*11-CLA only decreased VEGF-A^{121} mRNA expression of MCF-7 cells in co-culture with NSC but not with CASC; v) moreover, *t*10,*c*12-CLA appeared to be more active than *c*9,*t*11-CLA in down-regulating VEGF-A^{121} and VEGF-A^{165} mRNA expressions. Our results suggested that surrounded stromal cells provide paracrine signals that play important roles in increasing VEGF-A^{121} and VEGF-A^{165} mRNA expressions in malignant epithelial cells, which may lead to tumor progression. CLA isomers, especially *t*10,*c*12-CLA, can interrupt breast microenvironment paracrine signals which may suppress characteristic of human breast neoplasms.

c9,t11-CLA and t10,c12-CLA decreased VEGF-A protein expression in co-cultured MCF-7 cells. To compare the effects of *t*10,*c*12-CLA and *c*9,*t*11-CLA on VEGF-A protein expression in co-cultured MCF-7 cells, MCF-7 cells were cultured alone, or co-cultured with CASC, or co-cultured with NSC. The VEGF-A protein levels in MCF-7 cells were determined by Western blot analysis after the cells had been treated by both CLA isomers for 3 days and the results are shown in Figure 4.

VEGF-A^{121}, VEGF-A^{165} and VEGF-A^{189} are three major VEGF-A isomers in human cells and, among them, VEGF-A^{121} and VEGF-A^{165} are the predominant isomers (19). Therefore, detected VEGF-A protein might represent

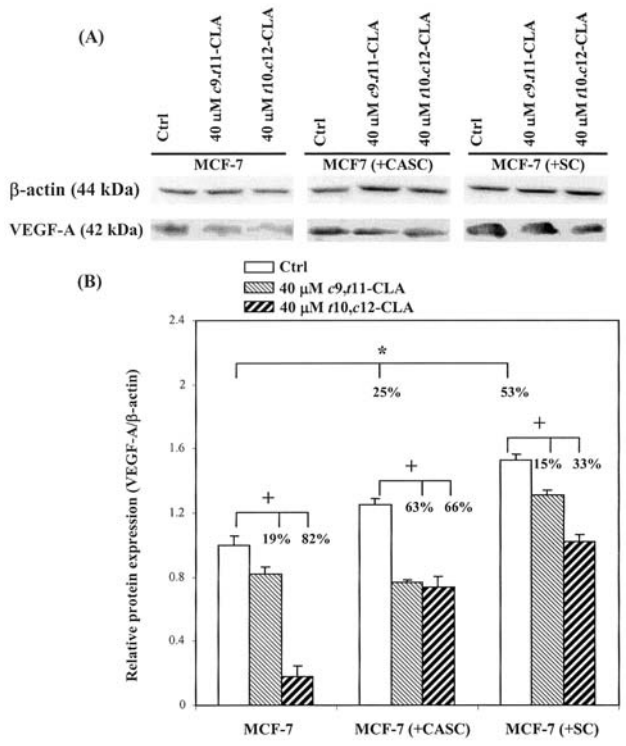

Figure 4. *CLA down-regulated VEGF-A protein expression in co-cultured MCF-7 cells. (A) Western blot analysis of CLA on VEGF-A protein expression. MCF-7, MCF-7 (+CASC) and MCF-7 (+SC) represent MCF-7 cells cultured alone, MCF-7 cells co-cultured with breast cancer stromal cells (CASC), and MCF-7 cells co-cultured with normal breast stromal cells (SC), respectively. The cells were treated by two CLA isoforms and whole cells lysate from MCF-7 cells was isolated from each group separately. β-actin was used as loading control. (B) The protein ratio of VEGF-A to β-actin was measured by densitometry. Bars represent mean±SD, n=3. *p<0.05 stands for control group in MCF-7 (+CASC) and MCF-7 (+SC) versus control group in MCF-7 cells cultured alone; +p<0.05 for control versus 40 μM c9,t11-CLA and 40 μM t10,c12-CLA.*

the sum of VEGF-A^{121} and VEGF-A^{165}. VEGF-A protein is detected as precursor 21 kDa, the one considered to be biologically inactive, and mature 42 kDa, the one considered to be active (19, 35). Both the VEGF-A precursor and mature VEGF-A protein were detected by Western blotting analysis in our system; nonetheless, the precursor band density was considerably weaker than the mature one (data not shown). The results shown in Figure 4 were mature VEGF-A protein (42 kDa). The experimental data demonstrated that: i) VEGF-A protein levels of co-cultured MCF-7 cells were significantly ($p<0.05$) higher in comparison with MCF-7 cells cultured alone; ii) *c*9,*t*11-CLA and *t*10,*c*12-CLA both down-regulated VEGF-A protein in cultured MCF-7 cells; iii) *t*10,*c*12-CLA is more potent in down-regulating VEGF-A protein levels.

Discussion

Anti-VEGF-A as one of CLA's anti-angiogenesis mechanisms in the breast cancer microenvironment. It has been suggested that different microenvironments affect a tumor's angiogenic response (36). Paracrine interactions between stromal cells and epithelial cells are known to play an essential role in neoplasia (37). Our work mimicked the breast tumor microenvironment by utilizing a co-culture system in which MCF-7 cells were either co-cultured with CASC or NSC. This method allowed signaling molecules secreted by either cell type to act on target cells through cultured medium and molecular markers changes to be examined in specific cell types. Furthermore, it has been shown that molecules activating angiogenesis, such as VEGF-A, are mainly secreted by cancer epithelial cells and act on surrounding stromal cells (18-19). Thus, the VEGF-A molecular biomarker was only examined in MCF-7 cells in the current study.

Our findings showed that as yet undefined factor(s), which were speculated to derive from the interactions of two cell types in the co-cultured system, contained in co-cultured medium were able to increase proliferation, VEGF-A^{121} and VEGF-A^{165} mRNA and VEGF-A protein levels in MCF-7 cells; and *c*9,*t*11-CLA and *t*10,*c*12-CLA were able to interrupt these paracrine signals (Figures 1-4). Whether *c*9,*t*11-CLA and *t*10,*c*12-CLA were targeted on specific cells types in this co-cultured system can not yet be answered; however, we also observed that CLA showed an inhibitory effect on breast stromal cells cultured alone (data not shown). Alternatively, MCF-7 cells were treated with conditioned medium prepared from human breast stromal cells. We did not observe stimulated cell proliferation and up-regulated VEGF-A mRNA expression in conditioned medium-treated MCF-7 cells. Therefore, we speculated that CLA inhibited MCF-7 cell proliferation and down-regulated VEGF-A^{121} and VEGF-A^{165} mRNA. We also speculated that CLA was able to interrupt VEGF-A autocrine and paracrine signals in the breast microenvironment which may contribute to its anti-angiogenesis effects in breast cancer.

c9,t11-CLA and t10,c12-CLA exerted isomer-specific effects on modulating VEGF-A in the breast cancer microenvironment. Dietary CLA isomers have been shown to decrease VEGF-A levels in serum and mammary glands in a mouse model (28, 38). Both *t*10,*c*12-CLA and *c*9,*t*11-CLA decreased the size of white adipocytes; however, only *t*10,*c*12-CLA, but not *c*9,*t*11-CLA, induced adipocyte apoptosis. Isomer specific characteristics of CLA suggested that *c*9,*t*11-CLA was reserved in mammary stromal compartment for long-acting effects; *t*10,*c*12-CLA can disrupt preexisting fenestrated vasculature and cause mammary stromal apoptosis, which could be used therapeutically (28, 38).

The roles of different VEGF-A isomers in tumor blood vessel formation and tumorigenicity have been examined by researchers in various tumor models. Findings from Dr. Dvorak's group (33, 34), on the effects of angiogenesis cytokines (VEGF-A^{120} and VEGF-A^{164}) in generating substitute tumor vessels, demonstrated that the vascular response to VEGF-A^{164} was heterogeneous inducing angiogenesis, vascular malformations, arteriogenesis and lymphangiogenesis. By contrast, the vascular response to VEGF-A^{120} was simpler, inducing angiogenesis but not lymphangiogenesis. Dr. Grunstein's group (39) utilized transformed murine fibrosarcoma cells to express specific VEGF-A isomers and found that only VEGF-A^{164} could fully rescue tumor growth; in contrast, VEGF-A^{120} partially rescued tumor growth. In glioma (40) and melanoma (41) models, VEGF-A^{165} strongly augmented neovascularization, which generated dense and highly heterogeneous vessel networks in mice; in contrast, VEGF-A^{121} formed poorly-vascularized and necrotic vessel networks. More recently, Fenton *et al.* (42) proposed a model in which VEGF-A^{121}-overexpressing human breast MCF-7 cancer cells improved oxygenation and decreased vessel spacing to a larger extent than VEGF-A^{165} tumor, despite the VEGF-A^{121} tumor having a smaller tumor volume. It was suggested that oxygen, nutrient, or chemotherapeutic agent delivery between VEGF-A^{165} tumor and VEGF-A^{121} tumor would be different and contribute to the ultimate therapeutic outcome (42).

To date, the anti-angiogenesis studies of CLA have not been focused on either of the VEGF-A isomers. Our work showed that both *t*10,*c*12-CLA and *c*9,*t*11-CLA appeared to decrease the dominant VEGF-A isomers, VEGF-A^{121} and VEGF-A^{165} mRNA expressions through interrupting these cytokines' autocrine and paracrine signals. These findings suggested that, by modulating VEGF-A protein isomers, CLA might be able to block angiogenesis. The consumption of foods produced from ruminant dairy and beef products containing CLA has been suggested to have a chemo-preventive or/and chemo-therapeutic influence (27). The experimental data described here yield new and potentially important information regarding the down-regulation of VEGF-A, a potential angiogenesis bio-marker, by CLA in the breast cancer microenvironment. Further studies investigating CLA-regulated VEGF-A, involving breast cancer epithelial cells and stromal cells in 3-D matrigel, are in progress in our laboratory.

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