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RESEARCH ARTICLE

CONJUGATED LINOLEIC ACID (*CIS*-9, *TRANS-11* ISOMER) MODULATED TNF-α INDUCED CELL ADHESION MOLECULES EXPRESSION IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)

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ABSTRACT

Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid (PUFA) that has been shown in recent years to have numerous potential benefits for human health. The vascular cell adhesion molecules play principal role in inflammatory diseases. Blocking the expression of these molecules or preventing their interaction with the receptors has been shown to be important in controlling various inflammatory diseases. However, the effect of CLA on endothelial cell adhesion molecules is unknown. We investigated the effects of c*is*-9, *trans-11* Isomer of CLA on expression of intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial selectin (E-selectin) in HUVECs and comparison with, linoleic acid (LA). Endothelial cells co-incubated with or without TNF-α (0.001μg/ml) in the presence of LA or CLA (100μM/L) for 16-h before assessing the expression of adhesion molecules. The expression of adhesion molecules in HUVEC was assayed by ELISA and RT-PCR technique. CLA but not LA, decreased VCAM-1 and ICAM-1 expression in both mRNA and soluble levels in TNF-α activated cells (P<0.001). Furthermore, CLA modulated mRNA concentration of E-selectin, without any effect in soluble level of this molecule (P> 0.05). While LA didn't show significant effect on TNF-α induced expression of adhesion molecules, a progressively increasing inhibitory activity was observed, for CLA. Therefore, CLA could be used as a novel agent for controlling various pathological conditions associated with up-regulation of inflammatory adhesion molecules at least in HUVECs. The role of CLA in the modulation of inflammation in vivo requires further study.

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INTRODUCTION

Vascular endothelium is an active and dynamic tissue involved in the control of leukocyte recruitment by expressing membrane molecules for adherence of leukocytes (1). Migration of leukocytes from systemic circulation into tissues is mediated through interactions between leukocytes surface integrins and endothelial cells adhesion molecules. ICAM-1, VCAM-1 and E-selectin are among the most prominent adhesion molecules involved in this process (2-4).

The adhesion molecules present at very low levels on resting endothelial cells and up-regulate via Bacterial Lipopolysaccharides (LPS), and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (2, 5). The increased expression of cell adhesion molecules on the endothelial cells alters the adhesive property of the vasculature leading to indiscriminate infiltration of the leukocytes across the blood vessels and hence causes inflammation (6). Study of the expression of the molecules on the surface of various cells or of the soluble form in the plasma may provide insights into their role(s) in pathophysiology in cardiovascular, connective tissue and neoplastic diseases (7, 8) and differences in levels of soluble cell adhesion molecules in the plasma may be useful tools in stratifying disease severity or prognosis (9, 10). A promising therapeutic approach for the management of various inflammatory conditions would be to inhibit the cytokine induced expression of cell adhesion molecules (11). Although various synthetic drugs, antibodies and peptides have been demonstrated to inhibit the expression of these molecules, they have their own limitations for usage because of unwanted side effects (12). Endothelial activation is considered to be an early and critical event in the pathology of atherogenesis which can be modified by environmental factors such as diet, pollutants, and lifestyle habits (13-15). CLA is a group of naturally-occurring, essential fatty acid (EFA) and refers to a mixture of positional and geometric conjugated dienoic isomers of linoleic acid that are found in milk, cheese, and beef (16). CLA is attracting great interest among nutritionists because it is a natural fat component that appears to have a number of health-giving properties (17). CLA isomers are being produced as health-food supplements, and many natural conjugated fatty acids are available from plant sources, whose therapeutic properties have never been investigated. When these are ingested by humans or other animals, further conjugated metabolites may be produced. 9-*Cis*.11-*trans*-octadecadienoate is the common natural isomer found in milk and dairy products, where it has arisen as a by-product or intermediate in the microbial bio-hydrogenation of linoleic acid in the rumen. Some scientists reported that CLA was an intermediate in the microbial hydrogenation of linoleic cid (18) and also, a rumen bacterium, *Butyrivibrio fibrisolvens*, converted linoleic acid to oleic acid with CLA as an intermediate (19). CLA has been shown to have anticancer effects against breast, colon and prostate cancer cell lines (20–24).

These actions may include its ability to interfere with the proliferation of cancer cells, inhibition of angiogenesis through effect in biosynthesis of adhesion molecules, or increased oxidative stress. Cheng et al. indicated a role of NF-κB in regulation of anti-inflammatory actions of CLA (25). As the interest in beneficial effects of CLA is increasing, so are the efforts to increase its dietary intake. Various strategies are being employed, which include increasing the content of CLA in eggs, milk, and meat. While scientists conducting research on CLA are very optimistic about being able to demonstrate its health benefits eventually, most scientists interviewed are extremely cautious about making statements that are definitive in claiming health benefits outright (26). With respect to CLA both beneficial and pro-atherogenic effects have been described (27, 28). However, the effect on expression of cell adhesion molecules on HUVECs is not yet established. To establish the effects of CLA on cardiovascular risk factors in humans, more research is needed using pure isomers or standardized mixture of CLA isomers. The aim of the experiments was to evaluate the influence of CLA in comparison with linoleic acid regarding the TNF-α induced expression of endothelial leukocyte adhesion molecules.

METHODS

Chemicals and cells

75cm^2 culture flasks, culture microplates (NUNC, Roskilde, Denmark), trypsin/EDTA solution, M199, penicillin, streptomycin, L-glutamin, heat-inactivated fetal calf serum (FCS), DMSO (Gibco BRL), free essential fatty acid bovine serum albumin (FEFA-BSA), Linoleic acid (LA) (*cis*-9,*cis*-12-18:2), Conjugated linoleic acid (CLA) (*cis*-9,*trans*-11-18:2), human recombinant TNF-α , vascular endothelial growth factor and heparin(Sigma, St. Louis, MO, USA), agarose, ELISA-based kits (96wells) for sICAM-1, sVCAM-1, and sE-selectin were purchased from BioSource International, Inc. (invitrogen Corp. USA), RNA extraction kit, Ex Taq hot start version and Syber Green (Takara Bio, Inc.; Otsu, Japan) von-willebrand factor antibody (Abcam plc.) cDNA synthesis kit and RQ1 RNase-free DNase purchased from promega (Promega, Madison, WI).

HUVECs isolation and culture

Endothelial cells were isolated from human umbilical veins by applying a collagenase solution according to a standard procedure described elsewhere (29). The cells were grown onto gelatin-coated 75-cm^2 flask in M199 containing 5 units/ml heparin, 10% FCS, 1000 U/ml penicillin, 1000 µg/ml streptomycin, endothelial cell growth factor (50 *m*g/mL), and 3 mmol/l L-glutamine, at 37°C under 5% CO2. HUVEC were characterized by morphologic criteria, by their constitutive expression of von Willebrand factor immunoreactive protein, and by their cytokine-induced expression of E-selectin (30). Cell density at the beginning of incubation experiments was chosen in order to reach confluency 72 h later, i.e., at the time of assessment of adhesion molecule expression. Only confluent monolayers between passages 1 and 3 were used for the experiments described below. Cells were grown in 96-, 12-, or 6-well flat-bottom cluster plates for control of FA toxicity, assessment of adhesion molecule expression by ELISA or for RT-PCR, respectively.

Cell viability assay

The cytotoxicity of FAs was analyzed by colorimetric MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay (31). Briefly, endothelial cells were treated with different time courses (12, 18, 24 h) and concentrations (25, 50, 100, 200 µM/L) of CLA. Four hours before the end of incubation, medium was removed and 100 ml MTT (2.5 mg/ml in phosphate buffered saline, pH 7.4) was added to each well. The MTT solution was removed after 4 h and 100 ml Dimethyl sulfoxide (DMSO) was added to each well to dissolve water insoluble formazan crystals. Absorbance was recorded at 570 nm in an ELISA reader (Bio-Rad, Model 680, USA). From this assay, the % viability of the cells at various concentrations of each compound was determined by normalization to cells incubated in vehicle (0.5% DMSO in cell culture medium) and which were considered 100% viable. The highest concentration at which the viability of the cells was >90% was denoted as the maximum tolerable concentration for that compound. All experiments were performed at least 3 times in triplicate wells.

Experimental design

The best concentrations of reagents that had maximum viability for each TNF-α and fatty acids are selected. Finally, Cultured HUVEC were incubated with TNF-α (0.001 µg/ml) and/or co-incubated with TNF-α and either LA or CLA (100 µM/L) for 16 hours. At the time of the experiments, fatty acids were further dissolved in FEFA-BSA at the final desired concentrations. The filter-sterilized fatty acids-BSA mixture (0.5ml) was added to 4.5 ml fresh M199 medium supplemented with 10% FCS (final concentration of fatty acids 100µM/L). Cells incubated with M199 and BSA (20mM) supplemented with 10 % FCS in absence of fatty acids and TNF-α was used as controls (32).

Supernatant ELISA for measurement of soluble cell adhesion molecule Expression

HUVEC were plated on to 75cm2 culture flasks and allowed to reach confluence. After adding the stimulants and fatty acids, soluble forms (in supernatant) of ICAM-1, VCAM-1 and E-selectin were quantified using the ELISA kits, which based on a sandwich format as described previously (32). Absorbance was recorded at 450 nm in an ELISA

reader (Bio-Rad, Model 680, USA). The data presented were means of triplicate determinations.

Total RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was isolated from treated cells with a fast pure RNA kit (Takara Bio, Inc.; Otsu, Japan). Contaminating genomic DNA was removed by treatment with RQ1 RNase-free DNase (Promega, Madison, WI) and its concentration was determined by spectrophotometry at 260 nm. The expression of the transcripts for ICAM-1, VCAM-1, E-Selectin and β-actin was evaluated by RT-PCR that performed following the manufacturer's protocol (Access RT-PCR system; Promega). Briefly, 100 ng of the total RNA was reverse transcribed using AMV reverse transcriptase, oligo dT, and 25 mM dNTP at 48 ^{0}C for 40 min. The temperature of the reaction was then raised to 94°C for 5 minutes to inactivate the enzyme and finally reduced to 4°C. PCR were performed using 1 ml of the cDNA product as template, 0.2 mM of each primer, 25 mM of dNTP, 0.125 U of Ex Taq hot start version (Takara Bio, Inc.; Otsu, Japan), and the standard buffer (final Mg^{2+} concentration was 1.5 mmol/L) in a total reaction volume of 5 ml for 25-30 cycles. The primers were synthesized according to the published cDNA sequences (33, 34). The sequences for the target molecules were as follows: β-actin (ATGTTTGAGACCTTCAACAC, CACGTCACACTTCATGATGG, 489 bp), VCAM-1 (CGTCTTGGTCAGCCCTTCCT, ACATTCATATACTCCCGCATCCTTC, 460 bp), ICAM-1 (AGGCCACCCCAGAGGACAAC, CCCATTATGACTGCGGCTGCTA, 406 bp) and E-selectin (CCAGTGCTTATTGTCAGC, CACATTGCAGGCTGGAAT, 610 bp). The conditions for PCR were as follows: denaturation at 93 ^{0}C for 45s, primer annealing at 52 ^{0}C for 90s, extension at 72 ^{0}C for 2 min, and a final extension at 72 ^{0}C for 10 min. The PCR products were separated on 2% agarose gel (Invitrogen, USA) and visualized by Syber Green (Takara Bio, Inc.; Otsu, Japan). The intensity of ICAM-1, VCAM-1 and E-selectin transcripts was densitometrically scanned using AlphaImager gel documentation system (Biorad, USA) and normalized with that of β-actin levels expressed under similar conditions.

Statistical analysis

Results are expressed as arithmetic mean (±SEM), and analyzed by using either paired two-tailed student's test for comparison between two groups or by ANOVA (analysis of variance), for multiple comparisons. The difference was considered to be significant when $P < 0.001$.

RESULTS

Viability assay

Studies through MTT assay indicated that the best time and concentration (with higher viability) for incubation of ECs with FAs was 16h and 100 μM/L respectively (Fig. 1a and b). We observed that effect of FAs in CAMs expression in lower concentrations have dose depended manner but with dose increasing over than 100 μM/L cell toxicity blocked normal ECs growth via unknown mechanisms (data not showed).

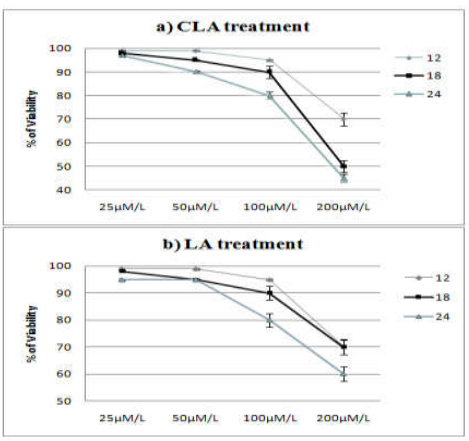

Fig. 1. Best concentration of FAs for treatment of HUVECs. After measurement of viability through MTT assay in several time and concentrations, the best of them are choose with attention to more viability (100μM/L for 16h). Three individual assays were performed and the averages are shown (mean ± SD).

CLA but not LA inhibits the expression of sICAM-1 and sVCAM-1 on HUVECs

As cell adhesion molecules play an important role during inflammation, we analyzed the effect of CLA on expression of these molecules. The effect of CLA in secretion of adhesion molecules on un-

stimulated or active HUVECs was analyzed using supernatant-ELISA as detailed in Material and Methods and compared with LA. Our results demonstrate that these adhesion molecules were expressed at low levels on un-stimulated HUVECs (control) and there was over three to eight-fold increase in their expression upon stimulation with TNF-α (Fig. 2). Pre-treatment of endothelial cells with CLA had no important effect on the constitutively expressed levels of sVCAM-1 ($P > 0.05$) and sE-selectin ($P < 0.05$) but reduced sICAM-1 ($P < 0.001$) significantly lower than un-stimulated cells (Fig. 2). When TNF-α primed HUVECs was incubated with CLA for at least 16 hours, the concentrations of sVCAM-1 and sICAM-1 considerably diminished in contrast with active phenotype ($P < 0.001$). In contrast, LA maintained (sICAM-1) or even enhanced (sVCAM-1 and sE-selectin) expression of these adhesion molecules when co-treatment with TNF-α (Fig. 2). The inhibitory activity of co-Incubation with TNF-α and CLA on expression of sICAM-1 in HUVECs was dramatically more than its inhibitory effect on sVCAM-1 (Fig. 2). CLA didn't have inhibitory effect on TNF-α induced sE-selectin expression in these conditions (Fig. 2).

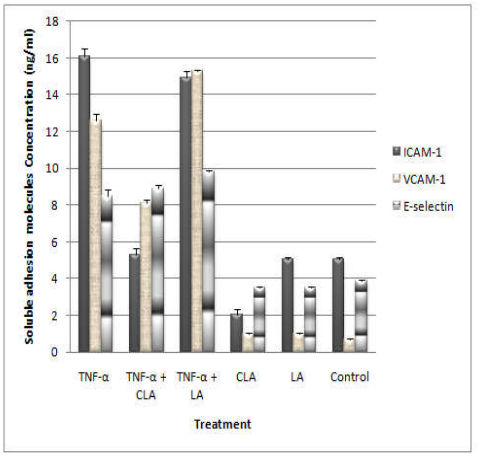

Fig. 2. Induction of cell expression of sICAM-1, sVCAM-1 and sE-selectin by fatty acids (FAs) in TNF-α stimulated HUVECs. The endothelial cells were co-treated with or without 100μM/L concentration of each FAs (CLA or LA) and TNF-α (0.001μg/ml) for 16 h. The cells supernatant were set apart and the cell surface expression of adhesion molecules were analyzed with ELISA assay. Each column shows the mean ±S. D. of triplicate determinations.

The transcript levels of ICAM-1, VCAM-1 and E-Selectin are decreased by CLA

The expression of membrane VCAM-1 and ICAM-1 along with E-selectin, upon TNF-α induction to the human endothelial cells, is also required for the firm adhesion and transmigration of lymphocytes to the site of tissue injury. Thus we have tested the ability of CLA to inhibit the TNF-α induced mRNA expression of these molecules use of RT-PCR method. A significant increase in ICAM-1(5.6-fold), VCAM-1(10.5-fold) and E-selectin (6.5-fold) mRNA expression over basal levels was observed with TNF-α (0.001 μg/ml) stimulation compared to control, as shown in Figure 3 ($P < 0.001$). It was found that CLA significantly decreased the TNF-α induced mRNA expression of these adhesion molecules in contrast LA (Fig. 3). Although LA (100 μM/L) alone did not enhance adhesion molecules mRNA expression, a combination of LA and TNF-α showed a slight additive effect on ICAM-1 mRNA expression ($P < 0.001$). In contrast, VCAM-1 mRNA expression was decreased by LA (Fig. 3). A combination of TNF-α and CLA resulted in the greatest down-regulation of VCAM-1(36%), ICAM-1(71%) and E-selectin (24%) mRNA expression compared to TNF-α treatment cells, as shown in Figure 3.

DISCUSSION

An isomer of linoleic acid that also may have important consequences for atherosclerosis, as well as cancer, is conjugated linoleic acid (CLA). CLA is unique because unlike most antioxidants which are components of plant products, it is present in food from animal sources such as dairy foods and meats. CLA concentrations in dairy products typically range from 2.9 to 8.92 mg/g fat of which the 9-*cis*, 11-*trans* isomer makes up to 73% to 93% of the total CLA. Low concentrations of CLA are found in human blood and tissues (35, 36). The physiological role of CLA in normal and immune-stimulated animals was studied by Cook and Pariza in the early 1990s (37, 38). In a study by Huang *et al.* (39) on nine men, plasma CLA increased 19% to 27% following a four weeks feeding of cheddar cheese, but no appreciable changes in linoleic and arachidonic acids, cholesterol or phospholipid

levels were observed. In the present study we demonstrate for the first time that CLA can be used for controlling expression of cell adhesion molecules and thus may be useful in the regulation of cellular trafficking. We analyzed its ability to inhibit TNF-α induced ICAM-1, VCAM-1 and E-selectin expression on endothelial cells. The receptors to ICAM-1, VCAM-1 and E-selectin are present on the circulating leukocytes, which then get arrested and migrate across the endothelium into the underlying tissues. Soluble ICAM-1 is the major ligand for localization of leukocytes to activated endothelium, and its expression has been documented on atherosclerotic plaques (40, 41). Expression of VCAM-1, however, occurs only on activated endothelial thus sVCAM-1 may serve as a specific marker of plaque burden or activity, underlying its potential use as a prognostic marker in the secondary preventative setting (42, 43, 44). Similarly, E-selectin, along with L- or P-selectin, mediates cell tethering and rolling interactions through the recognition of sialo-fucosylated Lewis carbohydrates expressed on structurally diverse protein-lipid ligands on circulating leukocytes or tumor cells (45). There is some controversy recently about molecular mechanisms that mediated benefit effects of CLA in system biology. Previous study showed that either CLA isomer or linoleic acid couldn't modulate the cytokine-induced expression of ICAM-1, VCAM-1 in human aortic endothelial cells (46). In the another study reported that reduction of ICAM-1 and VCAM-1 protein expression by n-3 PUFA was less dependent on the NF-*κ*B pathway than reduction by CLA which reflected the parallel attenuation of NF-*κ*B activity (47). Furthermore, The CLA decreases the atherogenesis-related genes in HUVECs and alters adhesion of macrophages (48). CLA supplementation also decreased the levels of the proinflammatory cytokines, TNF-α and IL-1β, but increased the levels of the anti-inflammatory cytokine, IL-10 (49). As we reported in previous studies FAs alone don't showed significant effect on CAMs protein expression pattern in ECs (32). Furthermore, treatment of ECs with concentrations more than 100 µM/L dramatically decreased ECs viability (32). In our study, CLA alone at a concentration of 100µM/L is found to be a potent inhibitor of sICAM-1 expression in HUVECs. Although the role of diet in the etiology of endothelial dysfunction is not fully understood, human epidemiological studies provide some evidence that dietary fat quality may influence the function of the endothelium (50-55). This finding may be of importance in changing the levels of CLA in biological fluids by altering specific dietary foods and fatty acids as sources of CLA and, thereby, protecting against inflammatory disease. CLA was also found to significantly reduce the transcript levels of ICAM-1, VCAM-1 and E-selectin. The ability of CLA to effectively block the induced expression of cell adhesion molecules demonstrates its potential that may be tested in various inflammatory conditions where down-regulation of cell adhesion molecules is required. The results of both RT-PCR and ELISA collectively indicated that CLA significantly down-regulate ICAM-1 and VCAM-1 induced by TNF-α. In contrast, LA maintained the activated phenotype of HUVEC cells. In addition, results of ELISA showed that sICAM-1 showed high down expression on response to CLA. On the other hand, CLA suppress expression of ICAM-1on activated HUVECs more than VCAM-1.

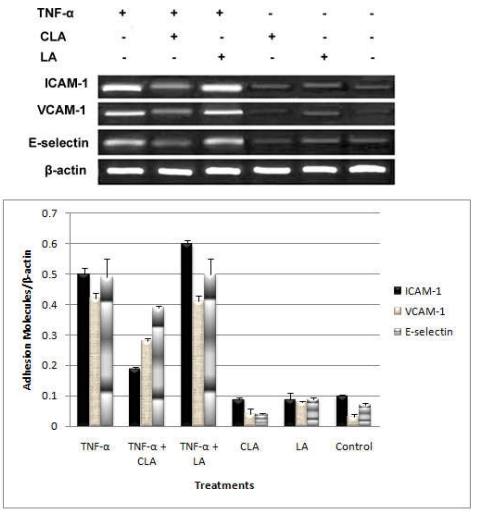

Fig. 3. Effects of CLA in the transcript levels of ICAM-1, VCAM-1 and E-selectin on HUVECs. The endothelial cells were co-treated with or without 100µM/L concentration of each FAs (CLA or LA) and TNF-α (0.001µg/ml) for 16 h. The total RNA of the cells was isolated and analyzed by RT-PCR as described in material and methods. Transcripts was densitometrically scanned and normalized with that of β-actin levels expressed under similar conditions. Each column shows the mean ±S. D. of triplicate determinations.

CONCLUSIONS

In conclusion, we have shown that CLA (c*is*-9, *trans-11* Isomer), in contrast to its trans-counterpart LA, reduced TNF-α stimulated expression of ICAM-1 and VCAM-1 in HUVECs. These findings suggest that CLA, is promising lead compound for the development of anti-inflammatory agents. Interestingly, CLA significantly inhibited the sICAM-1 and sVCAM-1 in contrast to sE-selectin. CLA was also found to significantly reduce the transcript levels of ICAM-1, VCAM-1 and E-selectin. It also offers a novel target for controlling various pathological conditions associated with up-regulation of endothelial leukocyte adhesion molecules especially. It remains to be seen if levels of inflammatory biomarkers, including soluble cell adhesion molecules, become used as part of routine biochemical risk factor assessment in both primary and secondary prevention of cardiovascular disease. Given the high prevalence of cardiovascular diseases in Iran, the possible decrease in risk for those diseases caused by shifting to high CLA consumption should be carefully considered.

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